Hyalocytes in Tissue Engineering

First Steps Towards a Cell-based Vitreous Substitute

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Table of Contents

Chapter 1 Ocular Tissue Engineering 5

Chapter 2 Introduction and Goals of the Thesis 31

Chapter 3 Culture Conditions for Primary Hyalocytes.................. 37

Chapter 4 Analytical Methods to Quantify Extracellular Matrix Components Accumulated by Hyalocytes 53

Chapter 5 Ascorbic Acid Modulates Proliferation and Extracellular Matrix Accumulation of Hyalocytes 69

Chapter 6 Pyruvate Modulates the Effect of Ascorbic Acid on Hyalocytes 91

Chapter 7 Modulation of Hyalocyte Proliferation and ECM Accumulation via bFGF and TGF-β1 109

Chapter 8 Three-Dimensional Hyalocyte Culture Systems 129

Chapter 9 FACS as Useful Tool to Study Distinct Hyalocyte Populations 149

Chapter 10 Summary and Conclusions.. 163

Appendix
Abbreviations 171
Curriculum vitae.. 173
List of Publications.. 175
Acknowledgements 179

Chapter 1

Ocular Tissue Engineering

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Introduction

In the early 1990s, tissue engineering emerged as a new concept to overcome the problem of tissue and organ failure. It proposed to supply engineered, yet biological, organ and tissue substitutes. It was anticipated that this technology would soon allow us to overcome donor shortages and graft rejection, the major limitations of tissue and organ transplantation. Tissue engineering approaches that were developed on the basis of this paradigm relied on the use of cells and stem cells, preferably of autologous origin, the application of growth factors and cytokines, the design of biodegradable scaffolds and bioreactor technology[1, 2].

Over the past decades, there has been tremendous progress towards the regeneration of tissues such as bone[3], heart valves[4], myocardial tissue[5] and cartilage[6]. While these examples impressively show that tissue engineering technology holds great promise for the manufacture of tissue grafts, even more diverse applications have emerged in recent years. Tissue constructs have been used to investigate cellular and molecular mechanisms[7], are used for in vitro drug screening and can be expected to reduce the number of time and cost intensive in vivo experiments in drug development[8]. Despite this success, one may still question, why tissue engineering has not progressed even faster and further.

Obviously, we underestimated some of the obstacles on the way towards the development of functional tissue-engineered grafts. Frequently, the host tissue fails to support the integration of engineered tissue. In many cases wound healing processes leading to scar formation dominate over the intended tissue repair and biodegradable scaffolds frequently raise concerns due to the risk of inflammatory responses[9]. With increasing size, engineered tissues also suffer from insufficient nutrient availability and limited metabolic waste removal by passive diffusion, resulting in cell death and necrosis. A rapid and adequate vascularization of an implanted tissue has, therefore, been identified as an essential prerequisite for its survival and integration. Induction of angiogenesis is recognized as one of the most critical factors to the success of tissue engineering[10, 11]. Although growth factors, such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF), are potent angiogenic factors, their use is associated with problems spanning from limited in vivo stability to an abnormal growth of blood vessels resembling the vascularization of tumor tissue[12, 13].

For the reasons outlined above, it would be advantageous to focus our tissue engineering efforts on systems that display less complexity. With these role models, it would be possible to gather experience that helps in the future to solve problems related to the regeneration of more complex tissues. Ocular tissues seem an ideal candidate for this strategy. Most of them, such as the corneal epithelium or the retinal pigment epithelium (RPE), are not vascularized

and resemble more sheet-like than three-dimensional structures. Nutrients and oxygen are sufficiently supplied by diffusion from adjacent tissues and, finally, parts of the eye enjoy an immune privilege that adds additional degrees of freedom with respect to the choice of materials and cells.

Altogether, ocular tissues seem to be predestined for regeneration using tissue engineering approaches. But besides the scientific and strategic incentive for reconstructing ocular tissues, there is also a tremendous need for novel therapeutic options for treating numerous eye diseases related to tissue failure. Age-related macular degeneration (ARMD), glaucoma and diabetic retinopathy (DR) are leading causes of blindness. The prevalence of these diseases among persons aged over 50 is between 3 and 10 %[14], illustrating the significance of the problem. Despite the tremendous medical progress made in recent years, especially in ophthalmology, the prevalence of age-related blindness is still increasing, spurred by demographic trends[15, 16], outlining the need for alternative treatments.

This article will review the state of the art in ocular tissue engineering. The goal is to illustrate the progress already made and the strides still necessary to create clinically relevant tissue substitutes.

Corneal Tissue Engineering

The cornea is the transparent barrier between the eye and the environment, protecting the eye from pathogenic microbes and dryness. The cornea is comprised of three major cellular layers: an outermost stratified squamous epithelium, a stroma with corneal fibroblasts (keratocytes), and an innermost monolayer of specialized endothelial cells[17] (Figure 1). In severe diseases of the cornea, their transparency is no longer maintained, usually due to a malfunction of only one of the three parts of the cornea. Therefore, tissue engineering developments focus on the reconstruction of the damaged part to restore transparency of the whole cornea. These strategies, especially the regeneration of the corneal epithelium, will probably be clinically approved in the near future.

Corneal Epithelium

The corneal epithelium consists of five cell layers in the tissue center and about ten layers on its periphery. It shows a distinct physiological turnover; the cells are constantly renewed by proliferating cells of the basal epithelium, often termed transient amplifying cells[18, 19]. These cells can divide only a limited number of times[20] and are themselves replaced by slowly proliferating stem cells of the limbus[21]. The limbus is surrounding the cornea; it was

demonstrated to be a reservoir of corneal epithelial stem cells, cells that are, therefore, also termed limbal stem cells. If these corneal epithelial stem cells are completely absent due to limbal disorders from severe trauma (for example thermal or chemical burns) or eye diseases (for example Stevens-Johnson syndrome), the source of corneal epithelial cells is exhausted, resulting in opacification of the cornea and severe visual impairment[22]. Therefore, in patients with unilateral limbal stem-cell deficiency, an autologous limbal transplantation is performed to restore the corneal epithelium[23]. However, there is an associated risk of inducing limbal stem cell deficiency in the healthy eye[24]. In patients with bilateral lesions, autologous limbal transplantation is rarely possible, due to the large number of cells necessary for transplantation. Limbal or corneal allograft transplantation, however, is limited by the number of organ donors and requires long-term immunosupression associated with severe side effects[25]. To overcome these limitations, strategies to cultivate autologous corneal epithelium in vitro based on tissue engineering concepts have been developed. The general idea is to cultivate physiological corneal epithelium including a sufficient number of stem cells for physiological regeneration in a culture dish, starting with a small sample of cells[26]. Corneal epithelial stem cells seemed to be an optimal cell source, as the corneal epithelial cells are physiologically renewed by these stem cells.

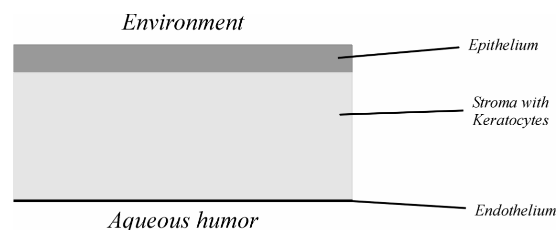

Figure 1: *Schematic survey of the three major cellular layers of the cornea: an outermost stratified epithelium, a stroma with corneal fibroblasts (keratocytes) and an innermost monolayer of endothelial cells.*

In 1997, Pellegrini et al. reported the first clinical success in two patients with complete loss of corneal-limbal epithelium of one eye using cultivated limbal stem cells[27]. After isolation and propagation of cells from a small biopsy of the limbus of the healthy eye, they cultured a sheet of cells for 19 days to prepare the epithelial graft. According to the authors, the resulting graft was microscopically similar to the cornea, stained positive for cytokeratin 3, a specific marker of the corneal lineage[28] and, therefore, represented an authentic in vitro cultured corneal epithelium. After release of the sheet from the culture plastic using the protease Dispase II, they transplanted the cultured cornea onto the patient’s prepared eye and patched it tightly for three days. After grafting of the cultured epithelium, both patients developed a stable and transparent corneal epithelium without vascularization. More than two years after

grafting, the patients were clinically stable and the authors strongly suggest that this was due to a successful engraftment of the stem cells.

In the following years, attempts were made to optimize this encouraging new therapy. The use of biomaterials was investigated to improve the handling and manipulability of the epithelial constructs, as well as their integration onto the corneal stroma[29]. Furthermore, as the use of proteolytic enzymes is associated with the destruction of cell-cell junctions and extracellular matrix, both critical to sheet integrity and function, new culture techniques were studied that allowed for the removal of the epithelial sheets from the culture plastic without using enzymes[30].

Searching for suitable biomaterials, amniotic membrane (AM) seemed suitable as a first cell carrier. AM is the inner layer of the fetal membranes and consists of a single layer of columnar cells firmly attached to an underlying basement membrane. It is known to suppress inflammation and scarring and serves as an anti-microbial barrier[31]. The successful transplantation of human AM to severely damaged rabbit cornea[32] has been reported. In 2000, Tsai et al. took a small limbal-biopsy specimen from the healthy eyes of six patients suffering from unilateral limbal epithelial cell deficiency and expanded them on AM to form an epithelial-cell sheet[33]. After about three weeks of culture, they transplanted the resulting epithelial-cell sheet, together with the membrane, to the damaged eyes of the same patient. Complete reepithelialization of the corneal surface occurred within two to four days in all of the patients, followed by improved clarification of the cornea after one month. No patient had recurrent neovascularization or inflammation in the transplanted area during the follow-up period of about 15 months and all patients demonstrated improved vision. The authors concluded that the use of autologous limbal epithelial cells grown on AM had all the benefits of AM transplantation, including the facilitation of epithelialization, reduction of inflammation and scarring, and replacement of substrate when the underlying stromal tissue is destroyed. Furthermore, in contrast to the report of Pellegrini et al.[34], the handling and suturing had been simplified.

In contrast to the work of Tsai et al.[35], Rama et al. used a fibrin glue for the preparation of epithelial cell sheets[36]. After transplantation of these sheets, all of these patients showed complete reepithelialization within the first week, similarly to the previous report.

The introduction of biomaterials as a cell carrier showed several advantages, as for example improved handling of the constructs, however, post-transplant effects from the carrier were expected to influence the clinical outcome. This was confirmed by the observation of eye-threatening complications in a patient after AM transplantation[37]. Therefore, Nishida et al.

focused again on the culture of epithelial sheets without a carrier. As temperature-responsive culture surfaces, established by Yamada et al. in 1990[38], were shown to allow the harvest of intact multilayered keratinocyte sheets without the use of proteolytic enzymes[39], this technology was used for the culture of corneal epithelial sheet grafts[40]. This method enabled them to obtain a well-structured, compact multilayered cell sheet architecture with the expected native cell microstructure, such as tight junctions, desmosomes and basement membrane, comparable to those in native corneal tissue. The resulting convenient and robust tissues could be transplanted onto the cornea of rabbits and adhered strongly to the corneal stroma within minutes, making sutures unnecessary. According to the authors, the grafts remained stable at the initial placement, exhibited a normal appearance and expressed the typical corneal marker cytokeratin 3.

This approach overcame a number of problems associated with other related techniques, however, there was still the need for autologous limbal stem cells for the culture of the corneal epithelium. To overcome this need, Konoshita et al. demonstrated the feasibility of using autologous mucosal epithelial cells for reconstruction of the ocular surface[41, 42]. Nishida et al. combined the culture of mucosal epithelial cells with the technique using temperature-responsive surfaces and established an alternative replacement strategy for damaged corneal epithelium[43] (Figure 2). According to the authors, the cultured sheets showed transparency equal to that of sheets originating from limbal stem cells and were microscopically similar to native corneal epithelium. The sheets could be transplanted onto the patients' corneas without suturing. During the follow-up period of 14 months, corneal transparency was maintained, visual acuity was improved and complications could not be observed. Therefore, the sheets of tissue engineered epithelial cells fabricated ex vivo from autologous oral mucosal epithelium seemed effective for reconstruction of the ocular surface, providing a possible therapy even for patients with bilateral total stem-cell deficiencies. However, it is still unclear whether stem cells of the mucosa can differentiate into corneal epithelium. It is also possible that the therapeutic success in this study was due to a stimulation and re-proliferation of a small number of still remaining autologous epithelial stem cells in the recipient's cornea[44]. Long-term studies and a larger number of patients will, therefore, be necessary to assess the benefits and risks of this therapy.

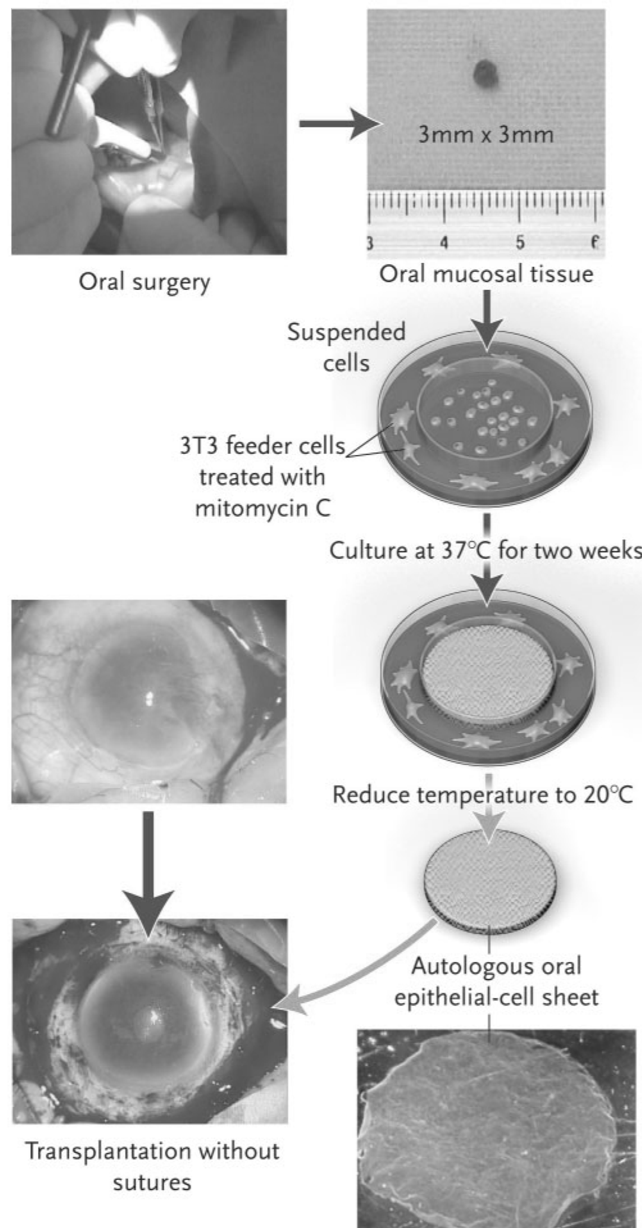

Figure 2: *In vitro culture of a corneal epithelial transplant using mucosal epithelial cells. After isolation of autologous oral mucosal epithelial cells, the cells were cultured in the presence of a feeder layer onto temperature-responsive culture surfaces at 37°C. Reduction of the temperature to 20°C leads to the removal of the cell sheet, which can subsequently be transplanted to the patient without the need for suturing. Reprinted from Nishida et al.*[45] *Copyright © 2004 Massachusetts Medical Society. All rights reserved.*

Corneal Stroma

The corneal stroma is the largest part of the cornea, underlying the epithelium and consisting of fibroblasts, also called keratocytes, embedded in a matrix of collagens and glycosaminoglycans. Blood vessels are absent in the central cornea in contrast to the limbus and conjunctiva, which are highly vascularized. Transparency of the tissue is caused by a small diameter and a distinct orientation of the collagen fibrils within the tissue[46]. Culture of

corneal stroma, in combination with epithelium, seems useful for clinical therapy of deep corneal lesions and failures in keratomileusis (the carving of the cornea to reshape it), furthermore the stroma displays the "backbone" of completely engineered cornea. Stroma engineering, however, could become a great challenge, as transparency of the stroma is essential because of the thickness of this corneal layer.

The successful cultivation of corneal stroma, even in combination with corneal epithelium and endothelium, has been reported[47, 48]. However, in many of the reports immortalized cell lines were used, cells that seem unsuitable for a clinical therapy. In 1999, Germain et al. reported the successful engineering of human cornea cultured with primary keratocytes and epithelial cells[49]. They reconstructed the corneal stroma by culturing keratocytes within collagen and cultured them for four days. After this cultivation, they seeded the gels with epithelial cells and cultured them for three more days. The resulting corneas were histologically similar to native cornea and expressed components of the epithelial basement membrane at the epithelium-stroma junction, but data about the transparency of the systems are missing. In 2004, Hu et al. reported the in vitro cultivation of corneal stroma for one week using rabbit keratocytes mixed with polyglycolic acid and the subsequent transplantation in vivo[50]. According to them, the tissue became transparent within eight weeks of transplantation of the cultured stroma and no differences in the diameters of native and engineered cornea could be observed. They confirmed that the cornea was formed by the cultured cells by transfecting them with GFP and detecting a green fluorescence within the whole stroma. Although the results for corneal stroma culture are encouraging, long-term in vivo data and clinical trials are still lacking.

Corneal Endothelium

The corneal endothelium consists centrally of a monolayer of endothelial cells underlying the corneal stroma and represents, from a medical point of view, the most important part of the cornea, as only an intact endothelium with a sufficient cell density can function properly and maintain clarity of the cornea by its dehydrating pump function[51]. In cases of intraocular surgery or inherited diseases, a drastic decrease in the number of cells can be observed. As the proliferative capacity of the endothelial cells is restricted[52], transplantation of isolated and cultured corneal endothelial cells (CEC) has been studied, however, the success of these experiments was limited[53] due to insufficient cell numbers or a lack of adherence. The first in vivo report of the transplantation of human CEC was published in 1991 by Insler and Lopez[54], who seeded human neonatal CEC on human corneas that were denuded of their native

epithelium. After implantation of the cultured corneas into African green monkeys, 75 % of the corneas cleared up and showed a clear decrease in diameter (large diameter indicates edema of the cornea) for up to twelve months. Ishino et al. reported the first in vivo study using adult cultured human CEC[55]. After propagation of the cells, they transplanted the endothelial cells onto amniotic membrane; they reached a sufficient cell density on the membrane by gently centrifugating the cells onto the membrane. After cultivation of these endothelial sheets for two weeks, they transplanted the sheets into rabbits' eyes and observed excellent transparency with little edema for at least seven days. Long-term consequences, however, could not be determined, as the corneal endothelium of rabbits proliferates in vivo, in contrast to human endothelium, and, therefore, this animal model seems not suitable for long-term evaluation. Similar results were reported by Mimura et al.[56] using adult human corneal endothelial cells in a rat model. Again, transparency of the cornea was restored by CEC after seeding them onto the excised cornea and subsequent transplantation of the cornea. In contrast to Ishino et al., no carrier membrane was used. Furthermore, Mimura et al. demonstrated that the corneal transparency was maintained for one month after transplantation.

Mimura et al. also evaluated a novel approach for corneal endothelial regeneration[57]. They exposed cultured CEC to iron powder and injected the cells after endocytosis of the iron into the anterior chamber of rabbits' eyes, subsequent to cryo-injury of the corneal endothelium. By fixing a magnet on the lid of animals, the injected CEC were attracted to the cornea for 24 h. They could demonstrate that the cells adhered to the Descemet's membrane, the native location of the CEC, resulting in decreased corneal edema over the whole investigation period of eight weeks. As this method could have several drawbacks associated with the iron powder, long-term observations have been performed. According to Mimura[58], the iron powder was not detectable after twelve months, however, in contrast to a negative control, sufficient numbers of CEC could be detected in the study group, resulting in a decreased edema score. Drawbacks, such as increased intraocular pressure or other ocular complications, could not be detected. Therefore, the authors conclude, the magnetic attachment of iron-endocytosing CEC can be an effective and safe method for corneal endothelial repair. This therapeutic option was the first to effectively restore corneal endothelium simply by injecting cells into the anterior chamber of the patient, however, no reports on human studies are published yet.

Besides the direct treatment of the patients' cornea, there is another interesting application of CEC transplantation: the improvement of corneas from organ donors. About 40 % of the corneas could not be transplanted, because they failed the quality criteria of the cornea banks, mostly due to their low endothelial cell density. To overcome this problem, several

approaches were performed to increase the cell density by transplanting CEC onto the corneas. These strategies, such as suitable isolation and cultivation conditions for human CEC, the use of growth factors or the transfection of endothelial cells with viral genes to enhance the cell proliferation, are discussed in detail by Engelmann et al.[59]

Summary

To conclude, the tissue engineering of the cornea is a promising field. Especially the reconstruction of the corneal epithelium seems to be a promising therapeutic option for the treatment of patients suffering from limbal stem cell deficiencies. To completely substitute corneal transplants, the culture of all three corneal layers, including epithelium, stroma and corneal endothelium is necessary. This will probably remain a challenging task, as optimal culture conditions for all three layers have to be established. Furthermore, for clinical approval, the use of serum or feeder layers of cells likely becomes problematic.

A future challenge will also be the innervation of the cultured cornea, as the cornea is one of the most innervated tissues and missing innervation could lead for example to the clinical syndrome of the "dry eye"[60]. Innervation of the cornea was already studied within biosynthetic tissue templates[61]; the control of complex interaction between materials, different corneal cell types and nerve conduits, however, remains a challenge ahead.

Retinal Pigment Epithelium Engineering

The retinal pigment epithelium (RPE) consists of a monolayer of cuboidal cells located between the choroidal layer of the eye and the neurosensory retina. It is part of the blood-retinal barrier and responsible for the attachment of the retina to the choroidal layer by a net transport of ions and water in an apical to basal direction. Further functions of the RPE are the absorption of stray light, the uptake, processing and transport of retinoids, and the phagocytosis of rod and cone outer segment fragments. Once differentiated, the RPE is not able to regenerate itself by cell division[62].

Disorders of the RPE are implicated in the pathogenesis of age-related macular degeneration (ARMD), the leading cause of blindness in people aged over 55[63], and other degenerative and hereditary ocular diseases. In "dry" (non-exudative) ARMD, which is the most common form, vision is impaired due to progressive atrophy of the RPE with subsequent loss of the choriocapillaris and photoreceptors within the macula. In contrast, loss of vision in "wet" (exudative) ARMD is associated with bleeding from abnormal blood vessels grown from the choriocapillaris beneath the RPE and macula (choroidal neovascularization, CNV). Currently,

there are no treatments for "dry" ARMD and the available therapies for "wet" ARMD, such as laser photocoagulation, are still controversially discussed because of their only moderate efficacy in preventing blindness[64].

As a potentially curative therapy, the concept of transplanting healthy RPE in the subretinal space has been extensively investigated in the past decades. Due to immune reactions, however, patients receiving transplants of homologous RPE had no visual benefit[65, 66]. Thus, the interest has been focused on autologous cells. The first prospective trials demonstrated that CNV membrane surgery combined with simultaneous transplantation of freshly isolated RPE cells resulted in clinically relevant improvements of vision compared to other surgical procedures. Potential drawbacks of this approach are the limited number of healthy cells that can be harvested from patients with degenerative eye diseases and the delivery of the cells in suspension[67, 68]. Since RPE cells are polar with distinct apical/basal characteristics and well established intracellular relationships[69], the implantation of an organized sheet of RPE cells with appropriate orientation is thought to be an important factor for a successful graft.

The concept of tissue engineering offers the chance to cope with the above mentioned problems: 1) Autologous cells are harvested from the patient and expanded in vitro to a sufficient number. 2) Dysfunctional donor cells can be manipulated to perform the required function in the retina by ex vivo gene manipulation[70]. 3) Culturing the cells under suitable conditions allows for the maintenance of a differentiated and epithelial phenotype of RPE. 4) Organized patches of tissue engineered RPE can be transplanted into the subretinal space of the patient in a proper orientation.

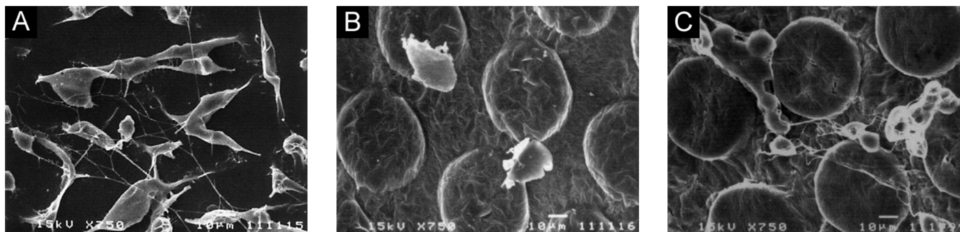

Figure 3: *Scanning electron micrographs of RPE cells adhering to plain PLGA films (adhesive) (A), PLGA surfaces modified with PEG/PLA (continuous region, non-adhesive) (B) and reversed patterns of PEG/PLA modified with PLGA (continuous region) after 8 h of cell seeding at 15 000 cells/cm2. Cells on the micropatterned surfaces (B, C) exhibited typical round RPE cell morphology. Scale bars are 10 µm. Reprinted from Lu et al.[71] Copyright © 2001 with permission from Elsevier.*

In accordance with this concept, the group headed by A. G. Mikos proposed the use of biodegradable polymer films as temporary substrates for RPE cell culture and the subsequent transplantation of these polymer-cell complexes into the subretinal space. However, RPE cells

cultured on thin films made of poly(lactic-co-glycolic acid) (PLGA) lost their characteristic cuboidal morphology during a 7-day culture period[72-77]. To retain normal RPE cell morphology and function in vitro, Lu et al., therefore, developed novel degradable micropatterned substrates from PLGA and block copolymers of poly(ethylene glycol) (PEG) and poly(lactic acid) (PLA) using a microcontact printing technique. The film surfaces consisting of adhesive (PLGA) and non-adhesive (PEG/PLGA) domains affected cell attachment and spreading, and allowed the maintenance of differentiated cell phenotype throughout the 8-h period of the study (Figure 3). The polymer substrate was thought to facilitate the handling during transplantation and to ensure the correct orientation of the graft in the subretinal space. During a period of several weeks, the matrix will be degraded into non-toxic products, which can be removed from the body by metabolic pathways[78-80].

Although PLA and PLGA have been shown to be biocompatible, their degradation products (lactic acid and glycolic acid) arouse concern due to their acidic nature. To meet these concerns, several research groups investigated the use of amniotic membrane (AM) as an alternative matrix substrate that modulates proliferation and differentiation of RPE cells in culture[81, 82]. Transplanted AM act as a suitable substrate for proper epithelialization and are widely used in ophthalmology for the treatment of persisting epithelial defects[83]. Stanzel et al. demonstrated that epithelially denuded AM promotes the formation of a RPE monolayer with tight junctions and, therefore, recommended its use as basement membrane-containing matrix to facilitate the clinical transplantation of RPE in treating ARMD[84].

Attempts to use RPE cell sheets without any supportive matrix are associated with various drawbacks. First, it is more difficult to handle the patches during transplantation[85]. In addition, the non-specific enzymatic detachment (using trypsin/ EDTA, for example) of cultured RPE sheets leads to a substantial decrease in the retinoid metabolism[86]. A novel type of detachable tissue culture substrate, developed in the group of T. Okano, holds the potential to overcome the latter problem. Those surfaces are grafted with thermally responsive polymers, such as poly(N-isopropylacrylamide-co- cinnamoylcarbamidemethylstyrene) and allow the detachment of cells as a continuous sheet by simply lowering the temperature to 20°C[87]. As von Recum et al. published later, the initial isolation of RPE cells using specific enzymes (such as collagenase type 3/ hyaluronidase) and the subsequent passaging on thermally responsive surfaces is an appropriate method to preserve metabolic activity in cultured RPE cells suitable for transplantation[88].

As an alternative approach, Ito et al. applied a novel methodology, termed “magnetic force-based tissue engineering”, that also aims at the construction and delivery of RPE cell sheets.

Briefly, ARPE-19 cells, a human RPE cell line, were magnetically labeled using magnetic cationic liposomes and seeded on ultra-low-attachment plates. In the presence of a magnetic force perpendicular to the culture plate, ARPE-19 cells formed multilayered sheet-like constructs that could be easily transferred into another tissue culture dish by a magnetic iron wire[89]. Even if this methodology provides various opportunities, especially for the delivery of tissue-engineered grafts, one should keep in mind that the multilayered structure of the constructed RPE sheets does not resemble the physiologic situation. It is quite questionable, whether the function of an epithelial monolayer, such as RPE, will be restored after the transplantation of multilayered RPE cell patches. As it is known from animal experiments that thickening of the RPE graft due to folding may reduce the width of the overlaying photoreceptor layer[90], further investigations using animal models will be necessary in order to evaluate the benefit of this recent approach.

Despite many advances in the past decades, especially in the field of cell culturing and material sciences, guaranteeing the long-term survival of an RPE graft still poses a big challenge. As epithelial cells generally fail to survive in suspension, RPE cells must reattach to a substrate to avoid apoptosis[91]. Unfortunately, age-related alterations, pathological processes during ARMD, or surgical treatments may inhibit the repopulation of Bruch's membrane (BM), the extracellular environment of RPE cells in the eye[92, 93]. To avoid graft failure and to enhance the medical benefit of RPE cell transplantation, Del Priore's group investigated ways to reengineering BM. They suggest the transplantation of extracellular matrix (ECM) prior to the transplantation of RPE[94] or the cleaning of BM with nonionic detergents and the subsequent coating with ECM proteins such as collagen, fibronectin, laminin and vitronectin[95]. However, the biological tolerability and the clinical applicability of these techniques have yet to be proven.

Against this background, it remains unclear, whether the transplantation of RPE sheets without any supportive matrix is superior to the injection of cell suspensions or not. Along with the RPE patches themselves, the utility of biodegradable polymers and amniotic membrane as temporary substrates must be evaluated after implantation in the subretinal space. Therefore, in order to determine the medical benefit of these promising strategies, in vivo examinations using animal models are mandatory.

Retina Regeneration

The neural retina is the key tissue of the eye, responsible for the conversion of light into electric signals that can be processed by the brain. The retina represents a highly specialized

part of the central nervous system that is frequently subject to both traumatic and genetic conditions. Retinitis pigmentosa[96] for example, the group of hereditary conditions involving death of retinal photoreceptors, is a common cause of blindness worldwide and effective therapeutic options are still lacking. As yet, only one report using tissue engineering strategies applied to retina regeneration has been published, however, the potential for retina tissue engineering will be addressed shortly in the following paragraph.

The discovery of neural stem cells in adult mammals[97], even in the eye[98, 99], raised the possibility for the development of powerful new therapeutic strategies, as the existence of these cells indicated a potential regenerative capacity of the retina. First evidence for the potential of neural stem cell transplantation to replace lost retinal cells emerged with the observation that adult hippocampus derived neural stem cells survived and integrated into the host retina after injection in the vitreous cavity of rats[100]. The cells, however, failed to express any retina-specific markers. Progenitor cells, isolated from rat embryonic retina, were demonstrated to express photoreceptor-specific markers after transplantation[101], but they did not show migration and integration into the host retina comparable to that of the hippocampus-derived stem cells. Therefore, conditions must be defined that promote structural as well as functional integration of the transplanted cells into the retina[102]. Injury-induced cues, for example, were demonstrated to play a significant role in promoting the incorporation of ocular stem cells/progenitors regardless of their origin or their differentiation along specific retinal sub-lineage[103]. By optimization of isolation, expansion and transplantation procedures of retinal progenitor cells, Qiu et al. were able to reach extensive rhodopsin expression as well as apparent integration of the cells within the host retina following subretinal transplantation into retina degeneration models[104]. The functional connections between grafted cells and the host retina, however, were not evaluated. These few examples can only give an indication of the large field of neural stem cell transplantation and its potential for retina regeneration; for more detailed information, we recommend the reviews by Klassen et al.[105] and Ahmad et al.[106]

The simple cell injection of retinal progenitor cells into the subretinal space or the vitreous is the most prominent experimental approach at the moment. A first report using retinal progenitor cells seeded on a highly porous scaffold was published by Lavik et al.[107] They could demonstrate that cells up-regulate markers of differentiation after seeding onto a scaffold with pores oriented normally to the plane of the scaffold. Therefore, they conclude that the scaffold likely provides a useful system for delivering retinal progenitor cells and may assist in the formation of photoreceptors. These first data suggest that further advances in

tissue engineering could play an important role in the development of strategies to treat complex retinal pathologies in the future. Towards a clinical application, the isolation of human retinal progenitor cells from fetal[108] as well as post mortem retina[109] were important steps. In our opinion, further characterization of these cells, using for example reaggregated neurospheres[110, 111] or 3D retina-like structures created in a bioreactor[112], combined with the improvements in the field of biomaterials research and scaffold technologies could result in retinal grafts that are able to restore vision.

Regeneration of the Lens

The bulk of the human lens is composed of lens fibers. These fibers are derived from an epithelial monolayer, which covers the anterior face of the lens. Opacification of the lens, termed cataract, is the most common cause of visual impairment world-wide[113]. In addition to genetic disposition, cataracts are induced as a result of aging. At present, cataracts are only treatable by surgical removal of the opacified lens and the subsequent replacement by an artificial substitute, which is held in place by the remaining lens capsule[114]. The major complication of cataract surgery is posterior capsule opacification (PCO). PCO is usually secondary to the proliferation and migration of remaining lens epithelial cells and often necessitates another surgery[115]. If lens regeneration were to be successful in humans, there would be no need for such an operation[116].

Among vertebrates, however, only some urodeles and fish can regenerate their lens into their adult life. After lensectomy, lens regeneration in the adult newt, for example, begins with the dedifferentiation and proliferation of dorsal iris pigment epithelial (PE) cells. Then these cells differentiate into lenticular cells and produce a new lens. The whole process of dedifferentiation and differentiation into another cell type has been called transdifferentiation[117]. In mammals, lens regeneration has been observed in rabbits, cats and mice, but only if the lens capsule is left behind. Obviously, lens regeneration is not achieved by transdifferentiation as in newts, but by differentiation of lens epithelial cells that remain attached to the lens capsule[118]. However, the potential of PE cells to transdifferentiate is not restricted to urodeles and corresponding culture systems using PE cells from embryonic chick retina have been well established (see the reviews by Eguchi et al. for further information)[119, 120].

In 2001, Tsonis et al. first reported on the differentiation of a human dedifferentiated retinal PE cell line (H80HrPE-6) into lentoids and lens-like structures. H80HrPE-6 cells cultured in MATRIGEL®, a commercially available basement membrane preparation extracted from a

murine tumor, were induced to synthesize crystallins and to form transparent structures resembling lentoids in vitro[121]. According to the authors, this cell line might provide an useful system for investigating the regeneration of the lens by human PE cells. Nevertheless, therapies based on these fascinating findings are still far away and one may question, if we will succeed in reconstructing the human lens with its outstanding abilities in the foreseeable future. Furthermore, with respect to the excellent outcomes achievable by the implantation of synthetic intraocular lenses, developing new therapeutic strategies in order to supersede cataract surgery may not be the urgent aim of the current research.

Concluding Remarks

The specific characteristics of the human eye, such as the sheet-like structure of many tissues and their diffusion-based nutrient supply, make it an ideal candidate for regeneration of diseased tissues using tissue engineering strategies. Consequently, significant progress has been made especially towards the regeneration of corneal epithelium. It seems feasible that engineered corneal grafts may be introduced into clinical therapy in the near future. Another promising field is the reconstruction of dysfunctional RPE. This could provide a curative therapy for degenerative diseases, such as ARMD. Long-term studies using animal models are currently under way.

Surprisingly, there are only few initiatives towards the regeneration of the vitreous body. Consisting mainly of collagens and glycosaminoglycans, this avascular gel-like system would be an ideal tissue to be regenerated using tissue engineering strategies. Elucidating the role of hyalocytes, the only cell-type lining the cortex of this tissue, which is currently investigated by our group, will be a first step towards that goal[122].

However, despite of these fascinating perspectives, we should still be aware of the numerous obstacles to be overcome in bringing this technology to the clinics. Minimally invasive techniques that require clever approaches to properly place delicate tissues or persisting disease-related factors that may also damage the regenerated tissue are just two examples of the numerous obstacles that have to be overcome.

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References

[1] Langer, R. and Vacanti, J. P. 'Tissue engineering'. *Science* (1993); 260: 920-926.

[2] Blunk, Torsten, Goepferich, A., and Tessmar, Jorg. 'Special issue Biomimetic Polymers'. *Biomaterials* (2003); 24: 4335-4335.

[3] Puelacher, W. C., Vacanti, J. P., Ferraro, N. F., Schloo, B., and Vacanti, C. A. 'Femoral shaft reconstruction using tissue-engineered growth of bone'. *Int J Oral Maxillofac Surg* (1996); 25: 223-228.

[4] Shinoka, T., Breuer, C. K., Tanel, R. E., Zund, G., Miura, T., Ma, P. X., Langer, R., Vacanti, J. P., and Mayer, J. E., Jr. 'Tissue engineering heart valves: valve leaflet replacement study in a lamb model'. *Ann Thorac Surg* (1995); 60: S513-S516.

[5] Leor, Jonathan, Amsalem, Yoram, and Cohen, Smadar. 'Cells, scaffolds, and molecules for myocardial tissue engineering'. *Pharmacol Ther* (2005); 105: 151-163.

[6] Vacanti, C. A., Langer, R., Schloo, B., and Vacanti, J. P. 'Synthetic polymers seeded with chondrocytes provide a template for new cartilage formation'. *Plast Reconstr Surg* (1991); 88: 753-759.

[7] Fischbach, C., Seufert, J., Staiger, H., Hacker, M., Neubauer, M., Goepferich, A., and Blunk, T. 'Three-dimensional in vitro model of adipogenesis: comparison of culture conditions'. *Tissue Eng* (2004); 10: 215-229.

[8] Reichl, S., Bednarz, J., and Mueller-Goymann, C. C. 'Human corneal equivalent as cell culture model for in vitro drug permeation studies'. *Br J Ophthalmol* (2004); 88: 560-565.

[9] Yang, Joseph, Yamato, Masayuki, Kohno, Chinatsu, Nishimoto, Ayako, Sekine, Hidekazu, Fukai, Fumio, and Okano, Teruo. 'Cell sheet engineering: Recreating tissues without biodegradable scaffolds'. *Biomaterials* (2005); 26: 6415-6422.

[10] Bouhadir, K. H. and Mooney, D. J. 'Promoting angiogenesis in engineered tissues'. *J Drug Target* (2001); 9: 397-406.

[11] Patel, Z. S. and Mikos, A. G. 'Angiogenesis with biomaterial-based drug- and cell-delivery systems'. *J Biomater Sci Polym Ed* (2004); 15: 701-726.

[12] Nomi, Masashi, Atala, Anthony, Coppi, Paolo De, and Soker, Shay. 'Principals of neovascularization for tissue engineering'. *Mol Aspects Med* (2002); 23: 463-483.

[13] Zisch, Andreas H., Lutolf, Matthias P., and Hubbell, Jeffrey A. 'Biopolymeric delivery matrices for angiogenic growth factors'. *Cardiovasc Pathol* (2003); 12: 295-310.

[14] Centers for Disease Control and Prevention (CDC), U. S. 'Prevalence of visual impairment and selected eye diseases among persons aged >/=50 years with and without diabetes'. *MMWR Morb Mortal Wkly Rep* (2004); 53: 1069-1071.

[15] Resnikoff, S., Pascolini, D., Etya'ale, D., Kocur, I., Pararajasegaram, R., Pokharel, G. P., and Mariotti, S. P. 'Global data on visual impairment in the year 2002'. *Bull World Health Organ* (2004); 82: 844-851.

[16] Frick, K. D. and Foster, A. 'The magnitude and cost of global blindness: an increasing problem that can be alleviated'. *Am J Ophthalmol* (2003); 135: 471-476.

[17] Pitts, D. G. and Kleinstein, R. N. '*Environmental Vision*', Butterworth-Heinemann, Boston (1993).

[18] Thoft, R. A. and Friend, J. 'The X, Y, Z hypothesis of corneal epithelial maintenance'. *Invest Ophthalmol Vis Sci* (1983); 24: 1442-1443.

[19] Lavker, R. M., Dong, G., Cheng, S. Z., Kudoh, K., Cotsarelis, G., and Sun, T. T. 'Relative proliferative rates of limbal and corneal epithelia. Implications of corneal epithelial migration, circadian rhythm, and suprabasally located DNA-synthesizing keratinocytes'. *Invest Ophthalmol Vis Sci* (1991); 32: 1864-1875.

[20] Schermer, A., Galvin, S., and Sun, T. T. 'Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells'. *J Cell Biol* (1986); 103: 49-62.

[21] Cotsarelis, G., Cheng, S. Z., Dong, G., Sun, T. T., and Lavker, R. M. 'Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells'. *Cell* (1989); 57: 201-209.

[22] Dua, H. S. and Forrester, J. V. 'The corneoscleral limbus in human corneal epithelial wound healing'. *Am J Ophthalmol* (1990); 110: 646-656.

[23] Kenyon, K. R. and Tseng, S. C. 'Limbal autograft transplantation for ocular surface disorders'. *Ophthalmology* (1989); 96: 709-722.

[24] Chen, J. J. and Tseng, S. C. 'Corneal epithelial wound healing in partial limbal deficiency'. *Invest Ophthalmol Vis Sci* (1990); 31: 1301-1314.

[25] Tsubota, K., Satake, Y., Kaido, M., Shinozaki, N., Shimmura, S., Bissen-Miyajima, H., and Shimazaki, J. 'Treatment of severe ocular-surface disorders with corneal epithelial stem-cell transplantation'. *N Engl J Med* (1999); 340: 1697-1703.

[26] Germain, Lucie, Carrier, Patrick, Auger, Francois A., Salesse, Christian, and Guerin, Sylvain L. 'Can we produce a human corneal equivalent by tissue engineering?'. *Prog Retin Eye Res* (2000); 19: 497-527.

[27] Pellegrini, G., Traverso, C. E., Franzi, A. T., Zingirian, M., Cancedda, R., and De Luca, M. 'Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium'. *Lancet* (1997); 349: 990-993.

[28] Schermer, A., Galvin, S., and Sun, T. T. 'Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells'. *J Cell Biol* (1986); 103: 49-62.

[29] Germain, Lucie, Carrier, Patrick, Auger, Francois A., Salesse, Christian, and Guerin, Sylvain L. 'Can we produce a human corneal equivalent by tissue engineering?'. *Prog Retin Eye Res* (2000); 19: 497-527.

[30] Nishida, K. 'Tissue engineering of the cornea'. *Cornea* (2003); 22: S28-S34.

[31] Tosi, Gian Marco, Massaro-Giordano, Mina, Caporossi, Aldo, and Toti, Paolo. 'Amniotic membrane transplantation in ocular surface disorders'. *J Cell Physiol* (2005); 202: 849-851.

[32] Kim, J. C. and Tseng, S. C. 'Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas'. *Cornea* (1995); 14: 473-484.

[33] Tsai, R. J., Li, L. M., and Chen, J. K. 'Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells'. *N Engl J Med* (2000); 343: 86-93.

[34] Pellegrini, G., Traverso, C. E., Franzi, A. T., Zingirian, M., Cancedda, R., and De Luca, M. 'Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium'. *Lancet* (1997); 349: 990-993.

[35] Tsai, R. J., Li, L. M., and Chen, J. K. 'Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells'. *N Engl J Med* (2000); 343: 86-93.

[36] Rama, P., Bonini, S., Lambiase, A., Golisano, O., Paterna, P., De Luca, M., and Pellegrini, G. 'Autologous fibrin-cultured limbal stem cells permanently restore the corneal surface of patients with total limbal stem cell deficiency'. *Transplantation* (2001); 72: 1478-1485.

[37] Schechter, Barry A., Rand, William J., Nagler, Robert S., Estrin, Irving, Arnold, Stephen S., Villate, Natalia, and Velazquez, Gabriel E. 'Corneal melt after amniotic membrane transplant'. *Cornea* (2005); 24: 106-107.

[38] Yamada, Noriko, Okano, Teruo, Sakai, Hideaki, Karikusa, Fumiko, Sawasaki, Yoshio, and Sakurai, Yasuhisa. 'Thermo-responsive polymeric surfaces; control of attachment and detachment of cultured cells'. *Macromol Rapid Commun* (1990); 11: 571-576.

[39] Yamato, Masayuki, Utsumi, Mika, Kushida, Ai, Konno, Chie, Kikuchi, Akihiko, and Okano, Teruo. 'Thermo-responsive culture dishes allow the intact harvest of multilayered keratinocyte sheets without dispase by reducing temperature'. *Tissue Eng* (2001); 7: 473-480.

[40] Nishida, K., Yamato, M., Hayashida, Y., Watanabe, K., Maeda, N., Watanabe, H., Yamamoto, K., Nagai, S., Kikuchi, A., Tano, Y., and Okano, T. 'Functional bioengineered corneal epithelial sheet grafts from corneal stem cells expanded ex vivo on a temperature-responsive cell culture surface'. *Transplantation* (2004); 77: 379-385.

[41] Kinoshita, S. and Nakamura, T. 'Development of cultivated mucosal epithelial sheet transplantation for ocular surface reconstruction'. *Artif Organs* (2004); 28: 22-27.

[42] Kinoshita, Shigeru, Koizumi, Noriko, and Nakamura, Takahiro. 'Transplantable cultivated mucosal epithelial sheet for ocular surface reconstruction'. *Exp Eye Res* (2004); 78: 483-491.

[43] Nishida, K., Yamato, M., Hayashida, Y., Watanabe, K., Yamamoto, K., Adachi, E., Nagai, S., Kikuchi, A., Maeda, N., Watanabe, H., Okano, T., and Tano, Y. 'Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium'. *N Engl J Med* (2004); 351: 1187-1196.

[44] Pellegrini, Graziella. 'Changing the cell source in cell therapy?'. *N Engl J Med* (2004); 351: 1170-1172.

[45] Nishida, K., Yamato, M., Hayashida, Y., Watanabe, K., Yamamoto, K., Adachi, E., Nagai, S., Kikuchi, A., Maeda, N., Watanabe, H., Okano, T., and Tano, Y. 'Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium'. *N Engl J Med* (2004); 351: 1187-1196.

[46] Doughty, Michael J. and Bergmanson, Jan P. G. 'Resolution and reproducibility of measures of the diameter of small collagen fibrils by transmission electron microscopy--application to the rabbit corneal stroma'. *Micron* (2005); 36: 331-343.

[47] Doillon, C. J., Watsky, M. A., Hakim, M., Wang, J., Munger, R., Laycock, N., Osborne, R., and Griffith, M. 'A collagen-based scaffold for a tissue engineered human cornea: physical and physiological properties'. *Int J Artif Organs* (2003); 26: 764-773.

[48] Griffith, M., Osborne, R., Munger, R., Xiong, X., Doillon, C. J., Laycock, N. L., Hakim, M., Song, Y., and Watsky, M. A. 'Functional human corneal equivalents constructed from cell lines'. *Science* (1999); 286: 2169-2172.

[49] Germain, L., Auger, F. A., Grandbois, E., Guignard, R., Giasson, M., Boisjoly, H., and Guerin, S. L. 'Reconstructed human cornea produced in vitro by tissue engineering'. *Pathobiology* (1999); 67: 140-147.

[50] Hu, Xiao, Wang, Min, Chai, Gang, Zhang, Yan, Li, Wei, Liu, Wei, and Cao, Yi. 'Reconstruction of rabbit corneal stroma using tissue engineering technique'. *Zhonghua Yan Ke Za Zhi* (2004); 40: 517-521.

[51] Maurice, D. M. 'The location of the fluid pump in the cornea'. *J Physiol* (1972); 221: 43-54.

[52] Waring, G. O., III, Bourne, W. M., Edelhauser, H. F., and Kenyon, K. R. 'The corneal endothelium. Normal and pathologic structure and function'. *Ophthalmology* (1982); 89: 531-590.

[53] McCulley, J. P., Maurice, D. M., and Schwartz, B. D. 'Corneal endothelial transplantation'. *Ophthalmology* (1980); 87: 194-201.

[54] Insler, M. S. and Lopez, J. G. 'Extended incubation times improve corneal endothelial cell transplantation success'. *Invest Ophthalmol Vis Sci* (1991); 32: 1828-1836.

[55] Ishino, Yutaka, Sano, Yoichiro, Nakamura, Takahiro, Connon, Che J., Rigby, Helen, Fullwood, Nigel J., and Kinoshita, Shigeru. 'Amniotic membrane as a carrier for cultivated human corneal endothelial cell transplantation'. *Invest Ophthalmol Vis Sci* (2004); 45: 800-806.

[56] Mimura, Tatsuya, Amano, Shiro, Usui, Tomohiko, Araie, Makoto, Ono, Kyoko, Akihiro, Hashizume, Yokoo, Seiichi, and Yamagami, Satoru. 'Transplantation of corneas reconstructed with cultured adult human corneal endothelial cells in nude rats'. *Exp Eye Res* (2004); 79: 231-237.

[57] Mimura, Tatsuya, Shimomura, Naoki, Usui, Tomohiko, Noda, Yasuo, Kaji, Yuichi, Yamgami, Satoru, Amano, Shiro, Miyata, Kazunori, and Araie, Makoto. 'Magnetic attraction of iron-endocytosed corneal endothelial cells to Descemet's membrane'. *Exp Eye Res* (2003); 76: 745-751.

[58] Mimura, Tatsuya, Yamagami, Satoru, Usui, Tomohiko, Ishii, Yasuo, Ono, Kyoko, Yokoo, Seiich, Funatsu, Hideharu, Araie, Makoto, and Amano, Shiro. 'Long-term outcome of iron-endocytosing cultured corneal endothelial cell transplantation with magnetic attraction'. *Exp Eye Res* (2005); 80: 149-157.

[59] Engelmann, Katrin, Bednarz, Jurgen, and Valtink, Monika. 'Prospects for endothelial transplantation'. *Exp Eye Res* (2004); 78: 573-578.

[60] Stern, M. E., Beuerman, R. W., Fox, R. I., Gao, J., Mircheff, A. K., and Pflugfelder, S. C. 'A unified theory of the role of the ocular surface in dry eye'. *Adv Exp Med Biol* (1998); 438: 643-651.

[61] Li, F., Carlsson, D., Lohmann, C., Suuronen, E., Vascotto, S., Kobuch, K., Sheardown, H., Munger, R., Nakamura, M., and Griffith, M. 'Cellular and nerve regeneration within a biosynthetic extracellular matrix for corneal transplantation'. *Proc Natl Acad Sci U S A* (2003); 100: 15346-15351.

[62] Bok, D. 'The retinal pigment epithelium: a versatile partner in vision'. *J Cell Sci Suppl* (1993); 17: 189-195.

[63] Hawkins, B. S., Bird, A., Klein, R., and West, S. K. 'Epidemiology of age-related macular degeneration'. *Mol Vis* (1999); 5: 26

[64] Tezel, Tongalp H., Bora, Nalini S., and Kaplan, Henry J. 'Pathogenesis of age-related macular degeneration'. *Trends Mol Med* (2004); 10: 417-420.

[65] Algvere, P. V., Berglin, L., Gouras, P., Sheng, Y., and Kopp, E. D. 'Transplantation of RPE in age-related macular degeneration: observations in disciform lesions and dry RPE atrophy'. *Graefes Arch Clin Exp Ophthalmol* (1997); 235: 149-158.

[66] Algvere, P. V., Gouras, P., and Dafgard, Kopp E. 'Long-term outcome of RPE allografts in non-immunosuppressed patients with AMD'. *Eur J Ophthalmol* (1999); 9: 217-230.

[67] Binder, S., Krebs, I., Hilgers, R. D., Abri, A., Stolba, U., Assadoulina, A., Kellner, L., Stanzel, B. V., Jahn, C., and Feichtinger, H. 'Outcome of transplantation of autologous retinal pigment epithelium in age-related macular degeneration: a prospective trial'. *Invest Ophthalmol Vis Sci* (2004); 45: 4151-4160.

[68] Binder, S., Stolba, U., Krebs, I., Kellner, L., Jahn, C., Feichtinger, H., Povelka, M., Frohner, U., Kruger, A., Hilgers, R. D., and Krugluger, W. 'Transplantation of autologous retinal pigment epithelium in eyes with foveal neovascularization resulting from age-related macular degeneration: a pilot study'. *Am J Ophthalmol* (2002); 133: 215-225.

[69] Gouras, P., Cao, H., Sheng, Y., Tanabe, T., Efremova, Y., and Kjeldbye, H. 'Patch culturing and transfer of human fetal retinal epithelium'. *Graefes Arch Clin Exp Ophthalmol* (1994); 232: 599-607.

[70] Acland, Gregory M., Aguirre, Gustavo D., Ray, Jharna, Zhang, Qi, Aleman, Tomas S., Cideciyan, Artur V., Pearce-Kelling, Susan E., Anand, Vibha, Zeng, Yong, Maguire, Albert M., Jacobson, Samuel G., Hauswirth, William W., and Bennett, Jean. 'Gene therapy restores vision in a canine model of childhood blindness'. *Nat Genet* (2001); 28: 92-95.

[71] Lu, L., Nyalakonda, K., Kam, L., Bizios, R., Goepferich, A., and Mikos, A. G. 'Retinal pigment epithelial cell adhesion on novel micropatterned surfaces fabricated from synthetic biodegradable polymers'. *Biomaterials* (2001); 22: 291-297.

[72] Thomson, R. C., Giordano, G. G., Collier, J. H., Ishaug, S. L., Mikos, A. G., Lahiri-Munir, D., and Garcia, C. A. 'Manufacture and characterization of poly(alpha-hydroxy ester) thin films as temporary substrates for retinal pigment epithelium cells'. *Biomaterials* (1996); 17: 321-327.

[73] Lu, L., Garcia, C. A., and Mikos, A. G. 'Retinal pigment epithelium cell culture on thin biodegradable poly(DL-lactic-co-glycolic acid) films'. *J Biomater Sci Polym Ed* (1998); 9: 1187-1205.

[74] Giordano, G. G., Thomson, R. C., Ishaug, S. L., Mikos, A. G., Cumber, S., Garcia, C. A., and Lahiri-Munir, D. 'Retinal pigment epithelium cells cultured on synthetic biodegradable polymers'. *J Biomed Mater Res* (1997); 34: 87-93.

[75] Lu, L., Kam, L., Hasenbein, M., Nyalakonda, K., Bizios, R., Goepferich, A., Young, J. F., and Mikos, A. G. 'Retinal pigment epithelial cell function on substrates with chemically micropatterned surfaces'. *Biomaterials* (1999); 20: 2351-2361.

[76] Lu, L., Nyalakonda, K., Kam, L., Bizios, R., Goepferich, A., and Mikos, A. G. 'Retinal pigment epithelial cell adhesion on novel micropatterned surfaces fabricated from synthetic biodegradable polymers'. *Biomaterials* (2001); 22: 291-297.

[77] Lu, L., Yaszemski, M. J., and Mikos, A. G. 'Retinal pigment epithelium engineering using synthetic biodegradable polymers'. *Biomaterials* (2001); 22: 3345-3355.

[78] Lu, L., Kam, L., Hasenbein, M., Nyalakonda, K., Bizios, R., Goepferich, A., Young, J. F., and Mikos, A. G. 'Retinal pigment epithelial cell function on substrates with chemically micropatterned surfaces'. *Biomaterials* (1999); 20: 2351-2361.

[79] Lu, L., Nyalakonda, K., Kam, L., Bizios, R., Goepferich, A., and Mikos, A. G. 'Retinal pigment epithelial cell adhesion on novel micropatterned surfaces fabricated from synthetic biodegradable polymers'. *Biomaterials* (2001); 22: 291-297.

[80] Lu, L., Yaszemski, M. J., and Mikos, A. G. 'Retinal pigment epithelium engineering using synthetic biodegradable polymers'. *Biomaterials* (2001); 22: 3345-3355.

[81] Ohno-Matsui, K., Ichinose, S., Nakahama, K., Yoshida, T., Kojima, A., Mochizuki, M., and Morita, I. 'The effects of amniotic membrane on retinal pigment epithelial cell differentiation'. *Mol Vis* (2005); 11: 1-10.

[82] Stanzel, B. V., Espana, E. M., Grueterich, M., Kawakita, T., Parel, J. M., Tseng, S. C., and Binder, S. 'Amniotic membrane maintains the phenotype of rabbit retinal pigment epithelial cells in culture'. *Exp Eye Res* (2005); 80: 103-112.

[83] Dua, Harminder S. 'Amniotic membrane transplantation'. *Br J Ophthalmol* (1999); 83: 748-752.

[84] Stanzel, B. V., Espana, E. M., Grueterich, M., Kawakita, T., Parel, J. M., Tseng, S. C., and Binder, S. 'Amniotic membrane maintains the phenotype of rabbit retinal pigment epithelial cells in culture'. *Exp Eye Res* (2005); 80: 103-112.

[85] Lu, L., Nyalakonda, K., Kam, L., Bizios, R., Goepferich, A., and Mikos, A. G. 'Retinal pigment epithelial cell adhesion on novel micropatterned surfaces fabricated from synthetic biodegradable polymers'. *Biomaterials* (2001); 22: 291-297.

[86] von Recum, H. A., Okano, T., Kim, S. W., and Bernstein, P. S. 'Maintenance of retinoid metabolism in human retinal pigment epithelium cell culture'. *Exp Eye Res* (1999); 69: 97-107.

[87] von Recum, H., Kikuchi, A., Okuhara, M., Sakurai, Y., Okano, T., and Kim, S. W. 'Retinal pigmented epithelium cultures on thermally responsive polymer porous substrates'. *J Biomater Sci Polym Ed* (1998); 9: 1241-1253.

[88] von Recum, H. A., Okano, T., Kim, S. W., and Bernstein, P. S. 'Maintenance of retinoid metabolism in human retinal pigment epithelium cell culture'. *Exp Eye Res* (1999); 69: 97-107.

[89] Ito, A., Hibino, E., Kobayashi, C., Terasaki, H., Kagami, H., Ueda, M., Kobayashi, T., and Honda, H. 'Construction and delivery of tissue-engineered human retinal pigment epithelial cell sheets, using magnetite nanoparticles and magnetic force'. *Tissue Eng* (2005); 11: 489-496.

[90] Gouras, P. and Algvere, P. 'Retinal cell transplantation in the macula: new techniques'. *Vision Res* (1996); 36: 4121-4125.

[91] Tezel, T. H. and Del Priore, L. V. 'Reattachment to a substrate prevents apoptosis of human retinal pigment epithelium'. *Graefes Arch Clin Exp Ophthalmol* (1997); 235: 41-47.

[92] Tezel, T. H., Del Priore, L. V., and Kaplan, H. J. 'Reengineering of aged Bruch's membrane to enhance retinal pigment epithelium repopulation'. *Invest Ophthalmol Vis Sci* (2004); 45: 3337-3348.

[93] Gullapalli, V. K., Sugino, I. K., Van Patten, Y., Shah, S., and Zarbin, M. A. 'Impaired RPE survival on aged submacular human Bruch's membrane'. *Exp Eye Res* (2005); 80: 235-248.

[94] Ho, T. C., Del Priore, L. V., and Kaplan, H. J. 'En bloc transfer of extracellular matrix in vitro'. *Curr Eye Res* (1996); 15: 991-997.

[95] Tezel, T. H., Del Priore, L. V., and Kaplan, H. J. 'Reengineering of aged Bruch's membrane to enhance retinal pigment epithelium repopulation'. *Invest Ophthalmol Vis Sci* (2004); 45: 3337-3348.

[96] Farrar, G. Jane, Kenna, Paul F., and Humphries, Peter. 'On the genetics of retinitis pigmentosa and on mutation-independent approaches to therapeutic intervention'. *EMBO J* (2002); 21: 857-864.

[97] Gage, F. H. 'Mammalian neural stem cells'. *Science* (2000); 287: 1433-1438.

[98] Ahmad, Iqbal, Tang, Lin, and Pham, Hao. 'Identification of Neural Progenitors in the Adult Mammalian Eye'. *Biochem Biophys Res Commun* (2000); 270: 517-521.

[99] Tropepe, V., Coles, B. L., Chiasson, B. J., Horsford, D. J., Elia, A. J., McInnes, R. R., and van der Kooy, D. 'Retinal stem cells in the adult mammalian eye'. *Science* (2000); 287: 2032-2036.

[100] Takahashi, M., Palmer, T. D., Takahashi, J., and Gage, F. H. 'Widespread integration and survival of adult-derived neural progenitor cells in the developing optic retina'. *Mol Cell Neurosci* (1998); 12: 340-348.

[101] Chacko, D. M., Rogers, J. A., Turner, J. E., and Ahmad, I. 'Survival and differentiation of cultured retinal progenitors transplanted in the subretinal space of the rat'. *Biochem Biophys Res Commun* (2000); 268: 842-846.

[102] Ahmad, I. 'Stem cells: new opportunities to treat eye diseases'. *Invest Ophthalmol Vis Sci* (2001); 42: 2743-2748.

[103] Chacko, David M., Das, Ani V., Zhao, Xing, James, Jackson, Bhattacharya, Sumitra, and Ahmad, Iqbal. 'Transplantation of ocular stem cells: the role of injury in incorporation and differentiation of grafted cells in the retina'. *Vision Res* (2003); 43: 937-946.

[104] Qiu, Guanting, Seiler, Magdalene J., Mui, Cathy, Arai, Shinichi, Aramant, Robert B., Juan, Jr, and Sadda, SriniVas. 'Photoreceptor differentiation and integration of retinal progenitor cells transplanted into transgenic rats'. *Exp Eye Res* (2005); 80: 515-525.

[105] Klassen, Henry, Sakaguchi, Donald S., and Young, Michael J. 'Stem cells and retinal repair'. *Prog Retin Eye Res* (2004); 23: 149-181.

[106] Ahmad, Iqbal, Das, Ani V., James, Jackson, Bhattacharya, Sumitra, and Zhao, Xing. 'Neural stem cells in the mammalian eye: types and regulation'. *Semin Cell Dev Biol* (2004); 15: 53-62.

[107] Lavik, E. B., Klassen, H., Warfvinge, K., Langer, R., and Young, M. J. 'Fabrication of degradable polymer scaffolds to direct the integration and differentiation of retinal progenitors'. *Biomaterials* (2005); 26: 3187-3196.

[108] Yang, Peng, Seiler, Magdalene J., Aramant, Robert B., and Whittemore, Scott R. 'In Vitro Isolation and Expansion of Human Retinal Progenitor Cells'. *Exp Neurol* (2002); 177: 326-331.

[109] Klassen, Henry, Ziaeian, Boback, Kirov, Ivan I., Young, Michael J., and Schwartz, Philip H. 'Isolation of retinal progenitor cells from post-mortem human tissue and comparison with autologous brain progenitors'. *J Neurosci Res* (2004); 77: 334-343.

[110] Layer, Paul G., Robitzki, Andrea, Rothermel, Andree, and Willbold, Elmar. 'Of layers and spheres: the reaggregate approach in tissue engineering'. *Trends Neurosci* (2002); 25: 131-134.

[111] Gamm, David M., Nelson, Aaron D., and Svendsen, Clive N. 'Human retinal progenitor cells grown as neurospheres demonstrate time-dependent changes in neuronal and glial cell fate potential'. *Ann N Y Acad Sci* (2005); 1049: 107-117.

[112] Dutt, K., Harris-Hooker, S., Ellerson, D., Layne, D., Kumar, R., and Hunt, R. 'Generation of 3D retina-like structures from a human retinal cell line in a NASA bioreactor'. *Cell Transplant* (2003); 12: 717-731.

[113] Kocur, I. and Resnikoff, S. 'Visual impairment and blindness in Europe and their prevention'. *Br J Ophthalmol* (2002); 86: 716-722.

[114] Francis, Peter J., Berry, Vanita, Moore, Anthony T., and Bhattacharya, Shomi. 'Lens biology: development and human cataractogenesis'. *Trends Genet* (1999); 15: 191-196.

[115] Apple, D. J., Solomon, K. D., Tetz, M. R., Assia, E. I., Holland, E. Y., Legler, U. F., Tsai, J. C., Castaneda, V. E., Hoggatt, J. P., and Kostick, A. M. 'Posterior capsule opacification'. *Surv Ophthalmol* (1992); 37: 73-116.

[116] Tsonis, Panagiotis A. and Rio-Tsonis, Katia. 'Lens and retina regeneration: transdifferentiation, stem cells and clinical applications'. *Exp Eye Res* (2004); 78: 161-172.

[117] Tsonis, P. A., Madhavan, M., Tancous, E. E., and Rio-Tsonis, K. 'A newt's eye view of lens regeneration'. *Int J Dev Biol* (2004); 48: 975-980.

[118] Call, M. K., Grogg, M. W., Rio-Tsonis, K., and Tsonis, P. A. 'Lens regeneration in mice: implications in cataracts'. *Exp Eye Res* (2004); 78: 297-299.

[119] Eguchi, Goro. 'Lens transdifferentiation in the vertebrate retinal pigmented epithelial cell'. *Prog Retinal Res* (1993); 12: 205-230.

[120] Eguchi, G. '*Transdifferentiation as the basis of eye lens regeneration*' in 'Cellular and Molecular Basis of Regeneration. From Invertebrates to Humans', Ferretti, P. and Geraudie, J. (ed.), John Wiley & Sons, Chichester (1998).

[121] Tsonis, P. A., Jang, W., Rio-Tsonis, K., and Eguchi, G. 'A unique aged human retinal pigmented epithelial cell line useful for studying lens differentiation in vitro'. *Int J Dev Biol* (2001); 45: 753-758.

[122] Sommer, F., Kobuch, K., Brandl, F., Wild, B., Weiser, B., Gabel, V.-P., Blunk, T., and Goepferich, A. 'Ascorbic acid for in vitro hyalocyte culture – an important factor towards a cellular vitreous substitute', presented at '2nd International Conference on Tissue Engineering', Crete, Greece (2005)

Chapter 2

Introduction and Goals of the Thesis

The eye is, undoubtedly, a fascinating and important organ, as it enables the perception of light and, therefore, the surrounding environment. Its structure and anatomy is thus completely dedicated to the task of focusing light onto the retina, the primary photosensitive tissue.

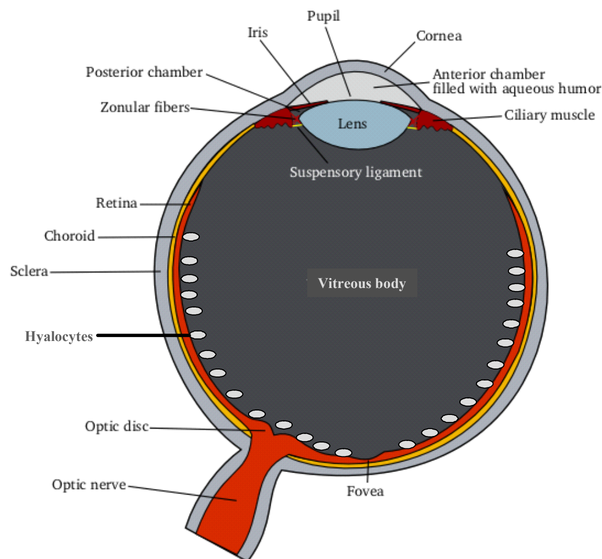

Figure 1: Schematic picture of a human eye

The vitreous body occupies two-thirds of the eye and, therefore, represents its main compartment (Figure 1). It has a volume of about 4 ml and is located in the posterior eye between the lens and the retina[1]. This unique tissue consists of different collagens, especially type II, V/XI and IX, and glycosaminoglycans, the most important one being hyaluronic acid[2]. The center of the vitreous body is free of cells[1, 3], however, in the cortex of the vitreous body, as well as in the vitreous base, there are a sparse number of cells, designated as hyalocytes[4, 5]. This gel-like system with a water content of about 98 % exhibits viscoelastic properties, with clearly elastically dominated behavior[6]. Therefore, the vitreous body is of significance for the eye due to its mechanical properties; it supports the shape of the eye and assists in holding the retinal tissues in place.

In an increasing number of clinical situations, mostly related to the dramatically growing number of diabetes patients, removal of the vitreous body becomes necessary to prevent blindness[7]. This is either due to dysfunctionality of the vitreous body itself due to such problems as opacification or hemorrhage, or more often, due to detachment of the retina[8]. Subsequent to this surgical procedure, the removed tissue has to be replaced by an artificial substitute[8, 9]. Currently, highly purified silicon oils or perfluorocarbons are in clinical use, however, these materials are associated with a plethora of side effects including retinal

toxicity, especially in the long-term use[9]. To overcome these limitations, a variety of alternative biomaterials have been studied over the last decades[8, 9], but none of them met clinical standards. The materials either showed severe side effects (found primarily in the hydrophobic materials studied) or they were degraded over time, thereby loosing their functionality (mainly observed for hydrophilic materials).

During the last 15 years, tissue engineering emerged as an interdisciplinary science dedicated to the regeneration or replacement of tissues and organs. This young discipline has successfully introduced several new therapeutic options into the clinic, including advances in ophthalmology **(Chapter 1)**. Based on these techniques, an innovative concept for vitreous replacement was developed in our group. By incorporation of the native cells of the vitreous body, namely hyalocytes, into a suitable and biocompatible material, a cellular vitreous substitute can eventually be developed. The combination of the vitreous body's own cells with a biocompatible, hydrophilic biomaterial could overcome the commonly observed progressive loss in functionality associated with replacements that elicit only minimal side effects, since the substitute may be reorganized by the embedded cells. The proposed cell-based vitreous substitute could thus yield a biocompatible vitreous replacement with long-term stability, providing a novel therapeutic option after vitrectomy in the future.

To pursue this promising development, it is mandatory to develop extensive knowledge about hyalocytes to precisely control them within a vitreous substitute. However, information about hyalocyte characteristics, their physiological and pathophysiological roles, as well as their suitability for tissue engineering applications is almost completely missing. To overcome these limitations, the presented work addresses some basic aspects of hyalocytes that are of importance for tissue engineering applications.

As a basis for these studies, optimal in vitro culture conditions for hyalocytes needed to be defined. Therefore, the first study aimed to establish isolation and propagation conditions that reliably result in the growth of a sufficient number of hyalocytes within a limited number of propagation steps **(Chapter 3)**. Furthermore, to facilitate hyalocyte characterization, markers indicating the functional properties of hyalocytes needed to be identified, since no such markers have been reported in the literature. The metabolic activity of the cells, namely the accumulation of glycosaminoglycans and collagens, seemed to represent suitable candidates that would concomitantly aid the assessment of these cells for tissue engineering applications. To that end, analytical methods that quantify these extracellular matrix components (ECM) accumulated by hyalocytes were established **(Chapter 4)**. The culture conditions as well as the analytical tools to characterize the cells provided the basis for the following studies.

Using the methods developed, the influence of bioactive substances and growth factors on hyalocytes was elucidated. Since high concentrations of ascorbic acid (vitamin C) are a characteristic of the vitreous body and, furthermore, this vitamin is widely acknowledged to be an important supplement for cell cultivation, the next study was dedicated to the effect of vitamin C on hyalocytes **(Chapter 5)**. The influence of the vitamin on cell proliferation and accumulation of extracellular matrix components (ECM) was investigated in two different culture systems, each of them mimicking some aspects of the native environment of hyalocytes. Additionally, the mRNA expression levels of various collagen genes was characterized using RT-PCR techniques. Because the effect of ascorbic acid on hyalocyte proliferation was found to be dependent upon the presence of pyruvate in the culture medium, a follow-up study was conducted to elucidate the interdependency of ascorbic acid and pyruvate on hyalocyte proliferation as well as ECM accumulation **(Chapter 6)**.

Control of hyalocyte behavior, especially with respect to proliferation and ECM accumulation, represents an important step towards the development of a cell-based vitreous substitute. Because basic fibroblast growth factor (bFGF) and transforming growth factor β-1 (TGF-β1) are reported to manipulate these parameters in other cell types, the effects of the two factors on hyalocytes were investigated in the next study **(chapter 7)**. In addition to proliferation and ECM production of the cells, hyalocyte morphology and internal actin organization were addressed. Because the ultimate goal of this research is clinical use of hyalocytes, fast cell expansion is desirable to minimize patient waiting times. To address this goal, sequential supplementation of bFGF followed by TGF-β1 was investigated.

Subsequent to these first steps towards the control of essential hyalocyte functions, the necessity for hyalocyte culture systems that allow for investigations into cell-biomaterial interactions became obvious, since such interactions represent a key issue in developing a cell-based vitreous substitute. To this end, the goal of the next chapter was to establish a hyalocyte culture system that allows investigations into cell-biomaterial interactions under conditions similar to the native environment of hyalocytes **(Chapter 8)**. Therefore, different hyalocyte in vitro culture systems were developed and studied using collagen type I as a model biomaterial. Furthermore, to clarify the suitability of the systems for studying growth factor effects, the influence of TGF-β1 on hyalocytes cultivated in these systems was elucidated.

To further characterize the potential of hyalocytes for tissue engineering applications, more detailed information about characteristics such as their exact metabolic activity or differentiation markers is required. Hyalocytes, however, may not represent a homogeneous

population, according to some reports in the literature, and no precise isolation method for these different populations is available at the moment. To overcome this limitation, the last study **(Chapter 9)** focused on the development of an isolation and separation method for distinct hyalocyte populations using fluorescence-activated cell sorting (FACS).

References

[1] Sebag, J. '*The vitreous*', Springer, New York (1989).

[2] Bishop, P. N. 'Structural macromolecules and supramolecular organisation of the vitreous gel'. *Prog Retin Eye Res* (2000); **19**: 323-344.

[3] Hamburg, A. 'Some investigations on the cells of the vitreous body'. *Ophthalmologica* (1959); **138**: 81-107.

[4] Uehara, M., Imagawa, T., and Kitagawa, H. 'Morphological studies of the hyalocytes in the chicken eye: scanning electron microscopy and inflammatory response after the intravitreous injection of carbon particles'. *J Anatomy* (1996); **188 (Pt 3)**: 661-669.

[5] Salu, P., Claeskens, W., De Wilde, A., Hijmans, W., and Wisse, E. 'Light and electron microscopic studies of the rat hyalocyte after perfusion fixation'. *Ophthalmic Res* (1985); **17**: 125-130.

[6] Sakuma, Toshiro, Won, Y. Y., Sueda, Jun, Usumoto, Nari, Weitz, D. A., and Hirose, Tatsuo. 'Rheology of Vitreous : Effects of Enzymes', presented at 'ARVO Annual Meeting', Fort Lauderdale, FL, USA (2004)

[7] Smiddy, W. E. and Flynn, H. W., Jr. 'Vitrectomy in the management of diabetic retinopathy'. *Survey of Ophthalmology* (1999); **43**: 491-507.

[8] Chirila, Traian V., Hong, Ye, Dalton, Paul D., Constable, Ian J., and Refojo, Miguel F. 'The use of hydrophilic polymers as artificial vitreous'. *Prog Polym Sci* (1998); **23**: 475-508.

[9] Soman, N. and Banerjee, R. 'Artificial vitreous replacements'. *Biomed Mater Eng* (2003); **13**: 59-74.

Chapter 3

Culture Conditions for Primary Hyalocytes

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Abstract

The eye is a highly specialized organ that has been heavily investigated over the last several decades. The main compartment of the eye, the vitreous body, is associated with physiological as well as pathophysiological processes within the eye. However, there is only limited knowledge about the physiology and pathology of the tissue itself, as well as the cells within, named hyalocytes. Because in vitro cell culture is widely used for the investigation of cells, this could be a useful tool to study hyalocytes. However, there are only sparse reports about in vitro cultivation of these cells.

The goal of the present study was the establishment of a reliable culture system for primary hyalocytes. To this end, a method based on enzymatic digestion of the vitreous body was established to isolate hyalocytes. Subsequently, the effects of different culture surfaces, culture media, seeding densities, and concentrations of fetal calf serum (FCS) on the proliferation of primary hyalocytes were elucidated. Although no differences between the culture surfaces or seeding densities investigated were observed, the culture medium clearly influenced hyalocyte proliferation. α-MEM or DMEM supplemented with 50 µg/ml ascorbic acid proved best. Furthermore, supplementation of 15% FCS favoured cell proliferation, whereas 5% FCS was more suitable for investigations on growth factor effects. Optimization of the investigated factors led to a cultivation method that allows for reliable in vitro cultivation of hyalocytes. The established culture conditions may enable further investigations into the characteristics of hyalocytes.

Introduction

Vision is, undoubtedly, the most important human sense. Since optimal vision is obviously connected to a healthy eye, this highly specialized organ has been thoroughly investigated, and many physiological and pathological processes of the eye are well understood[1, 2]. This knowledge has even resulted in a variety of therapeutic options to treat a number of ophthalmic diseases[3, 4]. However, although the cornea, lens and retina are well-described ocular tissues, there is much less known about the vitreous body. Its anatomical structure has been thoroughly described[5-8], but the physiology and pathology of the vitreous body and the cells within this tissue remain unclear[9].

The vitreous body is mechanically important for the shape of the eye because it fills the space between the lens and the retina. Furthermore, the viscoelastic system holds the retina in position, thus supplying the retina's nutritional requirements from the underlying choroid membrane. In addition to these purely mechanical functions, the vitreous body inhibits the infiltrative growth of cells from adjacent tissues. This inhibition is achieved by a) the dense collagen structure of the vitreous cortex that acts as a mechanical barrier for ingrowing cells[7] and b) the vitreous body itself[10], an effect that is probably caused by cytokines produced by hyalocytes[11]. Besides these physiological functions, the vitreous body is pathologically associated with vision-threatening ophthalmic diseases such as retinal detachment, which can be caused by mechanical forces induced by a degraded vitreous body[12, 13]. Furthermore, vitreous opacification inhibits light perception by the retina and can lead to blindness in severe cases[14, 15].

Although the vitreous body is associated with physiological as well as pathophysiological processes within the eye, there is little known about the physiology of this tissue and its cells. Hyalocytes are associated with the maintenance of the vitreous body as an avascular and transparent tissue[16-18] and are probably involved in vitreoretinal diseases such as epiretinal membrane formation[19, 20]. Since hyalocytes are the only cells within the vitreous body, it is reasonable to assume that they are responsible for at least some of the physiological functions of the vitreous body. Although initial steps have been taken to characterize hyalocytes, much information, including characteristic markers for the cells and their metabolic activity, is still unknown. Therefore, additional knowledge about these cells could be the key to a better understanding of the physiology of the vitreous body. Frequently the basis for the understanding and treatment of pathological situations is firmly built on a complete knowledge of normal physiological processes.

In vitro cell culture is widely used for investigations into a plethora of cellular characteristics, cell functions, and molecular factors influencing cell processes[21-24]. Because more in-depth knowledge about hyalocytes could be the key to understanding and treating several ocular diseases, we intended to use in vitro culture of hyalocytes to study their characteristics and functions. However, widely accepted culture conditions for hyalocytes are not described in current literature. Because of this, we established conditions that reliably allow cultivation of freshly isolated, primary hyalocytes. This study addresses the influence of culture surface, basal medium, seeding density and concentration of fetal calf serum on the proliferation of primary hyalocytes that were isolated using an enzymatic digestion of vitreous gels.

Materials and Methods

Materials

Dulbecco's modified Eagle medium (DMEM, low glucose, with glutamine and pyruvate) was obtained from Biochrom (Berlin, Germany). Fetal calf serum was bought from Gemini Bio-Products Inc. (Calabasas, CA, USA). Dulbecco's phosphate buffered saline (PBS), penicillin/streptomycin, 0.25 % Trypsin-EDTA, Minimum essential medium α-modification (α-MEM) and DMEM/Ham's-F12 were purchased from Invitrogen (Karlsruhe, Germany). Hoechst 33258 dye was obtained from Polysciences (Warrington, PA, USA). Papainase and collagenase type II were bought from Worthington (Lakewood, NJ, USA). L-Ascorbic acid in cell culture quality and hyaluronidase were obtained from Sigma (Steinheim, Germany). Cell culture plastics were purchased from Corning (Bodenheim, Germany) unless otherwise stated. Buffer for papainase digestion (PBE) was composed of 100 mmol Na_2HPO_4 (Merck, Darmstadt) and 10 mmol Na_2EDTA (Merck, Darmstadt) in water, adjusted to pH 6.5.

Methods

Freshly isolated porcine eyes were kindly provided by a local abattoir. Within 4 hours of slaughter, the adherent eye muscles were removed and the eyes were washed in PBS buffer containing penicillin/streptomycin. Under aseptic conditions, the vitreous bodies were excised, examined macro- and microscopically, and subsequently digested with different amounts of collagenase type II and, in some cases, hyaluronidase for 3 hours in culture medium on an orbital shaker. After digestion, cells from about 20 vitreous bodies were pooled, centrifuged at 200 g for 7 min to remove the enzyme solution, and subsequently cultured in culture medium containing 15% fetal calf serum (FCS) and 100 IU/ml penicillin/ 100 µg/ml streptomycin. For the investigation of FCS effects, the indicated concentrations of

FCS were used. Pictures of the primary cell isolate were taken after sedimentation of the cells for 1.5 hours in a T-25 flask using a Leica DM IRB inverted microscope with a phase contrast filter. Cells were cultured in an incubator at 37°C and 5% CO_2 in a humidified environment. Detailed concentrations of digesting enzyme solution, as well as the culture medium used for each study, are displayed in Table 1. The first medium exchange was performed as indicated in Table 1.

Varied parameter	Digestion solution	Culture medium	First medium exchange after	Culture period	Determination of cell number
Culture surface	Collagenase type II (0.5 mg/ml) Hyaluronidase (0.1 mg/ml)	DMEM	7 days	16 days	DNA-quantification
Culture medium	Collagenase type II (0.5 mg/ml)	DMEM / α-MEM / DMEM-F12	7 days	9 days	Neubauer chamber
Seeding density	Collagenase type II (1.0 mg/ml)	α-MEM	5 days	12 days	DNA-quantification
Serum concentration	Collagenase type II (1.0 mg/ml)	DMEM/50 µg/ml ascorbic acid	2 days	9 days	Neubauer chamber

***Table 1**: Detailed culture parameters used in each study*

After the first medium exchange, media was renewed three times a week. The cells were cultured until the sample proliferating most rapidly reached confluency (see Table 1) and were then harvested using trypsin. Cell number was determined either by counting the cell number using a Neubauer chamber or by measuring the DNA amount of the samples (Table **1**). Prior to determination of the DNA amount of the samples, any residual proteins were digested for 16 hours in 125 µg/ml papainase in PBE buffer containing 5 mmol cysteine at 60°C. DNA was quantified using the intercalating Hoechst dye 33258. Fluorescence of this dye is correlated with the amount of DNA in the sample. To extrapolate the number of cells from the amount of DNA measured in each sample, it was assumed there was 9.96 pg of DNA per cell, an amount, determined in a separate experiment correlating cell counts of a Neubauer chamber with DNA measurements (data not shown).

Culture surface

To study the influence of the culture surface, commercially available tissue culture plastics (TCP) from two different companies (Corning, Bodenheim, Germany, and Greiner Bio-One, Frickenhausen, Germany) and a collagen-coated surface were used. To coat TCP surfaces

with collagen, 100 µl of a 1 mg/ml Collagen A solution (Biochrom AG, Berlin, Germany) was used per 25 cm² of TCP and incubated at 37°C. After 10 min, the collagen solution was removed.

Culture medium

Dulbecco's modified eagle medium (DMEM), DMEM mixed with Ham's F12 in a ratio of 1 : 1 (DMEM-F12), and Minimum Essential Medium Alpha Modification (α-MEM) were used to elucidate the effect of the basal media on hyalocyte proliferation during the primary culture.

Cell seeding density

To elucidate the effect of different seeding densities on hyalocyte proliferation, cells isolated out of 14 porcine eyes were suspended in 1.9 ml of medium. 100, 200 and 300 µl of this cell suspension were seeded in each well of a 6-well plate and cultured in 2 ml of medium containing 15% FCS.

Concentration of fetal calf serum

Concentrations of 5, 10 and 15% fetal calf serum in DMEM containing 100 IU/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml ascorbic acid were used to investigate the influence of these serum concentrations on cell proliferation. After 9 days, cells were detached using trypsin and the cell number was determined using a Neubauer chamber. The influence of the serum concentration on hyalocyte proliferation was studied further after the first passage. Cells cultivated with 15% serum in the primary culture were subsequently cultured with 5%, 10% or 15% serum. Cells cultivated with 5% or 10% serum in the first proliferation phase were cultured again with 5% or 10% serum after the first passage. Therefore, hyalocytes were seeded in a density of 2 000 cells/cm². Medium was exchanged after 2 days, and the cells were harvested after 4 days. After digestion of the samples, the cell number was determined using Hoechst dye 33258.

Statistics

If not otherwise stated, all data are presented as the mean ± standard deviation. Single-factor analysis of variance (ANOVA) was used in conjunction with a multiple comparisons test (Tukey's test) to assess statistical significance at levels indicated in the figure captions.

Results & Discussion

Hyalocytes are assumed to play an important role within the vitreous body, however, there is still not much known about these cells' basic characteristics. To overcome these limitations, a number of parameters that influence primary cultures of hyalocytes were studied to establish a reliable in vitro hyalocyte culture system.

Isolation of hyalocytes

There are currently no known molecular markers specific for the cells of the vitreous body, known as hyalocytes. Lacking this basic tool for hyalocyte characterization, it was crucial to avoid any cross-contamination with cells from adjacent tissues during hyalocyte isolation. Cross-contamination was avoided by precise preparation of the tissues and subsequent washing of the excised vitreous bodies with sterile phosphate buffered saline. Because hyalocytes are attached to the vitreous gel, they were retained, while other cell types were washed away. This method yielded a preparation of vitreous bodies devoid of cells from adjacent tissues, such as the retinal pigment epithelium (RPE), as verified macro- and microscopically.

A widely used cultivation technique for hyalocytes was described by Francois et al.[25, 26] and was further improved by Kobuch et al.[27]. This isolation method, which requires mechanical dissociation of the tissue, did not reliably yield a sufficient number of hyalocytes within an acceptable culture time. We thus decided to digest the collagen structure of the vitreous body with collagenase type II and isolate the hyalocytes by centrifugation of the cell suspension. A similar method was described by Hilwig et al.[28] and is widely used for the isolation of chondrocytes from native cartilage[29, 30]. Digestion of the isolated vitreous bodies approached completion after 3 hours, and digestion for 24 hours showed no beneficial effects. Addition of hyaluronidase to the digesting enzyme solution, as done in previous isolation methods, clearly did not enhance digestion. However, increasing the concentration of collagenase type II from 0.5 mg/ml to 1 mg/ml increased the number of hyalocytes retrieved after centrifugation of the digested vitreous bodies. Therefore, after testing a series of enzyme solutions (Table 1), for all further studies hyalocytes were isolated by digesting vitreous bodies using a 1 mg/ml collagen type II solution for 3 hours.

Culture surface

After successful isolation of hyalocytes, it was next necessary to determine the most suitable culture surface for cultivation and proliferation of the cells. Therefore, we studied the effectiveness of two different commercially available tissue culture plastics (TCP) as well as a collagen coating for primary culture of hyalocytes.

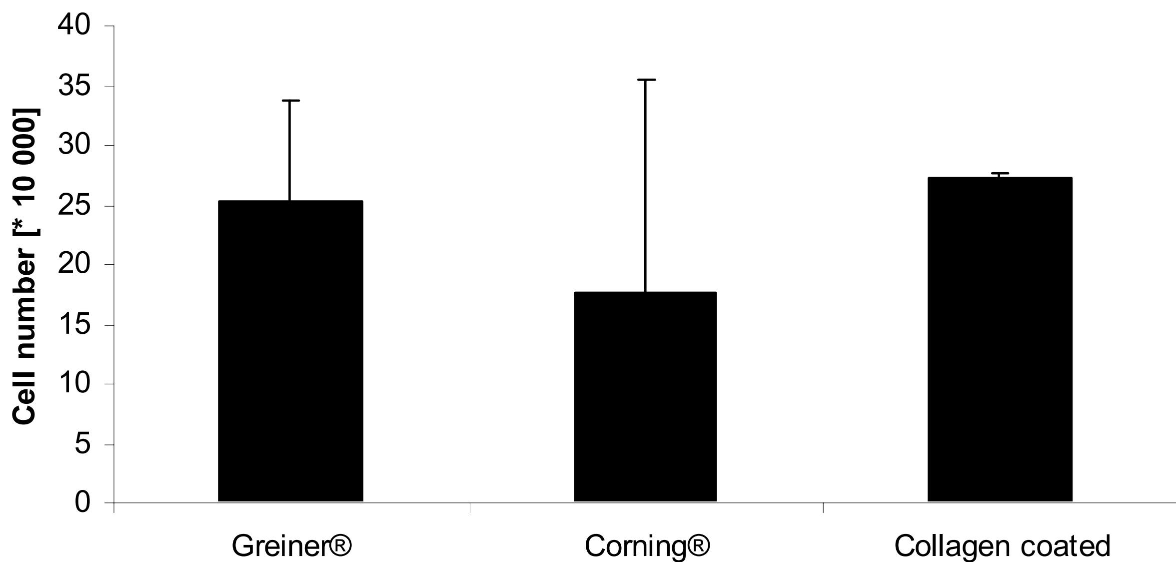

Figure 1: *Adhesion and proliferation of primary hyalocytes on different culture surfaces, n = 3. No significant differences could be observed.*

We did not observe any significant differences in cell adhesion and proliferation between the three different culture surfaces. In all groups, about 250 000 cells could be detected after 16 days of culture (Figure 1). Therefore, we decided to use TCP plates from Corning® for further experiments. The high standard deviations observed in all primary culture experiments were due to the proliferation behavior of hyalocytes, since only a couple of cells started to proliferate during the primary culture, but these cells showed a high proliferation capacity and built large colonies (cf. Figure 3 in Chapter 9). The small standard deviation observed for the collagen-coated surface is assumed to be caused by chance. This behaviour has been previously described[26, 31] and was also observed in subsequent studies. In this set of experiments, we exchanged medium for the first time after 7 days, because we wanted to give the cells additional time for adhesion to the culture surface. In subsequent studies, we shortened the time between cell seeding and first medium exchange to two days because we found the cells already attached to the culture surface by this time.

Culture medium

The next important factor for hyalocyte culture was the choice of a suitable culture medium. We tested three different media that are all widely used for the cultivation of primary cells and cell lines. Dulbecco's modified eagle medium[32] (DMEM) was chosen as the medium with the smallest amount of nutrients, containing a low level of glucose (1.0 g/l), a small range of amino acids, some vitamins, and pyruvate. In addition, DMEM mixed with Ham's F12[32, 33] (DMEM-F12) in ratio of 1:1 was tested. It contains smaller amounts of the same amino acids and vitamins, but more glucose (3.15 g/l), additional vitamins, such as biotin and vitamin B12, and more inorganic salts, such as cupric sulphate and zinc sulphate. Minimum Essential Medium Alpha Modification (α-MEM)[34] was the culture medium with the highest level of nutrients studied; this medium contained the highest concentrations of amino acids as well as a broad range of vitamins, most notably 50 µg/ml of ascorbic acid.

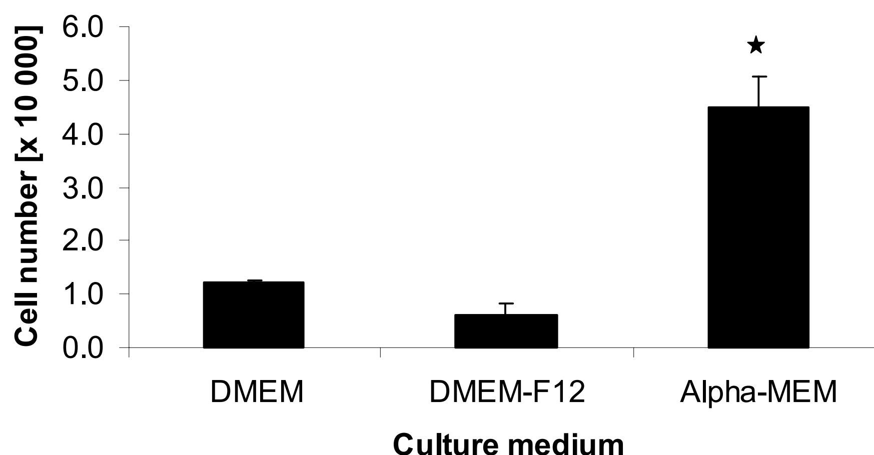

Figure 2: *Hyalocytes obtained after primary cultivation of the cells in different culture media; n = 3; * indicates statistical significance with p < 0.05. Greatest cell proliferation was observed in Alpha-MEM, probably due to the amount of ascorbic acid in this medium.*

After cultivation for 9 days, we observed no statistically significant difference between DMEM and DMEM-F12 (Figure 2). However, α-MEM significantly increased the cell number obtained after primary culture four-fold. This increase could be due to a variety of factors within the culture medium. As the native vitreous body contains about 100 µg/ml of ascorbic acid[35, 36], and both other media lack ascorbic acid, the increase in the cell number could have been caused by this vitamin. To clarify this assumption, further studies on the effect of ascorbic acid on hyalocytes were performed (cf. chapter 5).

Cell density

Another very important parameter for cell culture is the initial cell density. Cell seeding at very low densities theoretically supports optimum cell proliferation, because proliferation-inhibiting cell-cell interactions are minimized. However, it is known from a variety of cell types that some minimum cell density is necessary to induce cell proliferation, probably caused by factors secreted into the culture medium[37]. To elucidate a seeding density that allows for optimal hyalocyte proliferation, this parameter was investigated by changing the initial cell density. For this study, the absolute number of primary isolated hyalocytes had to be determined. However, this could not be done reliably, as the primary cell isolate is made up of a mixture of different cells.

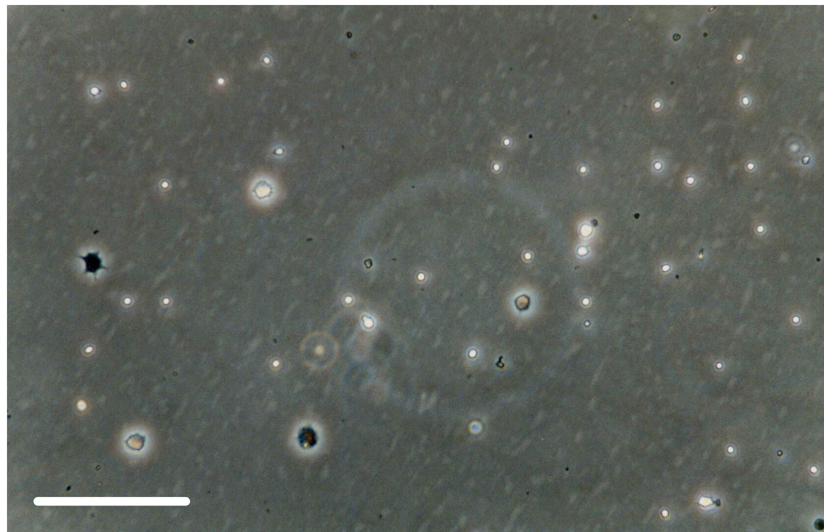

Figure 3: *Primary cell isolate out of porcine vitreous bodies. The cells were allowed to sediment for 1.5 hours. The image was taken on a phase-contrast microscope. Scale bar represents 50 µm. The primary isolate contains cells of different sizes and shapes.*

Figure 3 shows the primary cell isolate from vitreous bodies using phase-contrast microscopy after 1.5 hours of cell sedimentation. Cell sedimentation was necessary to obtain a clear picture of a high number of isolated cells while avoiding a second centrifugation step. According to Figure 3, the isolate contained both very small and large bright cells, as well as some dark cells that had already adhered to the culture surface. Because of this variety, it was difficult to reliably quantify the absolute cell number using non-destructive methods such as the Neubauer chamber. To that end, instead of a known cell number, different volumes (100µl, 200 µl, and 300 µl) of the same primary hyalocyte suspension were seeded in culture plates and cultivated using α-MEM as basal medium. This method allowed the variation of relative seeding densities. After 12 days of cultivation, in groups seeded with 100 or 200 µl of primary hyalocyte suspension, about 400 000 cells were found, as determined by

quantification of the DNA amount, whereas about 700 000 cells were observed in the 300 µl group (Figure 4, black bars). Because all of the groups started with a different number of cells, we divided the cell number determined after cultivation by the initial volume of cell suspension to calculate their proliferation rate (Figure 4, grey bars). The lowest seeding density showed the highest proliferation rate, however there was no statistically significant differences among the different seeding densities. In this study, standard deviations were once again very high, especially in the group with the lowest seeding density. This was due to the proliferation behavior of the primary cells mentioned above.

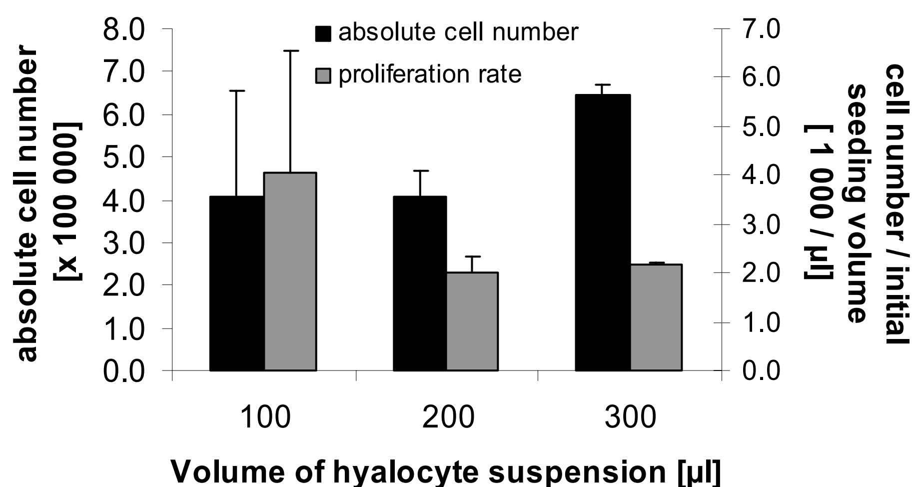

***Figure 4**: Effect of the relative seeding density on the number of hyalocytes obtained after primary culture, n = 3. Black bars and left y-axis indicate the absolute cell number; grey bars and right y-axis indicate the relative cell number (absolute cell number divided through the relative seeding density). No significant influence of the seeding density could be observed.*

To summarize, starting cell density had no significant effect on the proliferation behavior of the cells (Figure 4). One possibility for this null effect is that it could simply be a property of hyalocyte proliferation; hyalocyte proliferation may be independent of the starting cell density. Another possibility is that the seeding densities used were too low to allow for interactions between the cells. Since this second explanation seemed more plausible, we tried to optimize the primary culture for our subsequent studies by seeding approximately 900 µl of primary hyalocyte suspension on a comparable culture surface. We also cultivated all isolated cells in one culture flask to exclude possible variations between different flasks and, therefore, to provide a more homogeneous cell pool for subsequent experiments. This cultivation method yielded a reliable and reproducible primary culture of hyalocytes.

Fetal calf serum concentrations

In all of the above studies, the culture medium was supplemented with 15% fetal calf serum (FCS). Since FCS is a protein mixture containing a plethora of different growth factors, it seems advantageous to use high levels of serum to improve cell proliferation. However, for the investigation of growth factor supplements, high concentrations of FCS could be disadvantageous, since a high level of an uncharacterized mixture of growth factors could mask the effect of the supplemented factor. Therefore, we studied the influence of different concentrations of FCS on the proliferation of hyalocytes during primary culture (passage 0), as well as during the first passage. Since this study was also to provide a baseline for the investigation of ascorbic acid effects on hyalocytes (cf. chapter 5), ascorbic acid-free DMEM was used as basal medium instead of α-MEM. However, due to preliminary studies indicating that ascorbic acid enhances hyalocyte proliferation, we supplemented the media with 50 µg/ml of ascorbic acid during the first passages.

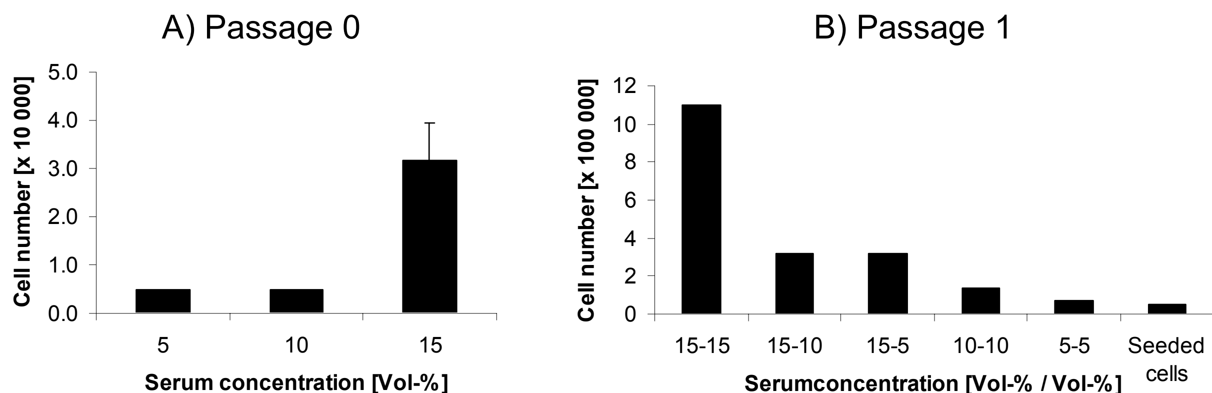

***Figure 5**: Influence of different serum concentrations on the proliferation of hyalocytes. A) Cell numbers obtained after primary culture of hyalocytes with different amounts of fetal calf serum (FCS). B) Cell numbers obtained after proliferation of first-passage hyalocytes with different amount of FCS. The two-digit combinations indicate the concentration of FCS during the primary culture (first digit) and after the first passage (second digit); concentrations of 15% of FCS during both passages clearly showed best results*

During the primary culture we observed an 8-fold increase in the cell number upon supplementation of 15% FCS to the medium when compared to supplementation of 5% or 10% FCS (Figure 5A). After passage 1 (Figure 5B), we again observed the highest cell proliferation with 15% serum supplementation (15-15). Supplementation of 10% or 5% FCS during the second passage, after cultivation with 15% FCS during passage 0 (groups 15-10 and 15-5), clearly decreased cell proliferation compared to the optimal conditions. The groups receiving 10% FCS (10-10) or 5% FCS (5-5) during both passages showed only a low

proliferation rate, however, the cells were still viable and showed proliferation. These results clearly indicate that 15% FCS in the culture medium is appropriate for augmentation of hyalocyte proliferation, as the cells are highly proliferative under these conditions. Supplementation of 5% serum, in contrast, is sufficient to keep the cells alive and proliferating slowly. Therefore, this low FCS concentration seems ideal for the investigation of growth factor effects on hyalocytes.

Summary

As a result of the above-mentioned studies, the following conditions were deemed optimal for primary culture of hyalocytes:

- Hyalocyte isolation is best done by digestion of excised vitreous bodies using a 1 mg/ml solution of collagenase type II for 3 h at 37°C while shaking;
- Hyalocytes should be seeded in a relatively high concentration on commercially available tissue culture treated plastics subsequent to centrifugation of the primary cell suspension for 7 min at 200 g;
- Primary cell culture is ideally performed in α-MEM (or DMEM supplemented with 50 µg/ml ascorbic acid, according to chapter 5) containing 15% fetal calf serum with the first medium change after two days;
- After first passage, 15% FCS should be supplemented to promote further hyalocyte proliferation.

Conclusions

To conclude, enzymatic digestion of vitreous bodies represents a useful technique for the isolation of hyalocytes. Furthermore, primary culture of these cells was optimized by studying various factors, such as culture surface, culture medium, seeding density and serum concentration. Taken together, the established cultivation procedure results in the reliable in vitro cultivation of hyalocytes. This allows for further investigations into the characteristics of hyalocytes, the determination of identification and differentiation markers for these cells, and the identification of influential factors on cell behavior.

Acknowledgment

Special thanks go to the Ophthalmology Department of the University of Regensburg, especially Karin Kobuch, M.D. and Brigitte Wild, for their help with the isolation of vitreous bodies.

References

[1] Langley, Tammy. '*Ocular anatomy and physiology*', Slack Inc.; U.S. (1999).

[2] Clyde, Oyster. '*The human eye: structure and function*', Sinauer Associates Incorporated (1999).

[3] Adler, Francis Heed. '*Adler's physiology of the eye. Clinical application*', Mosby (1981).

[4] Kanski, Jack J. '*Clinical ophthalmology: a systematic approach*', Butterworth-Heinemann Ltd (2003).

[5] Faulborn, J. and Bowald, S. 'Combined macroscopic, light microscopic, scanning, and transmission electron microscopic investigation of the vitreous body. 3. The structure of the anterior border layer of the vitreous'. *Ophthalmic Res* (1983); **15**: 11-18.

[6] Theopold, H. and Faulborn, J. 'Scanning electron microscopic aspects of the vitreous body'. *Mod Prob Ophthalmol* (1979); **20**: 92-95.

[7] Bishop, P. N. 'Structural macromolecules and supramolecular organisation of the vitreous gel'. *Prog Retin Eye Res* (2000); **19**: 323-344.

[8] Bos, K. J., Holmes, D. F., Meadows, R. S., Kadler, K. E., McLeod, D., and Bishop, P. N. 'Collagen fibril organisation in mammalian vitreous by freeze etch/rotary shadowing electron microscopy'. *Micron* (2001); **32**: 301-306.

[9] Qiao, H., Hisatomi, T., Sonoda, K. H., Kura, S., Sassa, Y., Kinoshita, S., Nakamura, T., Sakamoto, T., and Ishibashi, T. 'The characterisation of hyalocytes: the origin, phenotype, and turnover'. *Br J Ophthalmol* (2005); **89**: 513-517.

[10] Jacobson, B., Dorfman, T., Basu, P. K., and Hasany, S. M. 'Inhibition of vascular endothelial cell growth and trypsin activity by vitreous'. *Exp Eye Res* (1985); **41**: 581-595.

[11] Lazarus, H. S., Schoenfeld, C. L., Fekrat, S., Cohen, S., Carol, A., Hageman, G. S., Hackett, S., Chen, Y. S., Vinores, S. A., and Campochiaro et, al. 'Hyalocytes synthesize and secrete inhibitors of retinal pigment epithelial cell proliferation in vitro'. *Arch Ophthalmol* (1996); **114**: 731-736.

[12] Neal, R. E., Bettelheim, F. A., Lin, C., Winn, K. C., Garland, D. L., and Zigler, J. S., Jr. 'Alterations in human vitreous humour following cataract extraction'. *Exp Eye Res* (2005); **80**: 337-347.

[13] Kain, H. L. 'Experimental studies of proliferative vitreoretinopathy'. *Fortschritte der Ophthalmologie : Zeitschrift der Deutschen Ophthalmologischen Gesellschaft* (1991); **88**: 671-676.

[14] Thomas, R., Braganza, A., George, T., and Mermoud, A. 'Vitreous opacities in phacolytic glaucoma'. *Ophthal Surg Lasers* (1996); **27**: 839-843.

[15] Bradford, R. H., Jr. and Wilkinson, C. P. 'Vitreous opacification after cataract extraction'. *Am J Ophthalmol* (1987); **103**: 276-280.

[16] Boltz-Nitulescu, G., Grabner, G., and Forster, O. 'Macrophage-like properties of human hyalocytes'. *Adv Exp Med Bio* (1979); **121B**: 223-228.

[17] Zhu, M., Penfold, P. L., Madigan, M. C., and Billson, F. A. 'Effect of human vitreous and hyalocyte-derived factors on vascular endothelial cell growth'. *Aust N Z J Ophthalmol* (1997); **25**: 57-60.

[18] Lutty, G. A., Merges, C., Threlkeld, A. B., Crone, S., and McLeod, D. S. 'Heterogeneity in localization of isoforms of TGF-beta in human retina, vitreous, and choroid'. *Invest Ophthalmol* (1993); **34**: 477-487.

[19] Kampik, A., Kenyon, K. R., Michels, R. G., Green, W. R., and de la Cruz, Z. C. 'Epiretinal and vitreous membranes. Comparative study of 56 cases'. *Arch Ophthalmol* (1981); **99**: 1445-1454.

[20] Kampik, A., Green, W. R., Michels, R. G., and Nase, P. K. 'Ultrastructural features of progressive idiopathic epiretinal membrane removed by vitreous surgery'. *Am J Ophthalmol* (1980); **90**: 797-809.

[21] Esteban, Miguel A. and Maxwell, Patrick H. 'Manipulation of oxygen tensions for in vitro cell culture using a hypoxic workstation'. *Expert Rev Proteom* (2005); **2**: 307-314.

[22] Dimova, S., Brewster, M. E., Noppe, M., Jorissen, M., and Augustijns, P. 'The use of human nasal in vitro cell systems during drug discovery and development'. *Toxicol in Vitro* (2005); **19**: 107-122.

[23] Eldem, Turkan, Durlu, Yusuf, Eldem, Bora, and Ozguc, Meral. 'Cell cultures of the retinal pigment epithelium to model the blood-retinal barrier for retinal drug and gene delivery'. *Cell Culture Models of Biological Barriers* (2002); 271-288.

[24] Roguet, R. and Schaefer, H. 'Overview of in vitro cell culture technologies and pharmaco-toxicological applications'. *Toxicol in Vitro* (1997); **11**: 591-599.

[25] Francois J., Victoria-Troncoso V., and Eekhout M. 'Histochemical studies of hyalocytes in tissue culture'. *Ophthal Res* (1973); **5**: 215-221.

[26] Francois J., Victoria-Troncoso V., and Eekhout M. 'The micromorphology of Hyalocytes in tissue culture'. *Ophthalmic Res* (1973); **5**: 290-299.

[27] Kobuch, K., Wild, B., Eckert, E., Fischbach, C., and Gabel V.P. 'Expression of Collagen Type II by Hyalocytes In Vitro - First Steps Towards a Biological Vitreous Substitute', presented at ARVO Meeting, Fort Lauderdale, FL, 2002, Abstract No. 3495 (2002)

[28] Hilwig, I. 'Hyalocytes in tissue culture'. *Z Zellforsch Mikrosk Anat* (1971); **113**: 544-552.

[29] Kellner, K., Schulz, M. B., Gopferich, A., and Blunk, T. 'Insulin in tissue engineering of cartilage: A potential model system for growth factor application'. *J Drug Target* (2001); **9**: 439-448.

[30] Kellner, Karin, Lang, Kurt, Papadimitriou, Apollon, Leser, Ulrike, Milz, Stefan, Schulz, Michaela B., Blunk, Torsten, and Gopferich, Achim. 'Effects of hedgehog proteins on tissue engineering of cartilage in vitro'. *Tissue Eng* (2002); **8**: 561-572.

[31] Noda, Yoshihiro, Hata, Yasuaki, Hisatomi, Toshio, Nakamura, Yuka, Hirayama, Kumiko, Miura, Muneki, Nakao, Shintaro, Fujisawa, Kimihiko, Sakamoto, Taiji, and Ishibashi et, al. 'Functional Properties of Hyalocytes under PDGF-Rich Conditions'. *Invest Ophthalmol* (2004); **45**: 2107-2114.

[32] Dulbecco, R. and Freeman, G. 'Plaque production by the polyoma virus'. *Virology* (1959); **8**: 396-397.

[33] Ham, R. G. 'Clonal growth of mammalian cells in a chemically defined, synthetic medium'. *PNAS* (1965); **53**: 288-293.

[34] Stanners, C. P., Eliceiri, G. L., and Green, H. 'Two types of ribosome in mouse-hamster hybrid cells'. *Nature: New Biology* (1971); **230**: 52-54.

[35] Rose, R. C. and Bode, A. M. 'Ocular ascorbate transport and metabolism'. *Comp Biochem Physiol A* (1991); **100**: 273-285.

[36] Reiss, G. R., Werness, P. G., Zollman, P. E., and Brubaker, R. F. 'Ascorbic acid levels in the aqueous humor of nocturnal and diurnal mammals'. *Arch Ophthalmol* (1986); **104**: 753-755.

[37] Eagle, Harry and Piez, Karl A. 'The population-dependent requirement by cultured mammalian cells for metabolites which they can synthesize'. *J Exp Med* (1962); **116**: 29-43.

Chapter 4

Analytical Methods to Quantify Extracellular Matrix Components Accumulated by Hyalocytes

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Abstract

Although the vitreous body represents the main compartment of the eye, knowledge about the physiology of this tissue remains limited. Since it is speculated that hyalocytes, the cells of the vitreous body, are involved in the physiological functions of the vitreous body, a more complete characterization of these cells might yield a better understanding of the vitreous body. However, markers indicating the functional properties of the cells are still missing. Since hyalocytes are reported to produce glycosaminoglycans and collagens, the accumulation rate of these extracellular matrix components may provide a surrogate marker. Therefore, the work presented here aimed at the establishment of analytical methods to quantify the small amounts of these components accumulated by the cells. The commercially available ClinRep® Kit proved unsuitable for quantification of hyalocyte collagen accumulation. In contrast, the widely used method described by Woessner et al. was found satisfactory after downscaling to a microplate method, which decreased the detection limit by one order of magnitude. To achieve this, however, several influential factors such as the salt- and reagent-concentrations had to be optimized. For quantification of the accumulation of glycosaminoglycans, the method described by Farndale et al. proved adequate when chondroitin sulfate was used as a standard.

To conclude, in the present work, analytical tools that quantify glycosaminoglycan and collagen accumulation by hyalocytes were developed. These methods may allow for further characterization of hyalocytes, especially with respect to their potential for tissue engineering applications.

Introduction

The vitreous body represents the main compartment of the mammalian eye. This transparent, gel-like tissue consists of different collagens and glycosaminoglycans (GAG) and has a water content of about 98%[1]. The predominant collagen in the vitreous is the fibrillar type II collagen, accounting for approximately 75% of the total collagen, whereas the fibrillar collagen type V/XI, as well as the non-fibrillar collagen type IX, represent minor components[1]. Among the glycosaminoglycans, hyaluronan, chondroitin sulfate, and heparan sulfate were observed in the vitreous[1]. Non-sulfated hyaluronan is the predominant GAG in the mammalian vitreous body, whereas chondroitin sulfate predominates in avian vitreous[2]. In its center, the vitreous is an acellular system, however the vitreous cortex and the basal vitreous contain a low concentration of cells named hyalocytes[3].

Hyalocytes were shown to be derived from the bone marrow[4] and are thought to belong to the monocyte/macrophage lineage[5]. However, they differ significantly from other tissue macrophages in that they express S100 protein and do not express CD68[6]. Physiologically, these cells are thought to be involved in the maintenance of the vitreous as an avascular and transparent tissue[7-9]; pathologically, they are probably involved in diseases of the vitreoretinal interface[8]. Therefore, hyalocytes may have a house-keeping function within the vitreous.

Although hyalocytes are well-described histo- and microscopically, many of their fundamental functional properties remain to be elucidated. In this context, markers related to functional cell properties are necessary. Since hyalocytes were shown to produce the extracellular matrix (ECM) components that the vitreous body is built on, especially GAG[10-12] and collagens[13; 14], accumulation of these components by hyalocytes may be a suitable marker for the characterization of some functional cell properties, with important implications for tissue engineering applications.

For quantification of collagens and GAG in tissue samples, the methods described by Woessner et al.[15] and Farndale et al.[16] are widely used in cartilage tissue engineering[17; 18]. The amount of hydroxyproline (HYP), an amino acid that is present in significant amounts exclusively in collagen, is determined as a measure for collagen; the content of negatively charged GAG is quantified by a color reaction with a positively charged dye. However, these colorimetric methods showed limitations for the analysis of hyalocyte samples. The small amounts of HYP accumulated by hyalocytes were below the detection limit of the method. To quantify the GAG amounts accumulated by hyalocytes, a suitable standard substance is necessary. Therefore, the goal of the present study was to establish analytical methods that

allow for the quantification of ECM components produced by hyalocytes. To achieve this, an HPLC-based analytical method for HYP determination was developed. An alternative to this was also developed; the colorimetric quantification of HYP after oxidation with chloramine T and coupling with dimethylamino benzaldehyde as described by Woessner was downscaled to a microplate scale. For investigations into glycosaminoglycan accumulation by hyalocytes, a standard substance suitable for GAG quantification by the method of Farndale et al. was established.

Materials and Methods

Materials:

Unless otherwise stated, reagents were obtained from Sigma (Steinheim, Germany). ClinRep® Kit for determination of hydroxyproline in urine was an appreciated gift from Recipe® (Munich, Germany). Hydrochloric acid, dimethylaminobenzaldehyde, chloramine T, Na_2EDTA, Na_2HPO_4 and sodium chloride were bought from Merck (Darmstadt, Germany). Papainase was purchased from Worthington (Lakewood, NJ, USA). Phosphate buffer EDTA (PBE) was composed of 100 mmol Na_2HPO_4 and 10 mmol Na_2EDTA in water, adjusted to pH 6.5.

Methods:

To test the linearity of the established analytical methods, samples of hyalocytes cultivated with different amounts of ascorbic acid were analyzed. The cells were harvested and freeze-dried (Christ Beta 2-16, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at 20°C and 0.120 mbar for 16 hours. The dry samples were, subsequently, digested in 300 µl of a 125 µg/ml papainase solution in PBE buffer containing 5 mmol cysteine for 18 hours at 60°C.

Hydroxyproline determination using the ClinRep® Kit

The hydroxyproline content, an accepted measurement for collagen, of hyalocyte samples cultivated with 200 µg/ml ascorbic acid was determined using the reagents and the HPLC-column provided in the ClinRep® Kit according to the manufacturer's instructions. In brief, after addition of an internal standard, 100 µl of the samples were completely hydrolyzed with hydrochloric acid for 16 hours at 95°C, followed by a first derivatization at pH 8.5 – 9.0 and room temperature. Subsequent to a second derivatization with a UV/VIS-detectable

compound for 10 min at 70°C in a water bath, the samples were diluted and 20 µl of each sample were analyzed using an HPLC system with UV/VIS detection at 471 nm (Shimadzu, Duisburg, Germany). To identify the obtained peaks, different amounts of HYP up to 4 µg were supplemented to the cell sample prior to HYP quantification. For data acquisition and analysis, the ClassVP 6.12 software (Shimadzu) was used.

Photometric determination of hydroxyproline

Unless otherwise stated, 100 µl of HYP-containing sample were oxidized with 50 µl of a 0.05 M solution of chloramine T in a citrate buffer (pH = 6) in a microtiter plate at room temperature for 20 min. Subsequent to the addition and mixing of 50 µl of a 15% (m/m) dimethylaminobenzaldehyde solution in 4 mol perchloric acid in 70 % isopropanol/water (m/m), the plate was incubated for 30 min at 60°C. After cooling the plate to room temperature, the absorbance of the samples was immediately measured at 557 nm on a microplate reader (CS-9301 PC, Shimadzu, Duisburg, Germany).
To study the influence of the salt concentration on this analytical method, absorption of increasing amounts of HYP in different concentrations of sodium chloride were measured. To elucidate the effect of cysteine on the color formation, varying amounts of HYP either in water, in PBE containing 5 mM cysteine (PBE/cysteine), or in PBE/cysteine supplemented with an additional 10 mg/ml cysteine were analyzed. Furthermore, for investigations on the effect of chloramine T, the resulting absorptions of increasing amounts of HYP in PBE/cysteine after treatment with different concentrations of chloramine T (0.05 M, 0.1 M and 0.25 M) were determined.

Hydroxyproline determination in cell samples

One and two equivalents, exactly 50 and 100 µl, of cell samples cultivated with different amounts of ascorbic acid (Control, 50 and 200 µg/ml, n=3) were hydrolyzed with equal amounts of fuming hydrochloric acid at 105°C for 16 hours. After hydrolysis, hydrochloric acid was evaporated under a constant flow of nitrogen at about 40°C. The dry samples were dissolved in 500 µl double distilled water. Standard dilutions of hydroxyproline were prepared in PBE/cysteine buffer including 125 µg/ml papainase and treated under identical hydrolyzation conditions. Subsequent to hydrolysis, 100 µl of each cell sample and standard were analyzed as described above.

Determination of glycosaminoglycans

Glycosaminoglycan (GAG) content was measured photometrically as previously described[16; 19]. In brief, after digestion of interfering proteins with papainase, the glycosaminoglycan content was determined by a color reaction with dimethylmethylene blue at pH = 3, followed by measurement of the absorption at 525 nm after exactly 15 s. Standard curves were prepared with either chondroitin sulfate or hyaluronic acid.
To discriminate between sulfated glycosaminoglycans and hyaluronic acid as non-sulfated GAG, the sulfated compounds were removed by adsorption to diethylaminoethylsepharose CL-6B (DEAE) as previously described[20]. Therefore, equal amounts of sample and DEAE-sepharose [20 % (v/v) suspension in 1 M NaCl, 10 mM Tris, pH=7.4] were incubated for 15 min and, subsequently, centrifuged for 5 min at 13000g. Remaining GAGs in the supernatant were due to hyaluronic acid and quantified using the color reaction described.

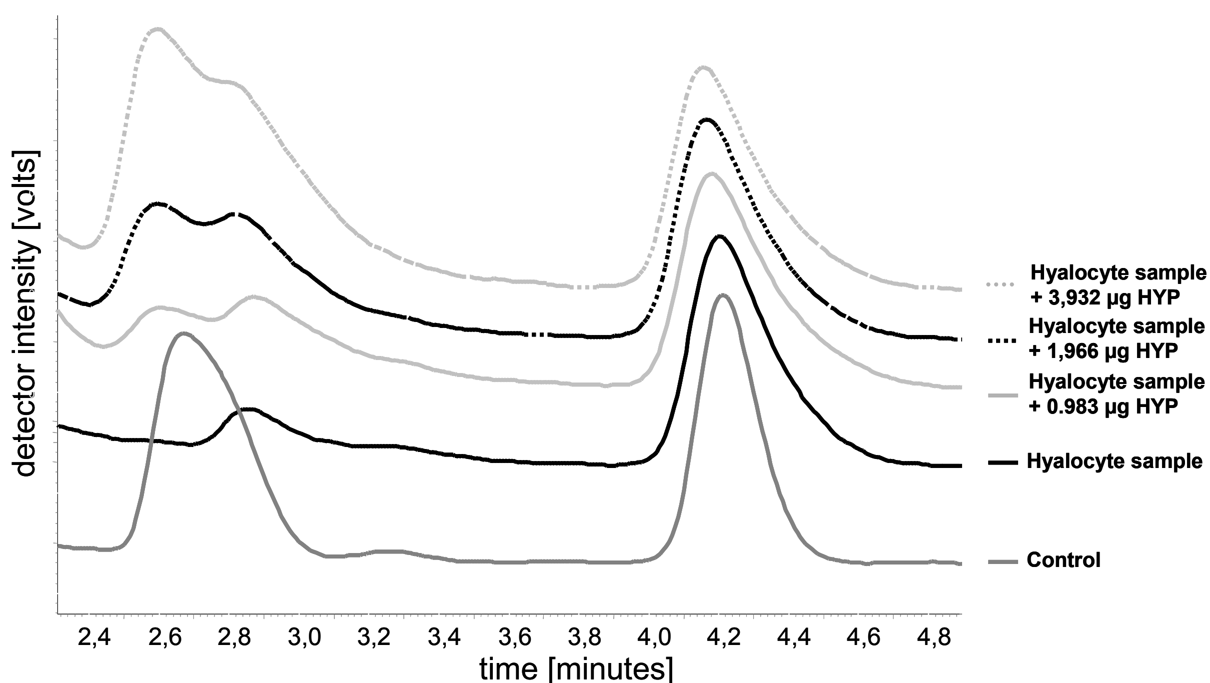

Figure 1: *HPLC chromatograms of the hydroxyproline determination using the ClinRep® Kit. Hydroyproline was eluated after 2.6 min, the internal standard after 4.2 min, as indicated by the control. Pure cell samples showed besides the internal standard a small peak after 2.9 min (Hyalocyte sample). This peak was not due to HYP, as supplementation of different amounts of HYP showed an additional increasing peak eluted after 2.6 min (Hyalocyte samples + HYP).*

Results

ClinRep® Kit for determination of hydroxyproline

The derivatisation conditions as well as the HPLC method for samples analysis were successfully established using calibration samples as well as controls provided by Recipe® (data not shown). The obtained chromatogram of the provided control showed, as denoted by the manufacturer, a peak for HYP eluted after 2.6 min and a peak of the internal standard eluted after 4.2 min (Figure 1). In chromatograms of hyalocyte samples, besides a distinct peak at 4.2 minutes indicating the internal standard, a small peak at 2.9 minutes could be observed (Figure 1). To clarify, whether this peak was due to HYP, different amounts of HYP up to about 4 µg were supplemented to the hyalocyte samples. Due to HYP addition an additional, increasing peak eluted after the expected 2.6 min was found. This indicates that the small peak in the hyalocyte sample observed at 2.9 min does not represent HYP.

Influence factors on colorimetric hydroxyproline determination

According to Woessner et al.[15], the concentrations of salts as well as some amino acids, foremost cysteine, within the sample have a clear impact on the colorimetric deterimation of HYP, as they influence the oxidation as well as coupling reaction with dimethylaminobenz-aldehyde. Therefore, these parameters were studied to establish the analytical method with minimized sample volumes.
Sodium chloride in concentrations up to 1 mol/l in the sample exhibited no influence on the colorimetric detection of HYP (Figure 2). A linear relation between the HYP amounts and the absorptions of the built dye could be observed in a range of 0.01 to 0.2 µg HYP per sample, independent of the NaCl concentration.
In contrast to sodium chloride, cysteine clearly affected the dye formation (Figure 3). Compared to HYP in water, identical amounts of HYP in PBE containing 5 mmol cysteine (PBE/cysteine) led to a clearly decreased absorption. Further supplementation of 10 mg/ml cysteine to PBE/cysteine blocked the color reaction almost completely, indicated by minimal absorptions even in the sample containing 0.3 µg HYP.

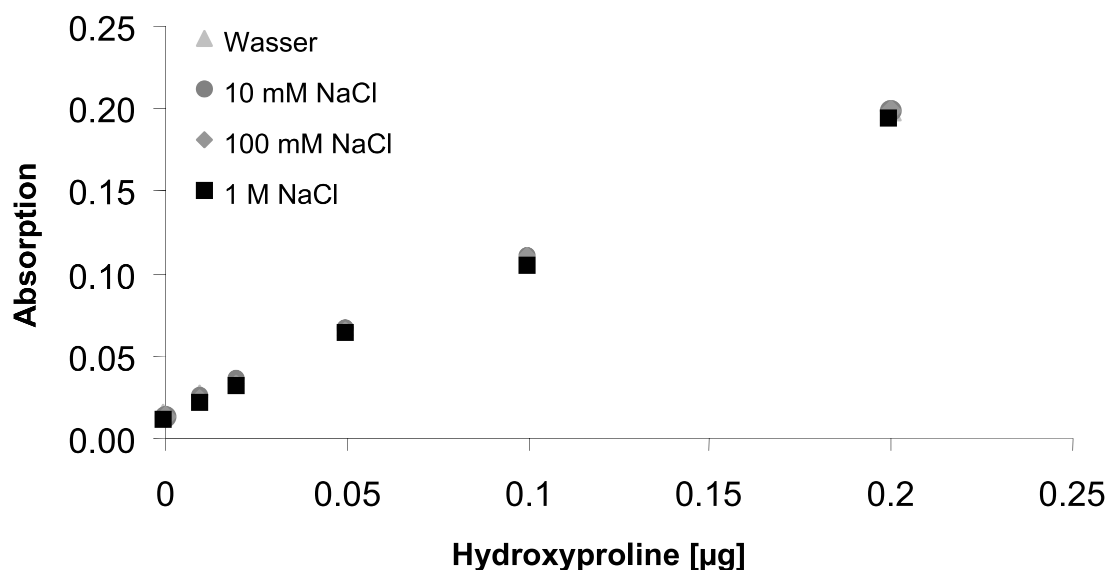

Figure 2: *Influence of different NaCl concentrations on the determination of hydroxyproline; similar absorptions of different hydroxyproline amounts were observed, independent of the NaCl concentrations within the samples.*

Chloramine T also influenced the colorimetric determination of HYP (Figure 4). Compared to 0.05 M chloramine T, concentrations of 0.1 and 0.25 mM decreased the measured absorptions of the built dye. However, even in the highest concentration of chloramine T tested, a dependency of the measured absorption to the HYP amount was observed.

Determination of hydroxyproline in cell samples

After hydrolysis and evaporation of the hydrochloric acid, the hydroxyproline content of different hyalocyte samples could reliably be determined using the established assay. As indicated in Figure 5, the HYP measurements of different cell samples led to consistent amounts of hydroxyproline per group, no matter whether one or two equivalents of the sample volume were used for the assay. Moreover, amounts of hydroxyproline as small as 0.1 µg in the control group could clearly be detected.

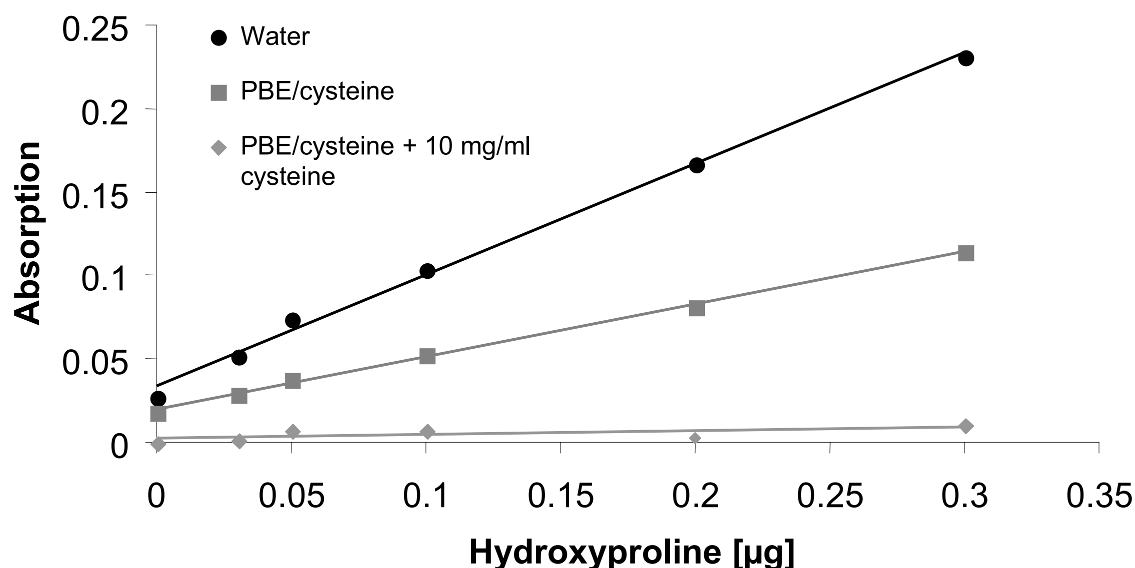

Figure 3: *Influence of cysteine on the measured absorption of hydroxyproline after colorimetric reaction; cysteine clearly decreased the absorptions of HYP samples measured after color reaction.*

Determination of glycosaminoglycans

Both important types of glycosaminoglycans (GAG) present in the vitreous body, hyaluronic acid and chondroitin sulfate, could be determined using the method previously described (Figure 6)[16]. Equal amounts of chondroitin sulfate showed, thereby, clearly higher absorptions compared to hyaluronic acid. Up to 5 µg, a linear relationship between the amounts of each glycosaminoglycan and the measured absorption were observed.

Glycosaminoglycans in cell samples

Clear amounts of GAG could be determined in hyalocyte samples after papainase digestion (Figure 7). By standardization of the measured absorptions to hyaluronic acid, significant differences in the amounts of glycosaminoglycans were observed, ranging from 30 to 90 µg in the group with 200 µg/ml ascorbic acid supplementation, dependent on the volume used for the analytics (Figure 7 A). After removal of the sulfated glycosaminoglycans in the samples by treatment with diethylaminoethylsepharose (DEAE), these differences could not be found. However, the measured amounts of hyaluronic acid were determined to be 25 µg in the group with ascorbic acid, and therefore, were significantly smaller compared to the untreated samples. In contrast to the standardization to hyaluronic acid, standardization to chondroitin sulfate exhibited reproducible amounts of glycosaminoglycan per sample independent of the used analytic volume even in the untreated sample (Figure 7 B). Using chondrotin sulfate as

standard, we found 30 µg of glycosaminoglycans in the cell samples cultivated with 200 µg/ml ascorbic acid.

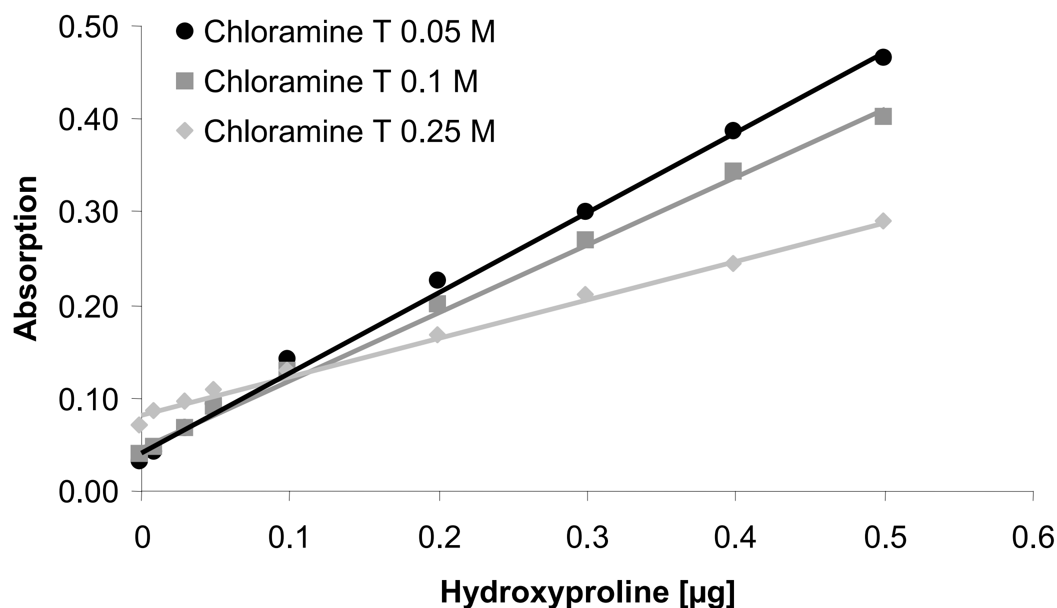

***Figure 4:** Influence of different chloramine T concentrations on the color formation used for hydroxyproline determination; concentrations above 0.05 M decreased the absorption of the built dye.*

Discussion

The production of extracellular matrix components by hyalocytes seems to be a suitable marker that indicates some functional properties of the cells. This study describes the establishment of analytical methods that allow for the determination of collagens and glycosaminoglycans accumulated by hyalocytes.

For collagen determination, most of the commonly used methods quantify hydroxyproline as marker for collagen, as this amino acid is present in considerable amounts exclusively in collagen. Quantification of HYP using the method described by Woessner et al.[15], however, was not suitable for hyalocyte samples due to its high detection limit of about 1 µg HYP per sample. The commercially available ClinRep® Kit, an HPLC based method for the clinical determination of HYP in urine, seemed to be an useful alternative, as this method, according to the manufacturer, has a lower detection limit of about 0.25 µg HYP per sample. Therefore, this analytical method was successfully established using controls and calibration samples provided by the manufacturer (data not shown). Hyalocyte samples, however, showed besides the peak for the internal standard only a small peak eluted at about 2.9 min (Figure 1). Addition of different amounts of HYP to the cell samples clarified this peak not to be related to HYP. This indicated that the peak at 2.9 min may be due to any other substance present

within the vast matrix of substances after digestion of the cell sample. However, as no clear peak at 2.6 min could be observed, the ClinRep® method seemed not suitable for determination of HYP in hyalocyte samples.

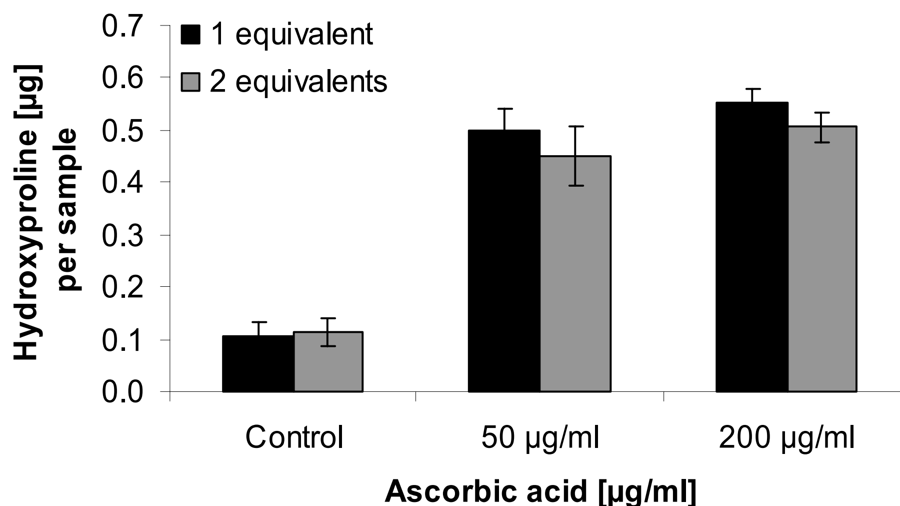

Figure 5: *Determination of hydroxyproline in hyalocyte samples cultured with different amounts of ascorbic acid using the down-scaled method according to Woessner et al.; similar amounts of HYP were calculated for the samples in one group, independent of the analytical volume of 1 or 2 equivalents.*

As alternative to the HPLC based method, the photometrical method by Woessner[15] was downscaled to allow detection of amounts as small as 0.05 µg HYP per sample. Therefore, the volume of analytical sample was reduced to a microplate scale with 100 µl. To achieve the necessary neutral pH of the sample after hydrolysis with HCl, the acid was evaporated under a flow of nitrogen at about 40°C instead of neutralization of the sample, as suggested by Woessner. This led to a dry sample and allowed, therefore, dissolution of the hydrolysed sample in small, distinct volumes of distilled water. As, according to Woessner, the salt as well as some amino acid concentrations, thereby most important cysteine, within the sample interfere with the color formation, this solution volume had to be optimized. Therefore, investigations into the influence of different concentrations of sample ingredients on the subsequent color reaction of HYP were performed; in detail, the effects of varying sodium chloride, cysteine, and chloramine T concentrations were studied. According to figure 2, different sodium chloride concentrations up to 1 M exhibited no influence on the color reaction of HYP. In contrast to this result, Woessner describes a slight decrease of about 3.5% in color formation due to 1 M NaCl. This discrepancy may be caused by the different amounts of HYP measured in the respective study; in the present study amounts up to 0.2 µg HYP were used whereas 5 µg were used in Woessner's experiments.

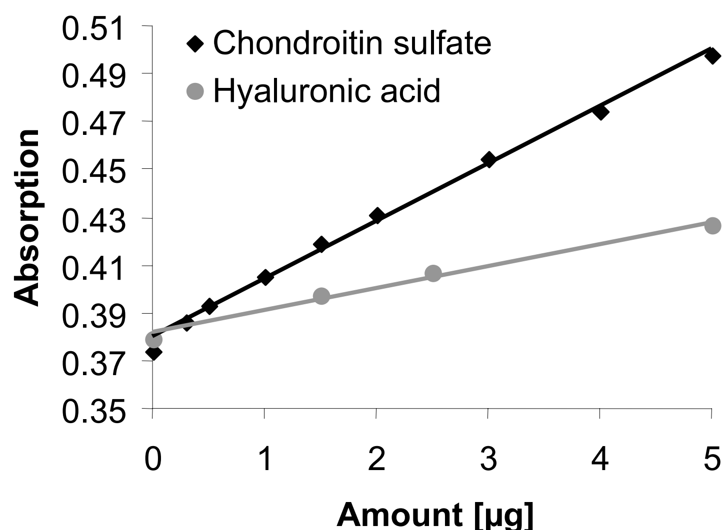

Figure 6: *Absorptions of different amounts of chondroitin sulfate and hyaluronic acid measured after addition of dimethylmethylene blue dye; both glycosmaminoglycans could be quantified using the method described by Farndale et al.*[16]*, chondroitin sulfate showed higher absorptions compared to hyaluronic acid.*

In contrast to sodium chloride, cysteine included in the digestion buffer PBE/cysteine clearly decreased the measured absorption of HYP subsequent to color formation (Figure 3); addition of 10 mg/ml of cysteine even blocked the color formation completely. This was most probably due to the reducing potential of the cysteine that counteracts the chloramine T. To overcome this problem, removal of cysteine prior to HYP determination seemed to be obvious. This, however, was not possible as cysteine represented a necessary cofactor for papainase digestion and this digestion, again, displayed a mandatory step for quantification of glycosaminoglycans within the hyalocyte sample. Because of this, increased amounts of chloramine T should compensate the cysteine effect. Chloramine T, for its part, however, was found to decrease the color formation itself in concentrations higher than 0.05 M (Figure 4). To this end, a compromise between the cysteine amount in the analytics, the used chloramine T concentration, and the necessary detection limit for HYP had to be found. This compromise could be achieved by dissolving the dry hydrolysate in 500 µl of water and subsequently, using 100 µl of this solution for the photometric assay and a 0.05 M chloramine T solution for oxidation.

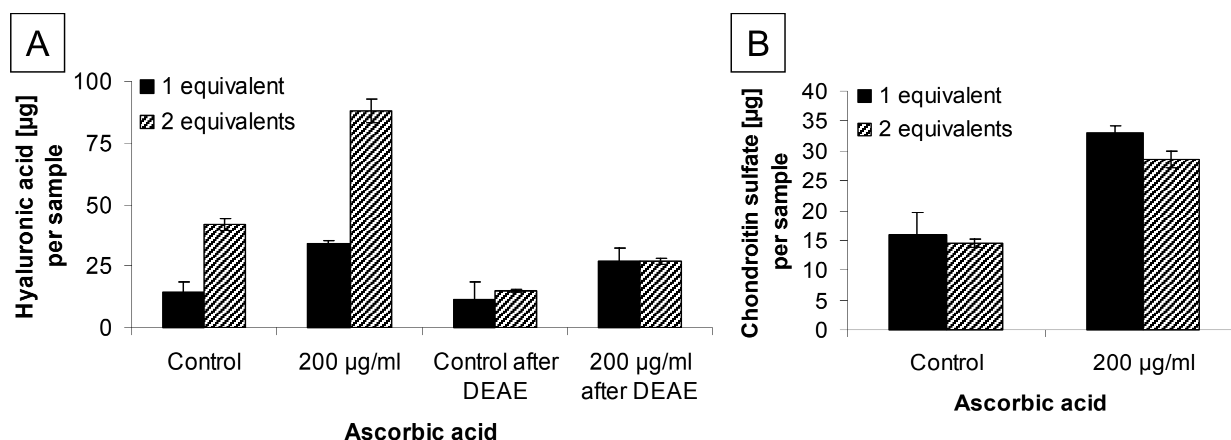

Figure 7: *Determination of glycosaminoglycans in hyalocyte samples cultured without or with 200 µg/ml of ascorbic acid; A) Values before and after removal of sulfated glycosaminoglycans with DEAE calculated after determination of 1 or 2 equivalents of analysis sample standardized to hyaluronic acid; B) Calculated values after determination of 1 or 2 equivalents of analysis sample standardized to chondroitin sulfate; GAG of untreated sample should be standardized to chondroitin sulfate, after treatment with DEAE standardization to both substances showed reproducible results.*

Determination of the hydroxyproline contents of hyalocyte samples that were cultivated with different amounts of ascorbic acid assured that the established analytical method reliably worked using the digested cell sample as analytical matrix (Figure 5). Furthermore, the linearity of the HYP determination was verified by analysis of different volumes of each sample and subsequent calculation of the whole amounts of HYP within the samples (Figure 5). Therefore, the outlined assay, a down-scaled variant of the method described by Woessner, enabled quantification of the HYP content of hyalocyte samples even in amounts as small as 0.1 µg per sample.

Besides accumulation of collagen, the production of GAG seems to be a suitable marker for functional characterization of hyalocytes. Using the widely accepted method described by Farndale et al.[16], clear amounts of GAG produced by hyalocytes could be determined. However, as accumulated GAGs probably present not a single defined substance, the question arose, which type of GAG suits best for standardization of the measured values. Chondroitin sulfate as well as hyaluronic acid showed both clear absorptions in the mentioned assay and, therefore, contribute both to the measured absorptions (Figure 6); the observed absorptions of chondroitin sulfate, however, were higher compared to equal amounts hyaluronic acid. To identify the better suited standard substance for characterization of GAGs accumulated by hyalocytes, samples of digested cells cultivated under different conditions were analyzed and the observed absorptions were subsequently standardized to chondroitin sulfate as well as to hyaluronic acid (Figure 7). Similar to the collagen analytics outlined above, again one and

two equivalents of sample volume were analyzed and the whole amount of GAG per sample was calculated. Identical values of GAG per whole sample indicated linearity of the analytical method. As, in contrast to hyaluronic acid, standardization to chondroitin sulfate resulted in comparable amounts of GAG per sample (Figure 7), this type of GAG seems to be a suitable standard to characterize the complete amount of GAG accumulated by hyalocytes. To further characterize the accumulation of GAGs by hyaloyctes, differentiation between sulfated and non-sulfated GAGs seemed useful. This was possible after removal of sulfated GAGs by DEAE treatment. The remaining amount of hyaluronic acid per sample could also be quantified using the method by Farndale.

Conclusions

The present work outlines analytical methods that allow for quantification of collagen and glycosaminoglycans accumulated by hyalocytes. As these ECM components seem to be markers that enable conclusions to the functional properties of hyalocytes, the established analytics may allow for functional characterization of hyalocytes. Moreover, a better knowledge about ECM accumulation of hyalocytes and influence factors thereon probably enable the elucidation of their potential for tissue engineering.

References

[1] Bishop, P. N. 'Structural macromolecules and supramolecular organisation of the vitreous gel'. *Prog Retin Eye Res* (2000); **19**: 323-344.

[2] Smith, G. N., Jr. and Newsome, D. A. 'The nature and origin of the glycosaminoglycans of the embryonic chick vitreous body'. *Dev biol* (1978); **62**: 65-77.

[3] Hamburg, A. 'Some investigations on the cells of the vitreous body'. *Ophthalmologica* (1959); **138**: 81-107.

[4] Qiao, H., Hisatomi, T., Sonoda, K. H., Kura, S., Sassa, Y., Kinoshita, S., Nakamura, T., Sakamoto, T., and Ishibashi, T. 'The characterisation of hyalocytes: the origin, phenotype, and turnover'. *Br J Ophthalmol* (2005); **89**: 513-517.

[5] Boltz-Nitulescu, G., Grabner, G., and Forster, O. 'Macrophage-like properties of human hyalocytes'. *Adv Exp Med Bio* (1979); **121B**: 223-228.

[6] Lazarus, H. S. and Hageman, G. S. 'In situ characterization of the human hyalocyte'. *Arch Ophthalmol* (1994); **112**: 1356-1362.

[7] Zhu, M., Penfold, P. L., Madigan, M. C., and Billson, F. A. 'Effect of human vitreous and hyalocyte-derived factors on vascular endothelial cell growth'. *Aust N Z J Ophthalmol* (1997); **25**: 57-60.

[8] Noda, Yoshihiro, Hata, Yasuaki, Hisatomi, Toshio, Nakamura, Yuka, Hirayama, Kumiko, Miura, Muneki, Nakao, Shintaro, Fujisawa, Kimihiko, Sakamoto, Taiji, and Ishibashi 'Functional Properties of Hyalocytes under PDGF-Rich Conditions'. *Invest Ophthalmol Vis Sci* (2004); **45**: 2107-2114.

[9] Lutty, G. A., Merges, C., Threlkeld, A. B., Crone, S., and McLeod, D. S. 'Heterogeneity in localization of isoforms of TGF-beta in human retina, vitreous, and choroid'. *Invest Ophthalmol Vis Sci* (1993); **34**: 477-487.

[10] Jacobson, B. 'Degradation of glycosaminoglycans by extracts of calf vitreous hyalocytes'. *Exp Eye Res* (1984); **39**: 373-385.

[11] Osterlin, S. E. and Jacobson, B. 'The synthesis of hyaluronic acid in vitreous. I. Soluble and particulate transferases in hyalocytes'. *Exp Eye Res* (1968); **7**: 497-510.

[12] Osterlin, S. E. and Jacobson, B. 'The synthesis of hyaluronic acid in vitreous. II. The presence of soluble transferase and nucleotide sugar in the accellular vitreous gel'. *Exp Eye Res* (1968); **7**: 511-523.

[13] Newsome, D. A., Linsenmayer, T. F., and Trelstad, R. L. 'Vitreous body collagen. Evidence for a dual origin from the neural retina and hyalocytes'. *J Cell Biol* (1976); **71**: 59-67.

[14] Kobuch, K., Wild, B., Eckert, E., Fischbach, C., and Gabel V.P. 'Expression of Collagen Type II by Hyalocytes In Vitro - First Steps Towards a Biological Vitreous Substitute', presented at ARVO Meeting, Fort Lauderdale, FL, 2002, Abstract No. 3495 (2002)

[15] Woessner, J. F., Jr. 'The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid'. *Arch Biochem Biophys* (1961); **93**: 440-447.

[16] Farndale, R. W., Buttle, D. J., and Barrett, A. J. 'Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue'. *Biochim Biophys Acta* (1986); **883**: 173-177.

[17] Kellner, Karin, Lang, Kurt, Papadimitriou, Apollon, Leser, Ulrike, Milz, Stefan, Schulz, Michaela B., Blunk, Torsten, and Gopferich, Achim. 'Effects of hedgehog proteins on tissue engineering of cartilage in vitro'. *Tissue Eng* (2002); **8**: 561-572.

[18] Martin, Ivan, Suetterlin, R., Baschong, W., Heberer, M., Vunjak-Novakovic, G., and Freed, L. E. 'Enhanced cartilage tissue engineering by sequential exposure of chondrocytes to FGF-2 during 2D expansion and BMP-2 during 3D cultivation'. *J Cell Biochem* (2001); **83**: 121-128.

[19] Taylor, K. B. and Jeffree, G. M. 'A new basic metachromatic dye, I:9-dimethyl methylene blue'. *Histochem J* (1969); **1**: 199-204.

[20] van den Hoogen, Bianca M., van Weeren, P. Rene, Lopes-Cardozo, Matthijs, van Golde, Lambert M. G., Barneveld, Ab, and van de Lest, Chris H. A. 'A Microtiter Plate Assay for the Determination of Uronic Acids*1'. *Anal Biochem* (1998); **257**: 107-111.

Chapter 5

Ascorbic acid Modulates Proliferation and Extracellular Matrix Accumulation of Hyalocytes

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Submitted to 'Tissue Engineering'

Abstract

Ascorbic acid is known to modulate the proliferation and functional properties of several intraocular cell types. In this study, the effect of ascorbic acid on the proliferation and functional properties of hyalocytes was evaluated. To this end, hyalocytes were cultured with different amounts of ascorbic acid in classical 2-D cultures and a 3-D pellet culture system. Ascorbic acid enhanced hyalocyte proliferation dose-dependently in concentrations between 0.1 and 3 µg/ml; proliferation was constant over a wide concentration range up to 150 µg/ml, concentrations of 500 µg/ml showed toxic effects. In 2-D hyalocyte culture, the accumulation of glycosaminoglycans and collagens was increased in response to ascorbic acid supplementation of 10 or 200 µg/ml. Normalized to the cell number, GAG production was not influenced, whereas collagen production was increased. These results could be verified in a pellet-like 3-D culture system. Ascorbic acid also influenced hyalocytes on the mRNA-level; the expression of COL11A1 was clearly enhanced by ascorbic acid. To conclude, ascorbic acid modulates proliferation and collagen accumulation of hyalocytes; it also influences mRNA expression of the cells. Taken together with the fact that ascorbic acid influences several other intraocular cell types, this study further establishes its role as a modulator of the intraocular environment.

Introduction

The vitreous body is the main component of the vertebrate eye. This gel-like system with a water content of about 98% consists mainly of collagens and glycosaminoglycans[1] with a single layer of cells in the cortex of the vitreous[2, 3]. The most abundant collagen in the vitreous is the fibrillar collagen type II, accounting for approximately 75% of the total collagen. Other important collagens are the fibrillar collagen type V/XI, containing α1(XI) and α2(V) chains, and collagen type IX, a non fibrillar collagen. Among the glycosaminoglycans (GAG) within the vitreous, the non-sulfated hyaluronic acid plays a predominant role, whereas the sulfated GAGs, such as chondroitin sulfate or heparan sulfate, represent only minor components[1]. Because of its simplicity and the lack of blood vessels within the tissue, the vitreous body seems to be an ideal target for regeneration via tissue engineering strategies. Furthermore, the cells within the vitreous seem to hold potential for reorganization of this transparent tissue[4].

The cells in the cortex of the vitreous body were described for the first time by Hannover in 1840[5] and Henle in 1841[6]. These so-called hyalocytes show a macrophage-like structure[7-9], express antigens typical for monocytes and macrophages, and are, therefore, thought to belong to the reticuloendothelial system[10]. Recent studies investigating rodents with green fluorescent protein transfected bone marrow demonstrated the cells to be derived from the bone marrow and to be replaced totally within 7 months[11]. Although hyalocytes were identified over one and a half centuries ago, there is limited knowledge about the function of the cells within the adult vitreous. In the literature, hyalocytes are often referred to as resting cells[8, 12, 13], however, they have been demonstrated to produce extracellular matrix (ECM) components like hyaluronic acid[14-19] and collagen[4, 20]. Physiologically hyalocytes are associated with the maintenance of the vitreous as an avascular and transparent tissue[7, 21-23]; pathologically the cells are thought to be involved in diseases of the vitreoretinal interface[22]. The cells may, therefore, have a house-keeping function within the adult vitreous.

A characteristic of the mammalian vitreous body is its ability to accumulate ascorbic acid to about 150 µg/ml, a concentration about ten times higher than that in plasma[24-26]. These high concentrations of ascorbic acid play a major role in oxidative protection of the eye[27]. However, ascorbic acid also has an effect on cell proliferation in tissues adjacent to the vitreous body, such as retinal pigment epithelial cells (RPE). Ascorbic acid was shown to inhibit proliferation of RPE cells[28-30] as well as proliferation of cultured lens epithelial cells [31] and vascular endothelial cells[32]. Ascorbic acid has also been shown to influence the functional

properties of different cell types in the eye. Trabecular meshwork cells, for example, showed an increase in collagen type I expression due to ascorbic acid[33] and the pigmentation of RPE cells, a differentiation marker for these cells, was enhanced by ascorbic acid[30].
Furthermore, in diseases like proliferative diabetic rethinopathy or proliferative vitreorethinopathy, both caused by an abnormal proliferation and differentiation behavior of retinal cells, especially RPE cells[34], a reduced level of ascorbic acid within the vitreous could be detected[35]. These findings give rise to the hypothesis that ascorbic acid is an important factor for the regulation of the intraocular environment. However, the influence of ascorbic acid on hyalocytes, cells that seem responsible for the physiological properties of the vitreous, has hardly been studied so far[4]. Finally, as the vitreous body seems to be a candidate for regeneration, it appears obvious to elucidate the effect of ascorbic acid, an acknowledged factor widely used in tissue engineering, on these cells.
Therefore, the present study addressed the effect of ascorbic acid on the proliferation and functional properties of porcine hyalocytes. Proliferation was studied in a classical two-dimensional (2-D) cell culture system. The influence of ascorbic acid on functional cell properties was studied using a 2-D culture system and the results were verified in a three-dimensional (3-D) hyalocyte pellet culture system. As markers for the functional properties of the cells, we investigated the production of glycosaminoglycans (GAG) and collagens. To further characterize the production of collagen in 2-D culture, mRNA expression of collagen type I, type II and type V/XI was evaluated semi-quantitatively.

Materials and Methods

L-Ascorbic acid in cell culture quality, cysteine, hematoxylin, eosin, chondroitin sulfate A from bovine trachea, dimethylmethylene blue, and highly polymerized deoxyribonucleic acid from calf thymus were purchased from Sigma (Steinheim, Germany). Fetal calf serum (South America, Batch Nr. 40A0044K), Dulbecco's phosphate buffered saline (PBS), penicillin/streptomycin, 0.25% Trypsin-EDTA, and agarose in electrophoresis grade were obtained from Invitrogen (Karlsruhe, Germany). Dulbecco's modified Eagle medium (DMEM, low glucose, with glutamine and pyruvate) as well as Trizol Reagent were obtained from Biochrom (Berlin, Germany). Dimethylaminobenzaldehyde, chloramine T, formaldehyde, isopropanol, Na_2HPO_4, and Na_2EDTA were bought from Merck (Darmstadt, Germany); hydroxyproline and perchloric acid were purchased from Fluka (Neu-Ulm, Germany). Hoechst 33258 dye was obtained from Polysciences (Warrington, PA, USA), papainase and collagenase type II were purchased from Worthington (Lakewood, NJ, USA).

Tissue Tek was bought from Sakura Finetek Europe (Zoeterwoude, The Nederlands). All other cell culture materials were purchased from Corning (Bodenheim, Germany). Buffer for papainase digestion (PBE) was composed of 100 mmol Na_2HPO_4, 10 mmol Na_2EDTA, and 5 mmol cysteine in water, adjusted to pH 6.5.

Cell culture

Freshly enucleated porcine eyes were kindly provided by a local abattoir. Within 3 hours, the vitreous bodies were dissected, washed once in PBS buffer, and examined microscopically. Vitreous bodies were subsequently digested in 1 mg/ml collagenase type II solution in standard culture medium (SCM) for 3 hours under shaking in an incubator at 37°C. SCM was composed of DMEM supplemented with 15% fetal calf serum (FCS) and 100 IU/ml penicillin/streptomycin. Digested vitreous bodies from about 20 eyes were pooled. Following centrifugation at 200 g for 7 min, cells were seeded for primary culture in a 25 cm^2 flask and cultured at 37°C and 5% CO_2. Media was exchanged three times a week. Primary culture was performed in SCM supplemented with 50 µg/ml ascorbic acid. After 9 days, the proliferating cells were detached using Trypsin-EDTA for 5 min and centrifuged at 200 g for 5 min. These cells were used for RT-PCR experiments after the first passage. For proliferation studies and for 2-D as well as for 3-D hyalocyte culture, cells were used after the second passage. To this end, during the first passage cells were plated at 2 000 cells per cm^2 in a new flask for further proliferation. After the first passage, hyalocytes were cultured in SCM without ascorbic acid supplementation and allowed to grow until almost reaching confluency.

Proliferation assay

Hyalocytes were seeded with 2 000 cells per cm^2 in 25 cm^2 culture flasks and cultured in SCM supplemented with different amounts of ascorbic acid up to 500 µg/ml; SCM without ascorbic acid served as control. Media were exchanged for fresh, ascorbic acid containing media after 2 days. After 4 days, the cells were harvested by trypsinization for 30 min in Trypsin-EDTA. After digestion of the cells in 125 µg/ml papainase solution in PBE buffer for 18 hours at 60°C, the cell number was determined by quantifying the DNA content as described below.

2-D hyalocyte culture

Identically to the proliferation assay, hyalocytes were seeded with 2 000 cells per cm^2 in 25 cm^2 culture flasks and cultured as described above. SCM was supplemented with 10 or

200 µg/ml of ascorbic acid, SCM without ascorbic acid served as control. Media were exchanged after 2 days, after 4 days the cells were harvested by mechanical dissociation. Subsequently, the samples were freeze dried (Christ Beta 2-16, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at 20°C and 0.120 mbar for 16 hours. The dry samples were digested in 300 µl papainase solution as described above. Cell numbers as well as extracellular matrix (ECM) contents were determined as described below.

3-D hyalocyte culture:

600 000 hyalocytes harvested after the second passage were suspended in SCM containing 0, 10, or 200 µg/ml of ascorbic acid and centrifuged for 5 min at 200 g in a 15 ml Falcon tube. The resulting cell aggregates were cultured in the same Falcon tube with loosened lid in an incubator for 30 days. Media were exchanged three times a week; ascorbic acid was added with each medium change. After 30 days, pellets were washed once with water and subsequently freeze dried. The dry samples were digested with papainase as described above. The cell number and ECM contents were determined as described below.
For histological analysis, pellets were fixed in 10% formaldehyde and embedded in Tissue Tek. Histological sections with 10 µm thickness were obtained using a Microm Cryotom HM 550 (Walldorf, Gemany) and subsequently stained using Meyers hematoxylin followed by eosin counterstaining.

Determination of cell number and ECM content

Cell number was determined by measuring the DNA amount using Hoechst 33258 dye[36-38]. In brief, the emission intensity of the intercalating dye was measured at 458 nm upon excitation at 365 nm. The fluorescence of Hoechst 33258 dye is correlated with the absolute amount of DNA in the sample. To calculate the cell number, an average amount of 9.96 pg DNA per cell was assumed. This value was determined in a separate experiment correlating DNA measurement results of proliferating hyalocytes after second passage with cell counts using a Neubauer chamber (data not shown) and correlates well with values published for other cell types[39-41].
Glycosaminoglycan content was measured spectroscopically as previously described[42, 43]. In brief, after digestion of interfering proteins with papainase, the glycosaminoglycan content was determined by a colorimetric reaction with dimethylmethylene blue followed by

measurement of absorption at 525 nm. Chondroitin sulfate was used to produce the standard curve.

The collagen content was determined by measuring the amount of hydroxyproline, an amino acid that exists in significant amounts exclusively in collagen, according to Woessner et al.[44] with some modifications. 100 µl digested sample from 2-D or 3-D culture was hydrolyzed with 100 µl 12 N hydrochloric acid for 16 h at 105°C. After hydrolysis, hydrochloric acid was evaporated under a constant flow of nitrogen at about 40°C. The dry samples were dissolved in 500 µl double distilled water. Standard dilutions of hydroxyproline were prepared in PBE/papainase solution and treated under the same hydrolysis conditions. In a microtiter plate 100 µl of each sample as well as 100 µl of each standard sample were oxidized by 50 µl of a 0.05 M solution of chloramine T in a citrate buffer (pH 6) for 20 min. Afterwards, 50 µl of a 15% (m/m) dimethylaminobenzaldehyde solution in 4 M perchloric acid in 70% isopropanol/water (m/m) was added and after shaking the plate was incubated for 30 min at 60°C. The plate was cooled to room temperature and the absorbance of the samples was immediately measured at 557 nm on a microplate reader (CS-9301 PC, Shimadzu, Duisburg, Germany).

Reverse transcription – polymerase chain reaction (RT-PCR)

To study mRNA-expression of hyalocytes, cells from the first passage were seeded at a density of 10 000 cells per cm² in 6-well plates and cultured for 4 days in SCM with 0, 1, 10, and 200 µg/ml of ascorbic acid. After 2 days the media were exchanged against fresh SCM, and ascorbic acid was again supplemented in the same amounts. Total RNA was harvested from the cells using Trizol® reagent according to the manufacturer's instructions. First-strand cDNA was synthesized from total RNA using random hexamers (Roche Diagnostics, Mannheim, Germany) and Superscript II Rnase H Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). Samples were incubated at 42°C for 50 min and then heated at 70°C for 15 min to inactivate the enzyme. Subsequently, polymerase chain reaction was performed using Sawady Taq-DNA-Polymerase (PeqPab, Erlangen, Germany); initial denaturation occured at 94°C for 120 s, final extension at 72°C for 30 s. Amplification was performed under the following conditions: Denaturation at 94°C for 45 s, annealing for 45 s at 55°C and elongation at 72°C for 60 s. The amplification was carried out using the oligonucleotides and number of cycles indicated in Table 1.

Gene	Sense-Primer (5' – 3')	Antisense-Primer (5' – 3')	Cycles	Product length
β-Actin	gtgcccatctacgaggggta	atggtgatgacctggccgtc	34	263 bp
COL1A1	ctgctcctcttagcggccac	cagtgtctcccttgggtccc	33	334 bp
COL2A1	ggtcttcctggcaaagatgg	cctgggaaacctcgttcacc	33	227 bp
COL11A1	ggtcacaggggtgaacgagg	gttcccttttggtcctgggg	33	204 bp

Table 1: *Primers and cell cycles used for the analysis of mRNA expression of hyalocytes.*

The housekeeping gene β-Actin served as reference for comparison. Reverse transcription and polymerase chain reaction were performed using a Mastercycler Gradient (Eppendorf AG, Hamburg, Germany). The amplified products were analyzed by electrophoresis in 2% agarose gels and stained with ethidium bromide. Finally, the gels were subjected to imaging and densitometric scanning of the resulting bands under UV light (λ = 312 nm) using a Kodak EDAS 290 (Fisher Scientific, Schwerte, Germany). To assure specificity of the amplifications, the amplified products as well as an amplification product of a positive control for COL2A1 (porcine cartilage) were sequenced (data not shown).

Statistics

All data are presented as means ± standard deviation. Single-factor analysis of variance (ANOVA) was used in conjunction with a multiple comparisons test (Tukey's test) to assess statistical significance at levels of $p < 0.05$ or $p < 0.01$ as indicated.

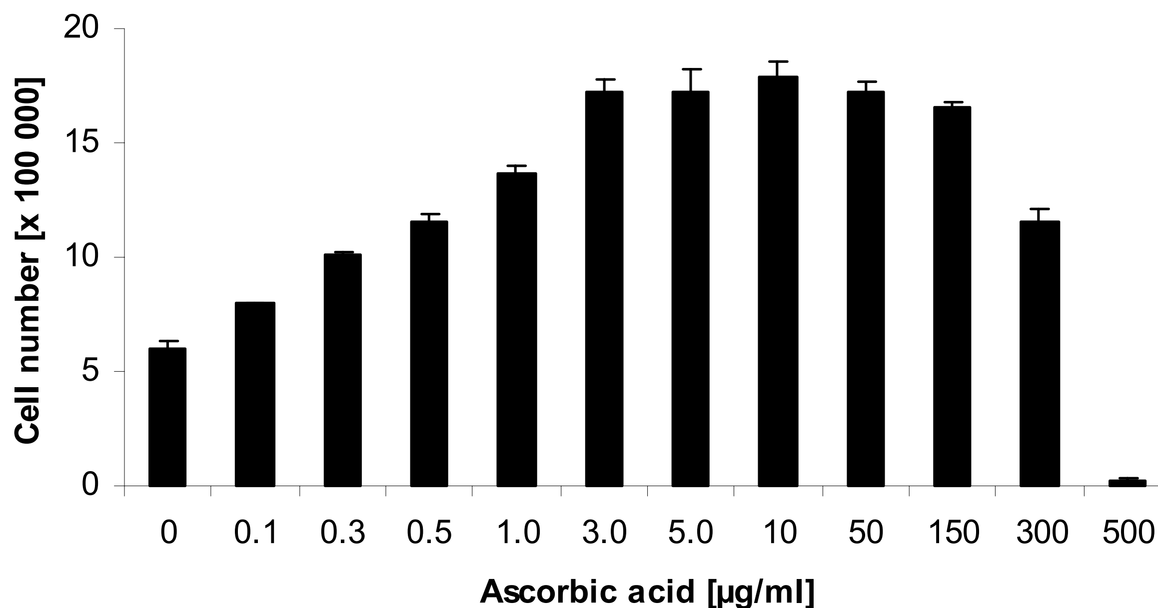

Figure 1: *Proliferation of hyalocytes in 2-D culture with various concentrations of ascorbic acid supplemented to the media; n=3; for all groups p < 0.01 compared to control; except for the plateau between 3.0 and 150 µg/ml, all groups showed statistically significance to each other (p < 0.01); experiment was performed in triplicate, representative data are shown.*

Results

Hyalocyte isolation and proliferation

After dissection halocytes were present mainly on the surface of the vitreous bodies, as described in the literature[3]. Due to the preparation method and subsequent washing with PBS, no pigmented cells could be observed on the isolated vitreous bodies either macro- or microscopically. After digestion of the vitreous bodies and cell seeding, most of the cells attached to the culture surface within 48 h. After 9 days, colonies of highly proliferating cells could be detected in the culture flask. After the first passage the cells grew homogenously on the culture surface and reached confluency within 7 days. Microscopically, cells showed fibroblast-like morphology with many lysosome-like granules mostly near the nucleus, as described earlier[45]. After the second passage, 8 – 10 million cells, a sufficient cell number for 2-D as well as for 3-D experiments, could be obtained.

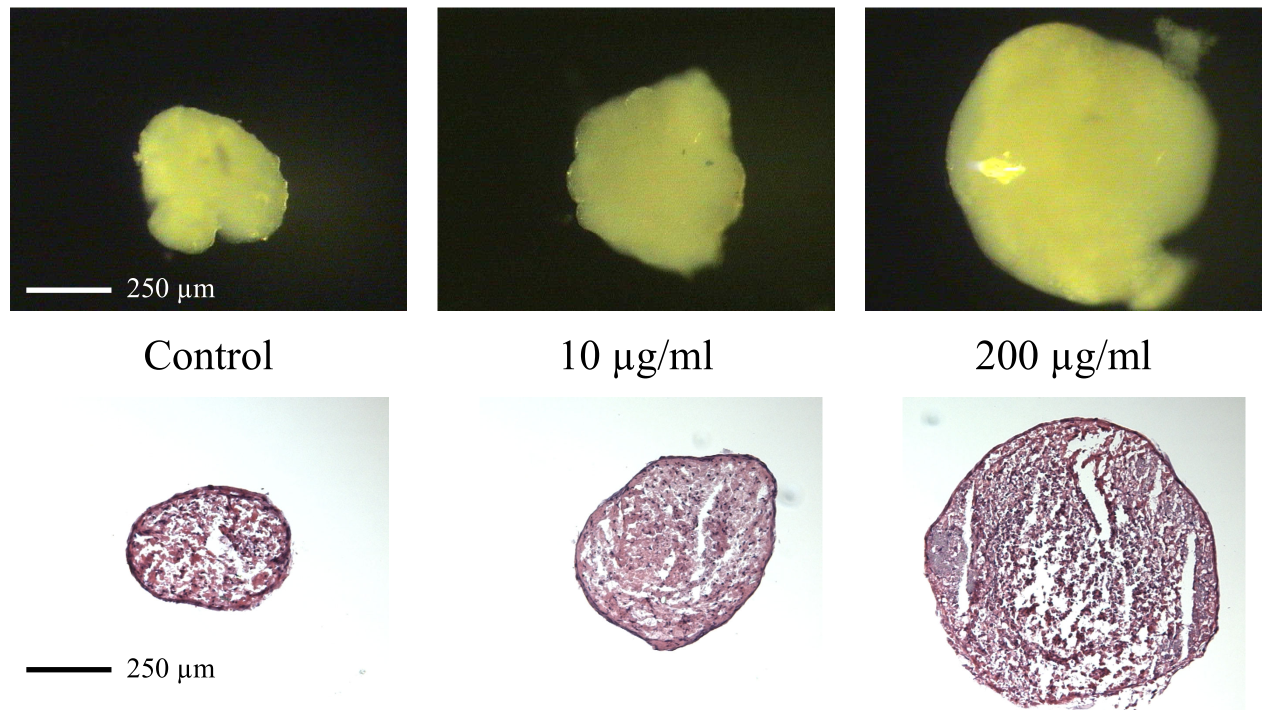

Figure 2: *Hyalocyte pellets after 30 days of cultivation. Pictures above were taken using a reflecting light magnifier, pictures below show histological sections of the pellets after H&E staining in 100-fold magnification; n = 3; each experiment was done in duplicate, representative data are shown.*

Ascorbic acid effect on proliferation of hyalocytes

Proliferation of hyalocytes in 2-D cell culture was significantly increased due to ascorbic acid supplementation (Fig. 1). Even in the minute concentration of 0.1 µg/ml, the proliferation of hyalocytes was significantly enhanced. In a concentration range up to 3.0 µg/ml, a dose-dependent effect of ascorbic acid on hyalocyte proliferation was observed. A wide plateau of constant cell proliferation (3-fold compared to control) was observed up to 150 µg/ml. Though concentrations of 300 µg/ml showed a significant increase in proliferation compared to the control, the proliferation was significantly decreased relative to the plateau. When adding 500 µg/ml ascorbic acid to the culture media, no cells could be detected after the cultivation time of 4 days.

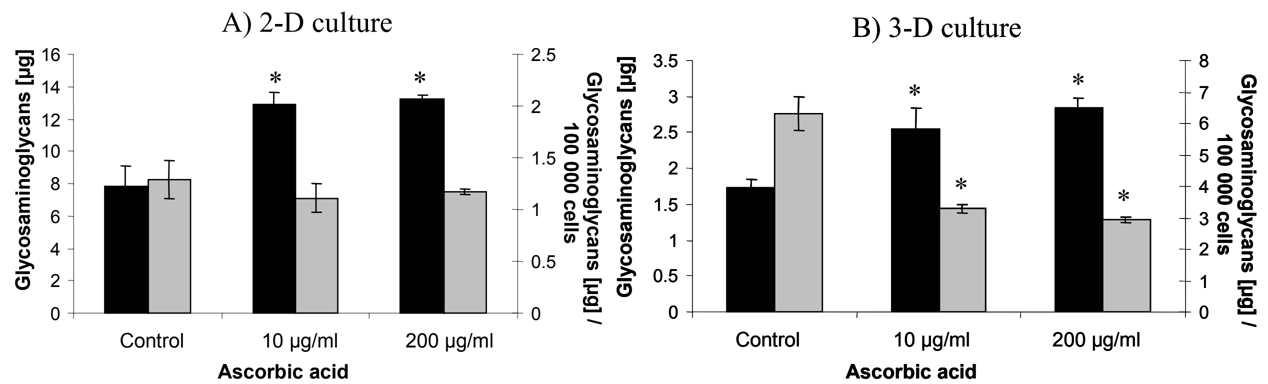

Figure 3: *GAG accumulation of hyalocytes, measured as chondroitin sulfate, depending on ascorbic acid supplementation. In both diagrams, black bars and left y-axis indicate absolute GAG content, whereas grey bars and right y-axis indicate the GAG amount accumulated per 100 000 cells; A) Influence of ascorbic acid on hyalocytes in 2-D culture, n = 4; B) Influence of ascorbic acid on hyalocytes in 3-D pellet culture after 30 days of cultivation; n = 3; * indicates p<0.01 compared to control; each experiment was performed in duplicate, representative data are shown.*

Pellet culture

For 3-D pellet culture, cells were centrifuged and subsequently cultured in Falcon tubes. After centrifugation of the cells, the resulting cell aggregates formed a spheroid within 48 hours. After the cultivation time of 30 days, pellets cultured without ascorbic acid reached a diameter of 350 µm. The pellet size was noticably increased due to ascorbic acid supplementation (Fig. 2). Supplementing 10 µg/ml ascorbic acid, the resulting pellet diameter was about 500 µm. 200 µg/ml ascorbic acid in the culture media increased the diameter of the pellets to about 750 µm, a more than 2-fold increase in diameter compared to the control. Calculations based on a spherical shape revealed that the increase in diameter corresponds to an 8-fold volume increase.

All pellets were dense and surrounded by a small, capsule-like structure, as indicated by histological sections (Fig. 2). However, the relation between cells in the cortex of the pellets and cells in the center of the pellets changed with increasing diameter of the pellets. Furthermore, with increasing pellet diameter a more distinct pellet core with densely packed cell nuclei could be observed.

GAG accumulation

The total amount of glycosaminoglycans accumulated by hyalocytes was enhanced by ascorbic acid in both culture systems. By adding 10 or 200 µg/ml ascorbic acid to the culture media, the absolute content of glycosaminoglycans, evaluated using a chondroitin sulfate standard curve, was increased to a similar extent (Fig. 3, black bars). As ascorbic acid

enhanced the proliferation of hyalocytes, we also calculated the amount of glycosaminoglycans accumulated per cell. This amount was differently affected by ascorbic acid in the two culture systems. In 2-D culture, the amount of glycosaminoglycans accumulated per cell was not influenced by ascorbic acid, whereas in the 3-D culture system this amount was significantly decreased (Fig. 3, grey bars). As observed for the absolute amounts of glycosaminoglycans, there was no significant difference between the two concentrations of ascorbic acid, 10 and 200 µg/ml.

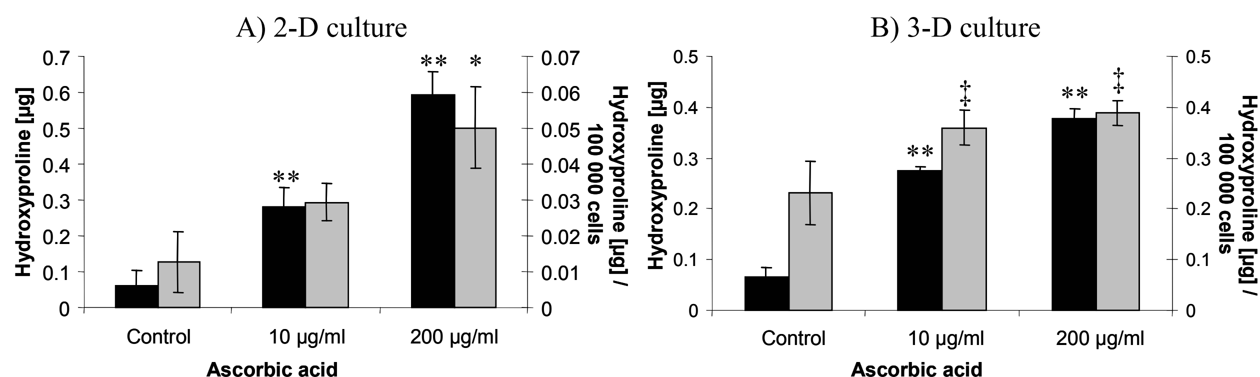

Figure 4: *Collagen accumulation of hyalocytes, measured as hydroxyproline, depending on ascorbic acid supplementation. In both diagrams, black bars and left y-axis indicate absolute collagen content, grey bars and right y-axis indicate collagen accumulation per 100 000 cells; A) Influence of ascorbic acid on hyalocytes in 2-D culture, n = 4; B) Influence of ascorbic acid on hyalocytes in 3-D pellet culture after 30 days of cultivation; n = 3; * indicates statistical significance (p<0.01) compared to control, ** compared to all groups; ‡ indicates statistical significance (p<0.05) compared to control; each experiment was performed in duplicate, representative data are shown.*

Collagen accumulation

The collagen accumulation of hyalocytes was influenced by ascorbic acid similarly in both culture systems (Fig. 4). Without ascorbic acid in the media, only small amounts of hydroxyproline could be detected. We observed an approximately 3-fold increase in the hydroxyproline content of the samples due to 10 µg/ml ascorbic acid supplementation. After adding 200 µg/ml ascorbic acid to the culture media, the absolute hydroxyproline content was even higher than in the 10 µg/ml group. In contrast to the GAG accumulation per cell, the hydroxyproline content normalized to the cell number was significantly increased by ascorbic acid in both culture systems. In 2-D culture, supplementation of 200 µg/ml ascorbic acid led to a 3-fold increase in hydroxyproline accumulation per cell. In the 3-D pellet system, this increase was about 1.5 fold.

Ratio of extracellular matrix components

To further characterize the influence of ascorbic acid on the composition of the accumulated ECM, we calculated the ratio of accumulated hydroxyproline to accumulated glycosaminoglycans. This value was clearly increased in both culture systems due to ascorbic acid supplementation. In 2-D culture, the ratio of ECM components produced by hyalocytes was increased by ascorbic acid supplementation up to a 5-fold value compared to control, in the 3-D pellet culture this value was enhanced 3-fold by supplementing the culture media with 200 µg/ml ascorbic acid (Fig. 5).

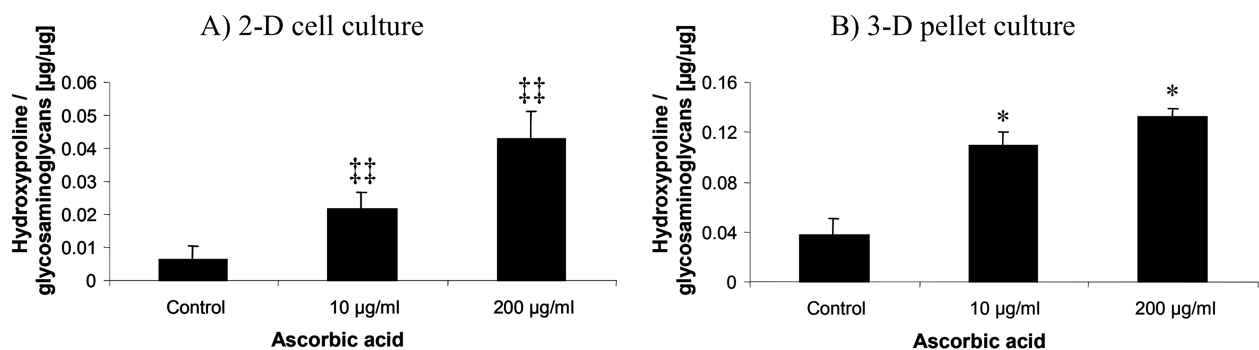

Figure 5: *Collagen to GAG ratio of accumulated extracellular matrix depending on ascorbic acid concentration; A) 2-D cell culture, n = 4; B) 3-D hyalocyte pellets after 30 days of cultivation; n = 3; ‡‡ indicates statistical significance (p<0.05) compared to all groups, * indicates statistical significance (p<0.01) compared to control; each experiment was performed in duplicate, representative data are shown.*

Expression of mRNA

To further investigate the effect of ascorbic acid on the collagen production of hyalocytes, semi-quantitative RT-PCR was performed in 2-D culture. COL1A1, coding for the α-1 chain in collagen type I, was clearly expressed in all groups, however, its expression was not affected by ascorbic acid (Fig. 6). No expression of COL2A1, coding for the α-1 chain in collagen type II, could be detected in any of the samples. In contrast, ascorbic acid enhanced the expression of COL11A1, a gene coding for the α-1 chain in collagen type V/XI. A slight increase in the expression level was detectable at a concentration as low as 1 µg/ml; at higher concentrations (10 and 200 µg/ml ascorbic acid), the increase in COL11A1 expression was distinct and significant.

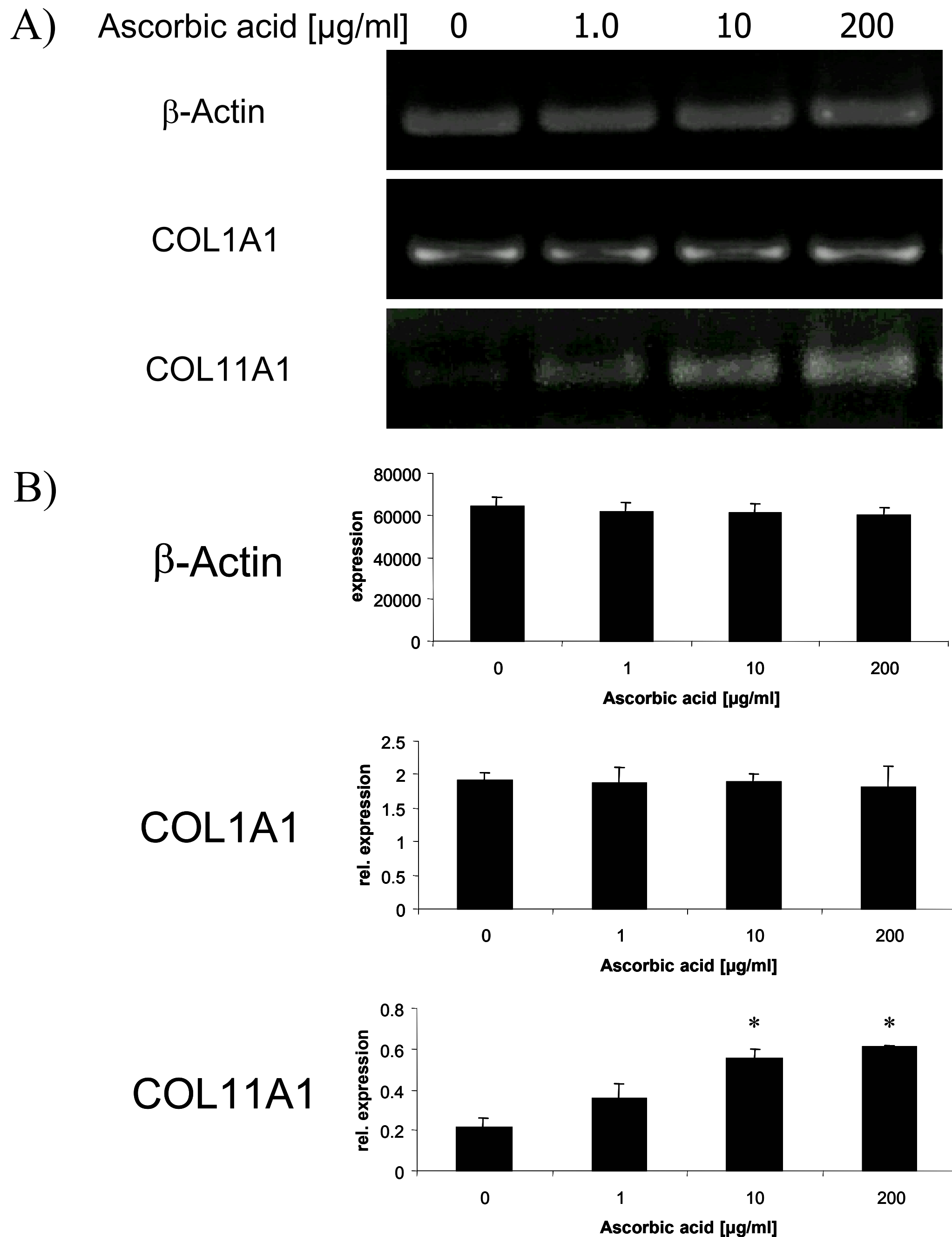

Figure 6: *Assessment of expression of genes COL1A1 and COL11A1 dependent on the ascorbic acid concentration using semi-quantitative RT-PCR technique; β-actin served as housekeeping-gene; A) Band intensity of representative bands; B) Data from semi-quantitative image analysis; n=3; * indicates statistical significance (p<0.01) compared to control; each experiment was performed in duplicate, representative data are shown*

Discussion

Ascorbic acid, one of the most important antioxidants in the eye, modulates proliferation and differentiation properties of several intraocular cell types and is, therefore, discussed as an intrinsic modulator of the intraocular environment[30, 46]. In the present study, we demonstrated that hyalocytes, cells that seem to modulate the functional properties of the vitreous, were also clearly influenced by ascorbic acid. Moreover, we elucidated the importance of ascorbic acid for in vitro hyalocyte culture.

The cells in the peripheral or cortical region of the vitreous body are designated as hyaloyctes; a widely used isolation technique for these cells is described by Francois et al.[47] and was further improved by Kobuch et al[4]. With this isolation method, however, we could not obtain a sufficient number of hyalocytes within a few passages to establish a three-dimensional pellet culture. As it is widely acknowledged that cells dedifferentiate more with increasing number of passages[48, 49], we intended to limit the number of propagation steps. Therefore, we used an isolation method based on enzymatic digestion of the vitreous gels, as described earlier[50]. This enabled us to harvest sufficient numbers of hyalocytes for our experiments after the second passage. Supplementation of ascorbic acid to the primary cells was performed because we observed a clear increase in hyalocyte proliferation in preliminary experiments. As we intended to study the influence of ascorbic acid on hyalocytes after second passage, we cultured the cells without ascorbic acid during the first passage. This assured that we do not measure the influence of ascorbic acid withdrawal in our experiments, but rather the effect of ascorbic acid supplementation.

As hyalocytes show a physiological turnover[11], we were first interested in the influence of ascorbic acid on their proliferation. To minimize the extent to which hyalocyte proliferation was influenced by cell-cell contact in the 2-D proliferation studies, we only allowed the cells to proliferate for 4 days, reaching about 90% confluency in the most rapidly proliferating groups. In contrast to many other intraocular cells types[28, 31, 32], in our experimental setup the proliferation of hyalocytes was clearly increased by ascorbic acid over a wide concentration range. As hyalocyte proliferation could be increased as much as 3-fold and even minute concentrations of 0.1 µg/ml of ascorbic acid showed significant effects (Fig. 1), ascorbic acid seems to be an important modulator of hyalocyte proliferation. In a concentration of 500 µg/ml, toxic effects on the cells could be observed. Despite the pronounced effects of ascorbic acid on hyalocytes, the exact mechanism remains to be elucidated. Furthermore, the behavior of the cells may be prone to modulations caused by the applied culture conditions.

In addition to proliferation, we also wanted to study the effect of ascorbic acid on functional properties of hyalocytes. As hyalocytes are known to produce the ECM components that form the vitreous body[14, 20], we studied the quantitative accumulation of extracellular matrix by hyalocytes. We found that ascorbic acid clearly affected the ECM accumulation of hyalocytes. This effect could be observed in two different culture systems: a classical 2-D as well as a novel 3-D hyalocyte culture system.

Neither of these two systems mimic the natural environment of hyalocytes completely, but each system displays some typical aspects of the physiological context. The classical 2-dimensional culture system mimics the natural environment of hyalocytes in terms of their isolated position in the vitreous and a minimum of cell-cell interactions. However, a disadvantage of the system is the partial interaction between cells and an unnatural synthetic culture surface. A disadvantage of the newly established 3-D hyalocyte pellet system is the unphysiological high cell density. In this system, however, the cells have only contact to one another and to the extracellular matrix they produced, which also partially imitates the natural environment.

Although the two culture systems were very different, we found comparable effects of ascorbic acid on hyalocytes; while it was not possible to distinguish between different types of GAGs, such as chondroitin sulfate, heparan sulfate or hyaluronic acid, ascorbic acid increased the absolute amounts of GAG and collagen that were accumulated by the cells. Furthermore, the production of collagen per cell was clearly enhanced by ascorbic acid, whereas the accumulation of GAG per cell was not affected. Regarding GAG-accumulation, however, there seems to be a difference between the two culture systems: while the accumulation of GAG per cell was constant in the 2-D culture, in the pellet culture this value was decreased upon ascorbic acid supplementation (Fig. 3, grey bars). This difference was most probably caused by a different nutrition supply of the cells within the pellet, as the pellet size was clearly increased by supplementation of ascorbic acid (Fig. 2). This is confirmed by the accumulated amounts of hydroxyproline per cell (Fig. 4, grey bars), which were affected in a similar way. In 2-D culture, there was a 3-fold increase in the accumulation of hydroxyproline per cell upon supplementation with 200 µg/ml ascorbic acid, while this increase was only 1.5-fold in the pellet culture. Furthermore, histological sections of the pellets indicate that cells on the edge of the pellets were clearly embedded in extracellular matrix, whereas cells in the middle of the pellets were densely packed and possibly necrotic (Fig. 2). The enhanced volume of the pellets in the ascorbic acid containing groups was due to

an increased cell number in the pellets (data not shown) as well as an increase in accumulated ECM.

Comparing the values for absolute and per cell ECM accumulation measured in the two systems, we observed clear distinctions. However, these differences were related to the culture conditions rather than in response to ascorbic acid supplementation. The higher values of absolute ECM contents in 2-D culture were due to the higher cell number in this system; the higher values of accumulated ECM per cell in the 3-D culture system could be explained by the longer cultivation time.

In both culture systems, ascorbic acid shifted the ratio of total hydroxyproline to total glycosaminoglycans towards the hydroxyproline and, therefore, to the collagen side (Fig. 5). That means, in two different culture systems ascorbic acid influenced hyalocytes in a similar way. This strongly supports the hypothesis that functional properties of hyalocytes are modified by ascorbic acid.

We could demonstrate that vitamin C affects hyalocyte properties influencing collagen accumulation. As ascorbic acid is an acknowledged cofactor in the synthesis of hydroxyproline[51], it is likely that in our experiments the enhanced collagen synthesis was at least partly due to a cofactor function of ascorbic acid. However, ascorbic acid is also known to modulate the mRNA expression of cells[52]. To clarify whether hyalocytes were also influenced on the molecular level with regard to the collagen production, we investigated the expression of mRNA coding for different types of collagen. The expression of collagen type I was not affected by ascorbic acid. The fact that we could detect any COL1A1 mRNA is likely due to the experimental setup including one passage, possibly leading to partial dedifferentiation of the cells. This is also known for other cell types, for example chondrocytes[53]. In our experimental setup, no expression of collagen type II, the most abundant collagen in vitreous, could be detected. This could also be explained by dedifferentiation of the cells caused by the proliferation step. However, we found evidence that the expression of COL11A1, coding for the α-1(XI) chain in collagen type V/XI, was strongly enhanced by ascorbic acid (Fig. 6). The fibrillar collagen type V/XI found in the vitreous body is composed of α-1(XI) chains and α-2(V) chains, but the exact stoichiometry remains unknown[54]. As the α-1(XI) chain is exclusively a part of the mixed collagen type V/XI and not part of any other type of collagen in the vitreous, it is reasonable to assume that the expression of COL11A1 is highly related to collagen type V/XI.

The function of collagen type V/XI in vitreous is not exactly known at the moment, however, there are hints that this type of collagen is involved in stabilizing the collagen network. The

Stickler syndrome, for example, an autosomal dominant disorder known to involve mutations in the COL11A1 gene, is associated, amongst others, with sparse and irregularly thickened bundles of fibers throughout the vitreous[55]. Furthermore, in cartilage the α-1(XI) chain is part of collagen type XI, a type of collagen important for stabilizing the collagen fibrils of cartilage[56]. Therefore, it appears likely that the vitreous collagen type V/XI is important for stabilizing the collagen type II fibrils of the vitreous. The observation that ascorbic acid has a clear influence on the expression of the COL11A1 coding for a part of collagen type V/XI suggests that ascorbic acid may influence the stability of the vitreous collagen fibers accumulated by hyalocytes. Whether these findings have any clinical significance remains to be elucidated. However, our experiments strongly support the hypothesis that ascorbic acid influences hyalocyte function on the gene expression level; not only the quantity, but also the quality of collagen produced by hyalocytes was influenced by ascorbic acid.

To conclude, we demonstrated that ascorbic acid has a significant influence on proliferation and functional properties of hyalocytes. Therefore, ascorbic acid seems to be a useful tool for in vitro hyalocyte culture and a key factor for reconstruction of the vitreous body using tissue engineering techniques. Furthermore, ascorbic acid is known to modulate proliferation and differentiation properties of cells within tissues close to the vitreous body[30-32]. This study clearly indicates that ascorbic acid is also an important factor influencing the cells of the vitreous body. These findings support the hypothesis that ascorbic acid is not only an important antioxidant for the eye, but also a modulator of the intraocular environment.

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References

[1] Bishop, P. N. 'Structural macromolecules and supramolecular organisation of the vitreous gel'. *Prog Retin Eye Res* (2000); **19**: 323-344.

[2] Haddad, Anto and Andre, Julio Cesar. 'Hyalocyte-Like Cells are more Numerous in the Posterior Chamber than they are in the Vitreous of the Rabbit Eye'. *Exp Eye Res* (1998); **66**: 709-718.

[3] Sebag, J. '*The vitreous*', Springer, New York (1989).

[4] Kobuch, K., Wild, B., Eckert, E., Fischbach, C., and Gabel V.P. 'Expression of Collagen Type II by Hyalocytes In Vitro - First Steps Towards a Biological Vitreous Substitute', presented at 'ARVO Annual Meeting', Fort Lauderdale, FL, USA (2002)

[5] Hamburg, A. 'Some investigations on the cells of the vitreous body'. *Ophthalmologica* (1959); **138**: 81-107.

[6] Henle, Jakob. '*Lehre von den Mischungs- und Formbestandtheilen des menschlichen Körpers*' in 'Vom Baue des menschlichen Körpers', Vos, Leipzig (1841).

[7] Boltz-Nitulescu, G., Grabner, G., and Forster, O. 'Macrophage-like properties of human hyalocytes'. *Adv Exp Med Bio* (1979); **121B**: 223-228.

[8] Salu, P., Claeskens, W., De Wilde, A., Hijmans, W., and Wisse, E. 'Light and electron microscopic studies of the rat hyalocyte after perfusion fixation'. *Ophthalmic Res* (1985); **17**: 125-130.

[9] Bloom, G. D. and Balazs, E. A. 'An electron microscopic study of hyalocytes'. *Exp Eye Res* (1965); **4**: 249-255.

[10] Lazarus, H. S. and Hageman, G. S. 'In situ characterization of the human hyalocyte'. *Arch Ophthalmol* (1994); **112**: 1356-1362.

[11] Qiao, H., Hisatomi, T., Sonoda, K. H., Kura, S., Sassa, Y., Kinoshita, S., Nakamura, T., Sakamoto, T., and Ishibashi, T. 'The characterisation of hyalocytes: the origin, phenotype, and turnover'. *Br J Ophthalmol* (2005); **89**: 513-517.

[12] Gloor, B. P. 'Development of the vitreous body and zonula'. *Albrecht Von Graefes Arch Klin Exp Ophthalmol* (1973); **187**: 21-44.

[13] Gloor, B. P. 'Radioisotopes for research into vitreous and zonule'. *Adv Ophthalmol* (1978); **36**: 63-73.

[14] Jacobson, B. 'Biosynthesis of hyaluronic acid in the vitreous. V. Studies on a particulate hyalocyte glycosyl transferase'. *Exp Eye Res* (1978); **27**: 247-258.

[15] Jacobson, B. 'Identification of sialyl and galactosyl transferase activities in calf vitreous hyalocytes'. *Curr Eye Res* (1984); **3**: 1033-1041.

[16] Jacobson, B. 'Degradation of glycosaminoglycans by extracts of calf vitreous hyalocytes'. *Exp Eye Res* (1984); **39**: 373-385.

[17] Osterlin, S. E. and Jacobson, B. 'The synthesis of hyaluronic acid in vitreous. I. Soluble and particulate transferases in hyalocytes'. *Exp Eye Res* (1968); **7**: 497-510.

[18] Osterlin, S. E. 'The synthesis of hyaluronic acid in the vitreous. IV. Regeneration in the owl monkey'. *Exp Eye Res* (1969); **8**: 27-34.

[19] Osterlin, S. E. 'The synthesis of hyaluronic acid in vitreous. 3. In vivo metabolism in the owl monkey'. *Exp Eye Res* (1968); **7**: 524-533.

[20] Newsome, D. A., Linsenmayer, T. F., and Trelstad, R. L. 'Vitreous body collagen. Evidence for a dual origin from the neural retina and hyalocytes'. *J Cell Biol* (1976); **71**: 59-67.

[21] Zhu, M., Penfold, P. L., Madigan, M. C., and Billson, F. A. 'Effect of human vitreous and hyalocyte-derived factors on vascular endothelial cell growth'. *Aust N Z J Ophthalmol* (1997); **25**: 57-60.

[22] Noda, Yoshihiro, Hata, Yasuaki, Hisatomi, Toshio, Nakamura, Yuka, Hirayama, Kumiko, Miura, Muneki, Nakao, Shintaro, Fujisawa, Kimihiko, Sakamoto, Taiji, and Ishibashi et, al. 'Functional Properties of Hyalocytes under PDGF-Rich Conditions'. *Invest Ophthalmol* (2004); **45**: 2107-2114.

[23] Lutty, G. A., Merges, C., Threlkeld, A. B., Crone, S., and McLeod, D. S. 'Heterogeneity in localization of isoforms of TGF-beta in human retina, vitreous, and choroid'. *Invest Ophthalmol* (1993); **34**: 477-487.

[24] Balazs, Endre A., Laurent, Torvard C., and Laurent, Ulla G. B. 'Structure of the vitreous body. VI. Biochemical changes during development'. *J Biol Chem* (1959); **234**: 422-430.

[25] Rose, R. C. and Bode, A. M. 'Ocular ascorbate transport and metabolism'. *Comp Biochem Physiol A* (1991); **100**: 273-285.

[26] Reiss, G. R., Werness, P. G., Zollman, P. E., and Brubaker, R. F. 'Ascorbic acid levels in the aqueous humor of nocturnal and diurnal mammals'. *Arch Ophthalmol* (1986); **104**: 753-755.

[27] Rose, Richard C., Richer, Stuart P., and Bode, Ann M. 'Ocular oxidants and antioxidant protection'. *Proc Soc Exp Biol Med* (1998); **217**: 397-407.

[28] Heckelen, A., Hermel, M., Kondring, B., and Schrage, N. F. 'Ascorbic acid reversibly inhibits proliferation of retinal pigment epithelial cells'. *Acta Ophthalmol Scand* (2004); **82**: 564-568.

[29] Hermel, M., Heckelen, A., Kirchhof, B., and Schrage, N. F. 'Inhibitory effect of ascorbic acid on human retinal pigment epithelial cell proliferation compared to cytostatic drugs - influence of histamine'. *Inflamm Res* (2001); **50**: S93-S95.

[30] Bohmer, J. A., Sellhaus, B., and Schrage, N. F. 'Effects of ascorbic acid on retinal pigment epithelial cells'. *Curr Eye Res* (2001); **23**: 206-214.

[31] Wunderlich, Kerstin, Knorr, M., Dartsch, P. C., Steuhl, H. P., and Thiel, H. J. 'Cytotoxic effect of ascorbic acid on cultured bovine lens epithelial cells'. *Ophthalmologe* (1992); **89**: 313-318.

[32] Hanashima, Carina and Namiki, Hideo. 'Cytotoxic and growth inhibitory effect of ascorbic acid on cultured bovine vascular endothelial cells'. *Zoolog Sci* (1999); **16**: 99-104.

[33] Sawaguchi, S., Yue, B. Y., Chang, I. L., Wong, F., and Higginbotham, E. J. 'Ascorbic acid modulates collagen type I gene expression by cells from an eye tissue--trabecular meshwork'. *Cell Mol Biol (Noisy-le-grand)* (1992); **38**: 587-604.

[34] Pastor, J. C., Rodriguez de la Rua, E., and Martin, F. 'Proliferative vitreoretinopathy: Risk factors and pathobiology'. *Prog Retin Eye Res* (2002); **21**: 127-144.

[35] Takano, S., Ishiwata, S., Nakazawa, M., Mizugaki, M., and Tamai, M. 'Determination of ascorbic acid in human vitreous humor by high-performance liquid chromatography with UV detection'. *Curr Eye Res* (1997); **16**: 589-594.

[36] Kim, Young Jo, Sah, Robert L. Y., Doong, Joe Yuan, and Grodzinsky, Alan J. 'Fluorometric assay of DNA in cartilage explants using Hoechst 33258'. *Anal Biochem* (1988); **174**: 168-176.

[37] McGowan, K. B., Kurtis, M. S., Lottman, L. M., Watson, D., and Sah, R. L. 'Biochemical quantification of DNA in human articular and septal cartilage using PicoGreen(R) and Hoechst 33258'. *Osteoarthritis Cartilage* (2002); **10**: 580-587.

[38] Ng, Kee W., Leong, David T. W., and Hutmacher, Dietmar W. 'The challenge to measure cell proliferation in two and three dimensions'. *Tissue Eng* (2005); **11**: 182-191.

[39] Kellner, K., Schulz, M. B., Gopferich, A., and Blunk, T. 'Insulin in tissue engineering of cartilage: A potential model system for growth factor application'. *J Drug Target* (2001); **9**: 439-448.

[40] Lieb, E., Milz, S., Vogel, T., Hacker, M., Dauner, M., and Schulz, M. B. 'Effects of transforming growth factor beta1 on bonelike tissue formation in three-dimensional cell culture. I. Culture conditions and tissue formation'. *Tissue Eng* (**10**: 1399-1413.

[41] Kim, Y. J., Sah, R. L., Doong, J. Y., and Grodzinsky, A. J. 'Fluorometric assay of DNA in cartilage explants using Hoechst 33258'. *Anal Biochem* (1988); **174**: 168-176.

[42] Farndale, R. W., Buttle, D. J., and Barrett, A. J. 'Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue'. *Biochim Biophys Acta* (1986); **883**: 173-177.

[43] Taylor, K. B. and Jeffree, G. M. 'A new basic metachromatic dye, I:9-dimethyl methylene blue'. *Histochem J* (1969); **1**: 199-204.

[44] Woessner, J. F., Jr. 'The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid'. *Arch Biochem Biophys* (1961); **93**: 440-447.

[45] Francois J. and Victoria-Troncoso V. 'Transplantation of vitreous cell culture'. *Ophthalmic Res* (1972); **4**: 270-280.

[46] Hanashima, Carina and Namiki, Hideo. 'Reduced viability of vascular endothelial cells by high concentrations of ascorbic acid in vitreous humor'. *Cell Biol Int* (1999); **23**: 287-298.

[47] Francois J., Victoria-Troncoso V., and Eekhout M. 'The micromorphology of Hyalocytes in tissue culture'. *Ophthalmic Res* (1973); **5**: 290-299.

[48] Mayne, Richard, Vail, Margaret S., Mayne, Pauline M., and Miller, Edward J. 'Changes in Type of Collagen Synthesized as Clones of Chick Chondrocytes Grow and Eventually Lose Division Capacity'. *PNAS* (1976); **73**: 1674-1678.

[49] der Mark, K, Gauss, V., der Mark, H., and Muller, P. 'Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture'. *Nature* (1977); **267**: 531-532.

[50] Hilwig, I. 'Hyalocytes in tissue culture'. *Z Zellforsch Mikrosk Anat* (1971); **113**: 544-552.

[51] Padh, H. 'Cellular functions of ascorbic acid'. *Biochem Cell Biol* (1990); **68**: 1166-1173.

[52] Hitomi, K. and Tsukagoshi, N. 'Role of ascorbic acid in modulation of gene expression'. *Subcell Biochem* (1996); **25**: 41-56.

[53] Sullivan, T. A., Uschmann, B., Hough, R., and Leboy, P. S. 'Ascorbate modulation of chondrocyte gene expression is independent of its role in collagen secretion'. *J Biol Chem* (1994); **269**: 22500-22506.

[54] Mayne, R., Brewton, R. G., Mayne, P. M., and Baker, J. R. 'Isolation and characterization of the chains of type V/type XI collagen present in bovine vitreous'. *J Biol Chem* (1993); **268**: 9381-9386.

[55] Snead, M. P. and Yates, J. R. 'Clinical and Molecular genetics of Stickler syndrome'. *J Med Genet* (1999); **36**: 353-359.

[56] Blaschke, U. K., Eikenberry, E. F., Hulmes, D. J., Galla, H. J., and Bruckner, P. 'Collagen XI nucleates self-assembly and limits lateral growth of cartilage fibrils'. *J Biol Chem* (2000); **275**: 10370-10378.

Chapter 6

Pyruvate Modulates the Effect of Ascorbic Acid on Hyalocytes

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Abstract

In vitro cell culture systems are widely employed to enable investigations into cells. Because the culture conditions have a significant influence on the obtained results, they have to be described exactly to allow evaluation of the results. In that context, the present study indicates that the label “DMEM” does not always describe identical media; the effects of ascorbic acid on hyalocytes, the cells of the vitreous body, were observed to be dependent on the DMEM type used. As this observation could be ascribed to pyruvate, the interdependent effects of ascorbic acid and pyruvate were further elucidated. Specifically, 200 µg/ml ascorbic acid was demonstrated to enhance hyalocyte proliferation in the presence of 110 µg/ml pyruvate, whereas it clearly inhibited cell proliferation in the absence of pyruvate. Pyruvate supplementation without ascorbic acid in the medium showed no effects. Furthermore, the accumulation of collagens and glycosaminoglycans was increased by supplementation of ascorbic acid without pyruvate in the medium. Pyruvate diminished this effect dose-dependently; with 110 µg/ml pyruvate in the medium, no effect of ascorbic acid on the accumulation of extracellular matrix by hyalocytes was apparent. The present study thus emphasizes the importance of the applied culture conditions on the results of in vitro studies. Ascorbic acid and pyruvate were demonstrated to be important factors for in vitro cultivation of hyalocytes.

Introduction

The vitreous body is the main compartment of the mammalian eye. It displays a transparent hydrogel consisting of different collagens, especially type II, and glycosaminoglycans with a water content of about 98 %[1]. The center of the vitreous body is free of cells, however, in the cortex of the tissue and near the vitreous base, there are a sparse number of cells referred to as hyalocytes[2-4]. Although these cells were described for the first time in the middle of the 19th century[5], they are still only poorly characterized[6]. Hyalocytes were shown to be derived from the bone marrow[7] and belong to the monocyte/macrophage lineage[2]. Physiologically, the cells are thought to be involved in the maintenance of the vitreous as a transparent tissue[8, 9], probably due to their ability to accumulate collagens[10, 11] and glycosaminoglycans[12-14]. Pathologically, hyalocytes are connected to severe vitreoretinal diseases such as epiretinal membrane formation, macular holes, and diabetic macular edema[15, 16]. However, further investigations into hyalocyte characteristics are needed to clarify both their physiological and pathological roles within the vitreous body.

In vitro cell culture systems are widely employed to investigate both cell characteristics and cell functions[17, 18]. In vitro cell culture is thus a valuable tool for further investigations into hyalocytes. It has to be kept in mind, however, that the culture conditions can have a significant influence on the performed study. To this end, a precise and detailed description of the culture conditions employed is mandatory within a scientific publication. In most of the recently published reports about in vitro hyalocyte culture, Dulbecco's modified Eagle medium (DMEM) was used as culture medium. However, as there are many DMEM variations provided by several companies (for example, high or low glucose, with or without glutamine and pyruvate), the name "DMEM" is not clear. In a preliminary study, the effect of ascorbic acid on hyalocyte proliferation differed when two different DMEM variations were used. Therefore, the present study addressed the influence of different DMEM compositions on the influence of ascorbic acid on hyalocyte proliferation. Because the preliminary data clearly indicated that pyruvate played a role in the observed differences, the effect of pyruvate in combination with ascorbic acid was studied on hyalocyte proliferation. Additionally, the effect of the two factors on accumulation of extracellular matrix components was addressed.

Materials and Methods

Materials

L-Ascorbic acid (cell culture quality), cysteine, chondroitin sulfate A from bovine trachea, dimethylmethylene blue, and highly polymerized deoxyribonucleic acid from calf thymus were purchased from Sigma (Steinheim, Germany). Fetal calf serum (South America, Batch Nr. 40A0044K), Dulbecco's phosphate buffered saline (PBS), penicillin/streptomycin, Dulbecco's modified Eagle medium (DMEM) without pyruvate [abbr. DMEM 2] (DMEM, 4.5 g/l glucose, with glutamine, without pyruvate), low glucose DMEM with pyruvate [abbr. DMEM 3] (DMEM, 1.0 g/l glucose, with glutamine and pyruvate), low glucose DMEM with pyruvate and glutamax® [abbr. DMEM 4] (DMEM, 1.0 g/l glucose, with glutamax® and pyruvate), and 0.25 % Trypsin-EDTA were obtained from Invitrogen (Karlsruhe, Germany). DMEM including pyruvate [abbr. DMEM 1] (DMEM, low glucose, with glutamine and pyruvate) was obtained from Biochrom (Berlin, Germany). Dimethylaminobenzaldehyde, chloramine T, isopropanol, sodium chloride, glucose, Na_2HPO_4, and Na_2EDTA were bought from Merck (Darmstadt, Germany). Hydroxyproline and perchloric acid were purchased from Fluka (Neu-Ulm, Germany). Hoechst 33258 dye was obtained from Polysciences (Warrington, PA, USA). Papainase and collagenase type II were purchased from Worthington (Lakewood, NJ, USA). All other cell culture materials were purchased from Corning (Bodenheim, Germany). Buffer for papainase digestion (PBE) was composed of 100 mmol Na_2HPO_4, 10 mmol Na_2EDTA, and 5 mmol cysteine in water, adjusted to pH 6.5.

Cell culture

Hyalocytes from freshly enucleated eyes were isolated as previously described (cf. chapters 3 and 5). In brief, after dissection of the vitreous bodies, the cells were isolated by enzymatic digestion of the tissue using collagenase type II. After centrifugation, hyalocytes obtained from about 20 eyes were pooled and seeded in a T-25 culture flask for cultivation in standard culture medium (SCM) supplemented with 50 µg/ml ascorbic acid. SCM was composed of DMEM 1 containing 15 % fetal calf serum and 100 IU penicillin/100 µg/ml streptomycin. Medium was exchanged three times a week. After 9 days the cells were passaged using trypsin and, subsequent to seeding with 2000 cells/cm², cultivated for another 7 days in SCM without ascorbic acid supplementation. To elucidate the influence of different DMEM compositions as well as the effects of ascorbic acid and pyruvate on hyalocytes, 50 000 cells after second passage were seeded per T-25 flask and cultured for 4 days with medium exchange after 2 days. After 4 days, the cells were completely trypsinized for 30 min, freeze-

dried and digested using papainase as previously described (cf. chapter 5). Cell number as well as glycosaminoglycan and collagen content were determined as described below.

For investigations into the influence of different DMEM compositions on the ascorbic acid effect on hyalocytes, four different DMEM compositions (displayed in Table 1) were used. Furthermore, 3.5 g/l glucose or 0.03 % NaCl was added to DMEM 1. To study pyruvate in combination with ascorbic acid, different concentrations (pyruvate: 0, 20, 110 µg/ml; ascorbic acid: 0, 10, 200 µg/ml) of the two factors alone, as well as in combination with each other, were supplemented in DMEM 2.

	Glucose [g/l]	Glutamine	Pyruvate [110 mg/l]	Osmolarity
DMEM 1	1.0	+	+	310 mOsmol
DMEM 2	4.5	+	-	336 mOsmol
DMEM 1 + gluc	4.5	+	+	330 mOsmol
DMEM 1 + NaCl	1.0	+	+	335 mOsmol
DMEM 3	1.0	+	+	317 mOsmol
DMEM 4	1.0	Glutamax®	+	322 mOsmol

Table 1: *Variations in DMEM media composition and osmolarity.*

Determination of cell number and ECM contents

Cell number was determined by measuring the DNA amount using Hoechst 33258 dye[19-21]. In brief, the emission intensity of the intercalating dye was measured at 458 nm upon excitation at 365 nm. The fluorescence of Hoechst 33258 dye is correlated with the absolute amount of DNA in the sample. To calculate the cell number, an average amount of 9.96 pg DNA per cell was assumed[22].

Glycosaminoglycan content was measured spectroscopically as previously described[23, 24]. In brief, after digestion of interfering proteins with papainase, the glycosaminoglycan content was determined by a colorimetric reaction with dimethylmethylene blue followed by measurement of absorption at 525 nm. Chondroitin sulfate was used to produce the standard curve (cf. chapter 4).

The collagen content was determined by measuring the amount of hydroxyproline, an amino acid that exists in significant amounts exclusively in collagen, according to Woessner et al. [25] with some modifications described earlier (cf. chapter 4 and 5). In brief, after hydrolysis of the sample, hydroxyproline was oxidized with chloramine T and, subsequent to the coupling

reaction with dimethylaminobenzaldehyde, the absorption of the resulting dye was measured. A standard curve of hydroxyproline in the PBE/cysteine served as reference.

Statistics

All data are presented as means ± standard deviation. One-way or two-way ANOVA analysis was used in conjunction with a subsequent post-hoc test (Tukey's test) to analyze the obtained results. SigmaStat for Windows (version 3.0.1) was used to calculate these statistics.

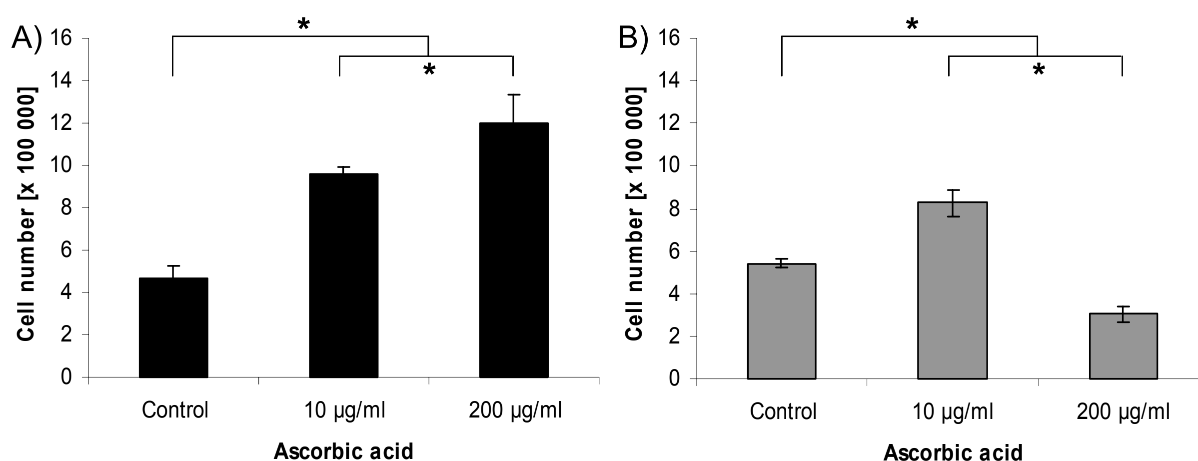

Figure 1: *Effect of ascorbic acid on hyalocyte proliferation is dependent on the composition of DMEM used in culture, n=4. A) Ascorbic acid supplementation to DMEM 1 clearly increased the cell number; B) Ascorbic acid supplementation to DMEM 2 had varied effects on the cell number: whereas 10 µg/ml increased the cell number, addition of 200 µg/ml decreased the cell number compared to control.*

Results

Effect of ascorbic acid on hyalocytes is dependent on the DMEM composition

Ascorbic acid influenced hyalocyte proliferation differently depending on which DMEM variation was used in cell culture. When using DMEM 1 as basal culture medium, ascorbic acid supplementation clearly enhanced the cell proliferation (Figure 1 A). Adding 10 µg/ml of ascorbic acid doubled the cell number, and the presence of 200 µg/ml within the culture medium led to an almost 3-fold increase. In contrast, addition of ascorbic acid to DMEM 2 resulted in a different effect (Figure 1 B). Although 10 µg/ml significantly enhanced hyalocyte proliferation, supplementation of 200 µg/ml clearly decreased the cell number compared to the control. To determine whether the high glucose or the osmotic pressure of DMEM 2 caused this effect, DMEM 1 was supplemented with glucose and sodium chloride. Furthermore, two DMEM types with low glucose levels were investigated. In all of these

media, a comparable increase in the cell number was observed when supplementing with either 10 or 200 µg/ml ascorbic acid (Figure 2), except when DMEM 2 was used. Here again, a decrease in cell proliferation was observed upon supplementation with 200 µg/ml ascorbic acid. Moreover, the increase in the cell number due to the addition of 10 µg/ml ascorbic acid was significantly smaller in DMEM 2 compared to all other DMEM types.

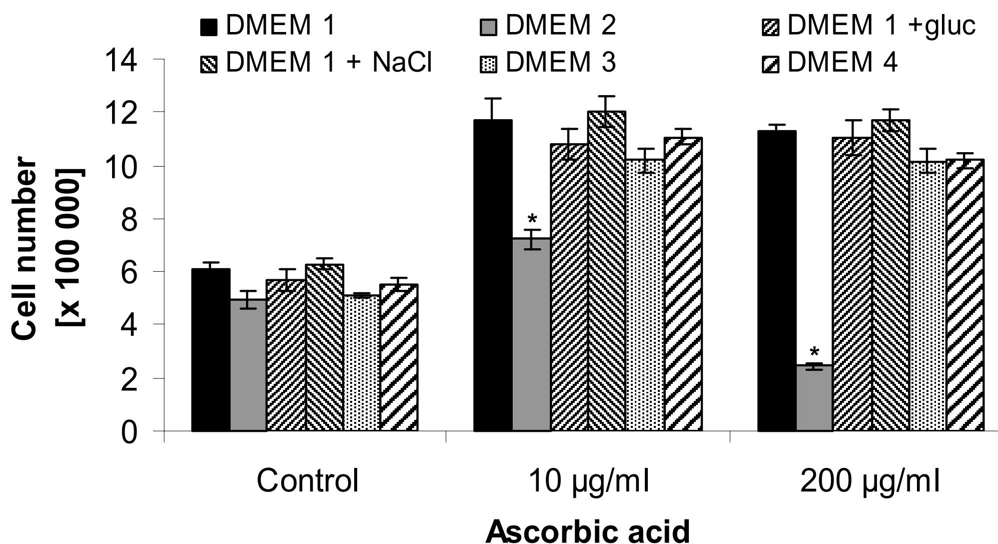

Figure 2: *Effect of ascorbic acid on hyalocytes is dependent on the variation of DMEM used, n=4; * indicates statistically significant difference compared to all other DMEM types supplemented with the same amount of ascorbic acid; ascorbic acid affected hyalocyte proliferation differently in DMEM 2 compared to all other DMEM types.*

Effect of varying ascorbic acid/ pyruvate supplementation on hyalocyte proliferation

Because ascorbic acid supplementation of DMEM 2 did not produce the same effect on hyalocytes as the other compositions of DMEM (Figure 2), and the only difference between DMEM 2 and all other DMEM compositions investigated is its lack of pyruvate, it seemed logical to investigate the effect of varying ascorbic acid/pyruvate concentrations on hyalocyte proliferation (Figure 3). For clarity, the obtained values are displayed in two different ways; Figure 3 A displays the effect of pyruvate with respect to the ascorbic acid concentration present in the medium, whereas Figure 3 B shows the effect of ascorbic acid with respect to the pyruvate concentration within the medium. Without ascorbic acid present in the culture medium, supplementation of pyruvate showed no effect on the cell proliferation (Figure 3 A). With 10 µg/ml ascorbic acid in the medium, 20 µg/ml pyruvate exhibited a slight, but not significant increase in the cell number, whereas this increase was clear and significant upon 110 µg/ml pyruvate supplementation. Moreover, pyruvate drastically increased hyalocyte proliferation in DMEM containing 200 µg/ml ascorbic acid up to a 4-fold value. Looking at

the data from a different perspective, ascorbic acid modulated the cell proliferation differently depending on the pyruvate concentration in the medium (Figure 3 B). Without pyruvate in the medium, 10 µg/ml ascorbic acid slightly increased the cell number, whereas 200 µg/ml decreased the obtained number of cells. In medium containing 20 µg/ml pyruvate, similar effects of ascorbic acid were observed. In contrast, with 110 µg/ml pyruvate in the medium, the measured cell number was about double due to supplementation of either 10 or 200 µg/ml of ascorbic acid.

According to the two way analysis of variance, the difference in the mean values among the different levels of ascorbic acid or pyruvate, respectively, were greater than would be expected by chance after allowing for the effects of the other factor ($p<0.001$), indicating both factors influence hyalocyte proliferation. Furthermore, the effects of different levels of ascorbic acid depended on the pyruvate level present in the medium, indicating a statistically significant interaction between both factors ($p<0.001$).

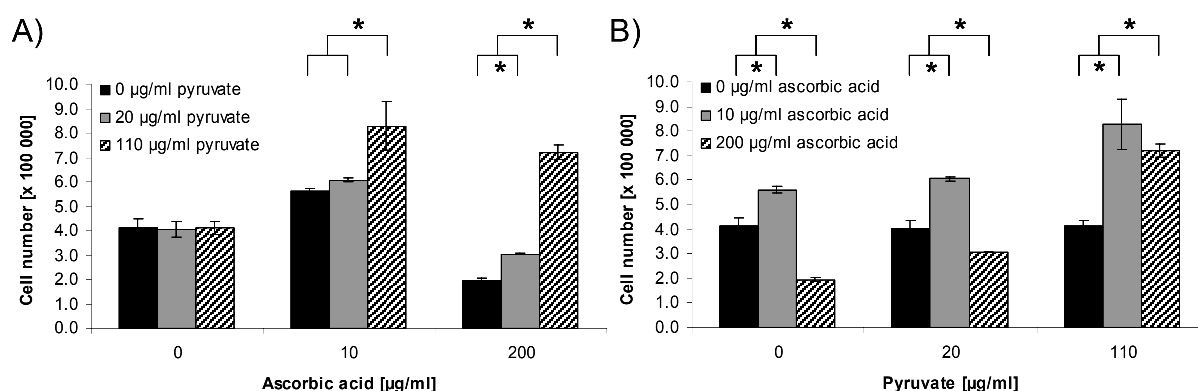

Figure 3: *Influence of combinations of ascorbic acid and pyruvate concentrations on the proliferation of hyalocytes, n=3. The study was performed in duplicate; representative data are shown. A) Effect of varying pyruvate concentrations dependent on the ascorbic acid concentration. B) Effect of varying ascorbic acid concentrations dependent on the pyruvate concentration. Both factors influenced cell proliferation, and a clear interaction between the factors was observed.*

Effect of varying ascorbic acid/ pyruvate supplementation on ECM production of hyalocytes

Pyruvate exhibited no effect on the GAG accumulation per cell if no or only 10 µg/ml ascorbic acid was available in the culture medium (Figure 4). However, with 200 µg/ml of ascorbic acid in the medium and without pyruvate, a significantly higher production of GAG per cell was observed compared to the groups with low ascorbic acid. Increasing amounts of pyruvate decreased these high GAG levels to values observed in the groups with no or 10 µg/ml of ascorbic acid in the medium. In other words, pyruvate diminished the enhancing effect of ascorbic acid on the GAG accumulation per cell.

Two way ANOVA indicated both factors significantly influence the GAG production per cell ($p<0.001$). Furthermore, a significant interaction between ascorbic acid and pyruvate was found ($p<0.001$).

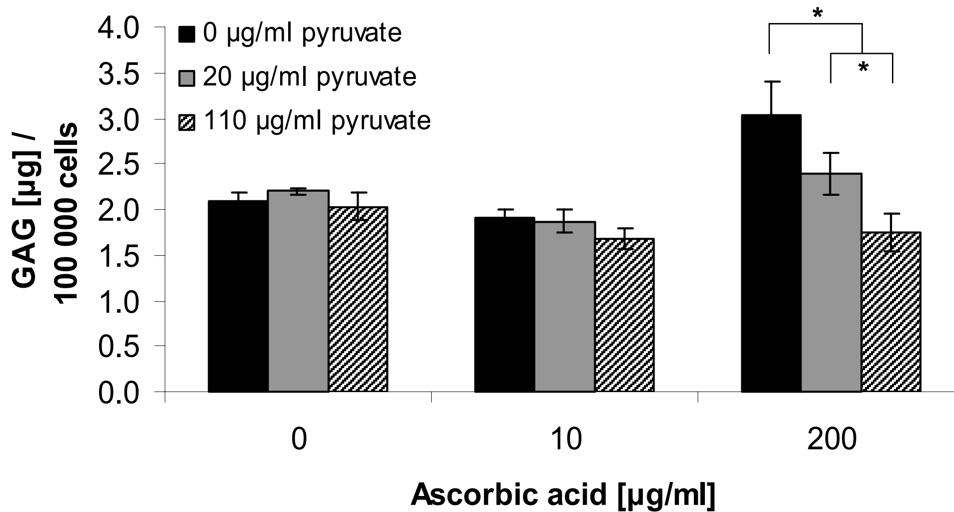

Figure 4: *Influence of ascorbic acid/pyruvate combinations on the GAG accumulation per 100 000 hyalocytes, n=3. Study was performed in duplicate; representative data are shown. With 200 µg/ml ascorbic acid in the medium, an enhanced GAG production per cell was observed; increasing amounts of pyruvate, however, diminished this enhancing effect of ascorbic acid.*

As seen with GAG accumulation, varying ascorbic acid/pyruvate supplementation affected the collagen accumulation per hyalocyte (Figure 5). When no more than 10 µg/ml of ascorbic acid was present in the medium, increasing amounts of pyruvate showed no influence on the collagen production per cell. In contrast, without pyruvate in the medium, 200 µg/ml ascorbic acid enhanced the collagen production of the cells significantly. Addition of 20 µg/ml pyruvate diminished the enhancing effect of ascorbic acid slightly, whereas supplementation of 110 µg/ml pyruvate completely eliminated the enhancing effect.

According to two way analysis of variance, ascorbic acid ($p<0.001$) as well as pyruvate ($p<0.005$) significantly influenced the collagen accumulation per cell. Furthermore, the interaction between these two factors was again significant ($p<0.005$).

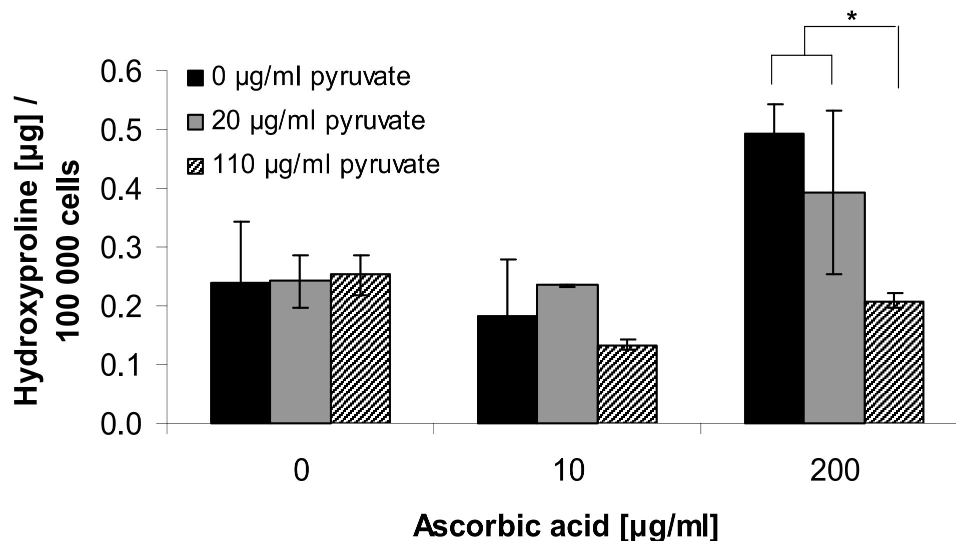

***Figure 5:** Influence of ascorbic acid/pyruvate combinations on the collagen accumulation per hyalocyte, n=3. With 200 µg/ml ascorbic acid in the medium, an enhanced collagen production per cell was observed; increasing amounts of pyruvate, however, diminished this enhancing effect of ascorbic acid.*

Discussion

In a previous study performed in our lab, proliferation of hyalocytes was demonstrated to be clearly influenced by ascorbic acid (Chapter 5). However, as indicated by a preliminary study using slightly different DMEM variations (Figure 1), this influence was affected by the culture medium the study was performed with. To clarify which property of the media caused this discrepancy, a variety of factors were studied. Neither the glucose level nor the osmotic pressure played a role in the effect of ascorbic acid on hyalocyte proliferation, as no differences between DMEM 1 supplemented with glucose or sodium chloride and pure DMEM 1 were obvious (Figure 2). The identical effects of ascorbic acid on hyalocytes cultivated in DMEM 1, DMEM 3 and DMEM 4 (Figure 2) led to the hypothesis that pyruvate was responsible for the modified effects of ascorbic acid in DMEM 2, as this factor represented the only difference between DMEM 2 and all other tested variations. This assumption could be verified by supplementation of pyruvate in different combinations with ascorbic acid to DMEM 2 (Figure 3).

More detailed investigations into ascorbic acid/pyruvate combinations indicated hyalocyte proliferation to be highly dependent on both factors; fastest cell proliferation was observed when the highest pyruvate concentration (110 µg/ml) was used in combination with either 10 or 200 µg/ml ascorbic acid (Figure 3 A). Pyruvate alone had no significant impact on proliferation, however, its presence was mandatory for ascorbic acid to exert its significant

enhancing effect on proliferation. In the absence of pyruvate, high ascorbic acid concentrations no longer enhanced cell proliferation but actually had a negative effect (Figure 3 B). In similar studies devoted to investigating the influence of pyruvate alone on cell proliferation, effects were dependent on the cell type under study; whereas proliferation of Ehrlich's ascites tumor cells, for example, was inhibited by high concentrations of pyruvate[26], small concentrations of pyruvate were essential for cell growth and survival in osteoblast cultures[27]. In both studies, however, the effect of pyruvate without ascorbic acid was measured and the media used did not contain ascorbic acid at all. Moreover, although there are a number of studies on the effect of pyruvate on cell proliferation[28-30], to the best of our knowledge, no work addressing the interaction between ascorbic acid and pyruvate has been published to date.

In addition to affecting hyalocyte proliferation, ascorbic acid and pyruvate combinations modified the accumulation of extracellular matrix components (Figure 4 and 5). For the two measured ECM components, GAG and collagen, pyruvate exhibited no influence when no or only small amounts of ascorbic acid were present. However, with 200 µg/ml of ascorbic acid present in the medium, pyruvate clearly decreased the accumulation of GAG and collagen per cell. Therefore, provided that high amounts of ascorbic acid were available, pyruvate seemed to favor cell proliferation (Figure 3) while inhibiting the accumulation of ECM components (Figure 4 and 5). Similar results were observed by Wasilenko and Marchok studying primary cultures of rat trachea epithelial cells[31]. For these cells, cultivated in Waymouth's medium MB 752/1 which contains 17.5 µg/ml ascorbic acid, pyruvate promoted proliferation of the epithelial cells while inhibiting their differentiation.

According to the presented data, pyruvate seems to modulate ascorbic acid's effects on hyaloycte proliferation as well as ECM accumulation. Although the exact mechanism of this interaction remains to be elucidated, some possibilities will be discussed below. Pyruvate is known to be an alternative energy substrate for certain cells, such as, for example, retina pigment epithelial cells[32]. Therefore, the observed effects might be linked to hyalocyte metabolism. However, because this study was performed in DMEM containing 4.5 g/l glucose, one of the most important energy sources for cells, it seems unlikely that the effect is related to pyruvate's ability to serve as an alternative cellular energy source.

In addition to its function as an energy source, pyruvate exhibits oxidative protection in several cell types such as neurons[33], thymocytes[34], and bovine pulmonary epithelial cells[35]. This is at least partially due to its ability to scavenge reactive oxygen species (ROS)[36].

Pyruvate reacts non-enzymatically with hydrogen peroxide to form carbon dioxide, water and carboxylic acid, thereby preventing the formation of hydroxyl radicals (Figure 6)[37].

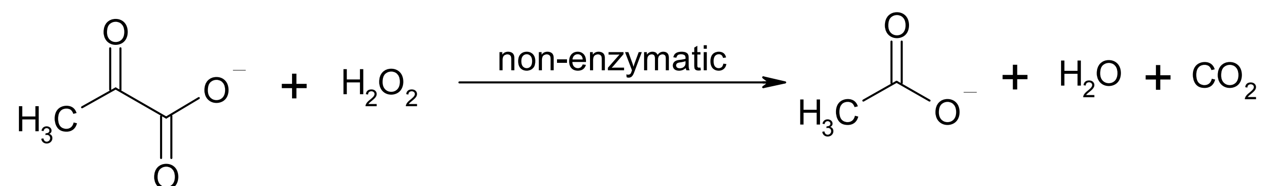

Figure 6: *Non-enzymatic detoxification of hydrogen peroxide by reaction with pyruvate*

Ascorbic acid, a widely acknowledged antioxidant, can also act prooxidatively[38-40], especially when aided catalytically by trace amounts of copper[41]. To that end, it is important to consider whether the observed interaction with the antioxidative pyruvate is at least partly based on redox processes. Redox processes may explain the observed effects on the ECM accumulation of hyalocytes. With media containing 200 µg/ml ascorbic acid, a concentration double the physiological one and, therefore, eventually prooxidative[42, 43], the antioxidative pyruvate exerted an effect on the ECM production per cell. However, this assumption does not explain the observed effects on hyalocyte proliferation, since proliferation was already affected by pyruvate with only 10 µg/ml ascorbic acid in the medium. This small amount represents only one-tenth of the physiological concentration of ascorbic acid and is therefore unlikely to exhibit any prooxidative effect. Mechanisms other than redox processes must thus be considered when seeking to explain the interplay between ascorbic acid and pyruvate.

In a prior study (chapter 5), the accumulation of collagen per cell was significantly increased by the addition of 200 µg/ml ascorbic acid to media (DMEM 1) containing 110 µg/ml of pyruvate. This is in direct contrast to the results obtained in the presently described study (Figure 5). As the medium in the prior study contained only 1.0 g/l glucose compared to 4.5 g/l in the present study, glucose may be playing a role in the observed cellular processes. This assumption is supported by the fact that the inhibition of the proliferation of Ehrlich's ascites tumor cells was found to depend on the presence of glucose in the medium[26].

As a last hypothesis, ascorbic acid and pyruvate could influence the cellular uptake of each other and, thereby induce the observed cellular effects. The cellular uptake of radiolabeled ascorbate into osteoblast-like cells[44] and astrocytes[45], however, was not affected by pyruvate in concentrations similar to the ones used in the present study. Therefore, these observations contradict the hypothesis that pyruvate inhibits the cellular uptake of ascorbic acid. The other alternative is that ascorbic acid modulated the cellular uptake of pyruvate, as the effects of pyruvate were only observed in the presence of ascorbic acid. Recent studies investigating the radical scavenger effect of pyruvate identified monocarboxylate transporters (MCT),

especially the MCT 1 to MCT 4 isoforms, as mediating the transport of pyruvate across plasma membranes[46], thereby playing a pivotal role in mechanisms related to the cytoprotective property of pyruvate[47, 48]. However whether ascorbic acid has any influence on the transport of pyruvate via MCTs is currently unknown.

To summarize the possible mechanisms proposed above, the observed effects of ascorbic acid and pyruvate seem to be, at least in part, due to their ability to influence processes induced by radicals such as reactive oxygen species. Furthermore, there are also indications that other mechanisms, dependent on glucose and/or on transport processes across the plasma membrane, are involved. To verify or reject these hypotheses, further investigations into the exact mechanisms are necessary. Moreover, it should be determined whether the displayed effects of ascorbic acid and pyruvate have any clinical relevance for the treatment of the vitreoretinal diseases that hyalocytes are assumed to be involved in[6]. Pyruvate, for example, was shown to inhibit the cataract-inducing effect of high galactose concentrations on lens epithelial cells in vitro[49, 50] and in vivo[51]. Additionally, α-keto-carboxylates, especially pyruvate, were shown to enhance the antioxidant power of ascorbic acid and thereby prevent oxidation of low-density lipoproteins as well as cell death of macorphages in vitro[52]. According to the authors of that study, the combination of ascorbic acid and pyruvate could, therefore, have implications for strategies aimed at attenuating atherosclerosis.

Conclusions

The proliferation of hyalocytes, as well as their accumulation of glycosaminoglycans and collagens, was found to be influenced by ascorbic acid and pyruvate. This has significant implications for the choice of cell culture media such as DMEM that may contain both factors. Moreover, a clear interaction between the two factors could be observed. The mechanism of this interaction remains to be clarified, however, the present study indicates that both ascorbic acid and pyruvate modulate hyalocyte behavior and are both, therefore, important for in vitro hyalocyte culture. Even more importantly, since pyruvate is being studied for the prevention of cataracts, a disorder connected to opacifications of the physiologically clear lens, it may also have implications for diseases of the vitreous body.

References

[1] Bishop, P. N. 'Structural macromolecules and supramolecular organisation of the vitreous gel'. *Prog Retin Eye Res* (2000); **19**: 323-344.

[2] Lazarus, H. S. and Hageman, G. S. 'In situ characterization of the human hyalocyte'. *Arch Ophthalmol* (1994); **112**: 1356-1362.

[3] Ogawa, K. 'Scanning electron microscopic study of hyalocytes in the guinea pig eye'. *Archives Histol Cytol* (2002); **65**: 263-268.

[4] Salu, P., Claeskens, W., De Wilde, A., Hijmans, W., and Wisse, E. 'Light and electron microscopic studies of the rat hyalocyte after perfusion fixation'. *Ophthalmic Res* (1985); **17**: 125-130.

[5] Hamburg, A. 'Some investigations on the cells of the vitreous body'. *Ophthalmologica* (1959); **138**: 81-107.

[6] Noda, Yoshihiro, Hata, Yasuaki, Hisatomi, Toshio, Nakamura, Yuka, Hirayama, Kumiko, Miura, Muneki, Nakao, Shintaro, Fujisawa, Kimihiko, Sakamoto, Taiji, and Ishibashi et, al. 'Functional Properties of Hyalocytes under PDGF-Rich Conditions'. *Invest Ophthalmol* (2004); **45**: 2107-2114.

[7] Qiao, H., Hisatomi, T., Sonoda, K. H., Kura, S., Sassa, Y., Kinoshita, S., Nakamura, T., Sakamoto, T., and Ishibashi, T. 'The characterisation of hyalocytes: the origin, phenotype, and turnover'. *Br J Ophthalmol* (2005); **89**: 513-517.

[8] Zhu, M., Penfold, P. L., Madigan, M. C., and Billson, F. A. 'Effect of human vitreous and hyalocyte-derived factors on vascular endothelial cell growth'. *Aust N Z J Ophthalmol* (1997); **25**: 57-60.

[9] Lutty, G. A., Merges, C., Threlkeld, A. B., Crone, S., and McLeod, D. S. 'Heterogeneity in localization of isoforms of TGF-beta in human retina, vitreous, and choroid'. *Invest Ophthalmol* (1993); **34**: 477-487.

[10] Newsome, D. A., Linsenmayer, T. F., and Trelstad, R. L. 'Vitreous body collagen. Evidence for a dual origin from the neural retina and hyalocytes'. *J Cell Biol* (1976); **71**: 59-67.

[11] Kobuch, K., Wild, B., Eckert, E., Fischbach, C., and Gabel V.P. 'Expression of Collagen Type II by Hyalocytes In Vitro - First Steps Towards a Biological Vitreous Substitute', presented at 'ARVO Annual Meeting', Fort Lauderdale, FL, USA (2002)

[12] Osterlin, S. E. and Jacobson, B. 'The synthesis of hyaluronic acid in vitreous. I. Soluble and particulate transferases in hyalocytes'. *Exp Eye Res* (1968); **7**: 497-510.

[13] Osterlin, S. E. 'The synthesis of hyaluronic acid in the vitreous. IV. Regeneration in the owl monkey'. *Exp Eye Res* (1969); **8**: 27-34.

[14] Jacobson, B. 'Biosynthesis of hyaluronic acid in the vitreous. V. Studies on a particulate hyalocyte glycosyl transferase'. *Exp Eye Res* (1978); **27**: 247-258.

[15] Badrinath, S. S., Gopal, L., Sharma, T., Parikh, S., Shanmugam, M. P., Bhende, P., and Biswas, J. 'Vitreoschisis in Eales' disease: pathogenic role and significance in surgery'. *Retina* (1999); **19**: 51-54.

[16] Heidenkummer, H. P. and Kampik, A. 'Morphologic analysis of epiretinal membranes in surgically treated idiopathic macular foramina. Results of light and electron microscopy'. *Der Ophthalmologe: Zeitschrift Der Deutschen Ophthalmologischen Gesellschaft* (1996); **93**: 675-679.

[17] Dimova, S., Brewster, M. E., Noppe, M., Jorissen, M., and Augustijns, P. 'The use of human nasal in vitro cell systems during drug discovery and development'. *Toxicol in Vitro* (2005); **19**: 107-122.

[18] Roguet, R. and Schaefer, H. 'Overview of in vitro cell culture technologies and pharmaco-toxicological applications'. *Toxicol in Vitro* (1997); **11**: 591-599.

[19] Kim, Young Jo, Sah, Robert L. Y., Doong, Joe Yuan, and Grodzinsky, Alan J. 'Fluorometric assay of DNA in cartilage explants using Hoechst 33258'. *Anal Biochem* (1988); **174**: 168-176.

[20] McGowan, K. B., Kurtis, M. S., Lottman, L. M., Watson, D., and Sah, R. L. 'Biochemical quantification of DNA in human articular and septal cartilage using PicoGreen(R) and Hoechst 33258'. *Osteoarthritis Cartilage* (2002); **10**: 580-587.

[21] Ng, Kee W., Leong, David T. W., and Hutmacher, Dietmar W. 'The challenge to measure cell proliferation in two and three dimensions'. *Tissue Eng* (2005); **11**: 182-191.

[22] Sommer, F., Kobuch, K., Brandl, F., Wild, B., Weiser, B., Gabel V.P., Blunk, T., and Göpferich, A. 'Ascorbic acid modulates proliferation and ascorbic acid accumulation of hyalocytes'. *submitted to Tissue Eng* (2006);

[23] Farndale, R. W., Buttle, D. J., and Barrett, A. J. 'Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue'. *Biochim Biophys Acta* (1986); **883**: 173-177.

[24] Taylor, K. B. and Jeffree, G. M. 'A new basic metachromatic dye, I:9-dimethyl methylene blue'. *Histochem J* (1969); **1**: 199-204.

[25] Woessner, J. F., Jr. 'The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid'. *Arch Biochem Biophys* (1961); **93**: 440-447.

[26] Kroll, W., Postius, S., and Schneider, F. 'Proliferation kinetics and metabolic features of in vitro grown Ehrlich ascites tumor cells in the presence of exogenous pyruvate'. *Journal Of Biosciences* (1986); **41**: 787-794.

[27] Hinoi, Eiichi, Fujimori, Sayumi, Takemori, Akihiro, and Yoneda, Yukio. 'Cell death by pyruvate deficiency in proliferative cultured calvarial osteoblasts'. *Biochem Bioph Res Co* (2002); **294**: 1177-1183.

[28] Ham, R. G. 'Clonal growth of diploid Chinese hamster cells in a synthetic medium supplemented with purified protein fractions'. *Exp Cell Res* (1962); **28**: 489-500.

[29] Eagle, Harry and Piez, Karl A. 'The population-dependent requirement by cultured mammalian cells for metabolites which they can synthesize'. *J Experimental Medicine* (1962); **116**: 29-43.

[30] McGowan, Joan A. and Bucher, Nancy L. R. 'Pyruvate promotion of DNA synthesis in serum-free primary cultures of adult rat hepatocytes'. *In Vitro* (1983); **91**: 159-6.

[31] Wasilenko, W. J. and Marchok, A. C. 'Pyruvate regulation of growth and differentiation in primary cultures of rat tracheal epithelial cells'. *Exp Cell Res* (1984); **155**: 507-517.

[32] Wood, John P. M., Chidlow, Glyn, Graham, Mark, and Osborne, Neville N. 'Energy substrate requirements for survival of rat retinal cells in culture: the importance of glucose and monocarboxylates'. *J Neurochem* (2005); **93**: 686-697.

[33] Desagher, S., Glowinski, J., and Premont, J. 'Pyruvate protects neurons against hydrogen peroxide-induced toxicity'. *J Neurosci* (1997); **17**: 9060-9067.

[34] Ramakrishnan, N., Chen, R., McClain, D. E., and Bunger, R. 'Pyruvate prevents hydrogen peroxide-induced apoptosis'. *Free Radical Res* (1998); **29**: 283-295.

[35] Kang, Y. H., Chung, S. J., Kang, I. J., Park, J. H., and Bunger, R. 'Intramitochondrial pyruvate attenuates hydrogen peroxide-induced apoptosis in bovine pulmonary artery endothelium'. *Mol Cel Biochem* (2001); **216**: 37-46.

[36] Tsuchihashi, Yuriko, Hinoi, Eiichi, and Yoneda, Yukio. 'Pyruvate as an endogenous antioxidant'. *Rec Res Devel BiophysBiochem* (2004); **4**: 103-115.

[37] Bunton, C. A. 'Oxidation of a-diketones and a-keto acids by hydrogen peroxide'. *Nature* (1949); **163**: 444

[38] Stich, H. F., Karim, J., Koropatnick, J., and Lo, L. 'Mutogenic action of ascorbic acid'. *Nature* (1976); **260**: 722-724.

[39] Speit, G., Wolf, M., and Vogel, W. 'The SCE-inducing capacity of vitamin C: Investigations in vitro and in vivo'. *Mutat Res* (1980); **78**: 273-278.

[40] Levine, M., Daruwala, R. C., Park, J. B., Rumsey, S. C., and Wang, Y. 'Does vitamin C have a pro-oxidant effect?'. *Nature* (1998); **395**: 231-231.

[41] Atalay, A., Ogus, A., Bateman, O., and Slingsby, C. 'Vitamin C induced oxidation of eye lens gamma crystallins'. *Biochimie* (1998); **80**: 283-288.

[42] Utsumi, Sakae. 'Amounts of ascorbic acid (vitamin C) in the aqueous humor, lens, and vitreous humor of eyes of experimental massage-cataract rabbits'. *Sei-I-Kai Medical Journal* (1935); **54**: 2069-2088.

[43] Takano, S., Ishiwata, S., Nakazawa, M., Mizugaki, M., and Tamai, M. 'Determination of ascorbic acid in human vitreous humor by high-performance liquid chromatography with UV detection'. *Curr Eye Res* (1997); **16**: 589-594.

[44] Dixon, S. Jeffrey, Kulaga, Andrew, Jaworski, Ewa M., and Wilson, John X. 'Ascorbate uptake by ROS 17/2.8 osteoblast-like cells: substrate specificity and sensitivity to transport inhibitors'. *J Bone Mineral Res* (1991); **6**: 623-629.

[45] Wilson, John X. and Dixon, S. Jeffrey. 'Ascorbic acid transport in mouse and rat astrocytes is reversibly inhibited by furosemide, SITS, and DIDS'. *Neurochem Res* (1989); **14**: 1169-1175.

[46] Halestrap, A. P. and Price, N. T. 'The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation'. *Biochem J* (1999); **343 Pt 2**: 281-299.

[47] Nakamichi, Noritaka, Kambe, Yuki, Oikawa, Hirotaka, Ogura, Masato, Takano, Katsura, Tamaki, Keisuke, Inoue, Maki, Hinoi, Eiichi, and Yoneda, Yukio. 'Protection by exogenous pyruvate through a mechanism related to monocarboxylate transporters against cell death induced by hydrogen peroxide in cultured rat cortical neurons'. *J Neurochem* (2005); **93**: 84-93.

[48] Hinoi, Eiichi, Takarada, Takeshi, Tsuchihashi, Yuriko, Fujimori, Sayumi, Moriguchi, Nobuaki, Wang, Liyang, Uno, Kyosuke, and Yoneda, Yukio. 'A Molecular Mechanism of Pyruvate Protection against Cytotoxicity of Reactive Oxygen Species in Osteoblasts'. *Mol pharmacol* (2006); **70**: 925-935.

[49] Gupta, S. K., Mohanty, Ipseeta, Trivedi, Deepa, Tandon, Radhika, Srivastava, Sushma, and Joshi, Sujata. 'Pyruvate Inhibits Galactosemic Changes in Cultured Cat Lens Epithelial Cells'. *Ophthalmic Res* (2002); **34**: 23-28.

[50] Mohanty, Ipseeta, Joshi, Sujata, Trivedi, Deepa, Srivastava, Sushma, Tandon, Radhika, and Gupta, Suresh K. 'Pyruvate modulates antioxidant status of cultured human lens epithelial cells under hypergalactosemic conditions'. *Mol Cell Biochem* (2002); **238**: 129-135.

[51] Devamanoharan, P. S., Henein, M., Ali, A. H., and Varma, S. D. 'Attenuation of sugar cataract by ethyl pyruvate'. *Mol Cell Biochem* (1999); **200**: 103-109.

[52] Kang, Young Hee, Park, Sung Hee, Lee, Yong Jin, Kang, Jung Sook, Kang, Il Jun, Shin, Hyun Kyung, Park, Jung Han Yoon, and Bunger, Rolf. 'Antioxidant a-keto-carboxylate pyruvate protects low-density lipoprotein and atherogenic macrophages'. *Free Radical Res* (2002); **36**: 905-914.

Chapter 7

Modulation of Hyalocyte Proliferation and ECM Accumulation via bFGF and TGF-β1

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Abstract

In some cases of severe retinal diseases, the vitreous body has to be removed surgically and, subsequently, replaced by a suitable biomaterial. Currently, however, no satisfying long-term vitreous substitute is in clinical use. A novel therapeutic concept represents the combination of hyalocytes, the cells of the vitreous body, with suitable biomaterials. To this end, control of hyalocyte proliferation and accumulation of extracellular matrix (ECM) seems to be a key issue. Therefore, the present study elucidated the effect of bFGF and TGF-β1 on hyalocyte morphology, proliferation and ECM production.

Both growth factors affected hyalocyte morphology; small, round cells could be observed after bFGF supplementation, whereas the cells appeared more completely spread when cultured in the presence of TGF-β1. Hyalocyte proliferation was increased 3-fold by 10 ng/ml bFGF; 1 ng/ml TGF-β1 in contrast reduced cell proliferation to about 40 % of the control. Converse effects of the two growth factors could also be observed on the ECM accumulation of hyalocytes; whereas bFGF halved their accumulation, TGF-β1 enhanced the ECM production up to 3-fold. Precultivation of hyalocytes with bFGF for two passages had no influence on their subsequent accumulation of glycosaminoglycans. However, cells precultivated with bFGF exhibited a doubled accumulation of collagen compared to controls. Moreover, supplementation of 1 ng/ml TGF-β1 doubled the ECM accumulation, independent of the precultivation conditions.

To conclude, the observed opposite effects of bFGF and TGF-β1 on hyalocyte proliferation and ECM accumulation may allow for the control of hyaloycte properties. Therefore, these two growth factors seem to be valuable tools towards the development of a cell-based vitreous substitute.

Introduction

The vitreous body, the main compartment of the posterior eye, is a gel-like structure consisting mainly of collagen type II and hyaluronic acid with a water content of about 98%. Minor components of the vitreous are collagen type V/XI and type IX as well as chondroitin sulfate[1]. While the center of this highly transparent and avascular tissue is free of cells, the cortex of the vitreous is occupied by a single layer of cells, named hyalocytes[2, 3]. Although these cells have been identified for more than a century[4], their exact physiological function still remains unclear. While hyalocytes are often referred to as resting cells[5-7] in the older literature, more recent studies show that the cells are physiologically renewed every several months[8] and belong to the monocyte-macrophage lineage[9]. Physiologically, hyalocytes are thought to be involved in maintaining the vitreous as a transparent and avascular tissue[10-13]. Pathologically, the cells are associated with some vitreoretinal diseases, such as epiretinal membrane formation, diabetic macular endema, and macular holes[14, 15]. Hyalocytes have been shown to produce extracellular matrix components, such as the vitreous typical collagen type II, in in-vitro cell culture[16]. Furthermore, our group has demonstrated hyalocyte functions to be dependent on ascorbic acid in a previous study[17]. To sum up, these data indicate that hyalocytes may have a house-keeping function within the adult vitreous.

In some cases of severe ophthalmic diseases, the vitreous body has to be removed surgically[18, 19]. In the treatment of severe retinal detachment, for example, removal of the vitreous is necessary to allow for the reattachment of the retina. At the end of this procedure, the excised vitreous body must be replaced by a suitable biomaterial to stabilize the treated retina in its position. Although in recent decades a plethora of biomaterials has been studied as vitreous replacements, no satisfying substitute is currently available on the market[20]. Some of the biomaterials in use are degraded over time and lose thereby their functionality within the eye; others are associated with severe side effects, such as retinal toxicity[20]. Novel therapeutic options could emerge from the field of tissue engineering. This approach is used to regenerate or replace tissues using combinations of cells, biomaterials, and growth factors. A key issue in tissue engineering is the control of cell behavior, especially regarding proliferation and extracellular matrix accumulation. To achieve this goal, growth factors are of utmost importance, as they possess the ability to modulate cell function in a variety of aspects. The use of these delicate substances in vivo, however, is enabled by sophisticated release systems that allow for their controlled delivery to the site of need.

Among the plethora of growth factors utilized in tissue engineering, basic fibroblast growth factor (bFGF), for example, is known to enhance proliferation of many cell types, including mesenchymal stem cells[21], osteoblasts[22], adipocytes[23] and chondrocytes[24]. In addition to enhancing cell proliferation, bFGF also enabled the retention of the differentiation potential of different cell types[25-28]. Transforming growth factor β1 (TGF-β1) has also been shown to elicit versatile effects on cells dependent on the cell type and the characteristics of the extracellular matrix the cells are in contact with[29, 30]. Although TGF-β inhibited epithelial cell and leucocyte proliferation, it stimulated the proliferation of smooth muscle cells, skin fibroblasts, and stromal fibroblasts[31-34]. Aside from cell proliferation, TGF-β1 reportedly stimulates the extracellular matrix production of some cells like chondrocytes[35] and osteoblasts[36].

The goal of the present study was to evaluate the potential of bFGF and TGF-β1 as tools to control hyalocyte proliferation and the accumulation of extracellular matrix. To this end, we used an established two dimensional in-vitro hyalocyte culture system to characterize the effect of supplementation of either bFGF or TGF-β1 on hyalocyte morphology and proliferation as well as the accumulation of glycosaminoglycans and collagen. Furthermore, we also investigated the influence of sequential supplementation of bFGF and TGF-β1 on hyalocyte behavior.

Materials and Methods

L-Ascorbic acid in cell culture quality, chondroitin sulfate A from bovine trachea, dimethylmethylene blue, fluorescein-phalloidin, and highly polymerized deoxyribonucleic acid from calf thymus were purchased from Sigma (Steinheim, Germany). Fetal calf serum (Batch Nr. 40A0044K), Dulbecco's phosphate buffered saline (PBS), penicillin/streptomycin, and 0.25% trypsin-EDTA were obtained from Invitrogen (Karlsruhe, Germany). Dulbecco's modified eagles medium (DMEM, low glucose, with glutamine and pyruvate) was obtained from Biochrom (Berlin, Germany); bovine serum albumin was bought from Serva (Heidelberg, Germany). Basic fibroblast growth factor (bFGF) was purchased from R&D Systems (Minneapolis, MN, USA); transforming growth factor β1 (TGF-β1) was obtained from Peprotech (London, UK). Dimethylamino benzaldehyde, chloramine T, isopropanol, formaldehyde and Triton X-100 were bought from Merck (Darmstadt, Germany); hydroxyproline and perchloric acid were purchased from Fluka (Neu-Ulm, Germany). Hoechst 33258 dye was obtained from Polysciences (Warrington, PA, USA); papainase and collagenase type II were purchased from Worthington (Lakewood, NJ, USA). All other cell

culture materials were purchased from Corning (Bodenheim, Germany). The buffer used during the papainase digestion (PBE) was composed of 100 mmol Na_2HPO_4 (Merck) and 10 mmol Na_2EDTA (Merck) in water, adjusted to pH 6.5.

Cell culture

Hyalocytes were isolated from freshly enucleated porcine eyes as previously described[17]. In brief, vitreous bodies were excised from porcine eyes, washed once in PBS buffer and examined microscopically. They were subsequently digested with a 1 mg/ml collagenase type II solution in standard culture medium (SCM) while shaking in an incubator at 37°C. SCM was composed of DMEM supplemented with 15% fetal calf serum (FCS), 100 IU/ml penicillin, and 100 µg/ml streptomycin. After 3 hours, digested vitreous bodies from about 20 eyes were pooled and subsequently centrifuged at 200 g for 7 minutes. The obtained hyalocytes were seeded for primary culture in a 25 cm² flask and cultured at 37°C and 5% CO_2 in SCM supplemented with 50 µg/ml ascorbic acid. Media was exchanged three times a week. After 9 days, the proliferating cells were detached with trypsin-EDTA for 5 minutes and centrifuged at 200 g for 5 minutes. For further proliferation, hyalocytes were seeded again at a density of 2000 cells/cm² and cultured for 5 days in SCM containing 50 µg/ml ascorbic acid. For investigation of growth factor effects, cells were used after the second passage.

Cell morphology

To study the influence of the two growth factors on hyalocyte morphology, cells were seeded at 1000 cells/cm² in 8-well Lab-Tek™ chamber-slides (Nunc, Wiesbaden, Germany) after the second passage and cultivated in DMEM containing 5% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml ascorbic acid supplemented with different concentrations of each growth factor. Cells cultured in media without growth factor supplementation served as a control. After two days, the media was exchanged, and after four days the cells were washed once with PBS buffer and fixed with a 3.7% formaldehyde solution for 10 min at room temperature. After cell fixation, the actin structure of the cells was stained using fluorescein-labeled phalloidin. The cells were permeabilized with a 0.1% Triton X-100 solution in PBS buffer for 5 minutes. To reduce nonspecific background staining, cells were first blocked with a 1% bovine serum albumin (BSA) solution was added for 30 minutes. Subsequently, 200 µl of a 2 U/ml dilution of fluorescein-phalloidin in PBS/1% BSA was added to each chamber. After incubation for 20 minutes in the dark, excess dye was removed by washing twice with PBS. For storage, the chambers of the slides were removed and the stained cells were coverslipped using the mounting medium Vectashield H-

1000 (Vector Laboratories, Burlingame, CA, USA). Stained cells were photographed on a Zeiss Axiovert 200M microscope coupled to an AxioCam HRc digital camera.

Cell proliferation and ECM accumulation

To study the effect of bFGF and TGF-β1 on hyalocyte proliferation and ECM accumulation, 50000 cells after the second passage were seeded in a 25 cm² culture flask and cultured in DMEM containing 5% FCS, penicillin/streptomycin, and 50 µg/ml ascorbic acid supplemented with different concentrations of each growth factor. Cells cultivated in non-supplemented media served as a control. Medium was exchanged for fresh, growth factor-containing media after two days. After four days the cells were harvested by complete trypsinization for 30 minutes. Subsequently, the samples were freeze dried (Christ Beta 2-16, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at 20°C and 0.120 mbar for 16 hours. The dry samples were digested in 300 µl of a 125 µg/ml papainase solution in PBE buffer for 18 hours at 60°C. Cell number and ECM content were determined as described below.

Precultivation with bFGF

To investigate the influence of bFGF precultivation on hyalocytes, bFGF was supplemented to hyalocytes during the first two passages, while cells cultivated without bFGF served as control group. Freshly isolated cells were seeded for primary culture in two 6-wells and cultivated with (F) or without (C) 10 ng/ml bFGF in SCM containing 50 µg/ml ascorbic acid. After first passage, each group was split and, subsequently, cultured again with (F) and without (C) supplementation of 10 ng/ml bFGF, starting with a cell density of 2000 cells/cm². The four resulting groups of cells are indicated by a two-letter combination, according to their cultivation conditions (Figure 1). After the second passage the effect of 1 ng/ml TGF-β1 on the cells of each groups was studied using the culture conditions described above.

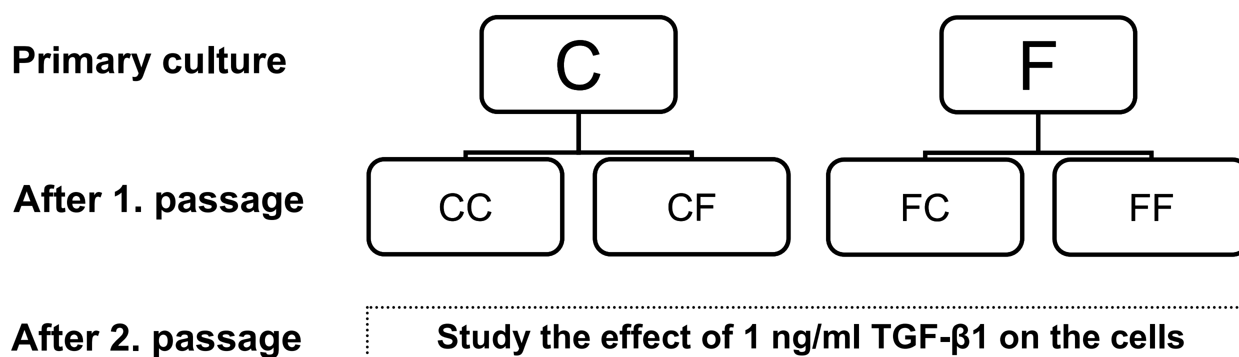

Figure 1: *Cultivation plan to study the influence bFGF precultivation on hyalocytes. During the primary culture, cells were divided into a control group (C) and a group supplemented with 10 ng/ml bFGF (F). After primary culture, each group was again split into a control group and a bFGF group. In the two-letter combinations, the first letter indicates conditions during primary culture, the second one the conditions after the first passage.*

Determination of cell number

Cell number was determined by measuring the DNA amount using Hoechst 33258 dye as previously described[37-39]. In brief, emission of the intercalating dye at 458 nm was measured using an excitation wavelength of 365 nm. The fluorescence of Hoechst 33258 dye is correlated with the absolute amount of DNA in the sample. To calculate the cell number, an average amount of 9.96 pg DNA per cell was assumed[17].

Glycosaminoglycan determination

The glycosaminoglycan content was measured photometrically as previously described[40, 41]. In brief, after digestion of interfering proteins with papainase, the glycosaminoglycan content was determined by a color reaction with dimethylmethylene blue followed by measurement of absorption at 525 nm. Chondroitin sulfate was used to prepare the standard curve.

Collagen determination

Collagen content was determined by measuring the amount of hydroxyproline, an amino acid that exists in significant amounts exclusively in collagen, according to a protocol described by Woessner et al.[42] with some modifications described elsewhere[17]. In brief, after hydrolysis of the sample, hydroxyproline was oxidized with chloramine T and, subsequent to coupling reaction with dimethylamino benzaldehyde, the absorption of the formed dye was measured. Hydroxyproline was dissolved in PBE/cysteine solution to prepare the standard curve.

Statistics

All data are presented as means ± standard deviation with $n = 3$. Single-factor analysis of variance (ANOVA) was used in conjunction with a multiple comparisons test (Tukey's test) to assess statistical significance at levels of $p < 0.05$ (*) or $p < 0.01$ (**).

Results

Cell morphology and actin organization

Hyalocytes were stained with fluorescein-phalloidin in order to asses the cell shape and the actin filament organization of the cells. Cells in the control group showed a spread and flattened phenotype with long and thick actin filaments (Figure 2). Upon 10 ng/ml bFGF supplementation, a higher number of small, rounded cells with diffuse actin staining appeared (arrows in the upper picture). Furthermore, these round cells showed small, bubble-like actin aggregations at the rim of the cells (arrows in the lower picture). Cells in the TGF-β1 group were well spread with clear and thick actin filaments; almost no rounded cells could be observed. Morphologically, cells cultured with 1 ng/ml TGF-β1 appeared similar to the control cells, however, the actin fibers within the cells seemed to be clearer and thicker compared to the control.

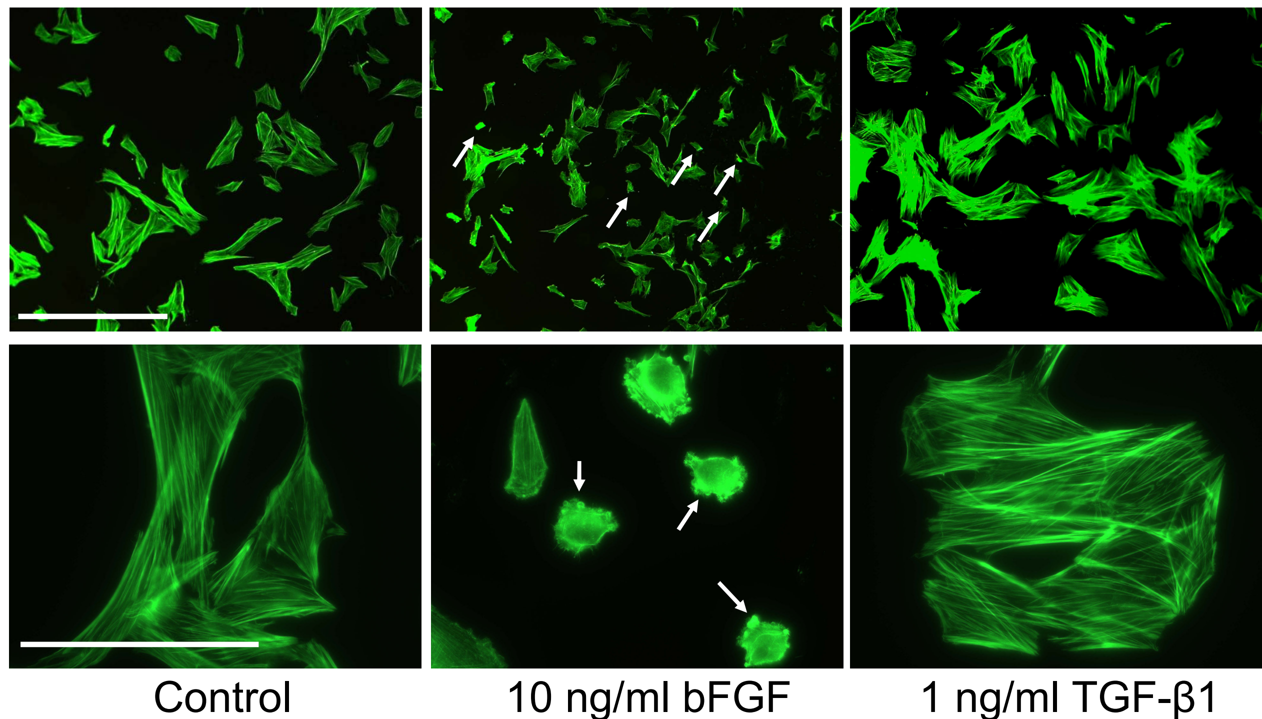

***Figure 2**: Influence of bFGF or TGF-β1 on cell morphology of hyalocytes after staining of F-actin using fluorescein-phalloidin. Scale bar in the upper pictures represents 500 µm, scale bar in the lower pictures represents 100 µm. Due to bFGF supplementation, an increased number of small, round and non-spread cells could be observed (arrows in the upper picture); furthermore, these cells showed bubble-like adhesion sites (arrows in the lower picture). TGF-β1 supplementation induced little enlargement in cell-spreading, however, compared to the control, actin filaments were more distinct.*

Hyalocyte proliferation

Hyalocyte proliferation was affected differently by the two growth factors studied. While 0.1 ng/ml bFGF supplemented to the culture medium induced only a small increase in the cell number, the increase was clear and significant upon supplementation with 1 ng/ml bFGF (Figure 3 A). The maximum effect of bFGF was observed with supplementation of 10 and 100 ng/ml bFGF, resulting in a 3-fold increase in proliferation. In contrast to bFGF, TGF-β1 inhibited cell proliferation even at concentrations as low as 0.1 ng/ml. Supplementation of 1 – 20 ng/ml TGF-β1 inhibited hyalocyte proliferation with a maximum effect of about 0.4 times the proliferation rate compared to control (Figure 3 B). However, starting with a cell number of 50 000 per sample, there was still about a 4-fold increase in the absolute cell number during the cultivation period.

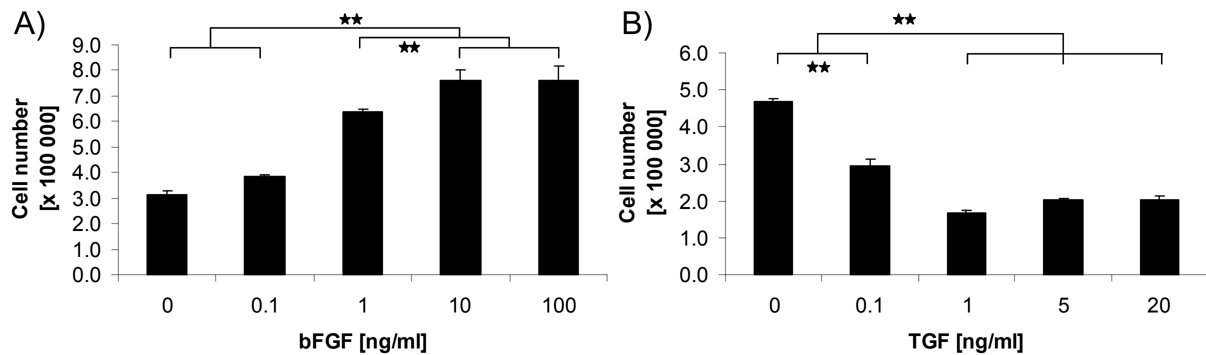

Figure 3: *Proliferation of hyalocytes dependent on the concentration of bFGF (A) and TGF-β1 (B) supplemented to the medium. Proliferation of hyalocytes was clearly enhanced due to supplementation of bFGF, whereas TGF-β1 inhibited the cell proliferation; study was performed in triplicate, representative data are shown*

Accumulation of extracellular matrix

Similarly to the cell proliferation, the production of extracellular matrix by hyalocytes was affected differently by the two growth factors. However, each factor influenced the production of glycosaminoglycans and collagen in a similar way.

The production of GAG per cell was clearly inhibited by bFGF (Figure 4 A). Concentrations of 0.1 ng/ml of bFGF decreased the GAG production per cell slightly, while supplementation of 1 – 100 ng/ml showed maximal inhibitory effect. In response to bFGF supplementation, the production of GAG per cell was decreased to half the value of the control. Similar results were obtained by measuring the collagen accumulation per cell (Figure 4 B). Under conditions of maximum inhibition, bFGF supplementation resulted in one third of the collagen production of the control. This effect of bFGF was also achieved with concentrations of 1 – 100 ng/ml.

In contrast to the inhibition of ECM accumulation per cell due to bFGF supplementation, TGF-β1 enhanced the production of both GAG and collagen per cell (Figures 4 C and D). Whereas supplementation of 0.1 ng/ml of TGF-β1 showed no effect on the GAG production of hyalocytes, concentrations of 1 – 20 ng/ml enhanced the GAG production of the cells up to a 1.5-fold value. Collagen accumulation was enhanced in a dose-dependent manner until 1 ng/ml and reached utmost values (up to an almost 3-fold increase) at concentrations of 1 – 20 ng/ml.

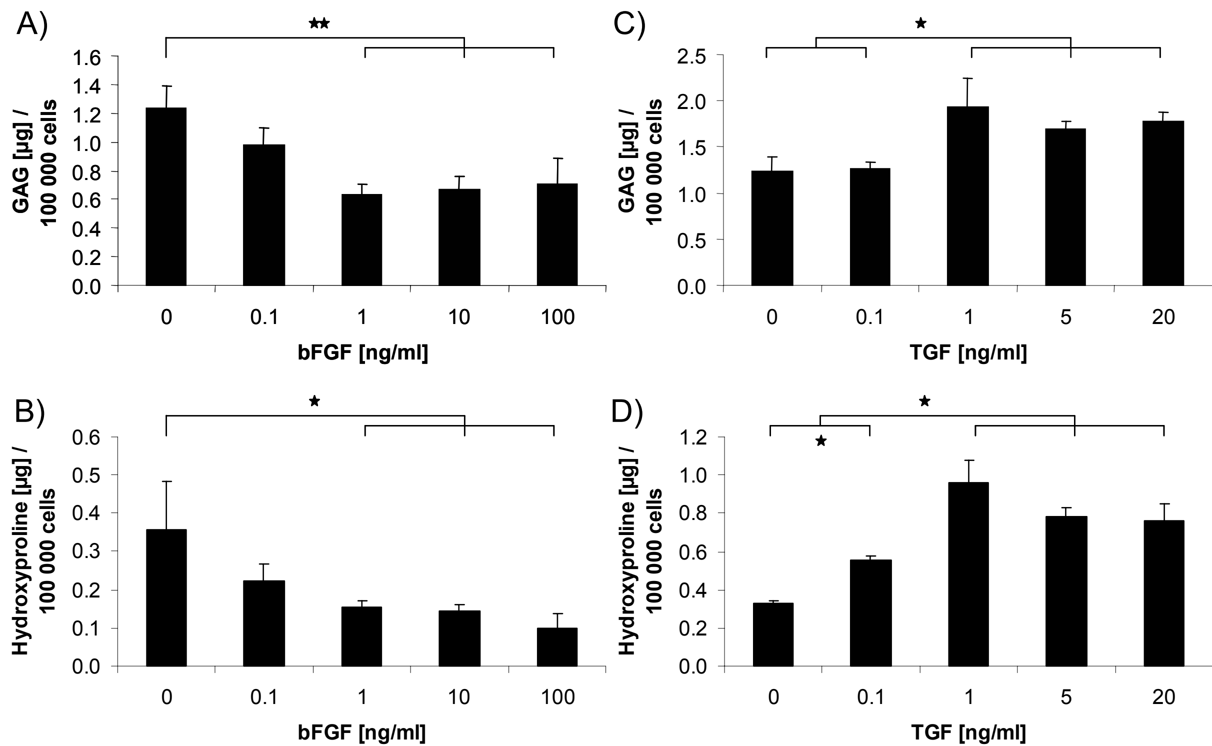

Figure 4: *Accumulation of extracellular matrix components dependent on the supplementation of bFGF and TGF-β1. Supplementation of bFGF reduced accumulation of glycosaminoglycans (A) and collagens (B); in contrast, TGF-β1 enhanced the accumulation of glycosaminoglycans (C) as well as of collagens (D); study was performed in triplicate, representative data are shown.*

Influence of bFGF during cell expansion

To study the effect of bFGF during hyalocyte expansion, 10 ng/ml of the growth factor was supplemented for two passages (Table 1). Subsequently, the influence of 1 ng/ml TGF-β1 on hyalocyte proliferation and ECM accumulation was investigated. In two independent studies, the number of hyalocytes obtained after primary culture was enhanced 1.5-fold due to supplementation of 10 ng/ml bFGF (data not shown). After trypsin detachment and seeding the cells of all groups at an equal density, proliferation was almost doubled upon bFGF supplementation. Again, compared to the control groups without supplementation of bFGF after the first passage (CC and FC) cell proliferation was clearly increased in the groups with 10 ng/ml bFGF supplementation (CF and FF). Precultivation with or without bFGF during the primary culture showed no effect on cell proliferation after the first passage (data not shown). After the second passage of all groups, we investigated the response of hyalocytes to supplementation of 1 ng/ml TGF-β1 dependent on the precultivation conditions. TGF-β1 inhibited the cell proliferation comparably in all groups, we observed no differences related to the precultivation conditions (Figure 5). Furthermore, the GAG accumulation of the cells was

comparably increased in all groups due to TGF-β supplementation; the precultivation conditions also showed no effect on the GAG production of hyalocytes (Figure 6 A). In contrast to the GAG accumulation, the collagen accumulation of hyalocytes was clearly influenced by the precultivation conditions (Figure 6 B). Whereas TGF-β supplementation increased the collagen production similarly in all groups compared to control, the groups receiving bFGF partially (CF and FC) or completely (FF) during cell expansion showed significantly higher levels of collagen accumulation per cell. Compared to the cells that were expanded completely without bFGF during the first two passages (CC), all other groups showed an almost doubled level of collagen production. In these groups, the levels of the controls as well as the levels of the TGF-supplemented groups were identically increased, indicating that the time interval of bFGF supplementation during the cell augmentation had no influence on the collagen accumulation of the cells.

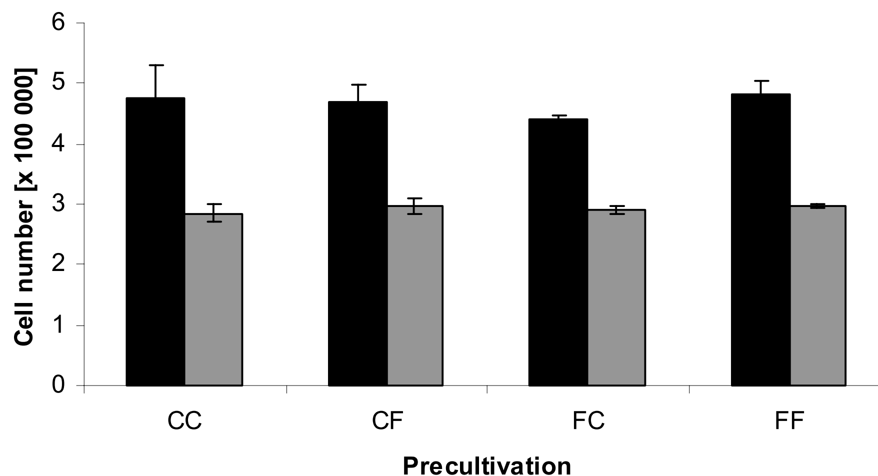

Figure 5: *Effect of 1 ng/ml TGF-β1 on the proliferation of hyalocytes after second passage, dependent on the precultivation with (F) or without (C) 10 ng/ml of bFGF. Black bars indicate controls; grey bars indicate supplementation of TGF-β1. The first letter indicates conditions during primary culture, the second one the conditions after the first passage. No differences related to the precultivation conditions could be observed; study was performed in duplicate, representative data are shown.*

Discussion

Novel concepts for vitreous replacement are needed to improve clinical therapy after vitrectomy[20]. Tissue engineering seems to be a promising strategy that could lead to the development of a suitable vitreous substitute in the future. For the development of a tissue-engineered, cell-based vitreous substitute, however, the control of hyalocyte behavior seems to be necessary. The presented study indicates that bFGF and TGF-β1 conversely influence

hyalocyte proliferation and their extracellular matrix accumulation. Therefore, these growth factors could be valuable tools to specifically control hyalocyte properties.

Hyalocytes are known as the cells of the vitreous body. To minimize individual variations between the cells of different animals, cells from about 20 eyes were pooled for each study. As ascorbic acid was previously demonstrated to be an important factor for the in vitro culture of hyalocytes[17], 50 µg/ml of ascorbic acid was supplemented to the media throughout the culture period. During the first two passages, the use of 15% FCS in the culture medium assured sufficient hyalocyte proliferation. However, during the investigation into the influence of the two growth factors, the FCS amount within the media was reduced to 5% in order to prevent the relatively uncharacterized mixture of growth factors in FCS from masking the effects of the supplemented growth factors. All investigations were performed using the culture system previously described by our group[17]. This system uses sub-confluent cell densities in two-dimensional culture, thereby mimicking the native environment of hyalocytes by ensuring an isolated position and a minimum of cell-cell contact.

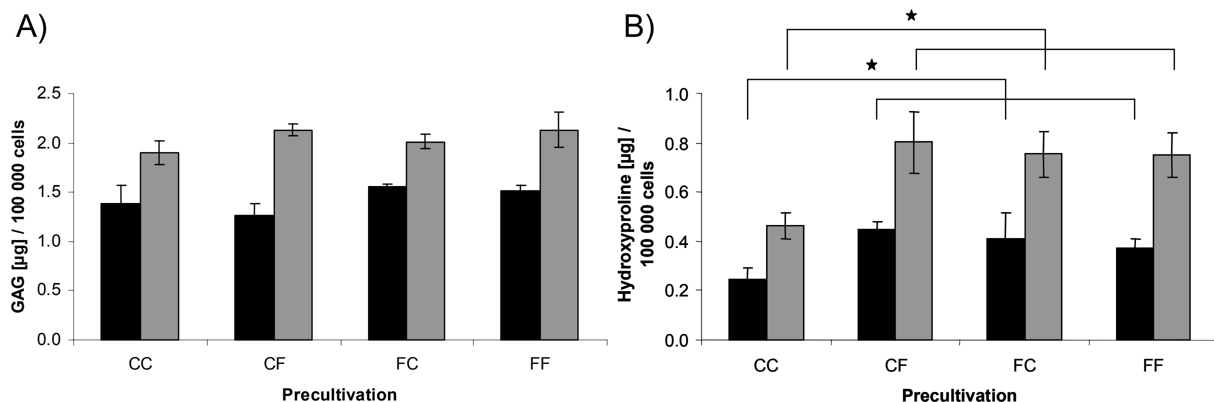

Figure 6: *Effect of 1 ng/ml TGF-β1 on the extracellular matrix accumulation of hyalocytes after second passage, dependent on the precultivation with (F) or without (C) 10 ng/ml of bFGF. Black bars indicate controls; grey bars indicate supplementation of TGF-β1. The first letter indicates conditions during primary culture, the second one the conditions after the first passage. Production of glycosaminoglycans per cell was not influenced by the precultivation conditions (A). In contrast, precultivation with bFGF enhanced the production of collagen per cell in the control groups as well as in the TGF-β1 supplemented cells (B); study was performed in duplicate, representative data are shown.*

The present study indicates that both the cell morphology and the actin structure of the cells are clearly affected by the investigated growth factors (Figure 2). TGF-β1 increased the thickness of the actin filaments within the cells, but had little effect on the morphology of hyalocytes, whereas bFGF increased the number of small, rounded cells displaying diffuse actin staining. Similar effects of TGF-β1 on the structure of the actin fibers are reported for example for mesenchymal stem cells[43] and for Leydig cells[44]. The observed effect of bFGF on

morphology and actin organization of the cells is also in accordance with other cell types, for example chondrocytes[25] or bone marrow-derived progenitor cells[27, 45]. FGFs are known to induce disassembly of actin filaments[46, 47], an effect that we observed with hyalocytes. Moreover, in the presented study small, bubble-like actin structures were partially observed at the rim of the cells after four days of cultivation with bFGF. These actin structures are probably linked to adhesion areas of the cells and, therefore, suggest that bFGF might change the adhesion behavior of hyalocytes. It is not unusual for chondrocytes, a well characterized and, due to its similar native environment consisting of collagen type II and GAG probably closely related cell type, to correlate cell morphology and actin fiber organization with the differentiation state of the cell[48-50]. However, as the characteristics of a differentiated hyalocyte are sill unclear, similar correlations for hyalocytes do not seem appropriate. An improved knowledge about hyalocytes may allow a more precise interpretation of these results in the future.

The present study clearly demonstrates that cell proliferation can be modulated by the use of bFGF and TGF-β1, as these two growth factors showed opposite effects on the proliferation of hyalocytes (Figure 3). Although TGF-β1 clearly decreased the obtained cell numbers compared to the controls, there was still an increase in the absolute cell number detectable after the cultivation time. Therefore, we conclude that the observed effect of TGF-β1 was due to inhibition of the cell proliferation. The described effects of bFGF and TGF-β1 on hyalocyte proliferation are in agreement with a report by Sakamoto et al.[51] and were similarly observed for other cell types, notably chondrocytes[45] and mesenchymal stem cells[36, 52]. Particularly retinal pigment epithelial cells (RPE) showed a comparable increase in their proliferation due to supplementation of 10 ng/ml bFGF, however, in contrast to the effect of TGF-β1 on hyalocytes, TGF-β2 showed no influence on proliferation of RPE cells[53].

In addition to cell proliferation, we were interested in the effect of the two growth factors on functional properties of the cells. As the cells are known to produce extracellular matrix, especially glycosaminoglycans[54] and collagens[16, 55], we quantified the accumulation of ECM per cell as a marker for the cell functions. We again found again opposite effects of the two growth factors on these markers. We could demonstrate that the bFGF-induced increase in the proliferation rate is associated with a decrease in the production of ECM components per cell (Figure 4). In contrast, the inhibition in cell proliferation due to TGF-β1 was correlated with an enhancement in the production of GAG and collagen per cell. Each growth factor affected the accumulation of GAG and collagen similarly by trend, however, the magnitude of the effect was not identical. As, for example, bFGF decreased the GAG accumulation per cell to

about half the value of control, the collagen accumulation was decreased to one third of the control. These differences were most probably due to the test system. It is known for chondrocytes, that the accumulation rate of GAG and collagen per cell changes with time during the cultivation period[56]. Furthermore, these ECM components show a different turnover. Therefore, the cultivation time that we limited to four days had a clear impact on the absolute amounts of accumulated ECM components. Although the absolute values measured are closely connected to the culture system, we observed clear effects of each investigated factor. As the factors affected the cells in an opposite manner, sensible use of bFGF and TGF-β1 would appear to allow for the control of ECM production of hyalocytes. Further studies are needed to clarify to what extend ECM production and, moreover, which specific types of GAG and collagens are ideal for the therapeutic use of the cells. In addition, as the presented data display the sum of all accumulated types of GAG and collagens, it remains to be elucidated whether the growth factors favor accumulation of some special subtype of these ECM components.

For the development of a tissue-engineered vitreous substitute, a sufficient population of hyalocytes of suitable quality will be needed. Supplementation with bFGF during cell expansion appears to be a promising approach to gaining sufficient cell numbers in a short timeframe, as bFGF clearly enhanced hyalocyte proliferation. However, it remains unclear whether this enhancement in the proliferation rate has an influence on the subsequent ECM accumulation of hyalocytes. To elucidate this, we studied the effect of TGF-β1 on hyalocyte proliferation and accumulation of extracellular matrix dependent on the precultivation with or without bFGF. To induce the maximum effects from the growth factors, we used, in accordance with the presented data (Figure 3 and 4), 10 ng/ml of bFGF during the precultivation phase and, subsequently, investigated the effect of 1 ng/ml of TGF-β1. To ensure the best conditions during the cell expansion phase for two passages, we used 15% FCS supplemented with bFGF; after the second passage the functional properties of the cells were investigated in 5% FCS to make differences between the studied groups clear. As expected, bFGF clearly enabled enhanced cell propagation, as higher cell numbers were detected in all groups receiving 10 ng/ml bFGF (F in primary culture, CF and FF after first passage) compared to controls (data not shown). Interestingly, measures of both cell proliferation and accumulation of GAG per cell (Figure 5 and 6 A) were comparable for all groups following supplementation with TGF-β, irrespective of their precultivation conditions. However, higher amounts of collagen per cell were observed upon precultivation with bFGF (Figure 6 B). Thus, the time point of bFGF supplementation does not seems to influence the

collagen production of the cell. These data suggest that an enhancement in the cell proliferation due to bFGF has no adverse effects on the ECM production of the cells. On the contrary, bFGF precultivation even appeared to induce positive effects on the total collagen production. To asses this observation, the pattern of accumulated types of collagens will need to be investigated. However, bFGF seems to be a useful tool for effective and sensible hyalocyte augmentation. Similar effects of bFGF have been reported for other cell types, including chondrocytes[25, 26] and mesenchymal stem cells[27, 28]. In addition to the supplementation of bFGF alone, there are also reports about combinations of growth factors, such as bFGF and TGF-β1, during cell propagation and differentiation[45, 57, 58]. Therefore, it seems obvious to systematically investigate the use of different growth factor combinations to further optimize hyalocyte expansion in the future. Moreover, further studies should clarify the mechanism of the influence of bFGF precultivation on hyalocytes. This could be either due to a direct effect on the cells, as for example an increase in the expression of certain genes, or due to preferential proliferation of a subpopulation of hyalocytes. However, our data demonstrate a successful step towards improving hyalocyte expansion, important progress not only towards a tissue-engineered vitreous substitute, but also an improvement for in vitro-cultivation of hyalocytes.

The sensitivity of growth factors to denaturation, connected with the loss of bioactivity, represents an important obstacle for their use. As bFGF was demonstrated to be a valuable tool for in vitro cell propagation, it may be predominantly used in vitro. Therefore, this limitation can easily be overcome using pump systems that supplement the factor continuously to the media. In contrast to bFGF, TGF-β1 seems to also display useful properties for in vivo use. In that setting, however, suitable intraocular release systems that allow for controlled delivery of the growth factor would be needed. Therefore, the appropriateness of the known intraocular release devices, for example implants, micospheres or rods[59 - 61], for the delivery of TGF-β1 should be evaluated in the future. Moreover, the development of biomaterials that combine suitability as a vitreous substitute with the ability to release drugs would represent significant progress.

Conclusions

To conclude, we identified bFGF and TGF-β1 as inducing converse effects on hyalocytes. Therefore, these two growth factors represent useful tools to influence functional properties of hyalocytes. For the development of a vitreous substitute based on tissue engineering strategies, it will be necessary to control hyalocyte functions, especially their proliferation and

the production of extracellular matrix. For that reason, bFGF and TGF-β1 may represent key factors towards this promising development.

References

[1] Bishop, P. N. 'Structural macromolecules and supramolecular organisation of the vitreous gel'. *Prog Retin Eye Res* (2000); **19**: 323-344.

[2] Bishop, P. N., Takanosu, M., Le Goff, M., and Mayne, R. 'The role of the posterior ciliary body in the biosynthesis of vitreous humour'. *Eye* (2002); **16**: 454-460.

[3] Sebag, J. '*The vitreous*', Springer, New York (1989).

[4] Henle, Jakob. '*Lehre von den Mischungs- und Formbestandtheilen des menschlichen Körpers*' in 'Vom Baue des menschlichen Körpers', Vos, Leipzig (1841).

[5] Salu, P., Claeskens, W., De Wilde, A., Hijmans, W., and Wisse, E. 'Light and electron microscopic studies of the rat hyalocyte after perfusion fixation'. *Ophthalmic Res* (1985); **17**: 125-130.

[6] Gloor, B. P. 'Development of the vitreous body and zonula'. *Albrecht Von Graefes Arch Klin Exp Ophthalmol* (1973); **187**: 21-44.

[7] Gloor, B. P. 'Radioisotopes for research into vitreous and zonule'. *Adv Ophthalmol* (1978); **36**: 63-73.

[8] Qiao, H., Hisatomi, T., Sonoda, K. H., Kura, S., Sassa, Y., Kinoshita, S., Nakamura, T., Sakamoto, T., and Ishibashi, T. 'The characterisation of hyalocytes: the origin, phenotype, and turnover'. *Br J Ophthalmol* (2005); **89**: 513-517.

[9] Lazarus, H. S. and Hageman, G. S. 'In situ characterization of the human hyalocyte'. *Arch Ophthalmol* (1994); **112**: 1356-1362.

[10] Boltz-Nitulescu, G., Grabner, G., and Forster, O. 'Macrophage-like properties of human hyalocytes'. *Adv Exp Med Bio* (1979); **121B**: 223-228.

[11] Zhu, M., Penfold, P. L., Madigan, M. C., and Billson, F. A. 'Effect of human vitreous and hyalocyte-derived factors on vascular endothelial cell growth'. *Aust N Z J Ophthalmol* (1997); **25**: 57-60.

[12] Noda, Yoshihiro, Hata, Yasuaki, Hisatomi, Toshio, Nakamura, Yuka, Hirayama, Kumiko, Miura, Muneki, Nakao, Shintaro, Fujisawa, Kimihiko, Sakamoto, Taiji, and Ishibashi et, al. 'Functional Properties of Hyalocytes under PDGF-Rich Conditions'. *Invest Ophthalmol* (2004); **45**: 2107-2114.

[13] Lutty, G. A., Merges, C., Threlkeld, A. B., Crone, S., and McLeod, D. S. 'Heterogeneity in localization of isoforms of TGF-beta in human retina, vitreous, and choroid'. *Invest Ophthalmol* (1993); **34**: 477-487.

[14] Badrinath, S. S., Gopal, L., Sharma, T., Parikh, S., Shanmugam, M. P., Bhende, P., and Biswas, J. 'Vitreoschisis in Eales' disease: pathogenic role and significance in surgery'. *Retina (Philadelphia, a)* (1999); **19**: 51-54.

[15] Heidenkummer, H. P. and Kampik, A. 'Morphologic analysis of epiretinal membranes in surgically treated idiopathic macular foramina. Results of light and electron microscopy'. *Der Ophthalmologe* (1996); **93**: 675-679.

[16] Kobuch, K., Wild, B., Eckert, E., Fischbach, C., and Gabel V.P. 'Expression of Collagen Type II by Hyalocytes In Vitro - First Steps Towards a Biological Vitreous Substitute', presented at 'ARVO Annual Meeting', Fort Lauderdale, FL, USA (2002)

[17] Sommer, F., Kobuch, K., Brandl, F., Wild, B., Weiser, B., Gabel V.P., Blunk, T., and Göpferich, A. 'Ascorbic acid modulates proliferation and ascorbic acid accumulation of hyalocytes'. *submitted to Tissue Eng* (2006);

[18] D'Amico, Donald J. 'Diseases of the Retina'. *N Engl J Med* (1994); **331**: 95-106.

[19] Frank, Robert N. 'Diabetic Retinopathy'. *N Engl J Med* (2004); **350**: 48-58.

[20] Soman, N. and Banerjee, R. 'Artificial vitreous replacements'. *Biomed Mater Eng* (2003); **13**: 59-74.

[21] Benavente, Claudia A., Sierralta, Walter D., Conget, Paulette A., and Minguell, Jose J. 'Subcellular distribution and mitogenic effect of basic fibroblast growth factor in mesenchymal uncommitted stem cells'. *Growth Factors* (2003); **21**: 87-94.

[22] Berrada, Saadia, Lefebvre, Francoise, and Harmand, Marie Francoise. 'The effect of recombinant human basic fibroblast growth factor (rhFGF-2) on human osteoblast in growth and phenotype expression'. *Dev Biol* (1995); **31**: 698-702.

[23] Hauner, H., Roehrig, K., and Petruschke, T. 'Effects of epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) on human adipocyte development and function'. *Eur J Clin Invest* (1995); **25**: 90-96.

[24] Cuevas, P., Burgos, J., and Baird, A. 'Basic fibroblast growth factor (FGF) promotes cartilage repair in vivo'. *Biochem Bioph Res Co* (1988); **156**: 611-618.

[25] Martin, I., Vunjak-Novakovic, G., Yang, J., Langer, R., and Freed, L. E. 'Mammalian chondrocytes expanded in the presence of fibroblast growth factor 2 maintain the ability to differentiate and regenerate three- dimensional cartilaginous tissue'. *Exp Cell Res* (1999); **253**: 681-688.

[26] Martin, Ivan, Suetterlin, R., Baschong, W., Heberer, M., Vunjak-Novakovic, G., and Freed, L. E. 'Enhanced cartilage tissue engineering by sequential exposure of chondrocytes to FGF-2 during 2D expansion and BMP-2 during 3D cultivation'. *J Cell Biochem* (2001); **83**: 121-128.

[27] Martin, Ivan, Muraglia, Anita, Campanile, Giuliano, Cancedda, Ranieri, and Quarto, Rodolfo. 'Fibroblast growth factor-2 supports ex vivo expansion and maintenance of osteogenic precursors from human bone marrow'. *Endocrinology* (1997); **138**: 4456-4462.

[28] Tsutsumi, Shinichi, Shimazu, Atsushi, Miyazaki, Kazuko, Pan, Haiou, Koike, Chika, Yoshida, Eri, Takagishi, Kenji, and Kato, Yukio. 'Retention of Multilineage Differentiation Potential of Mesenchymal Cells during Proliferation in Response to FGF'. *Biochem Bioph Res Co* (2001); **288**: 413-419.

[29] Attisano, L., Wrana, J. L., Lopez-Casillas, F., and Massague, J. 'TGF-beta receptors and actions'. *Biochim Biophys Acta* (1994); **1222**: 71-80.

[30] Massague, J. 'The transforming growth factor-beta family'. *Annu Rev Cell Biol* (1990); **6**: 597-641.

[31] Assoian, R. K., Fleurdelys, B. E., Stevenson, H. C., Miller, P. J., Madtes, D. K., Raines, E. W., Ross, R., and Sporn, M. B. 'Expression and secretion of type beta transforming growth factor by activated human macrophages'. *PNAS* (1987); **84**: 6020-6024.

[32] Cross, Michael and Dexter, T. Michael. 'Growth factors in development, transformation, and tumorigenesis'. *Cell* (1991); **64**: 271-280.

[33] Kay, E. P., Lee, M. S., Seong, G. J., and Lee, Y. G. 'TGF-beta s stimulate cell proliferation via an autocrine production of FGF-2 in corneal stromal fibroblasts'. *Curr Eye Res* (1998); **17**: 286-293.

[34] Song, Q. H., Klepeis, V. E., Nugent, M. A., and Trinkaus-Randall, V. 'TGF-b1 regulates TGF-b1 and FGF-2 mRNA expression during fibroblast wound healing'. *Mol Pathol* (2002); **55**: 164-176.

[35] Pangborn, Christine A. and Athanasiou, Kyriacos A. 'Effects of growth factors on meniscal fibrochondrocytes'. *Tissue Eng* (2005); **11**: 1141-1148.

[36] Lieb, E., Milz, S., Vogel, T., Hacker, M., Dauner, M., and Schulz, M. B. 'Effects of transforming growth factor beta1 on bonelike tissue formation in three-dimensional cell culture. I. Culture conditions and tissue formation'. *Tissue Eng* (2004); **10**: 1399-1413.

[37] Kim, Young Jo, Sah, Robert L. Y., Doong, Joe Yuan, and Grodzinsky, Alan J. 'Fluorometric assay of DNA in cartilage explants using Hoechst 33258'. *Anal Biochem* (1988); **174**: 168-176.

[38] McGowan, K. B., Kurtis, M. S., Lottman, L. M., Watson, D., and Sah, R. L. 'Biochemical quantification of DNA in human articular and septal cartilage using PicoGreen(R) and Hoechst 33258'. *Osteoarthritis Cartilage* (2002); **10**: 580-587.

[39] Ng, Kee W., Leong, David T. W., and Hutmacher, Dietmar W. 'The challenge to measure cell proliferation in two and three dimensions'. *Tissue Eng* (2005); **11**: 182-191.

[40] Farndale, R. W., Buttle, D. J., and Barrett, A. J. 'Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue'. *Biochim Biophys Acta* (1986); **883**: 173-177.

[41] Taylor, K. B. and Jeffree, G. M. 'A new basic metachromatic dye, I:9-dimethyl methylene blue'. *Histochem J* (1969); **1**: 199-204.

[42] Woessner, J. F., Jr. 'The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid'. *Arch Biochem Biophys* (1961); **93**: 440-447.

[43] Chu, P. K., Chen, J. Y., Wang, L. P., and Huang, N. 'Plasma-surface modification of biomaterials'. *Mat Sci Eng R* (2002); **36**: 143-206.

[44] Dickson, Cole, Webster, Daniel R., Johnson, Heather, Cecilia Millena, Ana, and Khan, Shafiq A. 'Transforming growth factor-beta effects on morphology of immature rat Leydig cells'. *Mol Cell Endocrinol* (2002); **195**: 65-77.

[45] Stevens, Molly M., Marini, Robert P., Martin, Ivan, Langer, Robert, and Prasad Shastri, V. 'FGF-2 enhances TGF-b1-induced periosteal chondrogenesis'. *J Orthop Res* (2004); **22**: 1114-1119.

[46] Pickering, J. G., Uniyal, S., Ford, C. M., Chau, T., Laurin, M. A., Chow, L. H., Ellis, C. G., Fish, J., and Chan, B. M. 'Fibroblast growth factor-2 potentiates vascular smooth muscle cell migration to platelet-derived growth factor: upregulation of alpha2beta1 integrin and disassembly of actin filaments'. *Circ Res* (1997); **80**: 627-637.

[47] Wroblewski, J. and Edwall-Arvidsson, C. 'Inhibitory effects of basic fibroblast growth factor on chondrocyte differentiation'. *J Bone Miner Res* (1995); **10**: 735-742.

[48] Borge, L., Lemare, F., Demignot, S., and Adolphe, M. 'Restoration of the differentiated functions of serially passaged chondrocytes using staurosporine'. *In Vitro Cellular & Developmental Biology Animal* (1997); **33**: 703-709.

[49] Brown, P. D. and Benya, P. D. 'Alterations in chondrocyte cytoskeletal architecture during phenotypic modulation by retinoic acid and dihydrocytochalasin B-induced reexpression'. *J Cell Biol* (1988); **106**: 171-179.

[50] Zanetti, N. C. and Solursh, M. 'Induction of chondrogenesis in limb mesenchymal cultures by disruption of the actin cytoskeleton'. *J Cell Biol* (1984); **99**: 115-123.

[51] Sakamoto, Taiji. 'Cell biology of hyalocytes'. *Nippon Ganka Gakkai Zasshi* (2003); **107**: 866-882.

[52] Neubauer, Markus, Hacker, Michael, Bauer-Kreisel, Petra, Weiser, Barbara, Fischbach, Claudia, Schulz, Michaela B., Goepferich, Achim, and Blunk, Torsten. 'Adipose tissue engineering based on mesenchymal stem cells and basic fibroblast growth factor in vitro'. *Tissue Eng* (**11**: 1840-1851.

[53] Spraul, Christoph W., Kaven, Corinna, Lang, Gerhard K., and Lang, Gabriele E. 'Effect of growth factors on bovine retinal pigment epithelial cell migration and proliferation'. *Ophthalmic Res* (2004); **36**: 166-171.

[54] Osterlin, S. E. and Jacobson, B. 'The synthesis of hyaluronic acid in vitreous. II. The presence of soluble transferase and nucleotide sugar in the accellular vitreous gel'. *Exp Eye Res* (1968); **7**: 511-523.

[55] Newsome, D. A., Linsenmayer, T. F., and Trelstad, R. L. 'Vitreous body collagen. Evidence for a dual origin from the neural retina and hyalocytes'. *J Cell Biol* (1976); **71**: 59-67.

[56] Kellner, K., Schulz, M. B., Gopferich, A., and Blunk, T. 'Insulin in tissue engineering of cartilage: A potential model system for growth factor application'. *J Drug Target* (2001); **9**: 439-448.

[57] Pei, M., Seidel, J., Vunjak-Novakovic, G., and Freed, L. E. 'Growth factors for sequential cellular de- and re-differentiation in tissue engineering'. *Biochem Bioph Res Co* (2002); **294**: 149-154.

[58] Inoue, H., Kato, Y., Iwamoto, M., Hiraki, Y., Sakuda, M., and Suzuki, F. 'Stimulation of cartilage-matrix proteoglycan synthesis by morphologically transformed chondrocytes grown in the presence of fibroblast growth factor and transforming growth factor-beta'. *J Cell Physiol* (1989); **138**: 329-337.

[59] Yasukawa, Tsutomu, Ogura, Yuichiro, Tabata, Yasuhiko, Kimura, Hideya, Wiedemann, Peter, Honda, Yoshihito. 'Drug delivery systems for vitreoretinal diseases'. *Prog Retin Eye Res* (2004); **23**: 253-281.

[60] Yasukawa, Tsutomu, Ogura, Yuichiro, Sakurai, Eiji, Tabata, Yasuhiko, Kimura, Hideya. 'Intraocular sustained drug delivery using implantable polymeric devices'. *Adv Drug Deliv Rev* (2005); **57**: 2033-2046.

[61] Haesslein, A., Ueda, H., Hacker, M. C., Jo, S., Ammon, D. M., Borazjani, R. N., Kunzler, J. F., Salamone, J. C., Mikos, A. G. 'Long-term release of fluocinolone acetonide using biodegradable fumarate-based polymers'. *J Control Release* (2006); **114**: 251-260.

Chapter 8

Three-Dimensional Hyalocyte Culture Systems

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Abstract

Hyalocytes, the cells of the vitreous body, seem to hold a significant potential for vitreous regeneration using tissue engineering techniques. Therefore, in order to overcome the limitations of currently available vitreous substitutes, the development of a cell-based approach may represent a promising concept. Towards this, the characterization of hyalocyte loaded biomaterials is necessary, as the interaction with a biomaterial could have an important influence on cells. To that end, the present study aimed at the development of a 3D culture system that allows for the characterization of hyalocytes in contact with biomaterials under conditions similar to the vitreal environment.

To achieve this, vitreous-like densities of hyalocytes were seeded in collagen type I gels with mechanical properties comparable to the mammalian vitreous. To mimic the native vitreous cortex, cells were seeded into the gels exclusively in a small layer near the surface or the bottom of the constructs. A homogenous distribution of cells served as a control. Furthermore, to elucidate the suitability of the culture system to study growth factor effects, TGF-β1 was supplemented to the media. During 30 days of cultivation, the impact of gel contraction became obvious. The highest rates of gel contraction were observed in the homogenous culture, whereas the systems that mimic the native environment of hyalocytes showed little or no contraction. The changed mechanical properties of the gels, caused by their contraction, seemed to exhibit an effect on the cell proliferation as well as their accumulation of extracellular matrix. This hypothesis, however, has to be clarified in the future.

To conclude, the presented systems seemed to allow for investigations into hyalocyte-biomaterial-interactions under conditions similar to the native environment of the cells. Furthermore, they seemed suitable to study growth factor effects on the cells. However, the usefulness of the established systems to mimic the in vivo environment remains to be elucidated.

Introduction

Tissue engineering represents a promising technique that aims for the regeneration of tissues and organs[1, 2]. This young scientific field combines knowledge from different disciplines, such as cell biology, biomaterials science, and engineering, to develop novel concepts for the treatment of diseases associated with cell-, tissue- or organ-failure[3, 4]. In a plethora of tissue engineering studies, the importance of the interplay between cells, biomaterials, and growth factors has become apparent[5, 6].

According to earlier studies (chapters 5, 6, 7), the cells of the vitreous body, termed hyalocytes, seem to hold potential for tissue engineering applications. As there is a tremendous need for novel vitreous substitutes[7], the development of a cell-based vitreous substitute using tissue engineering techniques seems promising. Towards this, however, characterization of hyalocytes within a three-dimensional (3D) material becomes necessary as cells are generally known to exhibit a different behavior when embedded in a biomaterial compared to 2D culture[8-10]. Furthermore, in vitro test systems for cell-biomaterial interactions are necessary for the development of such a cell-based vitreous substitute.

In the literature, studies on hyalocytes cultured in 3D collagen gels are reported[11, 12]. However, in these studies the hyalocyte-collagen-constructs were only used to quantify the contraction forces exhibited by the cells by measuring the gel contraction. Further data about the effects of hyalocytes on the gels and vice versa the effects of the material on the cells are missing.

Therefore, the present study aimed at the development of a 3D culture system that allows for the characterization of hyalocytes, the effect of growth factors on the cells, and hyalocyte-biomaterial-interactions under conditions similar to the vitreal environment. To achieve this, collagen type I gels with viscoelastic properties comparable to those found in the vitreous body were seeded with hyalocytes either homogenously or in a thin layer, mimicking the native position of the cells (Figure 1). After 7 and 30 days of dynamic cultivation, the constructs were characterized with regards to their cell numbers as well as their amounts of collagen and glycosaminoglycans. To furthermore characterize hyalocyte functions, the expression of mRNA of different collagen types was analyzed after 7 days of culture. Moreover, to elucidate the possibility of investigating growth factor effects using the studied systems, the influence of 1 ng/ml TGF-β1 on hyalocytes was analyzed.

Materials and Methods

Materials:

L-Ascorbic acid in cell culture quality, cysteine, chondroitin sulfate A from bovine trachea, dimethylmethylene blue, hematoxylin, $FeCl_3$, eosin, safranin O, nuclear fast green, SigmaCoat®, and highly polymerized deoxyribonucleic acid from calf thymus were purchased from Sigma (Steinheim, Germany). Fetal calf serum (South America, Batch Nr. 40A0044K), penicillin/streptomycin, phosphate buffer saline (PBS), and 0.25% Trypsin-EDTA were obtained from Invitrogen (Karlsruhe, Germany). Dulbecco's modified Eagle media (DMEM, low glucose, with glutamine and pyruvate) and TriZol® were obtained from Biochrom (Berlin, Germany). Dimethylaminobenzaldehyde, chloramine T, isopropanol, ethanol, xylene, acetic acid, Na_2HPO_4, and Na_2EDTA were bought from Merck (Darmstadt, Germany); hydroxyproline and perchloric acid were purchased from Fluka (Neu-Ulm, Germany). Tissue Tek was bought from Sakura Finetek Europe (Zoeterwoude, The Nederlands). Hoechst 33258 dye was obtained from Polysciences (Warrington, PA, USA); papainase, and collagenase type II were purchased from Worthington (Lakewood, NJ, USA). Collagen type I from bovine skin was bought from IBFB Pharma (Leipzig, Germany). Transwells (12 mm diameter polyester membrane with 3.0 µm pore size) as well as all other cell culture materials were purchased from Corning (Bodenheim, Germany). Buffer for the papainase digestion (PBE) was composed of 100 mmol Na_2HPO_4, 10 mmol Na_2EDTA, and 5 mmol cysteine in water, adjusted to pH 6.5.

Rheological characterization of collagen gels

To prepare collagen gels with concentrations of 0.75, 1.0 and 2.0 mg/ml collagen in phosphate buffer saline (PBS), suitable amounts of a 4 mg/ml collagen solution in 0.1 M acetic acid were diluted in PBS followed by neutralization with a 1 M sodium hydroxide solution under ice cooling; small volumes of DMEM served as a pH indicator. Subsequently, gelation of 1 ml of each collagen solution was investigated using a rheometer (AR 2000, TA Instruments, Alzenau, Germany) with a 20 mm steel plate and a gap of 500 µm. The temperature was increased from room temperature to 37°C while measuring the viscoelastic properties, namely G' (storage modulus) and G'' (loss modulus), of the aqueous solutions under a constant strain of 1% and a frequency of 1 Hz. After complete gelation of the sample, the mechanical properties of the gels were characterized in stress controlled mode by a strain sweep from 0.1 to 10% strain.

Cell culture

Hyalocytes were isolated enzymatically from freshly excised vitreous bodies as previously described (chapters 3 and 5). Subsequent to isolation, cells were propagated for two passages in standard culture medium (SCM) supplemented with 50 µg/ml ascorbic acid in a humidified atmosphere at 37°C and 5% CO_2; media was exchanged three times a week. SCM was composed of DMEM supplemented with 15% fetal calf serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin. For cell-biomaterial studies, hyalocytes were used after the second passage.

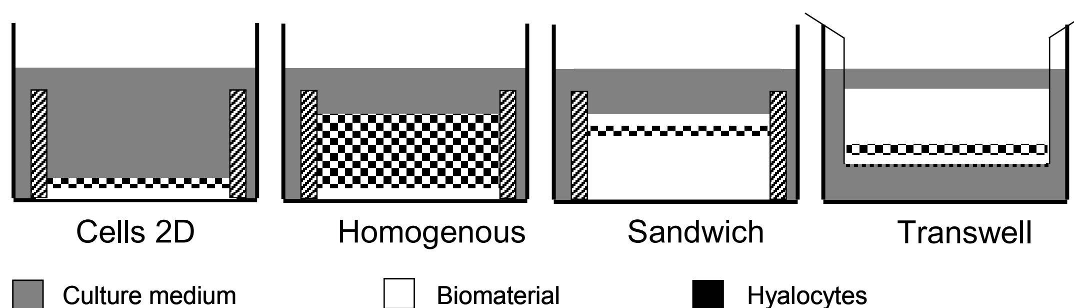

Figure 1: *Schematic picture of the different 3D culture systems as well as the cell control (2D); in the homogenous culture system, hyalocytes were evenly distributed throughout the biomaterial, although a small layer of pure biomaterial inhibited cell contact to the culture surface; in both the sandwich and transwell systems, the cells were cultivated in a small layer within the biomaterial, mimicking the native position of the cells in the vitreous; hyalocytes cultured on a thin layer of collagen served as control (Cells 2D). All systems were cultivated under three-dimensional shaking on a modified orbital shaker.*

Each 3D hyalocyte culture system consisted of 4 500 cells combined with 375 µl of a 0.75% collagen gel in DMEM and a small collagen layer of equal concentration (35 µl) with a calculated height of about 30 µm (Figure 1). Hyalocytes cultured on the small collagen layer without any additional biomaterial served as control (Cells 2D). Consistent with the transwell insert with an inner diameter of 12 mm, the homogenous and sandwich cultures were seeded in glass rings with an equal inner diameter; the glass rings were coated SigmaCoat prior to use. In all culture systems, the first sheet of collagen (the small layer in all systems except for the sandwich culture) was prepared in DMEM instead of PBS as described above and gelled for 1 h in an incubator at 37°C. Subsequently, the cells were seeded on the collagen. After 5 h of cell adherence under static conditions in an inbubator, the cells were capped with the second collagen sheet and again incubated for 1 h to allow for gelation. For homogenous cell cultivation, the cells were embedded in the neutralized collagen solution prior to gelation.

After preparation of the cell-biomaterial-combinations, they were cultivated in SCM/50 µg/ml ascorbic acid with or without supplementation of 1 ng/ml TGF-β1 on a slightly inclined orbital shaker that enabled shaking in three dimensions; media (2 ml for all systems except the

transwells, which required 1.5 ml in the lower chamber and 100 µl medium above the biomaterial) was exchanged three times a week. After 30 days, macroscopic pictures were taken with a commercial digital camera to determine gel contraction. Constructs were harvested after 7 and 30 days, followed by freeze drying and digestion of the samples in 300 µl of papainase buffer as previously described (cf. chapter 5). Cell number as well as collagen and glycosaminoglycan content were analyzed as follows.

Determination of cell number and ECM contents

Cell number was determined by measuring the DNA amount using Hoechst 33258 dye[13-15]. In brief, the emission intensity of the intercalating dye was measured at 458 nm upon excitation at 365 nm. The fluorescence of Hoechst 33258 dye is correlated with the absolute amount of DNA in the sample. An average amount of 9.96 pg DNA per cell was assumed for the cell number calculations.

Glycosaminoglycan content was measured spectroscopically as previously described[16; 17]. In brief, after digestion of interfering proteins with papainase, the glycosaminoglycan content was determined by a colorimetric reaction with dimethylmethylene blue followed by measurement of absorption at 525 nm. Chondroitin sulfate was used to produce the standard curve (cf. chapter 4).

The collagen content was determined by measuring the amount of hydroxyproline, an amino acid that exists in significant amounts exclusively in collagen, according to Woessner et al.[18] with some modifications described earlier (cf. chapters 4 and 5). In brief, after hydrolysis of the sample, hydroxyproline was oxidized with chloramine T and, subsequent to the coupling reaction with dimethylaminobenzaldehyde, the absorption of the newly formed dye was measured. A standard curve of hydroxyproline in the PBE/cysteine served as reference.

Histology

For histological analysis, samples cultivated for 30 days were washed once in PBS and fixed for 1 hour in 10% formalin at room temperature followed by embeddeding in Tissue Tek. Histological sections of 20 µm thickness were obtained using a Microm Cryotom HM 550 (Walldorf, Gemany) and subsequently stained with Meyers hematoxylin followed by eosin counterstaining. Additionally, sections were stained with safranin O for the detection of GAG and counterstained with fast green.

Semiquantitative mRNA analysis

For analysis of hyalocyte gene-expression, constructs were harvested after seven days in 1 ml TriZol®; mRNA was subsequently isolated according to the manufacturer's instructions. Reverse transcription as well as amplifications of COL1A1, COL2A1 and COL11A1 were performed using conditions and pairs of primers as described in chapter 5; β-actin served as a reference for comparisons. In contrast to chapter 5, COL11A1 was amplified for 38 cycles.

Statistics

All data are presented as means ± standard deviation with n=3. Single-factor analysis of variance (ANOVA) was used in conjunction with a multiple comparisons test (Tukey's test) to assess statistical significance with $p < 0.05$. A * indicates a statistical significance between day seven and day thirty, ‡ between control media and media supplemented with 1 ng/ml TGF-β1. Statistically significant differences compared to all other culture systems at identical time points and with the same media is indicated by $^{+}$. As assessed by ANOVA followed by a Dunnett's test, º indicates statistical significance compared to the pure collagen gels cultured for the same period of time.

Results

Rheological characterization of collagen gels

After preparation and neutralization of solutions with varying collagen concentrations, all solutions gelled within a few minutes of increasing their temperature to 37°C. After 15 minutes, no change in the viscoelastic properties of the gels could be observed, indicating that the gelation was complete (data not shown). All obtained gels exhibited a primarily elastic behavior within their linear viscoelastic region, indicated by a storage modulus (G') about one order of magnitude higher than the loss modulus (G'') (Figure 2). Both moduli of the gel samples were clearly dependent the collagen concentration: whereas the storage modulus of gels with 2.0 mg collagen was found to be 200 Pa, G' of 0.75% gels was about 45 Pa. A similar trend between 20 and 5 Pa was observed for the loss moduli G''. Signs of structural perturbations, indicated by increasing values for both moduli, were first observed at strains of about 5%; they were again dependent on the collagen concentration.

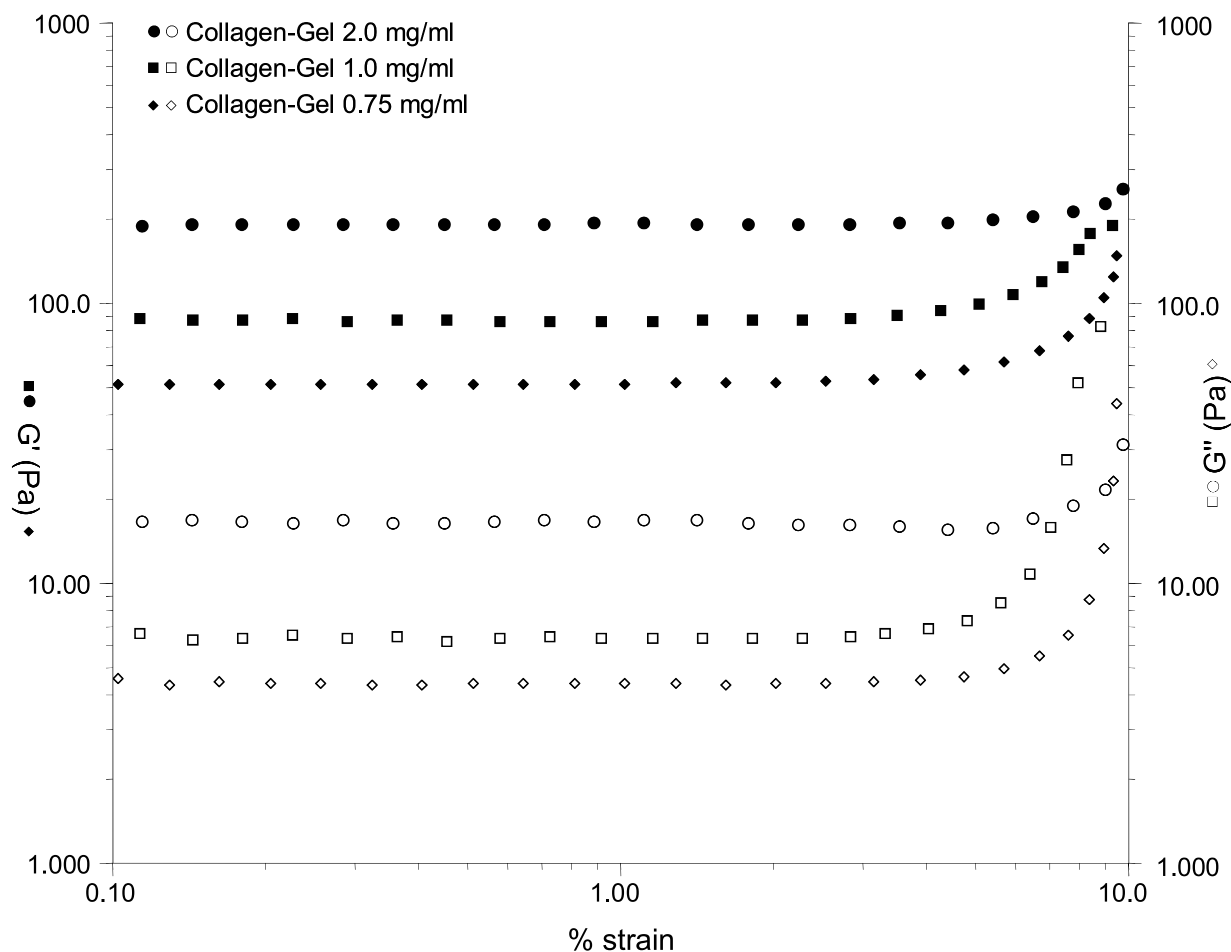

Figure 2: *Rheological characterisation of gels with different concentrations of collagen; all gels showed elastic dominated behavior, indicated by the clearly higher values of the storage modulus (G') compared to the loss modulus (G''); the gel with 0.75% collagen exhibited a storage modulus of about 45 Pa, a value similar to published data about the storage modulus of the vitreous body*[19].

Gel contraction

After 30 days of cultivation, some of the gels were clearly contracted by the embedded cells (Figure 3); control gels consisting of pure collagen, in contrast, showed no macroscopic signs of size alteration during the cultivation period. The cell controls cultivated without additional growth factor were still attached to the collagen coating, whereas cells cultured with TGF-β1 were clearly detached and built three-dimensional cell clusters (arrows in Figure 3). Among the 3D culture systems, the homogenous ones were most contracted, whereas in the transwell system no macroscopic gel contraction was observed. Furthermore, TGF-β1 supplementation seemed to enhance the contraction of the gels, as in the homogenous as well as the sandwich system the gels were more contracted compared to the non-supplemented controls.

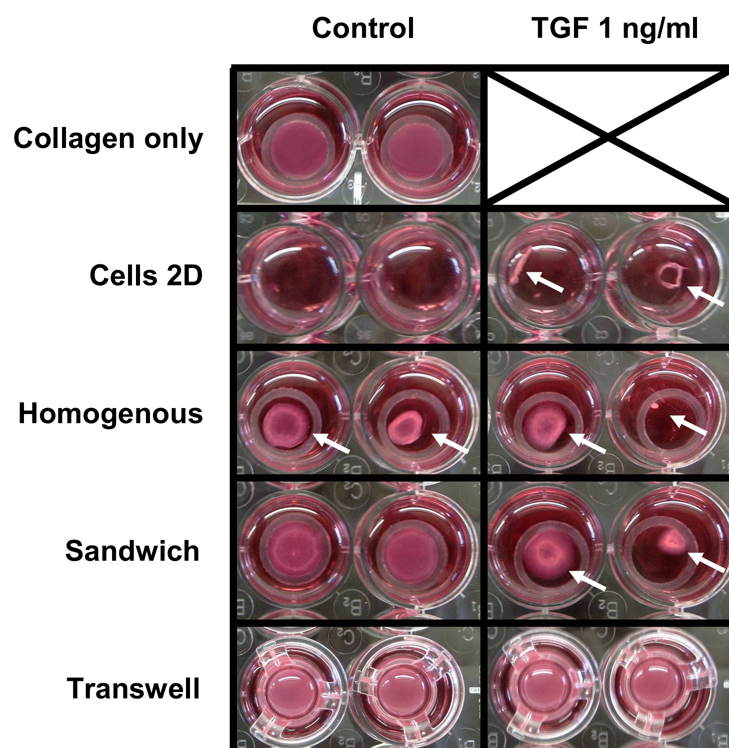

Figure 3: *Macroscopic pictures of the cell systems after 30 days of cultivation; whereas pure collagen gels appeared unaltered, some of the gels containing hyalocytes were clearly contracted (arrows); TGF-β1 seemed to enhance gel contraction.*

Histology

Due to the very high water content of the samples, it was difficult to obtain histological sections. Ruptures in the sections or the lifting of the cell sheet or the transwell membrane off the pure gel were unavoidable (Figure 4), probably caused by their different mechanical properties. Nevertheless, it was possible to get a crude picture of the microstructure of the samples by histological analysis. According to H&E-staining, hyalocytes were homogenously distributed throughout the gel in the homogenous culture system, whereas a small layer of cells adjacent to the gel surface in the sandwich culture or near the transwell membrane in these culture systems was observed (Figure 4). This indicated that the cells were predominantly present in the region in which they were seeded (compare to Figure 1).

After safranin O staining, regions with a slight red staining, mainly near the cells, were observed, indicating that small amounts of GAG must have been produced (Figure 5). Furthermore, regions with a green staining of the collagenous matrix were found in all constructs. No clear differences between the different culture systems or the growth factor supplementation on the GAG accumulation were obvious. However, histological analysis demonstrated GAG to be predominantly present near hyalocytes.

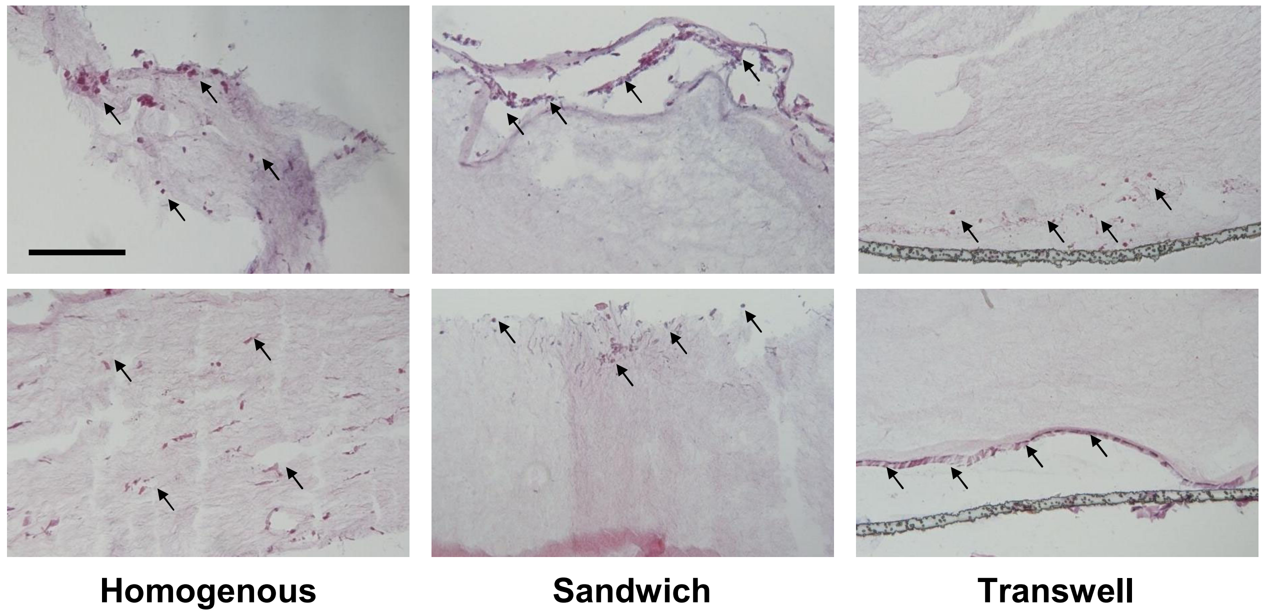

Figure 4: Histological sections of samples from different culture systems after H&E-staining; samples cultivated in control media are displayed above, pictures below show samples cultivated with 1 ng/ml TGF-β1; scale bar represents 200 µm; in the homogenous culture, hyalocytes (indicated by the arrows) were distributed throughout the gels, whereas in the sandwich and transwell systems, cells were predominantly present in a single layer near the surface of the constructs or near the transwell membrane, respectively (compare to Figure 1).

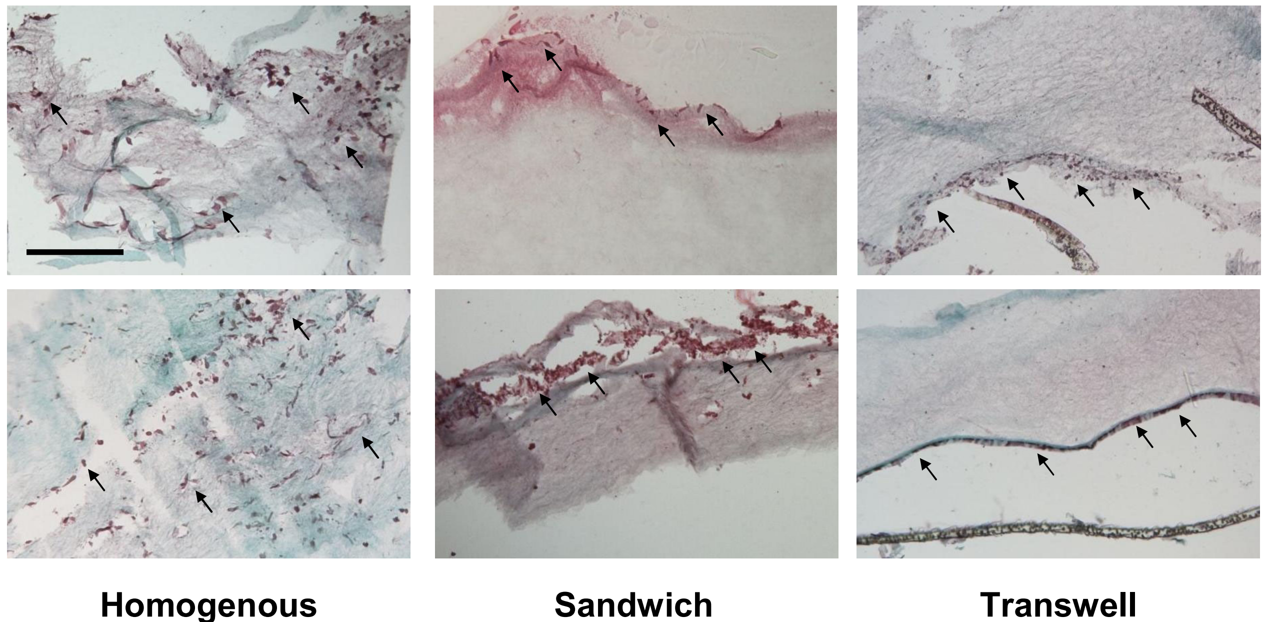

Figure 5: Histological sections of samples from different culture systems after safranin O staining; samples cultivated in control media are displayed above, pictures below show samples cultivated with 1 ng/ml TGF-β1; scale bar represents 200 µm; glycosaminoglycans were stained in red, collagenous structures in green; GAGs were predominantly observed near hyalocytes (indicated by the arrows).

Cell numbers

The number of cells within the constructs was clearly dependent on the time point and the culture system as well as the supplementation of TGF-β1 (Figure 6). In the 2D as well as in the homogenous and sandwich cultures, the cell number increased from day 7 to day 30, independent of the growth factor supplementation. In contrast, in the transwell system the number of cells obtained after 30 days was decreased compared to the number after 7 days. With the exception of the transwell system without TGF supplementation, all 3D culture systems showed a reduced cell proliferation compared to the 2D cells at day 7. At day 30, the cell numbers were also lower compared to the 2D cells when using control media. However, with TGF in the media, this trend was only clear for the transwell system, as the values for the other two 3D systems exhibited large standard deviations. Focusing on the effect of TGF-β1 on the cell number, the growth factor clearly decreased the number of cells in 2D, the sandwich, and the transwell systems at day 7 compared to control media, whereas it increased the cell number in the homogenous culture. After 30 days of cultivation, no influence of TGF-β1 on the cell number could be observed.

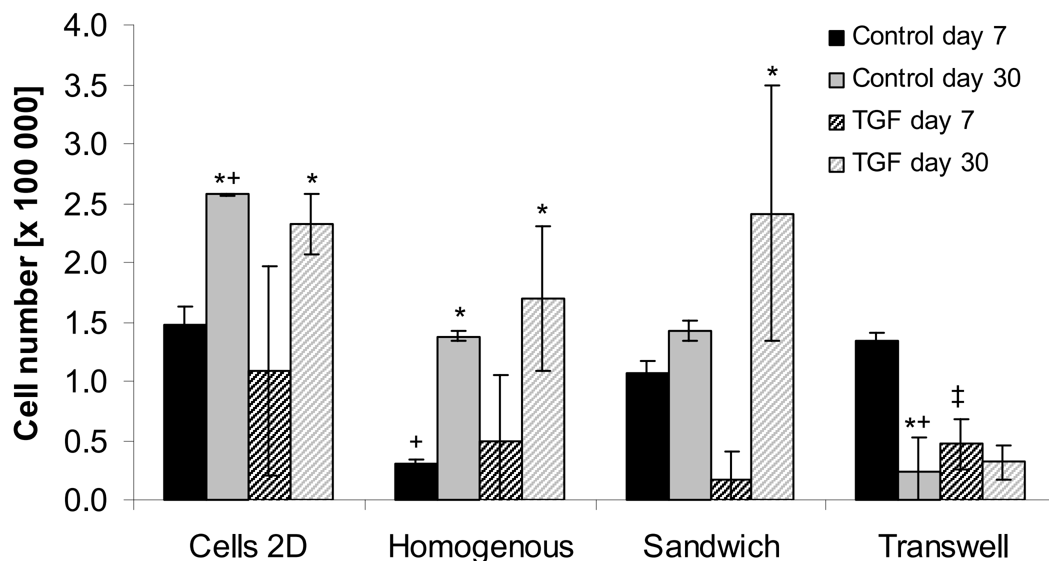

Figure 6: *Cell numbers obtained after cultivation of hyalocytes in different culture systems for 7 and 30 days with or without 1 ng TGF-β1 supplemented to the culture medium; signs indicating statistical significance are explained above.*

Glycosaminoglycan content

The GAG content within the samples was dependent on the cultivation period and the supplementation of TGF-β1. Among the cultivation systems, however, only the transwell system differed from the other systems (Figure 7). Similar to the cell numbers, the GAG contents were also increased from day 7 to day 30 in all culture systems except the transwell.

This tendency was not as distinct when TGF-β1 was supplemented. In contrast, the GAG amount within the transwell samples was clearly decreased at day 30 compared to day 7. Focusing on the culture systems, after day 7 higher values were measured in the transwell constructs compared to all other systems, independent on cultivation without or with TGF. At day 30 this trend was reverted; the GAG content of the transwell samples was apparently lower compared to the other systems. With the exception of the sandwich culture at day 7 and the transwell at day 30, GAG accumulation was increased due to supplementation of TGF-β1.

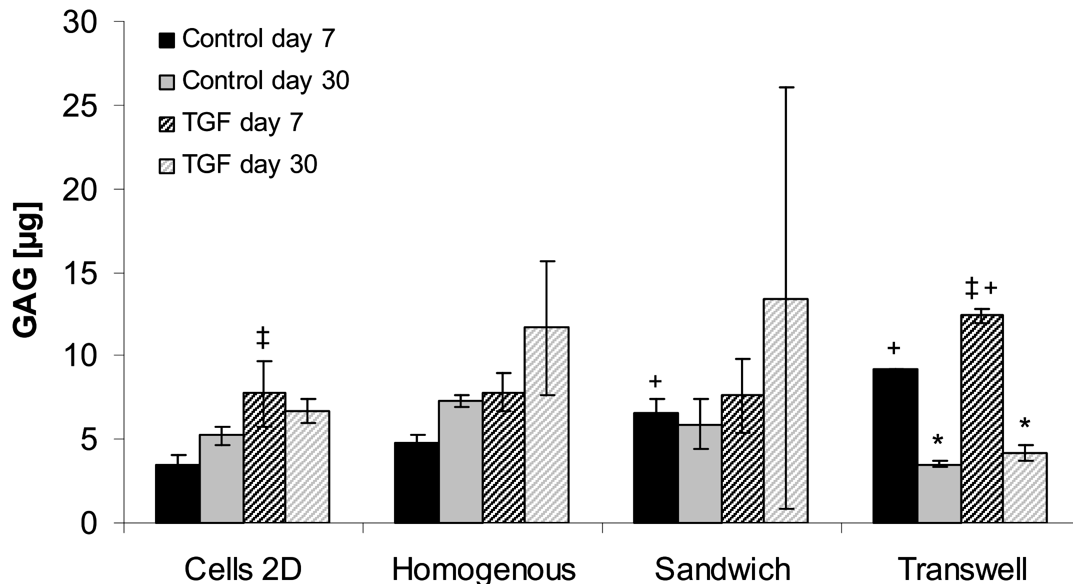

Figure 7: *Amounts of GAG measured after cultivation of hyalocytes in different culture systems for 7 and 30 days with or without 1 ng TGF-β1 supplemented to the culture media; signs indicating statistical significance are explained above.*

Collagen content

Whereas small amounts of hydroxyproline were observed in the 2D cell culture, clearly higher amounts of collagen were found in the 3D systems (Figure 8). This was due to the cultivation method, as the 3D systems started with about 10 times more collagen than the cell controls. Among the 3D systems, distinctly decreased hydroxyproline values were measured after 30 days of cultivation with TGF-β1 in the homogenous system. The other 3D groups produced values similar to the pure collagen samples cultivated without cells. The significant differences observed at day 7 in the sandwich and transwell systems seemed to be caused by chance, as they could not be repeated at 30 days. Regarding the different cultivation time points, there is a first slight, but not significant hint that cells seemed to decrease the collagen amount in the homogenous as well as the sandwich culture due to cultivation for 30 days compared to 7 days, whereas in the transwell system the cells seemed to enhance the collagen amounts. For the 2D cells cultivated with control media, the collagen amounts were doubled

during cultivation from day 7 to day 30. This tendency, however, was not apparent upon cultivation with TGF-β1. Focusing on the effect of TGF-β1 supplementation on hyalocytes cultured in the 3D systems, no influence was observed after 7 days, whereas after 30 days a decrease in the collagen amount was apparent, most prominently in the homogenous and the sandwich systems.

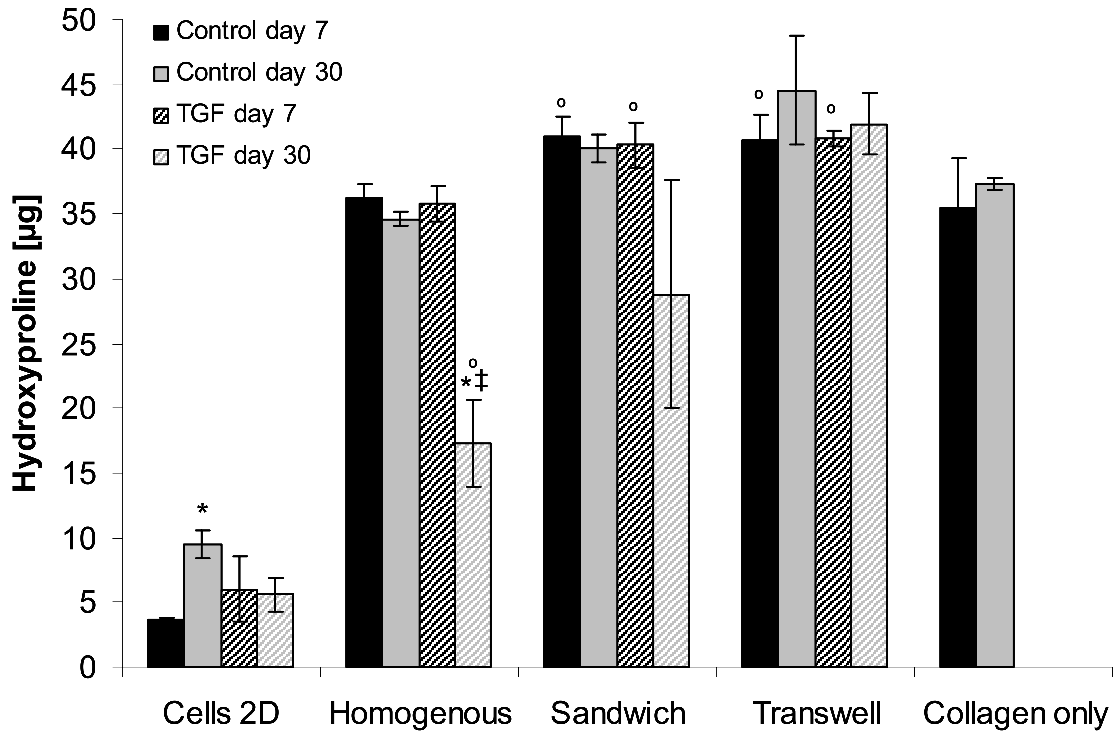

Figure 8: *Amounts of hydroxyproline as a measure for collagen determined after cultivation of hyalocytes in different culture systems for 7 and 30 days with or without 1 ng TGF-β1 supplemented to the culture media; pure collagen cultivated without cells served as control. Signs indicating statistical significance are explained above.*

mRNA expression

To further elucidate the influence of the different cultivation systems on hyalocytes, we studied the expression of different types of collagen after 7 days (Figure 9) using the housekeeping gene β-actin as reference. However, no differences between the culture systems or the growth factor supplementation were observed. In all groups, clear amounts of COL1A1, coding for the α-1 chain in collagen type I, and COL11A1, coding for the α-1 chain in the mixed collagen type V/XI, were observed. In contrast, no expression of COL2A1, coding for the α-1-chain in collagen type II, was found.

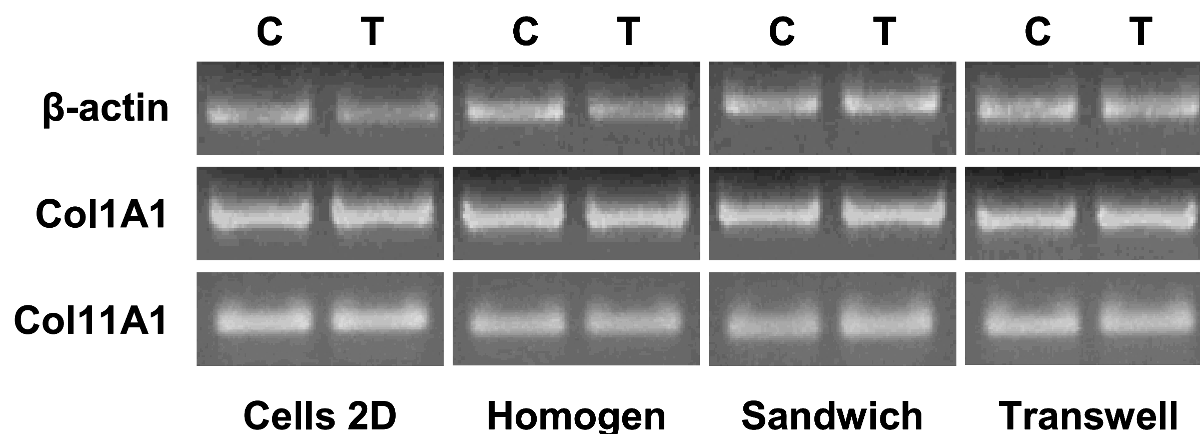

Figure 9: *mRNA expression of collagen type I (COL1A1) and collagen type V/XI (COL11A1) by hyalocytes after 7 days dependent on the culture system as well as TGF-β1 supplementation; C indicates control media, T indicates supplementation of 1 ng/ml TGF-β1; expression of the investigated genes was not influenced by the tested factors.*

Discussion

For the development of a “vitreous-like” in vitro culture system, the systems were modeled on the native environment of the cells. To this end, two different culture systems, namely the sandwich and the transwell systems, were developed (Figure 1). To mimic the cell density in the cortex of the vitreous[20], the seeding density was calculated providing a spherical shape of the vitreous with a volume of 4 ml and 50 000 cells per vitreous, a number determined from cell measurements of isolated vitreous bodies. As hyalocytes are natively present mostly in the cortex of the vitreous[21, 22], this situation was mimicked by embedding the cells into the biomaterial in a small layer either near the surface or near the bottom. Thereby, diffusion of nutrients was only inhibited by a small biomaterial layer with a calculated thickness of about 30 µm, similar to the physiological situation within the vitreous, where the cells mainly live on nutrients supplied by diffusion from the retinal blood vessels. Furthermore, as the eye is exposed to mechanical stress caused by the movement of the eye, this was imitated by shaking the samples continuously in all three dimensions. As control groups for the outlined “vitreous-like” culture systems, hyalocytes were on the one hand seeded homogenously into the biomaterial (homogenous) as performed in many other tissue engineering systems[23, 24]. In addition, cells were seeded on top of a small biomaterial layer, allowing the cells, thereby, only to adhere and interact with the material in a 2D manner in contrast to the other tested systems.

As the vitreous body mainly consists of collagens, especially collagen type II[25], it seemed reasonable to investigate collagen as a first biomaterial. Due to the poor availability of collagen type II, collagen type I was used as biomaterial for this initial study. Because it is

widely acknowledged that in addition to the chemical makeup of a biomaterial, the mechanics of a biomaterial have a tremendous impact on the cellular response[6], the mechanical properties of the collagen gels had to be adapted to that of native vitreous body. Using oscillatory rheology, storage moduli of 20 to 50 Pa and loss moduli between 4.5 and 11 Pa have been determined for vitreous bodies, dependent on the species the tissues were obtained from[19]. Furthermore, a recent report indicated that these values decline by roughly a factor of five to steady state values within an hour after removal from the eye[26]. Collagen gels with a concentration of 0.75% collagen exhibited viscoelastic properties similar to these reported values (Figure 2). Therefore, gels with this concentration of collagen were used as biomaterial.

After cultivation of the different systems for 30 days, cells were mainly present in the regions where they were seeded, as indicated by histological sections (Figures 4 and 5). This indicates the cells not to migrate within the gels. However, contraction of the collagen gels could be observed in most of the samples with exception of the transwell system (Figure 3). Contraction of collagen gels is a commonly used assay for characterization of cell-matrix interactions [27] and already reported for hyalocytes[11, 12]. However, for studying cell-biomaterial interactions, this is a critical phenomenon, as both the mechanical and diffusion properties of the material are dramatically changed, which has to be taken into consideration for the interpretation of the data.

The investigated hyalocyte culture systems led to different cellular responses regarding proliferation (Figure 6) and accumulation of glycosaminoglycans (Figure 7) and collagen (Figure 8). Whereas the sandwich and the homogenous culture showed similar results to the cell control, in the transwell system the opposite effects were observed. However, no differences in the gene expression were apparent; at day 7 the cells of all culture systems exhibited a similar expression pattern of varying collagen genes (Figure 9). As gel contraction was observed in all systems except the transwell (Figure 3), the changes in the cellular environment due to gel contraction might have had an important influence on the cell behavior. The systems with contracted gels, for example, seemed to favor hyalocyte proliferation over 30 days, whereas in the transwell system the high proliferation rate up to day 7 was even inversed, resulting in a lower cell number after 30 days compared to day 7. Similar effects were observed for the GAG content. Moreover, the systems showing the highest rate of contraction, the homogenous and sandwich systems with TGF-β1 supplementation, also exhibited the highest rate of collagen degradation. In contrast, in the transwell system the amount of collagen seems to have increased from 7 to 30 days of

cultivation. This observation was confirmed by preliminary studies investigating hyalocytes in more rigid gels with a collagen concentration of 2.0 mg/ml; a drastic decrease in the collagen content was observed in all groups in this study (unpublished data). As a result of the presented data, the hypothesis was developed, that hyalocytes take a housekeeping function within the systems and reorganize their environment. Thereby, the cells degrade non-physiological rigid collagen matrices in conjunction with an increased cell proliferation, whereas proliferation was inhibited and collagen accumulated under more physiological conditions. However, it has to be emphasized that the presented data give just a slight hint towards this hypothesis, which has to be substantiated in further studies. However, the report by Park et al.[28] supports this hypothesis, as they found chondrocytes to increase their expression of matrix metalloproteinase-13, an ECM-degrading enzyme, in non-degradable gels compared to degradable ones.

In addition to studying the different culture systems, the influence of 1 ng/ml TGF-β1 on hyalocytes was investigated in the different culture systems. The apparent effect of TGF-β1 on hyalocytes was overall comparable to the one observed in 2D culture experiments performed earlier on tissue culture treated plastic (cf. chapter 7). In general, TGF-β1 inhibited cell proliferation and enhanced the accumulation of glycosaminoglycans. In the previous studies the collagen accumulation of the cells was also increased by the growth factor. This effect could only be observed in the 2D cells after 7 days; in all other systems at this time point no differences due to TGF-β1 were found. Moreover, after 30 days the amounts of collagen within the samples were decreased in the TGF supplemented groups compared to controls in all systems. There was also a slight dependency of the TGF effect on the culture system; whereas the cell number obtained after 7 days, for example, was increased due to TGF supplementation in the homogenous one, in all other systems it was decreased. This dependency could again rely on the gel contraction, as TGF-β1 is known to enhance collagen gel contraction caused by fibroblasts [29] or tendon cells [30].

Conclusions

In the presented study, two different in vitro hyalocyte culture systems were established allowing for investigations into cell-biomaterial interactions under near-native conditions. Furthermore, the systems allow for investigations into growth factor effects on the cells in combination with biomaterials. During the first study on collagen type I as a biomaterial, the importance of gel contraction caused by the cells became obvious. At least partly due to that fact, the observed cellular response was found to be dependent on the culture system used. As

a possible explanation for these observations, the hypothesis that hyalocytes remodel the systems towards conditions similar to their physiological environment was developed. To clarify the validity of this hypothesis, additional studies will be necessary. Moreover, to elucidate the relevance of the established cell culture systems to predict in vivo situations, studies comparing in vitro results with in vivo observations should be performed.

References

[1] Langer, R. and Vacanti, J. P. 'Tissue engineering'. *Science* (1993); 260: 920-926.

[2] Vacanti, J. P. and Langer, R. 'Tissue engineering: The design and fabrication of living replacement devices for surgical reconstruction and transplantation'. *Lancet* (1999); 354 Supplement 1: SI32-SI34.

[3] Fuchs, J. R., Nasseri, B. A., and Vacanti, J. P. 'Tissue engineering: a 21st century solution to surgical reconstruction'. *Ann Thoracic Surgery* (2001); 72: 577-591.

[4] Langer, R. 'Tissue engineering'. *Mol Ther* (2000); 1: 12-15.

[5] Tabata, Yasuhiko. 'Tissue regeneration based on growth factor release'. *Tissue Eng* (2003); 9: S5-15.

[6] Brandl, F., Sommer, F., and Göpferich, A. 'Rational design of hydrogels for tissue engineering: impact of physical factors on cell behavior'. *Biomaterials* (2006); in press:

[7] Soman, N. and Banerjee, R. 'Artificial vitreous replacements'. *Biomed Mater Eng* (2003); 13: 59-74.

[8] Camps, J., About, I., Thonneman, B., Mitsiadis, T. A., Schmaltz, G., and Franquin, J. C. 'Two- versus three-dimensional in vitro differentiation of human pulp cells into odontoblastic cells'. *Connective Tissue Research* (2002); 43: 396-400.

[9] Hayman, M. W., Smith, K. H., Cameron, N. R., and Przyborski, S. A. 'Enhanced neurite outgrowth by human neurons grown on solid three-dimensional scaffolds'. *Biochem Bioph Res Co* (2004); 314: 483-488.

[10] Cukierman, Edna, Pankov, Roumen, Stevens, Daron R., and Yamada, Kenneth M. 'Taking cell-matrix adhesions to the third dimension'. *Science* (2001); 294: 1708-1712.

[11] Hirayama, Kumiko, Hata, Yasuaki, Noda, Yoshihiro, Miura, Muneki, Yamanaka, Ichiro, Shimokawa, Hiroaki, and Ishibashi, Tatsuro. 'The involvement of the rho-kinase pathway and its regulation in cytokine-induced collagen gel contraction by hyalocytes'. *Invest Ophthalmol Vis Sci* (2004); 45: 3896-3903.

[12] Noda, Yoshihiro, Hata, Yasuaki, Hisatomi, Toshio, Nakamura, Yuka, Hirayama, Kumiko, Miura, Muneki, Nakao, Shintaro, Fujisawa, Kimihiko, Sakamoto, Taiji, and Ishibashi et, al. 'Functional Properties of Hyalocytes under PDGF-Rich Conditions'. *Invest Ophthalmol Vis Sci* (2004); 45: 2107-2114.

[13] Kim, Young Jo, Sah, Robert L. Y., Doong, Joe Yuan, and Grodzinsky, Alan J. 'Fluorometric assay of DNA in cartilage explants using Hoechst 33258'. *Anal Biochem* (1988); 174: 168-176.

[14] McGowan, K. B., Kurtis, M. S., Lottman, L. M., Watson, D., and Sah, R. L. 'Biochemical quantification of DNA in human articular and septal cartilage using PicoGreen(R) and Hoechst 33258'. *Osteoarthritis Cartilage* (2002); 10: 580-587.

[15] Ng, Kee W., Leong, David T. W., and Hutmacher, Dietmar W. 'The challenge to measure cell proliferation in two and three dimensions'. *Tissue Eng* (2005); 11: 182-191.

[16] Farndale, R. W., Buttle, D. J., and Barrett, A. J. 'Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue'. *Biochim Biophys Acta* (1986); 883: 173-177.

[17] Taylor, K. B. and Jeffree, G. M. 'A new basic metachromatic dye, I:9-dimethyl methylene blue'. *Histochem J* (1969); 1: 199-204.

[18] Woessner, J. F., Jr. 'The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid'. *Arch Biochem Biophys* (1961); 93: 440-447.

[19] Nickerson, C. S., Karageozian, H. L., Park, J., and Kornfield, J. A. 'The Mechanical Properties of the Vitreous Humor'. *ARVO Meeting Abstracts* (2004); 45: 37

[20] Sebag, J. '*The vitreous*' Springer, New York (1989).

[21] Ogawa, K. 'Scanning electron microscopic study of hyalocytes in the guinea pig eye'. *Arch Histol Cytol* (2002); 65: 263-268.

[22] Uehara, M., Imagawa, T., and Kitagawa, H. 'Morphological studies of the hyalocytes in the chicken eye: scanning electron microscopy and inflammatory response after the intravitreous injection of carbon particles'. *Journal of Anatomy* (1996); 188 (Pt 3): 661-669.

[23] Salgado, Antonio J., Oliveira, Joao T., Pedro, Adriano J., and Reis, Rui L. 'Adult stem cells in bone and cartilage tissue engineering'. *Curr Stem Cell Res Ther* (2006); 1: 345-364.

[24] Bonfield, William. 'Designing porous scaffolds for tissue engineering'. *Philos Trans R Soc Lond A Math Phys Sci* (2006); 364: 227-232.

[25] Bishop, P. N. 'Structural macromolecules and supramolecular organisation of the vitreous gel'. *Prog Retin Eye Res* (2000); 19: 323-344.

[26] Nickerson, Charles S., Karageozian, Hampar L., Park, John, and Kornfield, Julia A. 'Internal tension: A novel hypothesis concerning the mechanical properties of the vitreous humor'. *Macromolecular Symposia* (2005); 227: 183-189.

[27] Ngo, Peter, Ramalingam, Punitha, Phillips, Jonathan A., and Furuta, Glenn T. 'Collagen gel contraction assay'. *Methods Mol Biol* (2006); 341: 103-109.

[28] Park, Yongdoo, Lutolf, Matthias P., Hubbell, Jeffrey A., Hunziker, Ernst B., and Wong, Marcy. 'Bovine primary chondrocyte culture in synthetic matrix metalloproteinase-sensitive poly(ethylene glycol)-based hydrogels as a scaffold for cartilage repair'. *Tissue Eng* (10: 515-522.

[29] Montesano, R. and Orci, L. 'Transforming growth factor beta stimulates collagen-matrix contraction by fibroblasts: implications for wound healing'. *PNAS* (1988); 85: 4894-4897.

[30] Chen, M. Y., Jeng, L., Sun, Y. L., Zhao, C. F., Zobitz, M. E., Moran, S. L., Amadio, P. C., and An, K. N. 'Contraction of collagen gels seeded with tendon cells'. *Biorheology* (2006); 43: 337-345.

Chapter 9

FACS as Useful Tool to Study Distinct Hyalocyte Populations

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To be submitted as 'Short note'

Abstract

Hyalocytes, the cells of the vitreous body, are assumed to be involved in physiological as well as pathophysiological processes within the eye. However, current knowledge about the cells is still limited. As different morphological types of hyalocytes are described in literature, it seems reasonable to try to isolate individual populations prior to characterization of single cell types. To achieve this, the present study investigated the utility of fluorescence activated cell sorting (FACS) for hyalocyte separation.

Subsequent to digestion of vitreous bodies using collagenase, the resulting cell suspension was analyzed and separated using FACS without any additional staining. Two-parameter dot plots of forward scatter (indicating size) against sideward scatter (indicating granularity) showed two distinct cell populations; staining with propidium iodide confirmed that both populations represent living cells. After sorting, cells of both populations were seeded on tissue culture plastic. Only one population attached and proliferated, whereas the other population was non-adherent. Even when seeding the native cell mix, only one population of cells was observed after two passages, as indicated by FACS. Furthermore, ascorbic acid increased proliferation of these cells similarly to the proliferation of the separated cell population. These data point out that only one of the two populations adheres and proliferates on tissue culture plastic.

To conclude, the established isolation technique allows for separation of clearly defined hyalocyte populations. Moreover, clear hints were obtained that only one of the two populations adheres and proliferates under the commonly applied culture conditions.

Introduction

The vitreous body is the main compartment of the mammalian eye. This transparent, hydrogel-like substance consists of different types of collagens and glycosaminoglycans[1] and is free of cells in its center; however, the cortex is lined by a single layer of cells, termed hyalocytes[2]. These cells exhibit two distinct morphologies, dependent on their localization on the vitreous surface. Hyalocytes on the inner limiting lamina appear elongated with a few stout processes, whereas cells on the epithelial surface of the ciliary body exhibit a stellate appearance with some short processes[3]. The elongated hyalocytes of the posterior vitreous are furthermore substantially larger than the cells on the ciliary epithelium[3]. Both cell types show a macrophage-like structure [4, 5] and are, therefore, thought to belong to the reticuloendothelial system[6]. According to older literature, the cells are often referred to as resting cells[5, 7, 8], however, in more recent publications their physiological role becomes apparent. The cells of the posterior vitreous body are associated with the maintenance of the vitreous body as a transparent and avascular system[9, 10]. Pathophysiologically, these cells are thought to be involved in diseases of the vitreoretinal interface, such as epiretinal membrane formation, diabetic macular endema, and macular holes[11, 12]. Furthermore, hyalocytes have been shown to be able to produce the extracellular matrix components the vitreous is built of, especially collagens[13, 14] and glycosaminoglycans[15, 16]. Although these studies indicate that hyalocytes may have a housekeeping function within the vitreous, the knowledge about hyalocytes is still limited.

To close this information gap, in vitro cell culture can be used to study cellular characteristics, cell functions, or the influence of specific factors on cells[17, 18]. In vitro cultivation of primary hyalocytes seems, thereby, to be a useful tool for investigations into these cells. As histological examinations indicated that these cells do not represent a homogenous population[3, 19], it seems reasonable to try to separate these different populations prior to characterization of each single cell type. However, no precise isolation method for different types of hyalocytes is published. The commonly used isolation technique is based on mechanical dissociation of the posterior part of the vitreous and assumes that only a single type of hyalocytes is present in the excised tissue[10, 14].

The present study investigated the utility of fluorescence activated cell sorting (FACS) for hyalocyte characterization and separation. To this end, the primary cell suspension obtained after digestion of porcine vitreous bodies was studied using FACS. Subsequent to sorting the cells into distinct cell populations, adhesion and proliferation of the different populations was

studied. To compare adherent and proliferating cells with each other, the influence of ascorbic acid on their proliferation behaviour was investigated.

Materials & Methods

Materials

Fetal calf serum (South America, Batch Nr. 40A0044K), Dulbecco's phosphate buffered saline (PBS), penicillin/streptomycin, and 0.25% trypsin-EDTA were obtained from Invitrogen (Karlsruhe, Germany). Dulbecco's modified Eagle medium (DMEM, low glucose, with glutamine and pyruvate) was obtained from Biochrom (Berlin, Germany). L-Ascorbic acid in cell culture quality, methylene blue, formaldehyde, boric acid, and propidium iodide were purchased from Sigma (Steinheim, Germany). Hoechst 33258 dye was obtained from Polysciences (Warrington, PA, USA); both collagenase type II and papainase were bought from Worthington (Lakewood, NJ, USA). All other cell culture materials were purchased from Corning (Bodenheim, Germany). Buffer for the papainase digestion (PBE) was composed of 100 mmol Na_2HPO_4 (Merck, Darmstadt) and 10 mmol Na_2EDTA (Merck) in water, adjusted to pH 6.5.

Cell isolation

Freshly enucleated porcine eyes were kindly provided by a local abattoir. Within 4 hours of slaughter, the vitreous bodies were excised under aseptic conditions, washed once in sterile PBS, and examined macro- and microscopically for adherent cells from adjacent tissues. Subsequently, the vitreous bodies were digested in a 1 mg/ml solution of collagenase type II in standard culture medium (SCM) with shaking at 37°C in a humidified atmosphere. SCM was composed of DMEM containing 15% fetal calf serum (FCS), 100 IU/ml of penicillin, and 100 µg/ml streptomycin. After digestion for 3 hours, the cell suspensions from about 20 vitreous bodies were pooled and centrifuged for 7 min at 200 g. To remove serum proteins, the cells were washed once with sterile PBS, followed by centrifugation at 200 g for 5 min. Cells were suspended in 1 ml PBS prior to further characterization.

FACS analysis and cell sorting

The primary cell isolate was analyzed without any additional staining using a FACSAria Flow Cytometer (BD Biosciences, Heidelberg, Germany) and WinMDI 2.8 software. For the determination of different cell populations, two-parameter density plots representing forward scatter against sideward scatter were used; events with a very low forward scatter were

assumed to be fragments and disregarded. The two observed populations of cells were gated out and, combined with quantification of the cell numbers, the cells within these gates were sorted into 1 ml of DMEM by a sort enhancement module (BD Biosciences, Heidelberg, Germany). After addition of 1 µg/ml propidium iodide to a fraction of the primary isolated cells, the gated cells were depicted in a two-parameter dot plot of forward scatter against the red fluorescence of propidium iodide measured with a 670 nm longpass filter (PE-Texas Red-A channel). Cells that showed a red fluorescence clearly above their autofluorescence at 670 nm were counted as dead cells.

Cell adhesion and proliferation

To study adhesion and proliferation of the two cell populations, 125 µl of cells of each sorted population (about 25 000 cells from population 1 and 12 500 cells from population 2) were seeded per 24-well either alone (population 1 and population 2) or mixed with each other (mixed populations) and subsequently cultivated in SCM supplemented with 50 µg/ml ascorbic acid. An equal amount of unsorted cells served as additional control group. Media was exchanged for the first time after two days in all groups, with the exception of the wells containing population 2. In two of four independent experiments, the population 2 media was first exchanged was exchanged after seven days to allow the cells more time to adhere to the culture plastic. After first media exchange, media was changed three times a week. After 14 days of cultivation, the cells present within the wells were fixed with 10% formaldehyde and stained with 1 ml of a 1% methylene blue solution in 10 mM borate buffer for 30 minutes. After washing of the wells three times with water, they were completely dried and photographed using a digital camera.

Effect of ascorbic acid on proliferating cells / FACS analysis of propagated cells

After nine days of cell cultivation, one well with proliferating cells from each group (population 1, mixed populations and native cell mix) was trypsinized followed by seeding of the cells at a density of 2 000 cells / cm² on tissue culture plastic. For further propogation, the cells were cultivated in SCM without ascorbic acid supplementation for seven days followed by trypsinization of the cells. An aliquot of trypsinized cells of the unsorted cell isolate ("native cell mix") was investigated using FACS as described above and analyzed by a two-parameter density plot of forward scatter against sideward scatter. Cells of all groups were seeded with 2000 cells / cm² in T-25 flasks and cultured in SCM containing 0, 10 or 200 µg/ml ascorbic acid. Subsequent to media exchange after two days, the cells were

harvested after four days by complete trypsinization for 30 minutes. After freeze drying the samples (Christ Beta 2-16, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at 20°C and 0.120 mbar for 16 hours, the dry samples were digested in 300 µl of a 125 µg/ml papainase solution in PBE for 18 hours at 60°C. Cell number was determined by measuring the DNA amount using Hoechst 33258 dye as previously described[20-22]. In brief, emission of the intercalating dye at 458 nm was measured at an excitation wave length of 365 nm. The fluorescence of Hoechst 33258 dye is correlated with the absolute amount of DNA in the sample. An average amount of 9.96 pg DNA per cell was assumed for the cell number calculations[23]. Obtained cell numbers were normalized to the cell number determined in the control groups.

Statistics

Cell sorting experiments as well as the adhesion and proliferation studies were reproduced independently four times, the influence of ascorbic acid on propagated cells was performed in duplicate. Representative data of each study are shown. All data are presented as means ± standard deviations. Single-factor analysis of variance (ANOVA) was used in conjunction with a multiple comparisons test (Tukey's test) to assess statistical significance at levels of $p < 0.01$.

Results & Discussion

In vitro cell culture proved to be a useful tool for the characterization of hyalocytes. As previous studies demonstrated that hyalocytes within the vitreous exhibit different morphologies[3, 19], a method to precisely isolate and separate these cells was deemed necessary. The present study demonstrated that FACS provides a means to separate two distinct populations of hyalocytes subsequent to digestion of vitreous bodies. Furthermore, the first evidence was found that only one of the two populations is cultivated under the commonly used culture conditions.

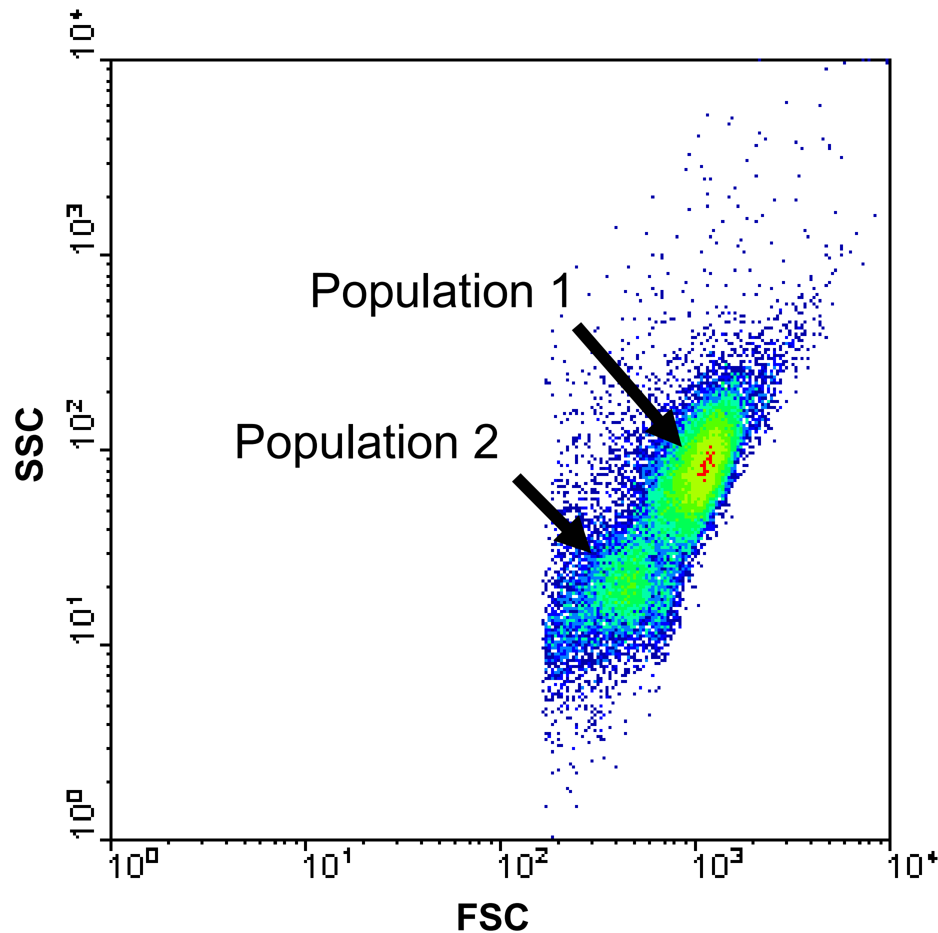

Figure 1: *Two-parameter density plot of forward light scatter (FSC, indicating cell size) against sideward light scatter (SSC, indicating cell granularity) of cells isolated out of vitreous bodies. Measurement of a single cell represents one event; dot colors indicate incidence of events, ranging from blue (low) to red (high). Two clearly distinguished regions with high event density were observed, indicating two populations of cells with different size and granularity.*

Isolation of primary hyalocytes

In contrast to the widely used hyalocyte cultivation technique described by Francois et al.[24], the established enzymatic digestion of the collagen structure of the vitreous led to a suspension of free floating cells and enabled, thereby, investigation and sorting of single cells using FACS. As displayed in Figure 1, two distinct populations of hyalocytes with different size (indicated by the forward scatter) and granularity (indicated by the sideward scatter) were detected in the primary cell isolate. The ratio of the larger cells of population 1 to the smaller cells of population 2 was reproducibly determined as 2:1. After gating for these two populations and staining with propidium iodide, the portion of dead cells within each population was quantified (Figure 2). As only about 3% of the cells of each group were identified as dead, we concluded that the established method allows isolation of two

populations of living hyalocytes, probably representing the morphologically different types described previously[3, 19]. The sufficient differences in size and granularity of the two populations allowed sorting of the cells without any additional staining and their subsequent cultivation.

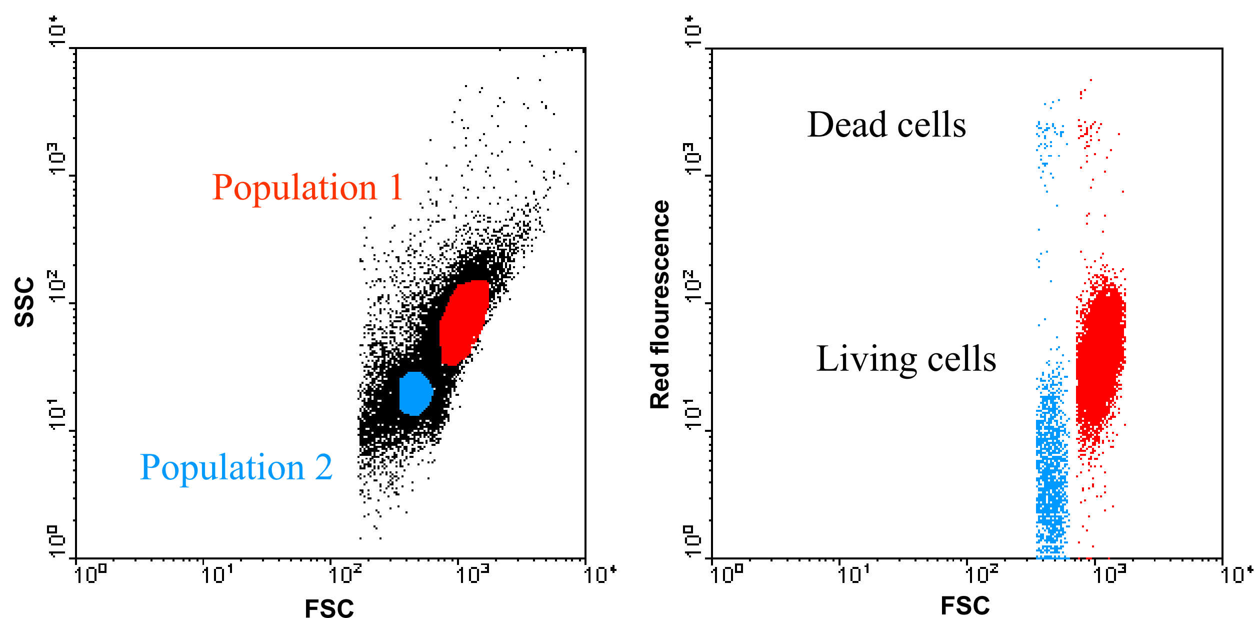

Figure 2: *Left: Two-parameter dot plot of forward light scatter (FSC) against sideward light scatter (SSC) of cells isolated out of vitreous bodies. Each dot indicates a single measurement representing a cell or a cell fragment. The observed populations of cells (according to Figure 1) were gated out; population 1 is displayed in red, population 2 in blue. Right: Two-parameter dot plot of forward light scatter (FCS) against red fluorescence measured at 670 nm of the gated populations. Living cells are indicated by a low fluorescence at 670 nm, dead cells by a high fluorescence. As only 3% of cells of each population were dead, both populations represented living cells.*

Cultivation of the different hyalocyte populations

Cells of population 1 adhered to the culture surface within two days and started to proliferate within seven days. After 14 days, colony-like proliferation was visible after methylene blue staining of the cells in an average of 60% of the seeded wells (Figure 3). In contrast, population 2 showed no adherence to the culture surface, regardless of the time point of first media exchange. Due to the cultivation method, this non-adherent population was subsequently lost during the media changes. A control group containing both populations in their native ratio exhibited adhesion and proliferation similar to population 1 (Figure 3). This indicated on the one hand that the sorting process does not kill the cells; on the other hand, interactions between the populations do not appear to influence adhesion and proliferation. Unsorted cells also showed adhesion and proliferation similar to population 1 (Figure 3).

These data gave a first hint that, even in the native cell mixture, only cells of population 1 adhere and proliferate under the applied culture conditions.

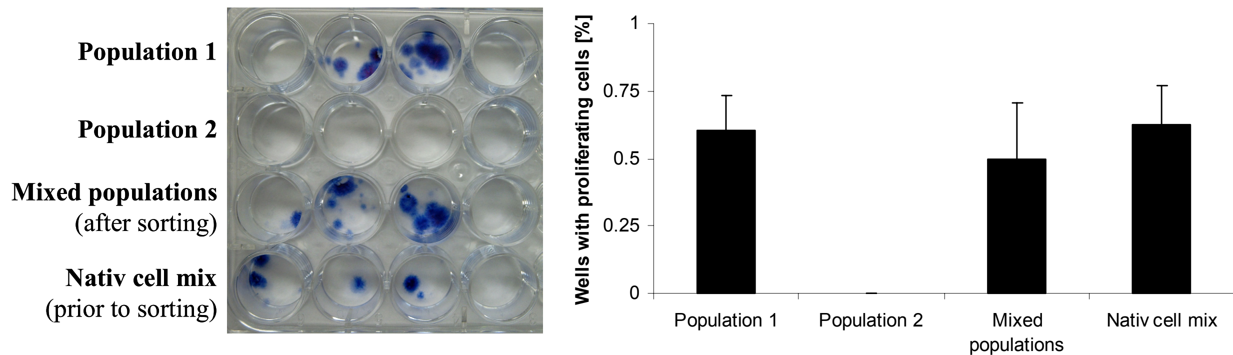

Figure 3: Left: Macroscopic picture of proliferating cells of different populations after cultivation for 14 days followed by fixation and methylene blue staining. Wells seeded with population 1 showed colony-like proliferation of the cells, whereas in the wells seeded with population 2 no proliferation could be observed. Control groups with both populations mixed in their native ratio as well as the unsorted, native cell mix exhibited cell proliferation similar to population 1. Right: Quantification of wells with proliferating cells after the cultivation period dependent on the seeded population. Average and standard deviation of four independent experiments, each n = 4, is displayed. Proliferation of population 1 was comparable to the two control groups, however, population 2 showed no adhesion and proliferation.

FACS of proliferated cells / Effect of ascorbic acid on proliferating and adherent cells

To address this assumption, we studied the cells obtained after proliferation of the native mix of isolated cells for two passages using FACS. As indicated in a two-parameter density plot of forward scatter against sideward scatter (Figure 4), only one distinct population of cells could be observed. To further support the hypothesis that only cells of population 1 adhere and proliferate, we compared the adherent cells of all groups with each other. To this end, we studied the effect of ascorbic acid on the proliferation rate of the cells after their propagation for two passages, as ascorbic acid was demonstrated to clearly influence the proliferation rate of hyalocytes[23]. Vitamin C increased the proliferation rate of all groups comparably up to a 3-fold value upon supplementation of 200 µg/ml ascorbic acid (Figure 5). This obviously similar response of all adherent cells to ascorbic acid furthermore substantiates the hypothesis that all propagated cells were derived from population 1.

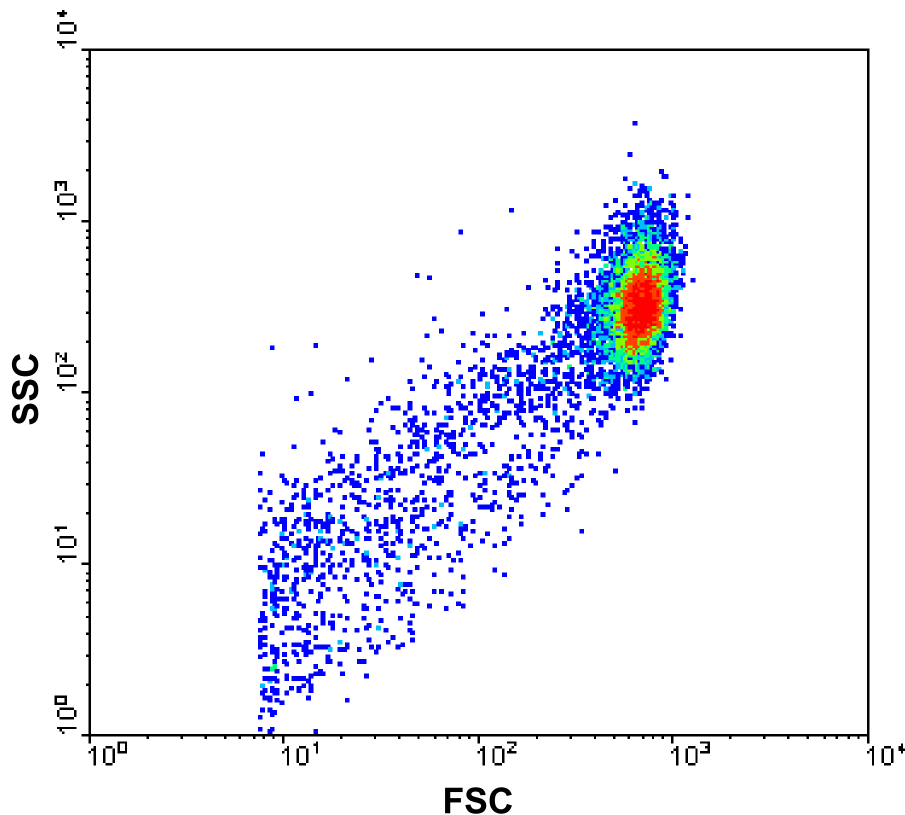

Figure 4: *Two-parameter density plot of forward light scatter (FSC, indicating cell size) against sideward light scatter (SSC, indicating cell granularity) of the native cell mix isolated out of vitreous bodies and proliferated for two passages. Measurement of a single cell represents one event; dot colors indicate incidence of events, ranging from blue (low) to red (high). Only one population could be observed after propagation of the native cell mix.*

To summarize, only cells of population 1 adhered to the culture surface and proliferated, whereas cells of population 2 showed no adherence to tissue culture plastics. Cell combinations of both populations as well as the native cell mix showed proliferation comparable to population 1 (Figure 3). Furthermore, after proliferation of the native mixture of two populations of hyalocytes (“native cell mix”) for two passages, only one population of cells could be observed (Figure 4). Moreover, all proliferating cells reacted similarly to supplementation of ascorbic acid (Figure 5). These data indicate that only cells of population 1 are cultivated under the culture conditions that are proposed by the contemporary literature. We assume that population 1 consists of hyalocytes from the posterior part of the vitreous body, as according to FACS this population is larger compared to the second one and hyalocytes within the posterior part of the vitreous are described to be larger than the ones observed near the ciliary body[3]. Additionally, in previous reports hyalocytes within the posterior part of the vitreous were cultivated using adherent cell culture[10, 25]. This fits well with our assumption that population 1 is derived from the posterior part of the vitreous.

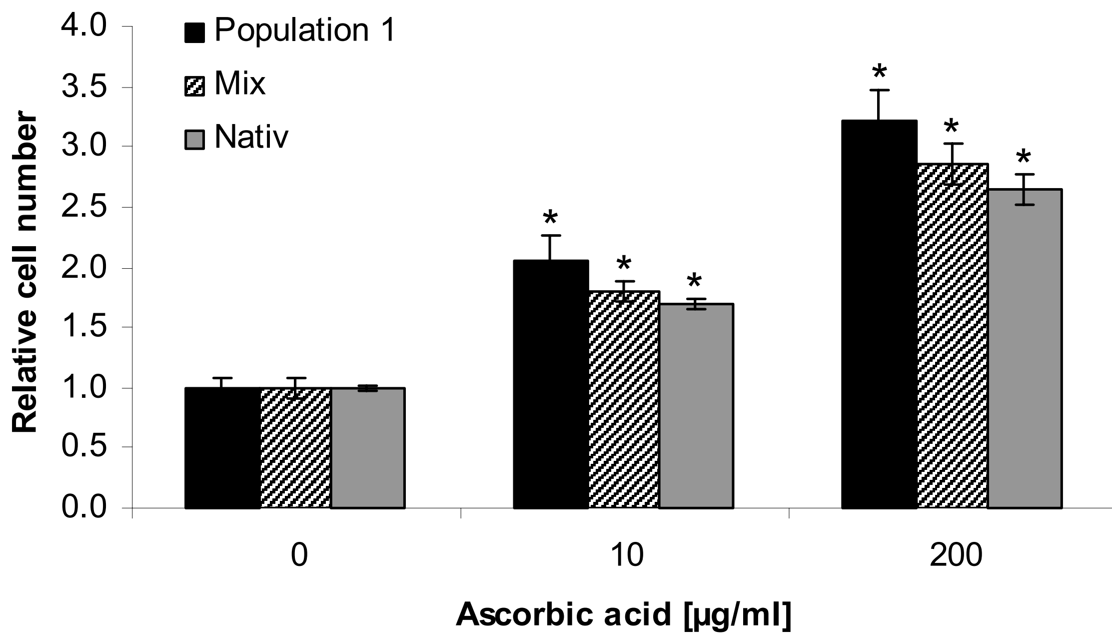

Figure 5: *Influence of ascorbic acid on the proliferation rate of the different populations after cell augmentation for two passages. Cell numbers were normalized to the control. Each group was investigated with n = 3; * indicates statistically significance to all groups except the ones supplemented with identical amounts of ascorbic acid. All populations exhibited similar response to ascorbic acid.*

Conclusions

To conclude, the established isolation technique using enzymatic digestion of the vitreous followed by FACS allows separation of clearly defined hyalocyte populations. The first evidence for different in vitro-characteristics of the previously described hyalocyte populations was found. Moreover, clear hints were obtained that only one of the two populations, probably the one observed in the posterior part of the vitreous, adhere and proliferate on tissue culture plastics. The presented isolation method, therefore, displays a valuable tool for characterization of distinct hyalocyte populations. A more complete characterization of hyalocyte properties, including their functions and their metabolic activity, may lead to a better understanding of physiological and pathophysiological processes within the eye.

References

[1] Bishop, P. N. 'Structural macromolecules and supramolecular organisation of the vitreous gel'. *Prog Retin Eye Res* (2000); **19**: 323-344.

[2] Sebag, J. '*The vitreous*', Springer, New York (1989).

[3] Ogawa, K. 'Scanning electron microscopic study of hyalocytes in the guinea pig eye'. *Arch Histol Cytol* (2002); **65**: 263-268.

[4] Boltz-Nitulescu, G., Grabner, G., and Forster, O. 'Macrophage-like properties of human hyalocytes'. *Adv Exp Med Bio* (1979); **121B**: 223-228.

[5] Salu, P., Claeskens, W., De Wilde, A., Hijmans, W., and Wisse, E. 'Light and electron microscopic studies of the rat hyalocyte after perfusion fixation'. *Ophthalmic Res* (1985); **17**: 125-130.

[6] Lazarus, H. S. and Hageman, G. S. 'In situ characterization of the human hyalocyte'. *Arch Ophthalmol* (1994); **112**: 1356-1362.

[7] Gloor, B. P. 'Development of the vitreous body and zonula'. *Albrecht Von Graefes Arch Klin Exp Ophthalmol* (1973); **187**: 21-44.

[8] Gloor, B. P. 'Radioisotopes for research into vitreous and zonule'. *Adv Ophthalmol* (1978); **36**: 63-73.

[9] Zhu, M., Penfold, P. L., Madigan, M. C., and Billson, F. A. 'Effect of human vitreous and hyalocyte-derived factors on vascular endothelial cell growth'. *Aust N Z J Ophthalmol* (1997); **25**: 57-60.

[10] Noda, Yoshihiro, Hata, Yasuaki, Hisatomi, Toshio, Nakamura, Yuka, Hirayama, Kumiko, Miura, Muneki, Nakao, Shintaro, Fujisawa, Kimihiko, Sakamoto, Taiji, and Ishibashi et, al. 'Functional Properties of Hyalocytes under PDGF-Rich Conditions'. *Invest Ophthalmol* (2004); **45**: 2107-2114.

[11] Badrinath, S. S., Gopal, L., Sharma, T., Parikh, S., Shanmugam, M. P., Bhende, P., and Biswas, J. 'Vitreoschisis in Eales' disease: pathogenic role and significance in surgery'. *Retina* (1999); **19**: 51-54.

[12] Heidenkummer, H. P. and Kampik, A. 'Morphologic analysis of epiretinal membranes in surgically treated idiopathic macular foramina. Results of light and electron microscopy'. *Der Ophthalmologe* (1996); **93**: 675-679.

[13] Newsome, D. A., Linsenmayer, T. F., and Trelstad, R. L. 'Vitreous body collagen. Evidence for a dual origin from the neural retina and hyalocytes'. *J Cell Biol* (1976); **71**: 59-67.

[14] Kobuch, K., Wild, B., Eckert, E., Fischbach, C., and Gabel V.P. 'Expression of Collagen Type II by Hyalocytes In Vitro - First Steps Towards a Biological Vitreous Substitute', presented at 'ARVO Annual Meeting', Fort Lauderdale, FL, USA (2002)

[15] Jacobson, B. 'Identification of sialyl and galactosyl transferase activities in calf vitreous hyalocytes'. *Curr Eye Res* (1984); **3**: 1033-1041.

[16] Jacobson, B. 'Degradation of glycosaminoglycans by extracts of calf vitreous hyalocytes'. *Exp Eye Res* (1984); **39**: 373-385.

[17] Esteban, Miguel A. and Maxwell, Patrick H. 'Manipulation of oxygen tensions for in vitro cell culture using a hypoxic workstation'. *Expert Rev Proteomics* (2005); **2**: 307-314.

[18] Dimova, S., Brewster, M. E., Noppe, M., Jorissen, M., and Augustijns, P. 'The use of human nasal in vitro cell systems during drug discovery and development'. *Toxicology in Vitro* (2005); **19**: 107-122.

[19] Uehara, M., Imagawa, T., and Kitagawa, H. 'Morphological studies of the hyalocytes in the chicken eye: scanning electron microscopy and inflammatory response after the intravitreous injection of carbon particles'. *J Anat* (1996); **188 (Pt 3)**: 661-669.

[20] Kim, Young Jo, Sah, Robert L. Y., Doong, Joe Yuan, and Grodzinsky, Alan J. 'Fluorometric assay of DNA in cartilage explants using Hoechst 33258'. *Anal Biochem* (1988); **174**: 168-176.

[21] McGowan, K. B., Kurtis, M. S., Lottman, L. M., Watson, D., and Sah, R. L. 'Biochemical quantification of DNA in human articular and septal cartilage using PicoGreen(R) and Hoechst 33258'. *Osteoarthritis Cartilage* (2002); **10**: 580-587.

[22] Ng, Kee W., Leong, David T. W., and Hutmacher, Dietmar W. 'The challenge to measure cell proliferation in two and three dimensions'. *Tissue Eng* (2005); **11**: 182-191.

[23] Sommer, F., Kobuch, K., Brandl, F., Wild, B., Weiser, B., Gabel V.P., Blunk, T., and Göpferich, A. 'Ascorbic acid modulates proliferation and ascorbic acid accumulation of hyalocytes'. *submitted to Tissue Eng* (2006);

[24] Francois J., Victoria-Troncoso V., and Eekhout M. 'The micromorphology of Hyalocytes in tissue culture'. *Ophthalmic Res* (1973); **5**: 290-299.

[25] Hirayama, Kumiko, Hata, Yasuaki, Noda, Yoshihiro, Miura, Muneki, Yamanaka, Ichiro, Shimokawa, Hiroaki, and Ishibashi, Tatsuro. 'The involvement of the rho-kinase pathway and its regulation in cytokine-induced collagen gel contraction by hyalocytes'. *Invest Ophthalmol* (2004); **45**: 3896-3903.

Chapter 10

Summary and Conclusions

Summary

Tissue engineering emerged as applied research in the 1970s[1] and gained increasing popularity in the late 1980s[2]. Since then, this interdisciplinary discipline developed a variety of approaches for the engineering of skin, bone, cartilage, liver, and many other tissues. In the field of ophthalmology, tissue engineering strategies gave birth to novel therapeutic concepts, especially for the treatment of the cornea **(Chapter 1)**. The vitreous body initially seemed to represent an ideal target for tissue engineering due to its simplicity and its lack of blood vessels, however, as of yet, no satisfactory approach for vitreous regeneration has been developed. This is all the more surprising, since, despite the clinical demand, no satisfying vitreous substitute is currently available[3]. This may be due, in part, to the limited knowledge about the cells of the vitreous body, known as hyalocytes.

To investigate hyalocyte cellular characteristics and functions, in vitro cultivation of hyalocytes under optimal culture conditions promises to be a useful tool. In vitro culture systems for hyalocytes have been reported in the literature, however, they have the distinct disadvantage of requiring a large number of propagation steps (4 – 8 passages) to obtain a sufficient number of cells for study[4]. Since cell propagation is widely known to result in dedifferentiation of cells, it is imperative to minimize the number of propagation steps. The established isolation and culture system outlined in this thesis is based on enzymatic digestion of vitreous bodies and guarantees a suitable yield of porcine hyalocytes after second passage **(Chapter 3)**.

In addition to defining suitable culture conditions, it was critical to identify markers that allow for the assessment of cell function, since differentiation markers for hyalocytes were still unknown. The quantitative accumulation of extracellular matrix components (ECM), of which the vitreous is made, was hypothesized to be a suitable surrogate. To that end, analytical tools that allowed quantification of glycosaminoglycans (GAG) and collagen accumulation by the cells were developed **(Chapter 4)**. A side benefit of this study was that the detection limit for hydroxyproline, indicative of collagen levels, was lowered by one order of magnitude compared to the widely used method by Woessner et al.[5]

These established methods enabled detailed investigations into the effects of bioactive substances or growth factors on hyalocytes. It was thus determined that ascorbic acid clearly increased hyalocyte proliferation and collagen accumulation **(Chapter 5)** in a two-dimensional (2D), as well as in a three-dimensional (3D) culture system. Further investigations into the mechanism of the ascorbic acid effect indicated that the enhanced

collagen production was at least partly due to an enhanced expression of mRNA coding for collagen type V/XI. Ascorbic acid was thus hypothesized to be an important modulator of the intraocular environment since the vitreous body physiologically accumulates ascorbic acid to concentrations several times higher than in plasma, and furthermore, this vitamin is known to inhibit proliferation of cells from tissues adjacent to the vitreous. In a follow-up study, the observed effect of ascorbic acid was found to be dependent upon the presence of pyruvate within the medium **(Chapter 6)**. Although the exact mechanism of this interaction remains to be elucidated, these two factors were identified as important supplements for in vitro hyalocyte culture. Moreover, combinations of the factors may allow for the modulation of hyalocyte behavior.

Further improvements in control of hyalocyte behavior were achieved by supplementation of basic fibroblast growth factor (bFGF) or transforming growth factor β-1 (TGF-β1) to the culture medium **(Chapter 7)**. Both factors were demonstrated to clearly affect the cell morphology as well as the actin organization. Furthermore, bFGF was demonstrated to enhance cell proliferation, thereby decreasing the ECM production, whereas TGF-β1 increased the accumulation of ECM while inhibiting cell proliferation. Moreover, accelerated cell expansion due to the use of bFGF was found to increase collagen production in the propagated cells, while the GAG accumulation remained unaffected.

In developing a cell-based vitreous substitute using tissue engineering strategies **(chapter 2)**, interactions between hyalocytes and biomaterials are important to consider. To enable investigations into these cell-biomaterial interactions, in vitro culture systems that mimic the native environment of hyalocytes were designed and tested with collagen type I gels of a mechanical stiffness similar to the native vitreous body **(chapter 8)**. The established systems proved suitable for studying cell-biomaterial interactions, although their relevance to in vivo situations remained to be elucidated. Furthermore, the importance of collagen gel contraction caused by the embedded hyalocytes became obvious.

According to current scientific literature, hyalocytes obtained from vitreous bodies represent a single cell population. However, using electron microscopy, two morphologically different types of hyalocytes were observed[6, 7]. Therefore, it seemed reasonable to separate these populations prior to cultivation and/or characterization. Using the already established enzymatic digestion of the vitreous **(Chapter 3)** in conjunction with fluorescence activated cell sorting (FACS), separation of clearly defined hyalocyte populations became possible without any additional staining **(Chapter 9)**. Further studies of the two populations indicated that only one population of cells adheres to and proliferates on tissue culture plastics. This

improved isolation method yielded a valuable tool for characterization of distinct hyalocyte populations.

Conclusions

In conclusion, the present thesis provided fundamental methods and techniques that allow for in vitro investigations into hyalocyte characteristics and functions in 2D, as well as 3D, culture systems. The quantitative accumulation of glycosaminoglycans and collagens, representative of the functional properties of hyalocytes, were studied for the first time. Using these methods, ascorbic acid and pyruvate were demonstrated to be key factors for in vitro cultivation. Furthermore, bFGF and TGF-β1 were identified as tools that may allow for the control of hyalocyte proliferation as well as accumulation of ECM. bFGF proved to be an especially valuable factor because it not only accelerates cell expansion but also increases the collagen production of the propagated cells. The new 3D hyalocyte culture system contributes significantly to the use of hyalocytes in future tissue engineering applications, because it allows for studies of cell-biomaterial interactions under conditions similar to the native environment of the cells. Finally, the outlined isolation and separation method using FACS enabled cultivation and characterization of distinct hyalocyte populations instead of a mixture of cells. More importantly, this resulted in the characterization of distinct cell populations isolated from the vitreous body in their native state for the first time.

In future studies, the complete expression of mRNA of both native populations should be screened using DNA array technology to gain insight into hyalocyte characteristics. This may lead to a better understanding of the physiological and pathological roles of the cells. A thorough understanding of the adhesion receptors or metabolic enzymes of hyalocytes could enable the rational design of a biomaterial that is tailored to the demands of the cells as well as to the intraocular environment. The detailed knowledge presented here regarding hyalocyte isolation, cultivation, and functional modulatory factors, as well as the analytical tools to analyze the accumulated ECM components, when combined with a rational biomaterial design, may help to establish a cell-based vitreous substitute in the future.

References

[1] Langer, R. 'Tissue engineering'. *Mo Ther* (2000); **1**: 12-15.

[2] Langer, R. and Vacanti, J. P. 'Tissue engineering'. *Science* (1993); **260**: 920-926.

[3] Soman, N. and Banerjee, R. 'Artificial vitreous replacements'. *Biomed Mater Eng* (2003); **13**: 59-74.

[4] Noda, Yoshihiro, Hata, Yasuaki, Hisatomi, Toshio, Nakamura, Yuka, Hirayama, Kumiko, Miura, Muneki, Nakao, Shintaro, Fujisawa, Kimihiko, Sakamoto, Taiji, and Ishibashi et, al. 'Functional Properties of Hyalocytes under PDGF-Rich Conditions'. *Invest Ophthalmol* (2004); **45**: 2107-2114.

[5] Woessner, J. F., Jr. 'The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid'. *Arch Biochem Biophys* (1961); **93**: 440-447.

[6] Uehara, M., Imagawa, T., and Kitagawa, H. 'Morphological studies of the hyalocytes in the chicken eye: scanning electron microscopy and inflammatory response after the intravitreous injection of carbon particles'. *J Anat* (1996); **188 (Pt 3)**: 661-669.

[7] Ogawa, K. 'Scanning electron microscopic study of hyalocytes in the guinea pig eye'. *Arch Histol Cytol* (2002); **65**: 263-268.

Appendices

List of Abbreviations

2D	2-dimensional
3D	3-dimensional
AM	amniotic membrane
α-MEM	Minimum Essential Medium Eagle, α-Modification
ANOVA	analysis of variance
ARMD	age-related macular degeneration
bFGF	basic fibroblast growth factor
BM	Bruch's membrane
BSA	bovine serum albumine
CEC	corneal endothelial cell(s)
CNV	choroidal neovascularization
CO_2	carbon dioxide
COL1A1	gene coding for the α1-chain of collagen type I
COL2A1	gene coding for the α1-chain of collagen type II
COL11A1	gene coding for the α1-chain of collagen type I
cDNA	complementary deoxyribonucleic acid
DAB	dimethylamino benzaldehyde
DEAE	diethylaminoethylseparose
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DR	diabetic rethinopathy
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence activated cell sorting
$FeCl_3$	Iron(III) chloride
FCS	fetal calf serum
FSC	forward scatter
GAG	glycosaminoglycans(s)
G'	storage modulus
G''	loss mudulus
H&E	hematoxylin & eosin
HPLC	high performance liquid chromatography

HYP	hydroxyproline
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cell
NaCl	sodium chloride
PBE	phosphate buffer with EDTA
PBS	phosphate buffered saline
PCO	posterior capsule opacification
PCR	polymerase chain reaction
PE	pigment epithelium
PEG	polyethylene glycol
PI	propidium iodide
PLA	poly(lactic acid)
PLGA	poly(lactic-co-glycolic acid)
RPE	retinal pigment epithelium
RT-PCR	reverse transcriptase – polymerase chain reaction
SafO	safranin O
SCM	standard culture medium
SSC	sideward scatter
T25-flask	25 cm^2 cell culture flask
Taq	thermus aquaticus
TGF-β	transforming growth factor β
Tris	tris(hydroxymethyl)aminomethane
UV/VIS	ultra violet / visible
VEGF	vascular endothelial growth factor

Curriculum vitae

Name:	Florian Sommer
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Education:

1984 – 1986	Elementary School, Hawangen
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1994 – 1997	Grammar School: Bernhard-Strigel-Gymnasium, Memmingen
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Professional Training and Experiences:

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11/01 – 04/02	Pharmaceutical Traineeship: "Mohren-Apotheke", Memmingen
05/02 – 10/02	Pharmaceutical Traineeship: Bayer AG, Leverkusen
11/02	Acquisition of the license to practise as pharmacist
01/03 – 09/06	PhD program at the Department of Pharmaceutical Technology, University of Regensburg, Regensburg, Prof. Dr. Achim Göpferich
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Scholarships:

Scholarship of the Konrad-Adenauer-Foundation for highly talented students during the pharmacy studies

Scholarship of the Bavarian Ministry of Sciences, Research and Arts at the beginning of the PhD programm

List of Publications

Publications

Sommer F, Brandl F, Göpferich A. 'Ocular Tissue Engineering'. Series: *Adv Exp Med Biol*, Fisher J P (ed.), **585** (2006); in press **(chapter 1)**.

Sommer F, Kobuch K, Brandl F, Wild B, Weiser B, Gabel V-P, Blunk T, Göpferich A. 'Ascorbic acid modulates proliferation and extracellular matrix accumulation of hyalocytes'. Submitted to *Tissue Eng* **(chapter 5)**.

Sommer F, Pollinger K, Brandl F, Weiser B, Teßmar J, Blunk T, Göpferich A. 'Modulation of hyalocyte proliferation and ECM accumulation via bFGF and TGF-β1'. To be submitted to *Eur J Pharm Biopharm* **(chapter 7)**.

Sommer F, Brandl F, Teßmar J, Blunk T, Göpferich A. 'Pyruvate modulates the effect of ascorbic acid on hyalocytes'. To be submitted **(chapter 6)**.

Sommer F, Brandl F, Weiser B, Teßmar J, Blunk T, Göpferich A. 'FACS as useful tool to study distinct hyalocyte populations'. To be submitted **(chapter 9)**.

Brandl F, Sommer F, Göpferich A. 'Rational design of hydrogels for tissue engineering: Impact of physical factors on cell behavior'. *Biomaterials* (2007); **28**: 134-146 doi:10.1016/j.biomaterials.2006.09.017

Abstracts

2003

Sommer F, Kobuch K, Wild B, Blunk T, Gabel V-P, Göpferich A. 'Influence of ascorbic acid on hyalocytes'. *DPhG Jahrestagung 2003*, Würzburg, Germany (Poster)

2004

Sommer F, Kobuch K, Wild B, Blunk T, Gabel V-P, Göpferich A. 'Influence of ascorbic acid on 2-D and 3-D cultured hyalocytes'. *1st Conference on Strategies in Tissue Engineering*, Würzburg, Germany [Cytotherapy 6 (3) 290] (Poster)

Sommer F, Kobuch K, Wild B, Weiser B, Blunk T, Gabel V-P, Göpferich A. 'First Steps towards a biological vitreous substitute – The influence of ascorbic acid on hyalocytes'. *DPhG Jahrestagung 2004 / Joint Meeting*, Regensburg Germany (Talk)

Weiser B, Neubauer M, Sommer F, Göpferich A, Blunk T. 'Ascorbic acid enhances adipogenesis of rat marrow stromal cells'. *DPhG Jahrestagung 2004 / Joint Meeting*, Regensburg Germany

2005

Kobuch K, Wild B, Sommer F, Fischbach C, Göpferich A, Gabel V-P. 'On the way to a cell-based vitreous substitute: proliferation and redifferentiation of hyalocytes in vitro'. *103rd Congress of the German Ophthalmic Society / 15th Congress of the European Society of Ophthalmology*, Berlin, Germany

Kobuch K, Wild B, Sommer F, Fischbach C, Göpferich A, Gabel V-P. 'Development of a cell-based vitreous substitute: proliferation, redifferentiation and expression of extracellular matrix of hyalocytes'. *2nd International Conference on Tissue Engineering*, Crete, Greece

Sommer F, Kobuch K, Brandl F, Wild B, Weiser B, Gabel V-P, Blunk T, Göpferich A. ‚Ascorbic acid for in vitro hyalocyte culture – an important factor towards a cellular vitreous substitute'. *2nd International Conference on Tissue Engineering*, Crete, Greece (Poster)

Sommer F, Kobuch K, Brandl F, Wild B, Weiser B, Gabel V-P, Blunk T, Göpferich A. ‘Ascorbic acid influences hyalocytes on the molecular level – increased expression of collagen type V/XI’. *European Tissue Engineering Society Conference*, Munich, Germany (Poster)

2006

Sommer F, Brandl F, Weiser B, Teßmar J, Blunk T, Göpferich A. ’Hyalocytes within the vitreous body – a homogenous population? First evidence for two distinct populations’. *2nd International Conference on Strategies in Tissue Engineering*, Würzburg, Germany [Cytotherapy 8 (Supp2) 53] (Poster)

Brandl F, Sommer F, Lungwitz U, Blunk T, Teßmar J, Göpferich A. ’In situ-gelling hydrogels based on poly(ethylene glycol)’. *Interface Biology of Implants*, Rostock, Germany

Sommer F, Pollinger K, Brandl F, Weiser B, Teßmar J, Blunk T, Göpferich A. ‘Towards a cell-based vitreous substitute – The effect of bFGF and TGF-β1 on hyalocytes’. *33rd Annual Meeting & Exposition of the Controlled Release Society*, Vienna, Austria (Talk)

Grants

Prof. Dr. Achim Göpferich, Dr. Torsten Blunk, Florian Sommer. ‘Development of a cell-based vitreous substitute for the therapy of vitreoretinal diseases’. Cooperation of the Pharmaceutical Technology as well as the Ophthalmology Department of the University of Regensburg with industrial partners. Bavarian Research Foundation, No 616/04

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