Development 111, 543-549 (1991)
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Primitive erythropoiesis of mouse teratocarcinoma stem cells PCC3/A/1 in serum-free medium

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Summary

Mouse teratocarcinoma stem cells PCC3/A/1 differentiated into various types of cells, such as red cells, when they were grown in serum-free medium containing transferrin and bovine serum albumin on a KCF cell feeder layer. These red cells were stained well with 2,7-diaminofluorene (DAF), and therefore were erythroid cells. They were nucleated and contained embryonic globin chains, immunologically identified with anti-embryonic hemoglobin antisera after acid urea Triton X-100 polyacrylamide gel electrophoresis (UT-PAGE). The addition of erythropoietin to the culture medium enhanced the production of both embryonic and adult globin chains. The addition of interleukin-3 also enhanced the production of embryonic globin chains, but not the production of adult globin chains. These results indicated that primitive erythropoiesis of PCC3/A/1 teratocarcinoma cells did not require exogenous addition of any hematopoietic factor such as erythropoietin or interleukin-3. This culture system will be a new model system for investigating the factors regulating the primitive erythropoiesis in yolk sac blood islands.

Key words: primitive erythropoiesis, teratocarcinoma stem cell, serum-free culture.

Introduction

During normal mouse embryogenesis, erythropoiesis begins in yolk sac blood islands on the 8th day of gestation. These blood islands produce primitive nucleated erythroid cells, which contain the embryonic hemoglobins, EI (x2y2), EII (α2y2) and EIII (α2z2) (Fantoni *et al.* 1967). On the 10th day of gestation, definitive enucleated erythrocytes, which contain adult type hemoglobin A ($\alpha 2\beta 2$), are produced in fetal liver continuing until near birth, and on the 12th day of gestation these are released into peripheral blood circulation (Craig and Russell, 1964; Barker, 1968).

Precursor cells for definitive erythrocytes such as erythroid colony-forming units (CFU-E) and erythroid burst-forming units (BFU-E) have been identified and characterized (Eaves *et al.* 1979). The factors regulating proliferation or differentiation of those precursor cells, such as erythropoietin (Epo) (Stephenson *et al.* 1971; Iscove *et al.* 1974), burst promoting activity (BPA) (Aye, 1977; Golde *et al.* 1980) and interleukin-3 (IL-3) (Goodman *et al.* 1985; Suda *et al.* 1986), have also been identified. On the other hand, nothing is known about precursor cells of primitive erythroid cells or the factors regulating their differentiation. One reason for this is the difficulty of collecting erythroid cells or their precursor cells from yolk sac blood islands.

Mouse teratocarcinoma stem cells (EC cells) and embryonic stem cells (ES cells) are considered a good model system for studying the mechanisms of early development and cell differentiation. Cudennec and Nicolas (1977) reported that PCC3/A/1 cells differentiated into primitive erythroid cells in organotypic cultures. Mouse EC cells PSA-1 (Martin *et al.* 1977) and ES cells (Doetschman *et al.* 1985) were also reported to differentiate into blood islands after they formed cystic embryoid bodies. In organotypic culture of PCC3/A/1 cells, the reproducibility of erythroid cell differentiation did not appear remarkable, perhaps due to the use of fetal calf serum-containing medium. In the formation of cystic embryoid bodies of ES cells, reproducible erythroid differentiation was observed only when human cord serum was used instead of fetal calf serum.

We previously reported that PCC3/A/1 cells constantly differentiated into chondrocytes and adipocytes after clonal growth for several days in serum-free medium on the KCF-feeder layer (Atsumi *et al.* 1985).

In the present study, we demonstrate that under the same culture condition, PCC3/A/1 cells can differentiate into erythroid cells.

Materials and methods

Cells and cell culture

Teratocarcinoma stem cells PCC3/A/1 and KCF fibroblastic cells were described elsewhere (Cudennec and Nicolas, 1977; Atsumi *et al.* 1985). Stock cultures of both cells were grown in DF medium, a 1:1 mixture of Dulbecco-modified Eagle's minimum essential medium (DMEM) and Ham F-12 containing 3×10^{-8} M sodium selenite, supplemented with 10% fetal calf serum, 10 $\mu g\,ml^{-1}$ bovine insulin (Sigma) and 10 $\mu g\,ml^{-1}$ iron-saturated human transferrin (Sigma). All cells were cultured at 37°C in a humidified atmosphere of 92.5% air and 7.5% CO_2.

Induction of differentiation

Detailed procedures for preparation of the KCF feeder layer and transfer of PCC3/A/1 cells into serum-free culture were described by Atsumi *et al.* (1985). KCF cells were treated with 20 $\mu g\,ml^{-1}$ mitomycin C (Sigma) for 4 hours, and were then dissociated by trypsin treatment. After adding soybean trypsin inhibitor, cells were washed three times and 2×10^5 cells were inoculated into a 35 mm tissue culture dish (Falcon) with DF medium. The following day, 20 PCC3/A/1 cells suspended in DF medium supplemented with 1 $mg\,ml^{-1}$ bovine serum albumin (BSA) and 10 $\mu g\,ml^{-1}$ iron-saturated human transferrin were inoculated on the KCF feeder layer. This is called KCF-feeder culture. If necessary, 2 i.u. ml^{-1} of erythropoietin (Epo, TOYOBO) and 50 i.u. ml^{-1} of recombinant murine interleukin-3 (IL-3, Genzyme) were added to the culture medium.

Histological section

Cultures were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, dehydrated in a graded series of ethyl alcohol and embedded in Epon 812. Sections (1 μm thickness) were stained in 0.1% toluidine blue in phosphate-buffered saline and observed with a light microscope.

Histochemical staining of erythroid cells

100 μl of 2,7-diaminofluorene (DAF, Sigma) staining reagent (0.01% DAF and 0.3% H_2O_2 in 200 mM Tris–HCl pH 7.0) was added to 2 ml of culture medium and incubated at room temperature for 15 min. Hemoglobin-containing cells were stained dark blue or brownish black (Worthington *et al.* 1987).

Cytological observation of erythroid cells

Cells were suspended in 300 μl of fetal calf serum and cytocentrifuged to be smeared directly onto a glass slide using Cytospin-2 (Shandon, United Kingdom) at 800 revs min^{-1} for 5 min. They were fixed with methanol, then stained by 50% May–Grünwald solution (Merck) for 10 min and 5% Giemsa solution (Merck) for 15 min.

Globin sample preparation

Erythroid cells released into culture medium were collected by centrifugation at 2000 revs min^{-1} for 5 min and washed three times with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4). They were then suspended in distilled water, lysed by three cycles of freeze-thawing and centrifuged at 3000 revs min^{-1} for 5 min. The supernatant was used as globin samples.

Acid urea Triton X-100 polyacrylamide gel electrophoresis (UT-PAGE)

The gel solution consisted of 12.5% acrylamide, 0.08% bisacrylamide, 5% acetic acid, 0.5% *N,N,N*^{1},*N*^{1}-tetramethylethylendiamine (TEMED), 6 M urea, 2% Triton X-100 and 0.06% ammonium persulfate. This solution was degassed for 10 min under vacuum and polymerized in a slab (130×140×1 mm) for 2 h. For a reservoir buffer, 5% acetic acid was used. The first prerunning was carried out at 200 V for 20 min. After 1 M cysteamine was applied onto the gel, the second prerunning was carried out at 150 V for 45 min, then cysteamine was removed from gel wells. 5 μl of globin sample was mixed with 20 μl of sample buffer (0.03% pyronin y, 6.7 M urea, 8.3% acetic acid and 8.3% 2-mercaptethanol). Electrophoresis was carried out at 7.5 mA for 14 h. The gel was stained with 0.5% Coomassie brilliant blue-R (Sigma) in 30% methanol and 7% acetic acid for 1 h. Then it was destained with 30% methanol and 7% acetic acid.

Isolation of embryonic hemoglobins

Embryonic hemoglobins (EI, EII and EIII) were isolated by isoelectric focusing, according to Cudennec *et al.* (1979). For isoelectric focusing, the gel solution (5% acrylamide, 0.13% bisacrylamide, 6.3% Pharmalite 6.5–9 (Pharmacia), 0.5% Nonidet P-40, 10% sucrose) was degassed for 10 min under vacuum then polymerize in a slab (85×50×1 mm) by the addition of 0.04% ammonium persulfate and 0.04% TEMED (final concentration) for 2 h. 3 μl of globin sample was mixed with 3 μl of sample buffer for isoelectric focusing (1% nonidet P-40, 20% sucrose and 12.6% Pharmalite 6.5–9) and was applied onto the gel. The upper reservoir buffer was 0.02 M phosphoric acid and the lower was 1 M sodium hydroxide. Isoelectric focusing was carried out at 200 V for 2 h.

Production of anti-hemoglobin antibody

Anti-embryonic hemoglobin antiserum was obtained from a rabbit immunized with embryonic hemoglobin EI which was isolated from circulating blood of a BALB/c mouse 12-day embryo by isoelectric focusing. Anti-adult mouse hemoglobin antiserum was purchased from Cappel (Pennsylvania).

Immunological detection of globin chains

After electrophoresis, the gel was immersed in blotting buffer (10 mM $NaHCO_3$, 3 mM Na_2CO_3) added with 0.02% sodium dodecyl sulfate (SDS) for 30 min and transferred from gel to nitrocellulose membrane using a blotting apparatus (Trans-Blot™SD, Bio-Rad) at 20 V for 40 min. Globin chains were detected by rabbit anti-mouse hemoglobin antiserum and a blotting detection kit for rabbit antibody (Amersham).

Results

Erythroid cell differentiation

When 20 PCC3/A/1 cells were inoculated onto KCF-feeder culture, 3 to 8 cells grew to form colonies. By the 7th day, these colonies contained several hundred cells that exhibited the morphology of undifferentiated stem cells. By the 10th day, cells formed three-dimensional aggregates at the center of colonies. By the 12th day, these aggregates formed cystic structures in which red cells became visible (Fig. 1A). Red cells were initially found in the colonies, and then most of them were released into the culture medium. These red cells continued to be released for about a week and, as they

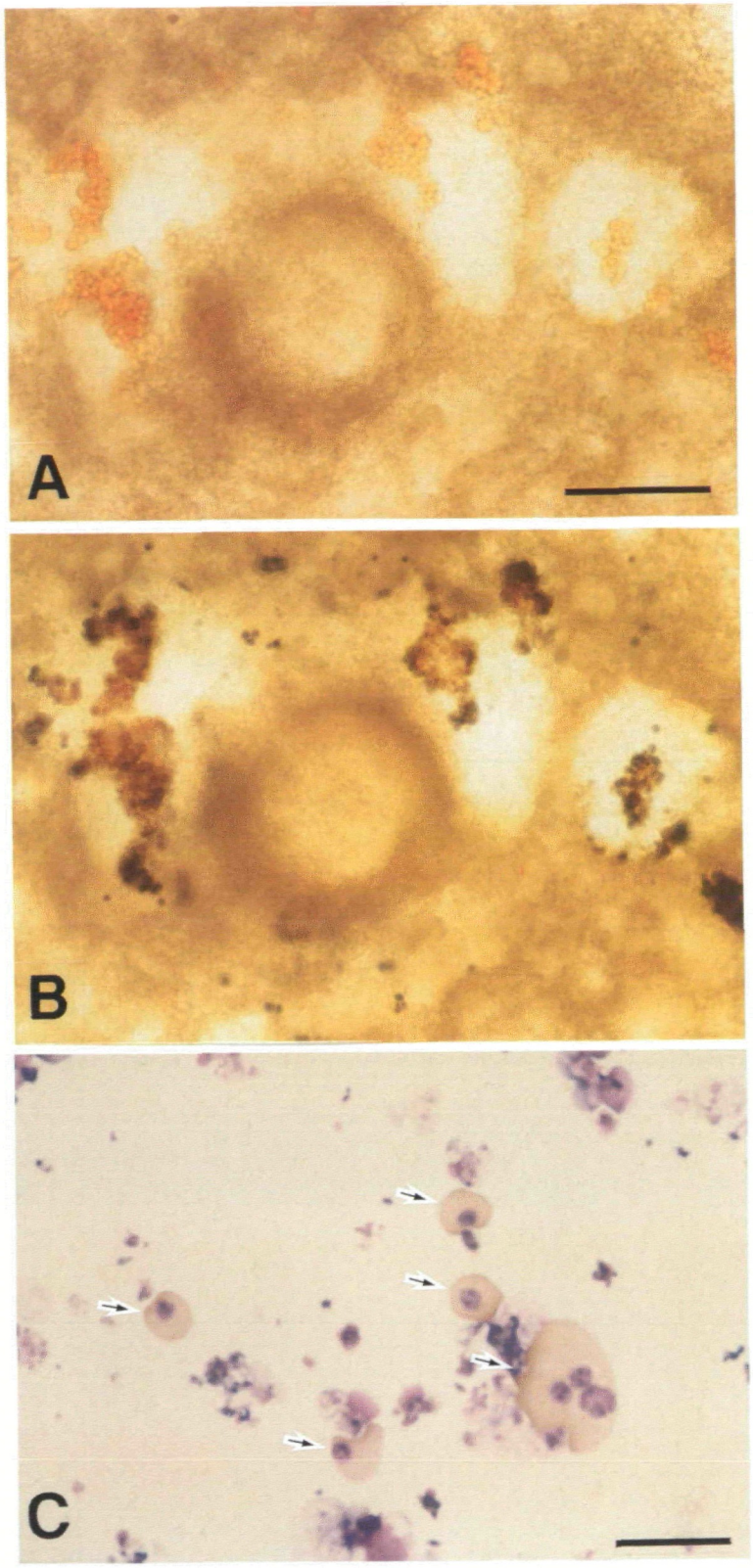

Fig. 1. (A) A photomicrograph of differentiating culture of PCC3/A/1 in KCF-feeder culture on the 12th day after inoculation. Red cells were found in cystic cell aggregates. Bar=100 μm. (B) The same field as A stained with DAF. Red cells in A were positively stained. (C) A photomicrograph of a cytocentrifuged smear of erythroid cells derived from PCC3/A/1 in KCF-feeder culture stained by May–Grünwald methods. Arrows indicate red nucleated erythroid cells. Bar=20 μm.

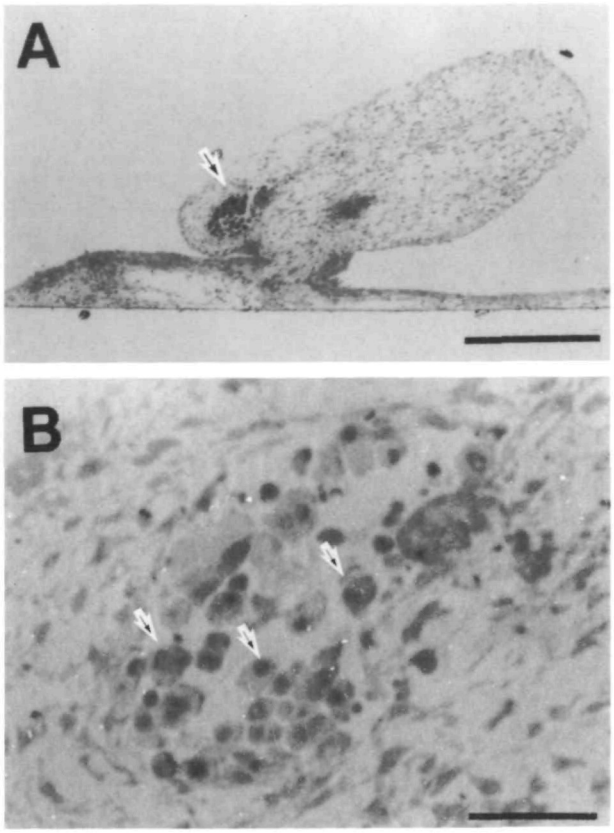

Fig. 2. (A) Photomicrograph of a histological section of a PCC3/A/1 colony in which erythroid cells differentiated. An arrow indicates a cavity in which erythroid cell differentiation occurred. Bar=1.2 mm. (B) High magnification of A. Arrows indicate erythroid cells. Bar=200 μm.

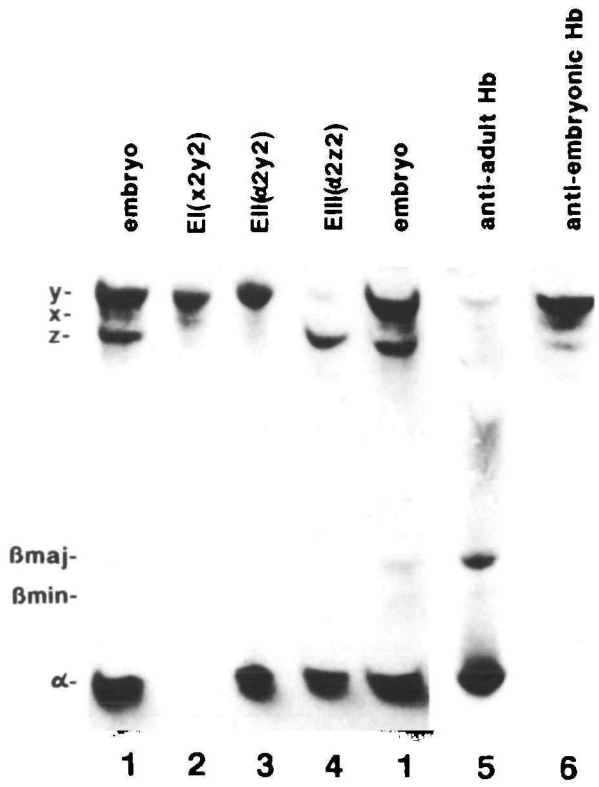

Fig. 3. UT-PAGE pattern of mouse globin chains and characterization of anti-adult or embryonic hemoglobin antiserum. Lane 1; hemolysate of circulating blood of 12-day embryos contained both adult and embryonic globin chains. Lower three bands correspond to β^{maj}-, β^{min}- and α-globin chains, respectively. As embryonic hemoglobin EI, EII or EIII gave y- and x- (lane 2), y- and α- (lane 3) or z- and α- (lane 4) globin chains, respectively, the upper three bands correspond to y-, x- and z-globin chains. After the same sample as in lane 1 was transferred to nitrocellulose membrane, anti-adult hemoglobin antiserum recognized β^{maj}-, β^{min}- and α-globin chains, whereas it scarcely recognized embryonic globin chains (lane 5). Anti-embryonic hemoglobin antiserum recognized strongly y- and x-globin chains and weakly z-globin chain, but hardly recognized adult globin chains (lane 6).

were well stained with DAF (Fig. 1B), were identified as erythroid cells. Typical features of differentiated cells are shown in Fig. 1C. Released erythroid cells were nucleated and sometimes polynucleated. Erythroid cells were always observed in the cavity bordered with flat cells (Fig. 2A,B). The cavity was sometimes surrounded with mesenchymal cells. Erythroid cell differentiation was not always accompanied by epithelial layers of endoderm cells.

Erythroid cells differentiated in all cultures where colonies were formed. In a special experiment, cultures in which single colonies were formed were selected and observed. In all 45 cultures, erythroid cells differentiated.

Supplementation of DF medium with 1 mg ml^{-1} of BSA and 10 μg ml^{-1} of iron-saturated human transferrin was not always necessary for erythroid cell differentiation. Though the efficiency of colony formation was very low, PCC3/A/1 cells did differentiate into erythroid cells in this medium without such supplement.

Chondrocyte differentiation followed the erythroid cell differentiation by about the 20th day.

Globin chain analysis

PCC3/A/1 cells were originated from mouse strain 129/Sv with a hemoglobin haplotype of diffuse (Hbb^d) (Russell and Gerald, 1958; Craig and Russell, 1963). Definitive erythrocytes from this strain gave adult globin chains (β^{maj}-, β^{min}- and α-globin chains), while yolk sac erythroid cells gave embryonic globin chains (y-, x- and z-globin chains) in addition to adult globin chain (α-globin chain). Circulating red blood cells of 12-day embryos contained both primitive and definitive erythroid cells. They gave embryonic and adult globin chains (Fig. 3, lane 1). The positions of β^{maj}-, β^{min}- and α-globin chains were well characterized and three lower bands were indicated (Fig. 3, lane 1) (Alter, 1979). To determine the position of embryonic globin chains and to characterize antiserum against adult or embryonic globin chains, embryonic hemoglobin EI (x2y2) (Fig. 3, lane 2), EII (α2y2) (Fig. 3, lane 3) or EIII (α2z2) (Fig. 3, lane 4) isolated by isoelectric focusing were separated by UT-PAGE. The positions of y-, x- and z-

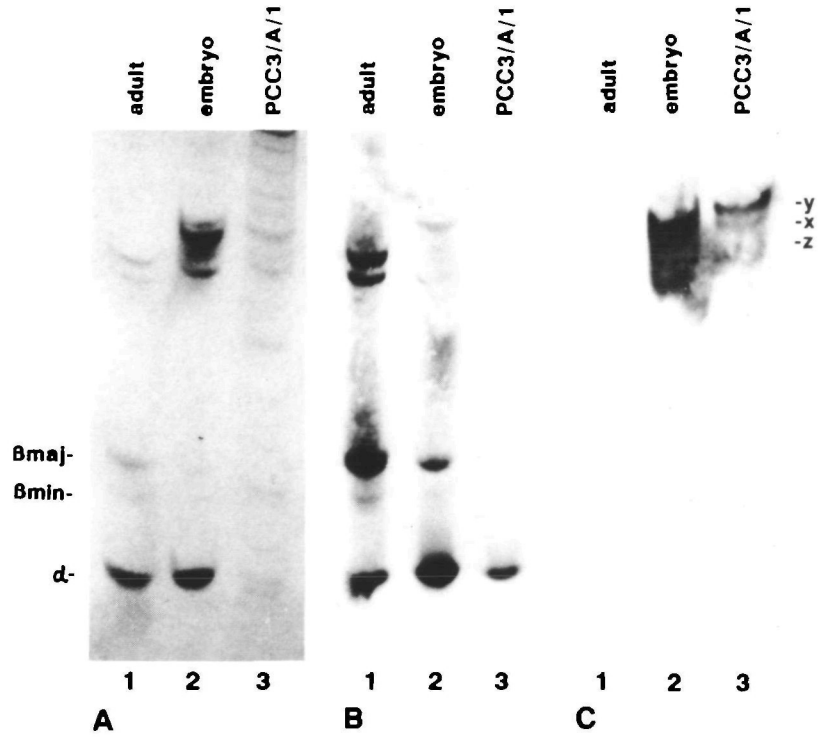

Fig. 4. Globin chain analysis of erythroid cells from PCC3/A/1. (A) Globin samples were separated by UT-PAGE and stained with Coomassie blue. Lane 1; adult mouse blood gave β^{maj}-, β^{min}-, α- and two unknown upper bands. Lane 2; circulating blood of 12 day embryos gave y-, x-, z- and α-globin bands. Weak β^{maj}- and β^{min}-bands are also visible. Lane 3; PCC3/A/1-derived erythroid cells gave bands at the same position as y-, x-, z- and α-globin bands. Numerous unknown bands are also visible. (B) After blotting to nitrocellulose membrane, samples were reacted with anti-adult mouse hemoglobin antiserum. Lane 1; β^{maj}-, β^{min}-, α- and two unknown upper bands were recognized with anti-adult hemoglobin antiserum. Lane 2; embryonic globins were hardly recognized with anti-adult hemoglobin antiserum. Lane 3; among numerous bands of PCC3/A/1-derived erythroid cells, only α-globin bands were recognized with anti-adult hemoglobin antiserum. (C) After blotting to a nitrocellulose membrane, samples were reacted with anti-embryonic hemoglobin antiserum. Lane 1; definitive erythrocytes gave no bands detected with anti-embryonic hemoglobin antiserum. Lane 2; y-, x- and z-bands were recognized with this antiserum. Lane 3; PCC3/A/1-derived erythroid cells gave y- and x-globin bands recognized with this serum. A band of z-globin chain was hardly detectable.

globin chains were then determined at the upper three bands. The results agreed with those reported by Farace *et al.* (1984*a*,*b*) and Vanin *et al.* (1981). Anti-adult hemoglobin antiserum recognized adult globin chains (α-, β^{maj}- and β^{min}-globin chains) strongly and embryonic globin chains (x-, y- and z-globin chains) very weakly (Fig. 3, lane 5). Anti-embryonic hemoglobin antiserum recognized embryonic y- and x-globin chains strongly and it also recognized embryonic z-globin chain weakly, but only faintly recognized adult globin chains (Fig. 3, lane 6).

Erythroid cells derived from PCC3/A/1 cells gave several bands after UT-PAGE (Fig. 4A, lane 3). The bands at the same position of y- and x-globin chains were recognized with anti-embryonic hemoglobin antiserum (Fig. 4C, lane 3) and α-globin chain was recognized with anti-adult hemoglobin antiserum (Fig. 4B, lane 3) after blotting to a nitrocellulose membrane. Thus the production of embryonic globin chains was evident in PCC3/A/1-derived erythroid cells. Adult β^{maj}- or β^{min}-globin chain was rarely detected even with anti-adult hemoglobin antiserum (Fig. 4B, lane 3). Bands other than adult or embryonic globin chains in Fig. 4A, lane 3, were not globin chains because they were not recognized with anti-adult hemoglobin antiserum (Fig. 4B, lane 3) or with anti-embryonic hemoglobin antiserum (Fig. 4C, lane 3) in western blotting.

Effects of the addition of erythropoietin or interleukin-3

As mentioned, PCC3/A/1 cells can differentiate into primitive erythroid cells without the addition of any hemopoietic growth factor such as Epo or IL-3. Thus the effects of the addition of Epo and/or IL-3 into this culture were examined. PCC3/A/1 cells were inoculated into KCF-feeder culture with or without Epo and IL-3. To compare the production of globin chains quantitatively, erythroid cells were collected from 20 dishes and applied for UT-PAGE (Fig. 5A). Adult globin chains were detected with anti-adult hemoglobin antiserum and embryonic globin chains were detected with anti-embryonic hemoglobin antiserum in western blotting. When Epo was added, the production of both embryonic (Fig. 5C, lane 3 or 5) and adult (Fig. 5B, lane 3 or 5) globin chains was enhanced. The addition of

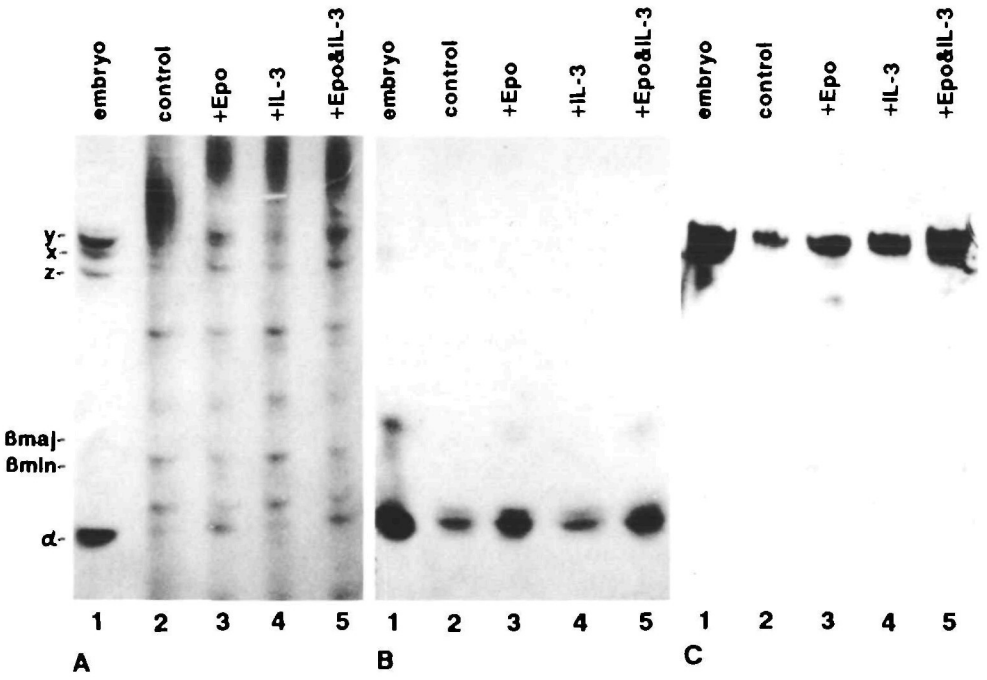

Fig. 5. Effects of the addition of Epo and/or IL-3 on the erythropoiesis of PCC3/A/1 in KCF-feeder culture. Released erythroid cells were collected from 20 dishes of KCF-feeder culture with no addition (lane 2), added with EOP (lane 3), added with IL-3 (lane 4) or added with both Epo and IL-3 (lane 5) and respectively applied for UT-PAGE. (A) The gel after staining with Coomassie blue. Lane 1, hemolysates from 12-day embryo blood gave mainly y-, x-, z- and α- globin bands. Bands at the same position of y-, x-, z- and α-globins are also visible in lane 2 to lane 5. The intensity of staining of those bands in lane 3 and lane 5 was higher than those in lane 2 and lane 4. (B) After UT-PAGE, samples were blotted to a nitrocellulose membrane and detected with anti-adult hemoglobin antiserum. The band of α-globin was detected in lanes 2 to 5. The intensity of staining in lane 3 or 5 is higher than that in 2 or 4. The band of β^{maj}-globin was detected in lane 3 and 5. These indicated that the addition of Epo enhanced the production of both β^{maj}- and α-globin chains. (C) After blotting to nitrocellulose membrane, samples were reacted with anti-embryonic hemoglobin antiserum. Embryonic globin chains (mainly y-globin chain) were detected in lanes 2 to 5. The intensity of staining in lane 3 (added with Epo) or 4 (added with IL-3) is higher than that in 2 (no addition). That in lane 5 (added with both Epo and IL-3) is the highest of all.

IL-3 also enhanced the production of embryonic globin chains (Fig. 5C, lane 4 or 5), but did not affect that of adult globin chains (Fig. 5B, lane 4 or 5) at all. The enhancement of embryonic globin production with Epo and with IL-3 were cumulative (Fig. 5C, lane 5).

Discussion

We reported here a new culture system (serum-free feeder culture of PCC3/A/1 cells) that provides a model system for studying the cellular and molecular mechanisms of primitive erythropoiesis. There are three characteristics of the system. First, PCC3/A/1 cells differentiate into erythroid cells in serum-free medium. Cudennec and Nicolas (1977) reported the erythroid cell differentiation of PCC3/A/1 cells in organotypic culture 13 years ago. However, we did not find the reproducibility of differentiation satisfactory under the conditions they described, partly because different batches of fetal calf serum were used. In our method, serum-free medium was used so that the erythroid cell differentiation was highly reproducible. Serum-free medium also allows identification of the factor(s) regulating the primitive erythropoiesis of PCC3/A/1 cells. Second, PCC3/A/1 cells differentiate into primitive erythroid cells in a submerged monolayer culture. Organotypic culture or the formation of cystic embryoid bodies has been reported to induce primitive erythroid cell differentiation of EC cells or ES cells. With those methods, it is not easy to observe the detailed process of the differentiation with a phase-contrast microscope, while in our system, it is possible to do so. Finally, PCC3/A/1 cells continued to produce primitive erythroid cells in KCF-feeder culture for about a week and these were finally released into the culture medium. These erythroid cells are easy to collect and can be biochemically characterized.

The present results demonstrate that the primitive erythropoiesis of PCC3/A/1 cells in KCF-feeder culture does not require the exogenous addition of any hematopoietic growth factors such as Epo and/or IL-3. This does not mean, however, that PCC3/A/1 cells can differentiate into primitive erythroid cells automatically and independently of any factor. If this were so, these cells could easily differentiate into erythroid cells spontaneously even in a stock culture; however, we never observed such spontaneous differentiation. Teratocarcinoma stem cells are able to differentiate into not only erythroid cells but also cells that provide a microenvironment and/or cells that produce factors necessary for primitive erythropoiesis. In our experiments, primitive erythropoiesis of PCC3/A/1 cells was usually accompanied by the formation of a cystic structure as was true in organotypic culture of these cells (Cudennec and Nicolas, 1977), or in the formation of embryoid bodies of ES cells (Doetschman *et al.* 1985). These cystic structures may produce factors and/or provide a microenvironment for primitive erythropoiesis.

We also obtained preliminary data that the culture

supernatant of differentiating PCC3/A/1 cells in KCF-feeder culture contained Epo-like activity that promoted the ^{59}Fe-uptake to mouse spleen cells, but no detectable Epo was found by radioimmunoassay using anti-Epo antiserum (unpublished data). Culture supernatant of KCF-feeder cells alone did not show that activity, though it had been found to contain the activity to support the growth of PCC3/A/1 teratocarcinoma stem cells in serum-free medium (Atsumi *et al.* 1985).

The addition of Epo into KCF-feeder culture of PCC3/A/1 cells enhanced the production of embryonic globin chains as well as adult globin chains. A similar result was reported by Kajigaya and Miura (1982) from an organotypic culture of PCC3/A/1 cells. As to the origin of β^{maj}-globin chain, there are two possibilities. Primitive nucleated erythroid cells originating from yolk sac were reported to produce adult globin chains later in gestation (Brotherton *et al.* 1979) or in *in vitro* methylcellulose culture (Wong *et al.* 1982). Therefore, one possibility is that primitive erythroid cells from PCC3/A/1 cells themselves produced adult β^{maj}-globin chain at the same time as embryonic globin chains. The other possibility is that not only primitive erythroid cells but also definitive erythrocytes differentiated from PCC3/A/1 cells when Epo was added, and that adult globin chains were produced by these definitive erythrocytes. PCC3/A/1 cells also differentiate into enucleated definitive erythrocytes (Cudennec and Salaün, 1979; Kajigaya and Miura, 1982), and Epo was reported to induce definitive erythropoiesis of yolk sac hemopoietic cells in organ culture, when the yolk sac microenvironment was maintained (Labastie *et al.* 1984). To determine whether definitive erythropoiesis of PCC3/A/1 cells is newly induced by the addition of Epo into KCF-feeder culture, it is necessary to identify an erythroid cell containing only adult hemoglobin using specific antibodies against adult hemoglobin or embryonic hemoglobin. The addition of IL-3 affected the production of embryonic globin chains differently to the production of adult globin chains. To determine the role of IL-3 in erythropoiesis of PCC3/A/1 cells, detailed experiments are necessary.

Other EC cell lines did grow in KCF-feeder culture but they did not differentiate into erythroid cells (data not shown). They had probably lost the capacity to differentiate into erythroid cells or into cells to form a microenvironment for erythroid cell differentiation. ES cells stopped growing in KCF-feeder culture within 3 or 4 days and erythroid cell differentiation was not observed (data not shown). ES cells require further supplements for growth and differentiation in this serum-free culture system.

PCC3/A/1 teratocarcinoma stem cells differentiate into erythroid cells on the KCF-feeder culture to which no exogenous addition of hematopoietic factors is necessary. This offers a new model system to study the cellular and molecular mechanisms of primitive erythropoiesis in yolk sac blood islands, especially to identify the factors regulating primitive erythropoiesis.

The authors thank Dr H. Urushihara for her critical reading of the manuscript and Drs H. Amanuma and S. Kobayashi for their useful suggestions. This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan.

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(Accepted 8 November 1990)