A Paracrine Role for IL6 in Prostate Cancer Patients: Lack of Production by Primary or Metastatic Tumor Cells

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Abstract

Correlative human studies suggest that the pleiotropic cytokine IL6 contributes to the development and/or progression of prostate cancer. However, the source of IL6 production in the prostate microenvironment in patients has yet to be determined. The cellular origin of IL6 in primary and metastatic prostate cancer was examined in formalin-fixed, paraffin-embedded tissues using a highly sensitive and specific chromogenic *in situ* hybridization (CISH) assay that underwent extensive analytical validation. Quantitative RT-PCR showed that benign prostate tissues often had higher expression of IL6 mRNA than matched tumor specimens. CISH analysis further indicated that both primary and metastatic prostate adenocarcinoma cells do not express IL6 mRNA. IL6 expression was highly heterogeneous across specimens and was nearly exclusively restricted to the prostate stromal compartment—including endothelial cells and macrophages, among other cell types. The number of IL6-expressing cells correlated positively with the presence of acute inflammation. In metastatic disease, tumor cells were negative in all lesions examined, and IL6 expression was restricted to endothelial cells within the vasculature of bone metastases. Finally, IL6 was not detected in any cells in soft tissue metastases. These data suggest that, in prostate cancer patients, paracrine rather than autocrine IL6 production is likely associated with any role for the cytokine in disease progression. *Cancer Immunol Res; 3(10); 1175–84. ©2015 AACR.*

Introduction

Interleukin 6 (IL6) is a pleiotropic cytokine produced by an array of cell types and affects diverse physiologic processes, including immune responses, hematopoiesis, and cellular proliferation and differentiation (1). Under normal conditions, IL6 levels in cells are typically low, although a number of stimuli result in induction of IL6 expression and secretion. For example, during acute inflammatory responses to infections, cellular production of IL6 is essential to the induction of acute phase proteins. Although the normal homeostatic response to inflammation is resolution and reversion of IL6 production to normal low levels, unrestrained production of IL6 drives chronic inflammation and increased systemic levels of IL6 have been associated with diseases, such as autoimmune disorders, arthritis, hepatitis, inflammatory bowel disease, pancreatitis, and cancer (2).

Early evidence for a role for IL6 in advanced prostate cancer came from studies examining serum levels of IL6 in relation to metastatic or hormone-refractory prostate cancer (3–5). These data showed that serum levels of IL6 are significantly elevated in prostate cancer patients with hormone-refractory disease compared with normal control subjects or men with prostatitis, benign prostatic hyperplasia, and localized and recurrent disease (3). Likewise, serum IL6 levels were correlated to patients with clinically evident metastases (4) or with extent of bone metastasis (5). Subsequent studies have consistently shown that elevated systemic IL6 levels confer poor prognosis (6–8) and may also serve as a marker of prostate cancer morbidity, including cachexia (9, 10). A key clinical question is precisely when during disease development and progression IL6 is expressed and what specific cell types are responsible for its production (e.g., prostate tumor cells or another cellular source). A secondary question is whether the elevated systemic levels of this cytokine actually drive disease progression, or whether the elevated levels of IL6 are a surrogate for tumor burden.

Along these lines, multiple studies have shown that some prostate cancer cell lines can secrete IL6 *in vitro*. The androgen-independent prostate cancer cell lines DU145 and PC3 have been shown to secrete IL6, whereas androgen-sensitive LNCaP cells do not (9, 11, 12). In primary prostate cancer, protein extracts prepared from prostate cancer tissues showed elevated IL6 levels compared with benign tissues in approximately 50% of cases when analyzed by ELISA (12). Furthermore, studies using immunohistochemistry (IHC) to detect IL6 in prostate tissues have reported IL6 production by both benign and malignant prostate epithelia (13, 14). An additional study using IHC to detect IL6 in a series of metastatic tissues from 26 prostate cancer patients

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reported that IL6 is produced in the majority of prostate cancer bone metastases and to a lesser extent in prostate cancer soft tissue metastases (15). Another series of studies proposed that IL6, along with a related member of the IL6 family of cytokines, oncostatin M (OSM), may activate androgen receptor (AR) in the absence of androgen, providing a potential mechanism whereby IL6 contributes to recurrent prostate cancer growth following androgen deprivation therapy (reviewed in ref. 16). Cumulatively, these studies have led to the hypothesis that IL6 serves as an *autocrine* growth factor in both primary and metastatic prostate cancer (12, 13, 16).

Additional studies have suggested a role for *paracrine* IL6 signaling in prostate cancer progression. Recent work using a human prostate dissociation and tissue recombination system identified a role for paracrine expression of IL6 or OSM specifically in the stromal compartment in concert with cell-autonomous oncogenic events, such as PTEN loss of function, in the promotion of an aggressive prostate cancer phenotype (17). Another recent study identified a role for paracrine signaling from IL6 upregulation in mesenchymal stem cells and promotion of adipogenesis and prostate cancer cell migration and invasion (18). Again, this study indicated a specific role for IL6 production from the stromal compartment in facilitating prostate cancer progression (18).

Cumulatively, the evidence to date indicates that IL6 may act as a key mediator in several steps in prostate carcinogenesis, including initiation, progression, metastases, and the development of castration resistance and/or resistance to chemotherapy. What is less well understood is what cell types are responsible for production of the cytokine in the tumor microenvironment in patients, and, by extension, whether IL6 in prostate cancer patients functions through autocrine or paracrine mechanisms.

Materials and Methods

Patient population and clinical samples

All specimens were acquired under Institutional Review Board–approved protocols at the respective institutions. RNA samples from matched tumor and benign tissues were obtained from 10 radical prostatectomy specimens using the standard operating procedure protocols for the Prostate Cancer Biorepository Network (PCBN) as previously described (19). Each case consisted of fresh frozen tumor and matched benign peripheral zone tissues obtained at radical prostatectomy. For RNA isolation, tissues containing cancer were dissected such that they contained at least 70% to 90% tumor cells. Recently collected formalin-fixed paraffin-embedded (FFPE) primary clinical prostate cancer tissues (<1 year old) were obtained from 21 prostatectomy specimens in addition to 12 biopsy or autopsy metastatic tissue samples from 9 cases at Johns Hopkins Hospital and 20 bone metastatic tissue samples from 10 cases at the University of Washington Medical Center (Seattle, WA) for use in chromogenic *in situ* hybridization (CISH) assays. One block containing the highest grade/index cancer and adjacent benign tissue was chosen for CISH analysis from each prostatectomy case. The clinical and pathologic details of the patient samples are listed in Supplementary Table S1. Tissue microarrays (TMA) containing metastatic tissues (bone and soft tissue metastases) from 21 cases (University of Washington Medical Center) and 15 cases (Johns Hopkins Hospital, Baltimore, MD) were used in IL6 IHC experiments.

Cell lines

LNCaP, VCaP, and CWR22Rv1 were obtained from the American Type Culture Collection. PC3, DU145, MCF7, and NCI-H460 cells were obtained from the NCI-Frederick. PrEC and PrSC cells were obtained from Lonza. LAPC4, RWPE-1, and C4-2B cells were obtained from J.T. Isaacs (Johns Hopkins University), and LNCaP-abl cells were obtained from Z. Culig (University of Innsbruck). All cell lines used were authenticated via short tandem repeat profiling of 9 genomic loci with the Powerplex 1.2 system (Promega) before use.

Quantitative real-time reverse transcription PCR

RNA was treated with DNase I (RNase-free; Ambion) followed by cDNA synthesis using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen) following standard protocol for "First-Strand Synthesis Using Random Primers." Quantitative PCR was performed with SYBR Green Supermix (Bio-Rad) and 0.4 µmol/L IL6 primers (IL6-F 5′-GGTACATCCTCGACGG-CATCT-3′ and IL6-R 5′-GTGCCTCTTTGCTGCTTTCAC-3′) or 1.0 µmol/L GAPDH primers (GAPDH-F 5′-CGCTCTC-TGCTCCTCCTGTT-3′ and GAPDH-R 5′-CCATGGTGTCTGAGC-GATGT-3′) in a real-time detection system. PCR conditions were as follows: 2 minutes at 94°C, 40 cycles of 30 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C, followed by a melt curve analysis. GAPDH was used as a housekeeping gene for normalization. The fold differences in expression levels of IL6 in tumor samples were determined using the $2^{-\Delta\Delta CT}$ method, relative to GAPDH and to the matched benign tissue.

CISH

CISH was performed using the RNAscope 2.0 FFPE Brown Reagent Kit or RNAscope 2-plex assay kit (Advanced Cell Diagnostics, Inc.). Briefly, FFPE tissues were first baked at 60°C for 1 hour followed by deparaffinization in two changes of 100% xylene for 5 minutes each and two changes of 100% alcohol for 3 minutes each. Next, the slides were treated with endogenous peroxidase-blocking pretreatment reagent for 10 minutes at room temperature. The slides were then added to boiling buffer for 30 minutes at 99°C to 104°C in a water bath and then treated with protease digestion buffer for 30 minutes at 40°C. The slides were incubated with a custom RNAscope target probe designed against IL6 mRNA (probe region 27-876, NCBI reference sequence Accession #NM_000600.3) or peptidyl prolyl isomerase B (PPIB), also known as cyclophilin B, as a positive control mRNA (probe region 139-989, NCBI reference sequence Accession #NM_000942.4) for 2 hours at 40°C, followed by signal amplification. DAB was used for colorimetric detection for 10 minutes at room temperature.

IHC

IHC was performed using the Power Vision+ Poly-HRP IHC kit (Leica Biosystems). Slides were steamed for 45 minutes in antigen retrieval solution (Dako; #S1700) and incubated with rabbit polyclonal anti-IL6 antibody (#6672; Abcam; lot #GR106735-5 at 1:1,000 dilution) for 45 minutes at room temperature. Poly-HRP–conjugated anti-rabbit IgG antibody was used as secondary antibody. Staining was visualized using 3,3′-diaminobenzidine (Sigma), and slides were counterstained with hematoxylin.

Western blot

Cells were lysed in RIPA buffer containing protease inhibitors (Sigma) and phosphatase inhibitors (Cell Signaling). Lysates were then centrifuged at $14,000 \times g$ for 10 minutes at $4°C$. Proteins were electrophoresed and transferred to nitrocellulose membranes for immunoblotting. Membranes were probed with anti-IL6 antibody (#6672; 1:500 dilution; Abcam; lot #GR106735-5). The blots were visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences). Recombinant mouse IL6 (R&D Systems) was used as a positive control for Western blot.

Controls for CISH, Western blot, and IHC

MCF7 (breast cancer cell line) cells were transfected with the IL6 cDNA clone expression vector (Origene; SC125236) using lipofectamine (Life Technologies). NCI-H460 cells were treated with monensin (Golgi-Stop; BD Biosciences) at a dilution of 1:1,000 for 4 hours.

Results

Variable expression levels of IL6 transcript are present in primary prostate adenocarcinoma when compared with matched benign tissues as assessed by qRT-PCR

To determine if IL6 expression levels in primary prostate cancer are associated with prognostic factors, we obtained a series of RNA samples from fresh-frozen cancer and matched benign tissues from 10 radical prostatectomy specimens. This series was enriched for cases of higher-grade (Gleason $\geq$ 7) cancer (Supplementary Table S1). IL6 message levels were not significantly elevated in tumors compared with matched benign tissues, as only 3 of 10 prostatectomy samples were observed to have higher expression of IL6 in tumor compared with benign tissue (Fig. 1). Conversely, there were more cases that showed higher levels of IL6 in the benign regions than in those containing cancer (Fig. 1). Based on this apparent discrepancy with published data (12, 13), we queried the Oncomine database (20) for datasets of prostate carcinoma versus normal tissues, and an analysis of 17 such datasets showed only one dataset with a greater than 2-fold increase in cancer versus benign tissues and a P value $\leq$ 0.05 (Supplementary Table S2). Due to our observation of a low frequency of IL6 overexpression in tumor compared with benign tissues as well as the elevated IL6 levels observed in benign tissues in our qRT-PCR studies (Fig. 1B), we next sought to determine the cellular origin of IL6 in prostate specimens.

Development of a highly sensitive and specific IL6 CISH assay

For the development of an IL6 CISH assay, we used the highly sensitive RNAscope 2.0 assay from Advanced Cell Diagnostics. This assay consists of a hybridization probeset complementary to a length of the protein coding region of the IL6 mRNA (see Materials and Methods) in which the hybridization event is subjected to signal amplification and chromogenic detection. To establish the specificity of the CISH approach, we transfected MCF-7 cells (which do not express IL6 mRNA) with an IL6 cDNA clone expression vector (Origene; SC125236). As shown in Fig. 2A, the transfected cells stained strongly positive using the CISH assay and thus served as a genetically defined positive control. To further verify the specificity of the probeset, we next quantified IL6 mRNA levels in a panel of prostate cancer cell lines (LAPC4, CWR22Rv1, PC3, LNCaP, C4-2B, VCaP, DU145, and
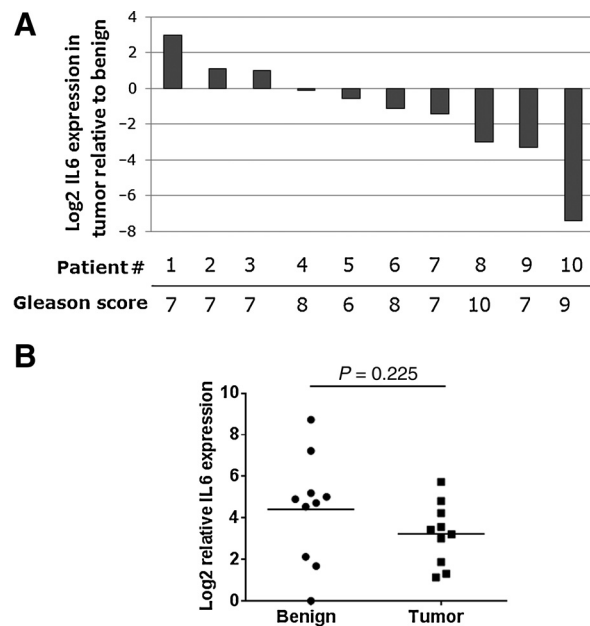
Figure 1.
IL6 mRNA expression in benign and malignant prostate tissues as assessed by qRT-PCR. RNA extracts were prepared from matched tumor and benign tissues from radical prostatectomy specimens. IL6 mRNA expression was then determined by qRT-PCR. A, log2 IL6 mRNA expression in tumor relative to matched benign tissue in 10 prostatectomy specimens. IL6 mRNA expression levels were normalized to GAPDH, and then tumor was compared with benign using the $2^{-\Delta\Delta CT}$ method followed by log2 transformation. B, log2 relative IL6 mRNA expression in benign samples versus tumor samples.

LNCaP-abl), benign prostate cultured cells (PrSC and PrEC), and a human papillomavirus (HPV)-transformed prostate cell line (RWPE-1) via qRT-PCR. Providing further evidence for the specificity of the CISH assay, we found that there was complete concordance between the two assays, i.e., all lines that were positive for IL6 by qRT-PCR were positive by CISH for IL6 mRNA expression (Fig. 2B and C). Of note, although previous studies reported that the PC3 cell line secretes IL6 (9, 11, 12), we did not find the line to be positive for IL6 mRNA expression in the present study (Fig. 2B and C). We hypothesize that this could be due to differences in cell culture conditions for this highly inducible cytokine. The CISH assay has been reported to have a detection limit of a single mRNA molecule (21).

Prostate adenocarcinoma cells in prostatectomy specimens do not express IL6 mRNA

We next used the IL6 CISH mRNA assay on FFPE tissues from a series of 21 radical prostatectomy specimens of varying Gleason grades and tumor stages (Supplementary Table S1). In each case, we examined full tissue sections containing the highest grade/ index cancer and adjacent benign tissue. We verified RNA integrity in the tissue sections used in this study with a positive control probeset against PPIB on adjacent sections. PPIB hybridization signals demonstrated expression in virtually all cells present on all slides that were used in this study (Fig. 3A), showing that the RNA in these specimens was intact. We did not detect positive IL6 mRNA expression in tumor cells in any of the cases examined, regardless of tumor grade (Fig. 3A; Table 1). Instead, IL6 mRNA
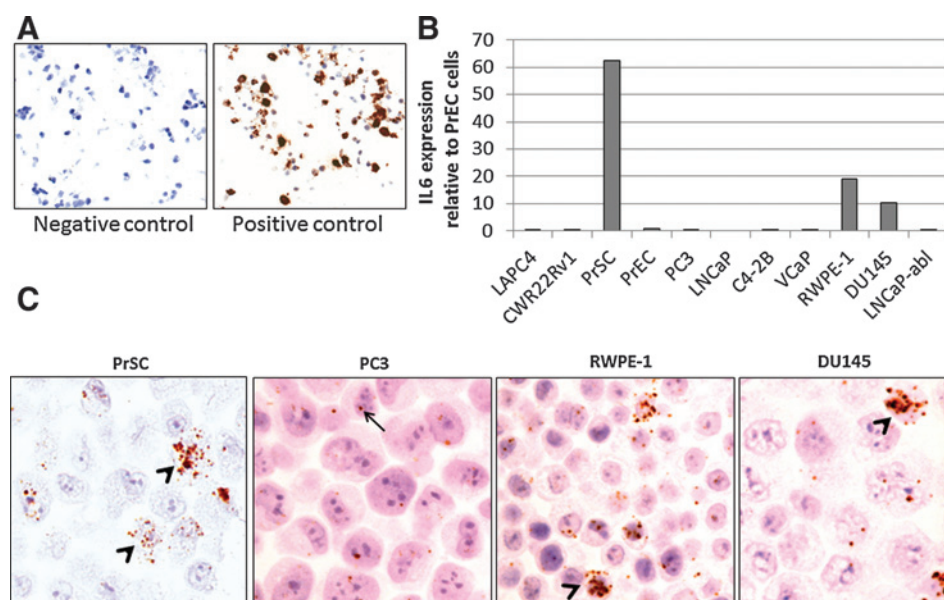
Figure 2.
Validation of IL6 CISH assay. A, IL6-transfected (positive control) or nontransfected (negative control) FFPE MCF7 cells assayed by IL6 CISH. B, IL6 qRT-PCR on 11 prostate cell lines. IL6 mRNA expression levels were normalized to GAPDH and then to PrEC cell expression level using the $2^{-\Delta\Delta CT}$ method. C, IL6 CISH was performed on the same 11 prostate cell lines, and representative examples are shown. Brown staining (arrowheads) represents positive IL6 mRNA expression. Single dots in the nucleus of cells are interpreted as the genomic copy of the gene (PC3 cells, arrow). Both qRT-PCR and CISH analyses indicated that three of the prostate cultured cells or cell lines (PrSC, RWPE-1, DU145) were positive for IL6 mRNA, indicating complete concordance between the two assays.

expression in areas containing prostate cancer was restricted to cells within the stromal compartment of the tumor and primarily in tumor-associated endothelial cells (Fig. 3B). The detection of IL6 mRNA in stromal cells, in combination with positive control staining of transfected MCF-7 cells (Fig. 2A) and prostate cultured cells and cell lines (Fig. 2C), confirms that the IL6 CISH probe was functioning as expected and that prostate adenocarcinoma cells in primary tumors do not express IL6 mRNA.

IL6 mRNA expression is highly upregulated in areas of acute inflammation and prostatic atrophy

As shown in Table 1, an assessment of the distribution of IL6 mRNA expression in the prostatectomy tissues as analyzed by IL6 CISH demonstrated that, overall, the expression was highly heterogeneous from case to case and by region within a given case. As shown in Fig. 4A and B, the number of IL6-positive cells was highly increased in the stroma in areas of acute inflammation (as evidenced by accumulation of neutrophils within glandular lumens), although the neutrophils were not positive. The cases with the highest numbers of IL6 mRNA-expressing cells were those in which acute inflammation was present (Table 1).

Increases in IL6-positive cells were also seen in areas of proliferative inflammatory atrophy (Fig. 4C and D). Positive cells in areas of prostatic atrophy were generally confined to the stroma surrounding atrophy. In some instances, positive IL6 mRNA expression was observed in select epithelial cells in atrophic glands (Table 1; Fig. 4D). Morphologically, these appeared to be epithelial cells; however, we did not perform a double label with an epithelial cell marker. In a number of cases, the IL6 mRNA-expressing cells could be identified as endothelial cells lining small blood vessels (presumably venules or lymphatics; Fig. 4C) as well as prostate smooth muscle cells. Other positive staining cells had an appearance consistent with macrophages. To more definitively identify IL6 mRNA-expressing cells, we performed costaining (2-plex) for IL6 and either CD68 (a macrophage marker) or CD31 (an endothelial cell marker). The results of this 2-plex staining clearly indicated that some of the IL6-positive cells are CD68-positive macrophages and some are CD31-positive endothelial cells (Supplementary Fig. S1). Interestingly, the majority of IL6-positive cells in areas of acute inflammation did not costain for CD68 or CD31, suggesting that most of the IL6 mRNA-expressing cells in these areas are of a different stromal cell
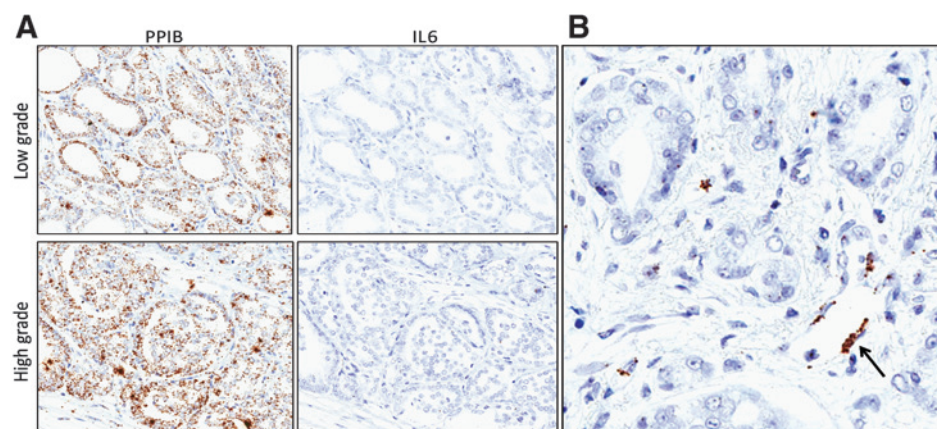

Figure 3.
IL6 mRNA is not detected in prostate adenocarcinoma cells in primary tumors. Twenty-one prostatectomy specimens were selected for IL6 CISH (Table 1). PPIB (housekeeping gene) is used as a positive control for the CISH assay. A, representative examples of low-grade (Gleason pattern 3) and higher-grade (Gleason patterns 4 and 3) prostate cancer with positive staining for PPIB (positive control) and negative staining for IL6. B, IL6-positive cells in endothelium (arrow) in an area of cancer.

Table 1. Assessment of levels and distribution of IL6 mRNA expression in prostatectomy tissues

Patient			IL6 epithelium			IL6 stroma		
Patient #	Gleason score	Clinical stage	Tumor	Normal	Atrophy	Noninflamed	Inflamed	Acute inflammation present?
1	3+3 = 6	T2N0MX	−	−	++	++	++++	Yes
2	3+4 = 7	T2N0MX	−	−	−	−	+++	Yes
3	4+5 = 9	T2N0MX	−	−	+	−	−	No
4	3+4 = 7	T2N0MX	−	−	+	−	+	No
5	3+3 = 6	T2N0MX	−	−	−	−	+	No
6	3+4 = 7[a]	T2N0MX	−	−	−	−	++	No
7	3+3 = 6[b]	T2N0MX	−	−	++	−	++	No
8	3+4 = 7	T2N0MX	−	−	−	−	++++	Yes
9	5+4 = 9	T2N0MX	−	−	−	−	++	No
10	3+3 = 6[b]	T2N0MX	−	−	−	−	−	No
11	3+4 = 7[a]	T2N0MX	−	−	−	−	−	No
12	3+4 = 7	T2N0MX	−	−	+	−	++	No
13	3+4 = 7	T3AN0MX	−	−	−	−	−	No
14	3+4 = 7	T2N0MX	−	−	++	−	++	No
15	3+3 = 6	T2N0MX	−	−	+	−	++	No
16	3+4 = 7	T2N0MX	−	−	+++	++	++++	Yes
17	3+3 = 6	T2N0MX	−	−	−	−	+++	Yes
18	4+3 = 7[a]	T3AN0MX	−	−	+	−	+	No
19	3+4 = 7	T3AN0MX	−	−	−	−	−	No
20	4+3 = 7	T3BN0MX	−	−	++	−	+	No
21	3+4 = 7[a]	T2N0MX	−	−	+	−	++	No

−, No cells positive for IL6 expression; +, areas with 5 to 10 positive cells per 20× field; ++, areas with 11 to 25 positive cells per 20× field; +++, areas with 26 to 50 positive cells per 20× field; ++++, areas of >50 positive cells per 20× field.
[a]Tertiary pattern 5.
[b]Tertiary pattern 4.

type, such as fibroblasts, smooth muscle cells, or other types of inflammatory cells.

Prostate adenocarcinoma cells in metastatic lesions do not express IL6 mRNA

Previous studies suggested that production of IL6 by metastatic prostate cancer cells may facilitate invasion and metastasis to bone and/or promote resistance to prostate cancer therapies (15, 22). Therefore, we next examined IL6 mRNA expression in biopsy samples from patients with castration-resistant metastatic prostate cancer (samples evaluated included 4 lymph node specimens and 3 liver biopsy samples) and 25 autopsy samples, which included metastases to the liver, lung, bone, and lymph node (Supplementary Table S1). Consistent with data from primary prostatectomy specimens, IL6 mRNA expression was not observed in prostate cancer cells in any of the metastatic tissues examined. Also consistent with the primary prostatectomy specimens, the metastatic tissues all showed strong hybridization signals with PPIB as a positive control (Fig. 5A), confirming RNA integrity. Interestingly, IL6 mRNA staining was observed in endothelial cells in blood vessels in 9 of 21 bone metastases (42.9%; Fig. 5B; Supplementary Fig. S2; Supplementary Table S3) but not in any of the 11 soft tissue metastases analyzed (P = 0.013, Fisher exact test).

IL6 IHC is only successful when Golgi export is blocked

Finally, we performed a series of analyses to examine IL6 protein production in cell lines and tissues using IHC. To perform these studies, we prepared FFPE blocks from a lung cancer cell line (NCI-H460) that was strongly positive for IL6 mRNA as assessed by CISH (Fig. 6A) and produces physiologic levels (e.g., not transfection levels) of IL6 protein as assessed by Western blot (Supplementary Fig. S3). Of interest, there appears to be heterogeneity in cells that are positive for IL6 expression (i.e., not all cultured cells appear to be positive) via CISH (Fig. 6A),

and this was consistent with what we observed for the IL6 mRNA-positive prostate cultured cells or cell lines as well (Fig. 2C). IHC on the NCI-H460 cells using the polyclonal anti-IL6 antibody (#6672; Abcam; lot #GR106735-5) showed no detectable signal above background levels (as established by negative control cell lines; Fig. 6B and C). In one sense, these data are not particularly surprising, because IL6 is a secreted protein and secreted proteins are generally only detectable by flow cytometry analysis when protein export from the Golgi apparatus is blocked using protein transport inhibitors, such as brefeldin A or monensin (23). As such, we next treated NCI-H460 cells with monensin prior to formalin fixation and preparation of FFPE blocks for use with IHC. IHC of "stopped" cell lines treated with monensin showed that NCI-H460 cells now stained positive for IL6 protein (Fig. 6D). We then performed IL6 IHC on a series of prostatectomy specimens, including cases that were strongly positive in areas of acute inflammation with IL6 CISH (Fig. 6E and F). We did not detect IL6 protein above background levels in the acutely inflamed areas, either in epithelial or stromal cells using IHC, and likewise we did not observe IL6 protein in prostate tumor cells using IHC (Fig. 6G). Similarly, we did not detect any IL6 protein in tumor cells via IHC on metastatic lesions from standard slides from cases used for CISH assays or in two separate metastatic prostate cancer TMAs, including one of bone metastases (Fig. 6H). The IHC signal in metastases was restricted to extracellular spaces between tumor cell nests (Fig. 6H), and no specific signal was detectable above background levels in the tumor cells in any of the cases. The lack of positive signal for IL6 protein using IHC in areas that were positive for IL6 mRNA using CISH is likely not due to lack of IL6 protein production by the IL6 mRNA-positive cells. Rather, these results, coupled with those from our experiments using monensin in the NCI-H460 cell line, suggest that detection of this particular cytokine in tissue sections using IHC could be severely limited without being able to block protein export prior to fixation.
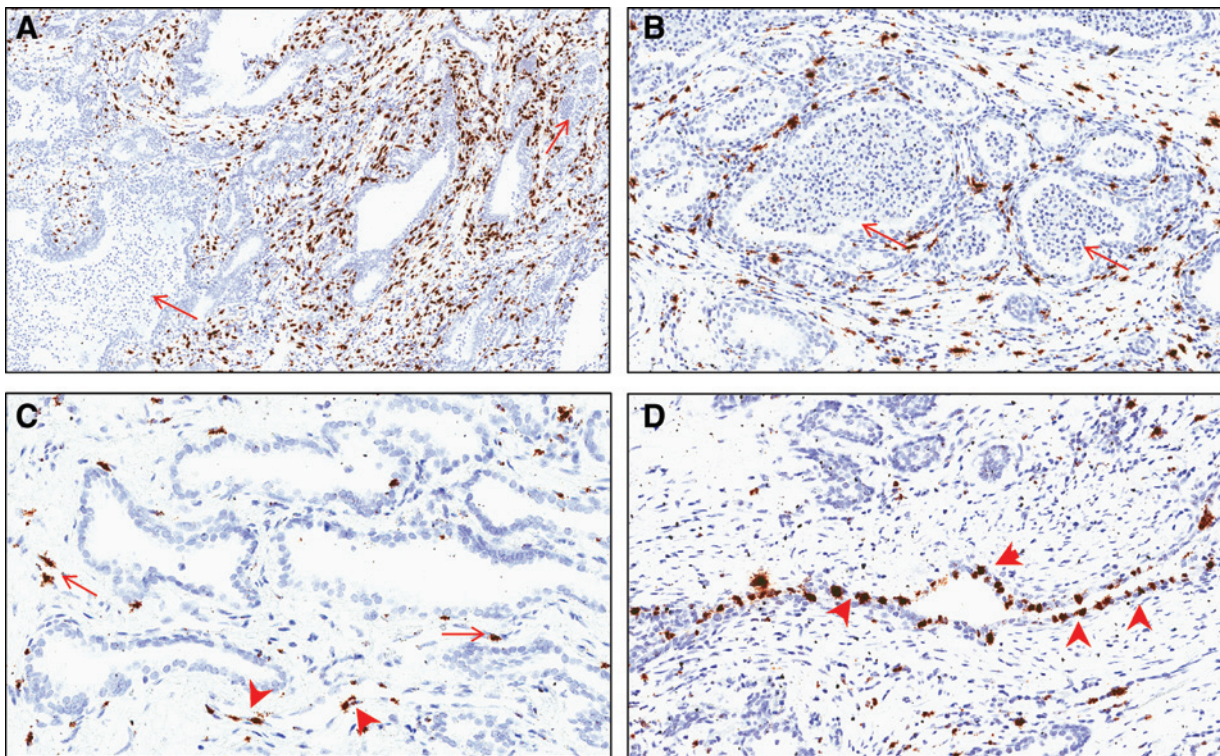
Figure 4.
IL6 mRNA expression is nearly exclusively restricted to the prostate stromal compartment. A and B, IL6 mRNA-expressing cells (brown staining) are highly enriched in the stroma in areas of acute inflammation as indicated by the presence of neutrophils in glandular lumens (red arrows). C, IL6-positive cells in the stroma (arrows) and endothelium (arrowheads) surrounding prostate atrophy. D, positive epithelial staining was rare and restricted to prostatic atrophy (arrowheads).

To further demonstrate the sensitivity of the CISH assay over IHC, we used qPCR to generate a standard curve with estimated copies of the IL6 cDNA vector (Supplementary Fig. S4A). qRT-PCR was then performed with RNA extracted from a known number of cells of the DU145 cell line (the lowest positive IL6-expressing cell line by qRT-PCR/CISH in Fig. 2B and C) and PC3 (determined to be negative in our hands by CISH). Comparison of the results from DU145 and PC3 to the standard curve indicated values of approximately 2 copies of IL6 mRNA per cell in the DU145 cell line and 0.017 copies per cell for PC3 (roughly 1 copy of IL6 mRNA per 59 cells). Cells from the same flasks of DU145 and PC3 used for RNA extraction were made into FFPE cell blocks. CISH analysis revealed (as also seen in Fig. 2C) positive detection of IL6 mRNA within the cytoplasm of DU145 cells (Supplementary Fig. S4B). Conversely, in PC3 cells, we see no cytoplasmic staining and generally one or two single dots in the nucleus. Since we have observed such signals at times in cells that have no corresponding mRNA (A.M. De Marzo, Q. Zheng, A.K. Meeker; unpublished data), and since it is clear that the CISH assay can at times detect genomic DNA as one or two dots in the nucleus (21), we do not consider one or two dots in the nucleus to be a bona fide positive signal and consider it below the limit of detection. IHC on adjacent slides was negative for IL6 protein (Supplementary Fig. S4B). We do not interpret this to mean that IL6 mRNA-expressing cells do not make IL6 protein, but that the IL6 protein cannot be detected using IHC without protein transport inhibition and, therefore, the CISH assay is more sensitive than our standard IHC assay.

Discussion

The cellular origin of IL6 in prostate cancer: a focus on the stromal compartment

In the present study, we analytically validated and used a CISH assay to detect IL6 mRNA in tissue sections to sensitively and specifically determine the cellular origin of IL6 in the prostate primary and metastatic tumor microenvironment. The results of our studies indicate that prostatic adenocarcinoma cells do not express IL6 mRNA. Rather, IL6 mRNA expression is restricted nearly exclusively to the cells in the stromal compartment, including endothelium, and is highly upregulated in areas of acute inflammation and prostatic atrophy.

Although prostate adenocarcinoma cells do not express IL6 mRNA, as evidenced by the results of our study, this does not eliminate a potential contributory role for IL6 signaling in prostate cancer development and/or progression. IL6 production by cells in the stromal compartment may signal in a paracrine fashion through the transmembrane IL6 receptor (IL6R) mediated by glycoprotein 130 (gp130) or via a soluble IL6 receptor (sIL6R) that signals through membrane-bound gp130. Therefore, IL6 potentially can signal through any cell that produces IL6R or gp130. In this respect, multiple studies have demonstrated the presence of IL6R and/or gp130 in prostate cancer cells (reviewed in ref. 24).

Interestingly, of the 11 prostate cultured cells or cell lines derived from cancer or benign tissues that we examined for IL6 mRNA expression levels in the present study, the cells that were found to express the highest levels of IL6 mRNA via qRT-PCR were PrSC
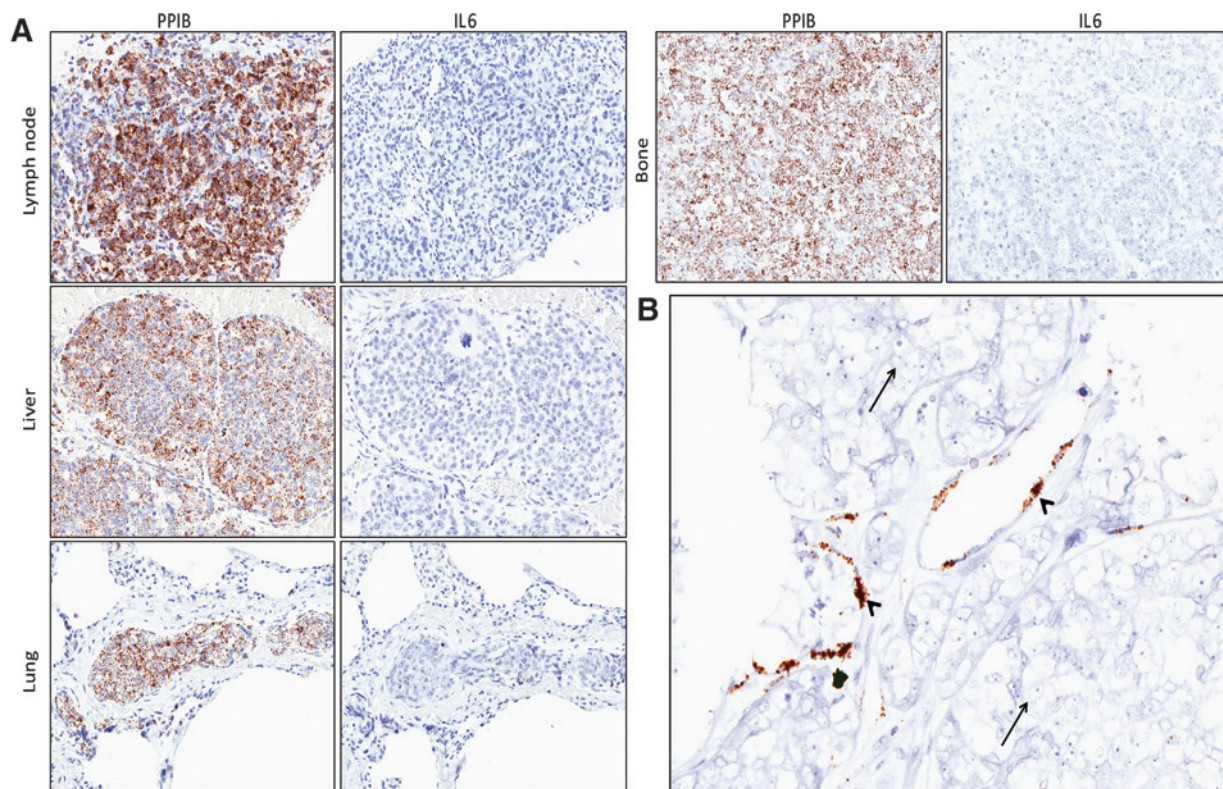
Figure 5.
IL6 mRNA is not detected in metastatic prostate cancer cells. In this study, metastatic prostate cancer in lymph node and liver biopsy samples and autopsy samples (liver, lung, bone, and lymph node) were assayed by IL6 CISH. No IL6-positive mRNA expression was observed in prostate cancer cells in any of the metastatic tissues. A, representative examples of lymph node biopsy and autopsy liver, lung, and bone metastases with positive staining for PPIB (positive control) and negative staining for IL6. B, example of IL6 mRNA-positive blood vessels (arrowheads) and IL6-negative tumor cells (arrows) in bone metastases.

cells (Fig. 2B). PrSC cells are a prostate stromal cell line; therefore, the high levels of IL6 mRNA expression in this cell line of stromal cell origin would correlate to our IL6 CISH results in prostate tissue sections where we found that IL6 mRNA expression was restricted almost exclusively to the stromal compartment. Of note, we also detected IL6 mRNA expression in RWPE-1 cells, which are HPV-immortalized benign prostate epithelial cells. It is possible that IL6 expression is induced in this line by the HPV infection (25). Whereas dual stains for IL6 and CD68 or CD31 confirmed that some of the IL6 mRNA-expressing cells in the stromal compartment are prostate-infiltrating macrophages or endothelial cells, respectively (Supplementary Fig. S1), this represented the minority of IL6-positive stromal cells in the highly positive areas surrounding acute inflammation (Fig. 4A and B). We predict that additional cells in the stromal compartment that express IL6 mRNA may include fibroblasts/myofibroblasts and smooth muscle cells. In this respect, Hobisch and colleagues detected IL6 secretion into the supernatant of *ex vivo*–cultured prostatic fibroblasts and smooth muscle cells, although no positive staining of these types of stromal cells was detected using IHC (14). These data are quite consistent with the results of the present study.

In areas of acute inflammation in some cases, a large proportion of cells in the stroma were positive for IL6 mRNA expression (Fig. 4A and B), arguing that a number of cell types in these areas may express IL6 mRNA in what may be part of a positive feedback loop as has been previously described (26). The stimulus for acute inflammation that is frequently observed in radical prostatectomy specimens (albeit to a lesser degree than chronic inflammation) is unknown (27), but may be caused in part by bacterial infections (27–29). A recent study also identified a role for IL6 upregulation by bone marrow–derived mesenchymal stem cells (MSC) in promotion of adipogenesis and prostate cancer progression (18), and it is possible that some of the IL6-positive cells identified in the present study may represent MSCs. This would be difficult to assess using the current CISH technologies that are limited to one to two markers per assay, as MSCs are typically identified using a number of surface markers, such as CD105, CD166, CD44, and CD29 (18). Future studies using such techniques as flow cytometry may help to further verify this possibility. Another recent study using a human prostate dissociation and tissue recombination system identified a role for paracrine expression of IL6 in the stromal compartment in concert with cell-autonomous oncogenic events in the promotion of an aggressive prostate cancer phenotype (17). In all, these studies, in parallel with the results of the present study, set the precedence for a potentially important role for paracrine IL6 signaling originating from the stromal compartment in the prostate tumor microenvironment.

The role of IL6 in metastatic disease

Studies have consistently shown that serum levels of IL6 are elevated in metastatic prostate cancer patients (3, 4, 6–9, 30), and that these levels may correlate to tumor burden (4, 5, 31) and/or
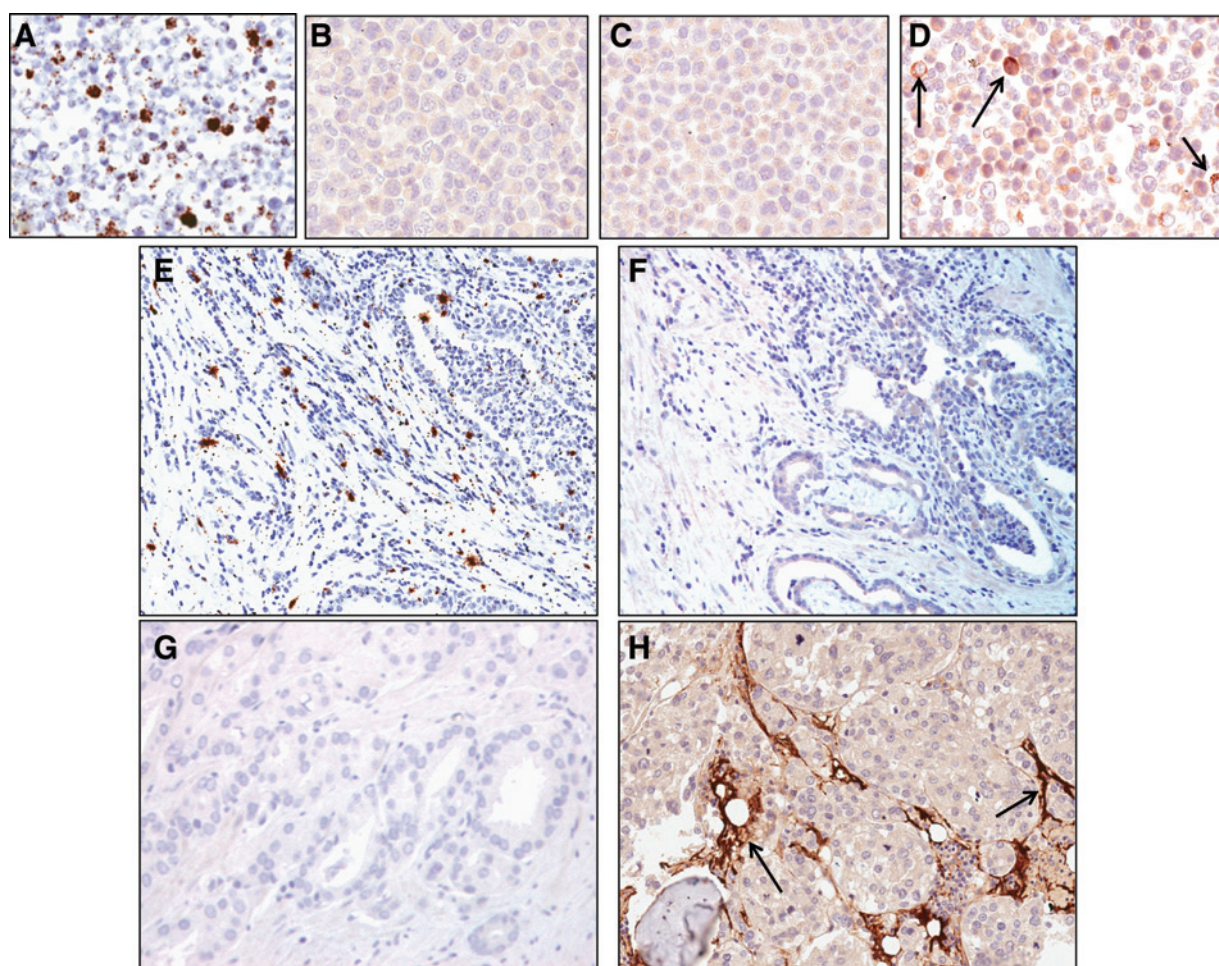
Figure 6.
A requirement for protein transport inhibition for IL6 IHC. A, IL6 CISH performed on the IL6-positive cell line NCI-H460. B and C, IL6 IHC performed on (B) PC3 cells (IL6 negative as assessed by qRT-PCR and CISH) and (C) NCI-H460 cells. D, marked difference in IHC results when NCI-H460 cells are treated with monensin prior to fixation. Arrows point to IL6-positive cells. E and F, the same prostatectomy case containing acute inflammation assayed for IL6 by CISH (E) and IHC (F). G and H, example of IL6 IHC on primary prostate cancer (G) and prostate cancer bone metastasis (H). All tumor cells were negative in all samples analyzed. Positive staining observed in extracellular spaces (arrows in H) was considered to be nonspecific.

may serve as a surrogate marker for morbidity associated with advanced prostate cancer including cachexia (9, 10). Interestingly, our studies of IL6 CISH in a series of metastatic prostate cancer biopsy or autopsy samples indicated that metastatic prostate cancer cells do not express IL6 mRNA. Although prostate tumor cells do not express IL6, we did observe a significant difference between IL6-positive blood vessels in bone metastases versus soft tissue metastases (Supplementary Table S3). Our data strongly suggest that the increased systemic levels of IL6 observed in advanced prostate cancer patients are not from production of the cytokine by metastatic tumor cells. Rather, elevated IL6 levels may be due at least in part to increased IL6 production by the tumor vasculature. Because the presence of bone metastases is also associated with morbidity, it is plausible that the presence of IL6 in vasculature in bone metastases could be a source of the elevated serum IL6 in patients with a high metastatic burden. The lack of IL6 mRNA expression by primary and metastatic prostate cancer cells observed in our study may help explain why minimal to no clinical activity has been observed to date when a monoclonal

antibody therapy targeting IL6 (siltuximab) has been tested in clinical trials (32, 33).

IL6 and IHC

IHC-based studies on IL6 production in prostate tissue sections have reported varying degrees of basal cell staining in benign epithelium as well as more pronounced staining of tumor epithelium (13, 14) that increases in intensity with increasing pathologic Gleason grade (13). Likewise, a previous study using IHC to detect IL6 production in prostate cancer metastases reported that over twice as many bone metastases samples are positive for IL6 than soft tissue metastases, and with much stronger staining intensity (15). Unfortunately, our data do not support those results. With a well-validated, positively controlled IL6 CISH assay, we did not detect IL6 mRNA in any of the primary or metastatic prostate cancer cells in our study. We were able to detect IL6 using IHC in positive control cell line specimens, but only when IL6 accumulation was augmented by blocking Golgi export using a protein transport inhibitor.

To help determine whether we could account for the discrepant results between a past study (15) and the present one, we compared by IHC the staining obtained with the polyclonal anti-IL6 antibody batch (#6672; Abcam; lot #385304) that was used previously to the currently commercially available batch (lot # GR169214-2). These analyses indicated that under identical conditions, the newer version of the antibody does not stain tumor cells in bone metastases, yet the older antibody batch gave similar results to those reported previously (15) with strong tumor cell staining (C. Morrissey; unpublished data). Taken together with the present study, it would appear that prior results showing high-level expression in tumor cells are related to antibody lot variability and it no longer appears with the newer currently available antibody from the same vendor. Also, traditional decalcification/fixation methods used for bone can have a significant impact on RNA integrity, although formic acid treatment, as was used for the metastatic bone samples in the present study, has been shown to result in improved nucleic acid recovery and quality compared with stronger acid treatment (34, 35). We acknowledge this potential limitation to the present study, and addressed this concern by including the positive control (PPIB) in the CISH assay for each sample analyzed. Likewise, we detected IL6 mRNA in the metastatic bone samples (albeit not in the tumor cells but in the blood vessels), indicating that at least there did not appear to be any generalized issues with detection of IL6 mRNA in the decalcified bone samples.

In conclusion, our results suggest that, in prostate cancer patients, paracrine rather than autocrine IL6 production is likely associated with any role for the cytokine in disease development and/or progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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