Prostate tumor CXC-chemokine profile correlates with cell adhesion to endothelium and extracellular matrix

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Abstract

Though chemokines of the CXC family are thought to play key roles in neoplastic transformation and tumor invasion, information about CXC chemokines in prostate cancer is sparse. To evaluate the involvement of CXC chemokines in prostate cancer, we analyzed the CXC coding mRNA of both chemokine ligands (CXCL) and chemokine receptors (CXCR), using the prostate carcinoma cell lines PC-3, DU-145 and LNCaP. CXCR proteins were further evaluated by Western blot, CXCR surface expression by flow cytometry and confocal microscopy. The expression pattern was correlated to adherence of the tumor cells to an endothelial cell monolayer or to extracellular matrix components. Based on growth and adhesion capacity, PC-3 and DU-145 were identified to be highly aggressive tumor cells (PC-3 > DU-145), whereas LNCaP belonged to the low aggressive phenotype. CXCL1, CXCL3, CXCL5 and CXCL6 mRNA, chemokines with pro-angiogenic activity, were strongly expressed in DU-145 and PC-3, but not in LNCaP. CXCR3 and CXCR4 surface level differed in the following order: LNCaP > DU-145 > PC-3. The differentiation factor, fatty acid valproic acid, induced intracellular CXCR accumulation. Therefore, prostate tumor malignancy might be accompanied by enhanced synthesis of angiogenesis stimulating CXC chemokines. Further, shifting CXCR3 and CXCR4 from the cell surface to the cytoplasm might activate pro-tumoral signalling events and indicate progression from a low to a highly aggressive phenotype.

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Introduction

Chemokines are a family of low molecular weight (8–10 kDa) pro-inflammatory cytokines, which bind to G-protein coupled receptors. More than 40 different chemokines have been isolated and structurally divided into four groups, defined by the arrangement of the conserved cysteine (C) residues of the mature proteins (Baggiolini et al., 1997; Baggiolini et al., 1994). The CXC chemokines have one amino acid residue separating the first two conserved cysteine residues. The primary function of CXC chemokines is chemoattraction and activation of specific leukocytes in various immuno-inflammatory responses. However, it has recently been shown that they also play key roles in neoplastic transformation and in the passage of tumor cells through the endothelial vessel wall and extracellular matrix in studies pertaining to melanoma (Payne and Cornelius, 2002), breast (Walser and Fulton, 2004), colon (Sharma et al., 2004) and lung carcinoma (Strieter et al., 2004).

Information about the involvement of CXC chemokines in prostate cancer, the second leading cause of cancer death in the United States and Europe, is sparse. CXCL8 (IL-8) has been found to be elevated in prostate cancer patients which might be coupled to tumor growth and local invasion (Uehara et al., 2005; Lehrer et al., 2004; Aalinkeel et al., 2004; Lee et al., 2004). There is also evidence that high expression levels of the CXCR4 receptor and positive staining for its ligand CXCL12 (SDF-1) correlates with the presence of metastatic disease in prostate cancer patients (Arya et al., 2004; Singh et al., 2004).

These results suggest that prostate cancer may be influenced by CXC chemokines during metastasis. However, a single chemokine might not be the sole trigger initiating the complex program of tumor dissemination. Rather, the balance of tumoral
CXC production and alterations of the CXC profile might determine whether a tumor cell will penetrate blood vessels to form a secondary tumor (Menten et al., 2002). In the present report, the CXC coding mRNA of both chemokine ligands (CXCL) and chemokine receptors (CXCR) was analyzed, using three different prostate carcinoma cell lines. CXCR proteins were further evaluated by Western blot, CXCR surface expression by flow cytometry and confocal microscopy. The expression pattern was correlated to the ability of the tumor cells to adhere to an endothelial cell monolayer or to extracellular matrix components.

Materials and methods

Cell cultures

Prostate carcinoma DU-145, LNCaP and PC-3 cells were purchased from DSMZ (Braunschweig, Germany). Tumor cells were grown and subcultured in RPMI1640 medium (Seromed, Berlin, Germany) supplemented with 10% FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified, 5% CO₂ incubator.

Endothelial cells (HUVEC) were isolated from human umbilical veins and harvested by enzymatic treatment with chymotrypsin. HUVEC were grown in Medium 199 (Biozol, Munich, Germany), 10% fetal calf serum (FCS; Gibco, Karlsruhe, Germany), 10% pooled human serum (Blood Bank of the German Red Cross, Frankfurt am Main, Germany), 20 μg/ml endothelial cell growth factor (Boehringer, Mannheim, Germany), 0.1% heparin (Roche, Basel, Switzerland), 100 ng/ml gentamycin (Gibco) and 2% 1 M HEPES-buffer (Seromed, Berlin, Germany). To control the purity of HUVEC cultures, cells were stained with fluorescein isothiocyanate (FITC)-labelled monoclonal antibody against Factor VIII-associated antigen (von Willebrand factor; clone 8-2/86; Dako, Hamburg, Germany), 0.1% heparin (Roche, Basel, Switzerland), 100 ng/ml gentamycin (Gibco) and 2% 1 M HEPES-buffer (Seromed, Berlin, Germany). To control the purity of HUVEC cultures, cells were stained with fluorescein isothiocyanate (FITC)-labelled monoclonal antibody against Factor VIII-associated antigen (von Willebrand factor; clone 8-2/86; Dako, Hamburg, Germany) and analyzed microscopically or by FACscan (Becton Dickinson, Heidelberg, Germany) in combination with reagents for unspecific cell adhesion. Thereafter, 0.5 × 10⁶ tumor cells/well were added for 60 min. Subsequently, nonadherent tumor cells were washed off using warmed (37 °C) PBS. The adherent cells were fixed with 1% glutaraldehyde and counted in five different fields (5 × 0.25 mm²) using a phase contrast microscope (20 × objective) to calculate the mean cellular adhesion rate which was defined by adherent cells/coated well – adherent cells/background.

Monolayer adhesion assay

HUVEC were transferred to 6-well multiplates (Falcon Primaria; Becton Dickinson, Heidelberg, Germany) in complete HUVEC-medium. When confluency was reached, 0.5 × 10⁶ tumor cells/well were carefully added to the HUVEC monolayer for 60 min. Subsequently, nonadherent tumor cells were washed off using warmed (37 °C) Medium 199. The adherent cells were fixed with 1% glutaraldehyde and counted in five different fields (5 × 0.25 mm²) using a phase contrast microscope (20 × objective) to calculate the mean cellular adhesion rate.

Attachment to extracellular matrix components

Six-well plates were coated with collagen (Seromed; diluted to 100 μg/ml in PBS), laminin (Becton Dickinson; diluted to 50 μg/ml in PBS), or fibronectin (Becton Dickinson; diluted to 50 μg/ml in PBS) overnight. Plastic dishes served as the background control. Plates were washed with 1% bovine serum albumin (BSA) in PBS to block nonspecific cell adhesion. Thereafter, 0.5 × 10⁶ tumor cells/well were added for 60 min. Subsequently, nonadherent tumor cells were washed off using warmed (37 °C) PBS. The adherent cells were fixed with 1% glutaraldehyde and counted in five different fields (5 × 0.25 mm²) using a phase contrast microscope (20 × objective) to calculate the mean cellular adhesion rate which was defined by adherent cells/coated well – adherent cells/background.

Chemoattractants

Cell migration toward CXCL12 was examined using 6-well Transwell chambers (Greiner, Frickenhausen, Germany) with 8-μm pores. DU-145 or LNCaP cells were removed from the culture flasks and resuspended at 0.1 × 10⁶ cells/ml in serum-free culture medium. CXCL12 (500 ng/ml) was placed in the lower chamber. Test cells were then placed in the upper chamber for 4 h. After incubation, the upper surface of the Transwell membrane was wiped gently with a cotton swab to remove nonmigrating cells. Cells which migrated to the lower surface of the membrane were stained using hematoxylin, counted in five different fields of a defined size (5 × 0.25 mm²) using a phase contrast microscope and the mean cellular migration rate was calculated.

Evaluation of CXCR surface expression

Tumor cells were washed in blocking solution (PBS, 0.5% BSA) and then incubated for 60 min at 4 °C with phycoerythrin (PE)-conjugated monoclonal antibody (mouse anti-human) anti-CXCR1 (IgG2b, clone 42705.111), anti-CXCR2 (IgG2a, clone 48311.211), anti-CXCR3 (IgG1, clone 49801.111), anti-CXCR4 (IgG2a, clone 12G5), anti-CXCR5 (IgG2b, clone 51505), or anti-CXCR6 (IgG2b, clone 56811; all: R&D Systems, Wiesbaden, Germany). CXCR expression of tumor cells was then measured using a FACscan (Becton Dickinson;
FL-2H (log) channel histogram analysis; $1 \times 10^4$ cells/scan) and expressed as mean fluorescence units (MFU). Mouse IgG1-PE, IgG2a-PE, or IgG2b-PE was used as an isotype control (all: Cymbus Biotechnology, Hofheim, Germany).

To analyze CXCR distribution on the cell membrane, tumor cells were transferred to round cover slips (pretreated with 2% 3-aminopropyl-triethoxysilan) placed in a 24-well multilipate. Upon reaching confluency, cell cultures were washed and fixed in cold ($-20^\circ$ C) methanol/acetone (60/40 v/v). Subsequently, cells were incubated for 60 min with unconjugated anti-CXCR monoclonal antibodies. Indocarbocyanine (Cy 3TM; Dianova; working dilution: 1:50) conjugated goat–anti-mouse IgG was then added as the secondary antibody. To prevent photobleaching of the fluorescent dye, cover glasses with stained cells were taken out of the wells and the residual liquid was removed. These were then embedded in an antifade reagent/mounting medium mixture (ProLongTM Antifade Kit, MoBiTec, Göttingen, Germany) and mounted on slides. The slides were viewed using a confocal laser scanning microscope (LSM 10; Zeiss, Jena, Germany) with a plan-neofluar ×100/1.3 oil immersion objective.

Western blot analysis

Total CXCR3 or CXCR4 content in LNCaP or DU-145 tumor cells was evaluated by Western blot analysis: tumor cell lysates were applied to a 7% polyacrylamide gel and electrophoresed for 90 min at 100 V. The protein was then transferred to nitrocellulose membranes. After blocking with nonfat dry milk for 1 h, the membranes were incubated overnight with the anti-CXCR3 or anti-CXCR4 antibody (dilution 1:100). HRP-conjugated goat–anti-mouse IgG (Upstate Biotechnology, Lake Placid, NY, USA; dilution 1:5000) served as the secondary antibody. The membranes were briefly incubated with ECL detection reagent (ECL™, Amersham) to visualize the proteins and exposed to an X-ray film (Hyperfilm™ ECTM, Amersham).

mRNA expression of CXCR and CXCL

mRNA expression of CXCR and CXCL was evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR). Tumor cells were seeded in 50 ml culture flasks (25 cm² growth area; Falcon Primaria, Becton Dickinson). Total RNA was extracted by using RNeasy kit (Qiagen, Hilden, Germany) and RNA samples were then treated with 80 U/ml of Rnase-free Dnase I (Boehringer Mannheim, Mannheim, Germany) for 20 min. Subsequently, samples were incubated for 10 min at 37°C to inactivate Dnase. Complementary DNA was synthesized from 1 g of total RNA per sample with a 60-min incubation at 42°C, using the Moloney murine leukemia virus reverse transcriptase (Invitrogen) in a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany). Reactions were performed in the presence of 0.5 μl cDNA, with an initial incubation step at 94°C for 5 min. Cycling conditions consisted of denaturation at 94°C for 60 s, annealing at 60°C for 60 s and extension at 72°C for 60 s over a total of 25 cycles. The reaction was completed by another 10-min incubation step at 72°C. The specific sequences for sense and anti-sense primers are shown in Table 1. The PCR products were subjected to electrophoresis in 1.5% agarose gel and visualized by ethidium bromide.

**Differentiation induction**

LNCaP cells were treated with the differentiation-inducing agent valproic acid (VPA; Sigma, München, Germany) at a final concentration of 1 mM for 3 or 5 days. CXCR4 coding mRNA, CXCR4 protein level, and CXCR4 surface expression were then measured in VPA-treated cells. Results were compared to untreated controls. Viability of tumor cells in presence of VPA was assessed by propidium iodide dsDNA-intercalation or quantitative fluorescence analysis of enzyme-catalyzed fluorescein-diacetate metabolism. Trichostatin A (TSA; 1 μM) was used as a reference compound, to demonstrate that changes of the tumor differentiation status are not limited to VPA.

**PSA secretion**

DU-145 or LNCaP tumor cells were grown in 6-well plates and treated with VPA or TSA for 3 days (control: untreated cell cultures). Release of free and active PSA into the cell culture supernatant was then measured after 3 days by the “two-step” sandwich type immunoassay (ELISA), according to the manufacturer’s recommendation (Diagnostic Systems Laboratories, Sinsheim, Germany).

**Table 1**

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Statistical analysis

All studies were performed 3–6 times. Statistical significance was investigated by the Wilcoxon–Mann–Whitney $U$-test. Differences were considered statistically significant at a $p$ value less than 0.05.

Results

Growth capacity of tumor cells

DU-145 cells grew as monolayers with an epithelial-like, cuboidal morphology. PC-3 cells were characterized by a flat morphology, cell borders were not clearly seen under phase contrast microscope. LNCaP cells showed loose intercellular adhesion and differentiated morphology with cell protrusions. Seventy-two hours of proliferation analysis revealed rapid cell growth of DU-145 and PC-3 cells with doubling every 24 h. In contrast, LNCaP exhibited significantly lower growth activity, compared to DU-145 and PC-3 cells (Fig. 1).

Tumor cell adhesion

Adhesion capacity of tumor cells to both endothelium or extracellular matrix components was evaluated. The 60-min adhesion rates of tumor cells to HUVEC were 116 ± 21 PC-3 cells/mm$^2$ (endothelium) or 104 ± 13 DU-145 cells/mm$^2$ (extracellular matrix) (Fig. 2). The adhesion capacity of LNCaP cells was significantly reduced, compared to DU-145 and PC-3 cells (64 ± 11 LNCaP cells/mm$^2$).

Both PC-3 and DU-145 cells equally and strongly bound to laminin coated surfaces (PC-3: 538 ± 71 cells/mm$^2$, DU-145: 540 ± 83 cells/mm$^2$), whereas LNCaP did not (Fig. 3A). In a similar fashion, PC-3 and DU-145 attached to the collagen matrix, whereas LNCaP did not. DU-145 cells (mean binding rate/mm$^2$: 442 ± 74 cells) bound to a higher extent than PC-3 cells (mean binding rate/mm$^2$: 339 ± 69 cells; Fig. 3B). PC-3 and DU-145 also bound to fibronectin, whereby more DU-145 cells attached to the substratum than PC-3 cells (640 ± 96 adherent cells/mm$^2$ versus 521 ± 92 adherent cells/mm$^2$). LNCaP attached to fibronectin as well. However, this effect was less intense than that of PC-3 or DU-145 (mean binding rate: 340 ± 80 cells/mm$^2$; Fig. 3C).

CXCL and CXCR mRNA expression pattern

Comparative analysis of mRNA coding for chemokines of the CXCL family was carried out by RT-PCR. Fig. 4A demonstrates close similarity between DU-145 and PC-3 CXCL expression pattern. However, it should be noted that CXCL12 mRNA was expressed by PC-3 cells only. CXCL12 mRNA was not active in DU-145 cells. Remarkably, LNCaP cells were characterized by a complete loss of CXCL1, CXCL2, CXCL6 and CXCL7 mRNA and a very low expression of CXCL 3–5 mRNA. On the other hand, CXCL9, CXCL12 and CXCL13 mRNA were distinctly expressed, compared to DU-145 cells.

The CXCR mRNA pattern was not similar between DU-145 and PC-3 cells. CXCR4 mRNA was expressed most strongly in DU-145 cells followed by CXCR5, CXCR6 and CXCR1...
mRNA. CXCR4 mRNA was clearly visualized in PC-3 cells as well. However, CXCR5 and CXCR1 mRNA were only weakly detected (Fig. 4B). CXCR4, CXCR5 and CXCR6 mRNA were expressed similarly in LNCaP cells. CXCR2 was also expressed distinctly in LNCaP cells, in contrast to DU-145 and PC-3 cells.

**CXCR surface expression**

CXCR surface expression was evaluated by flow cytometry. Only PE-labelled CXCR3 and CXCR4 evoked specific fluorescence, compared to the isotype controls in all cell lines. High CXCR3 expression level was detected on LNCaP, whereas DU-145 and PC-3 cells presented lower amounts of CXCR3 molecules (Fig. 5). CXCR4 was detected moderately on LNCaP plasma membranes and weakly on DU-145 and PC-3 cells (Fig. 5). Confocal analysis confirmed the presence of CXCR4 along cell boundaries, even in CXCR4 low expressing DU-145 cells (Fig. 6).

**CXCR protein level**

Fluorescence analysis demonstrated higher CXCR3 and CXCR4 expression levels (CXCR3 > CXCR4) in LNCaP cells than in DU-145 and PC-3 cells. Remarkably, intracellular CXCR protein content did not follow this rule. Rather, receptor proteins accumulated more in DU-145 cells than in LNCaP...
cells, and the amount of CXCR4 proteins exceeded the amount of CXCR3 proteins (Fig. 7).

Tumor cell differentiation

Based on the inverse correlation between CXCR surface expression and intracellular CXCR protein level, it was hypothesized that prostate tumor cell differentiation and malignancy might be characterized by a chemokine receptor translocation. To elucidate this speculation, LNCaP cells were treated with the differentiation-inducing compound VPA, and CXCR4 mRNA, CXCR4 protein and CXCR4 surface level was evaluated and compared to CXCR4 expression in nontreated cells. The data, presented in Fig. 8, demonstrate slight modifications of the CXCR4 coding mRNA. In good accordance to our hypothesis, the CXCR4 protein level was distinctly reduced after a 3-day and a 5-day VPA treatment, whereas the CXCR4 surface level was increased significantly. VPA application also reduced intracellular CXCR4 and enhanced CXCR4 surface expression in DU-145 tumor cells (data not shown). VPA did not exert any toxic effects on the cell cultures which might contribute to the CXCR4 protein down-regulation.

Chemotaxis, proliferation and adhesion assays confirmed the differentiation-inducing effects of VPA. VPA blocked tumor cell proliferation by 70% (DU-145) or 40% (LNCaP), respectively (Fig. 1), as well as tumor cell attachment to human endothelial cells (Fig. 2). VPA also blocked CXCL12 triggered migration of DU-145 cells (98.2 ± 17.4 cells/mm² versus 55.6 ± 14.6 cells/mm²; random migration of control cells: 45.6 ± 8.6 cells/mm²). Proliferation blocking effects after 72 h were also observed in presence of TSA, indicating that changes are not limited to VPA. TSA reduced the number of DU-145 cells by 48.7 ± 14.2% (mean ± S.D., n = 3), and the number of LNCaP cells by 41.1 ± 17.5% (mean ± S.D., n = 3).

Differentiation-inducing effects of VPA were also seen with respect to PSA release. VPA reduced the amount of active PSA in LNCaP culture supernatant from 40.28 ng/ml (control) to 6.62 ng/ml. The reference compound TSA reduced the amount of active PSA to 27.45 ng/ml. All values are related to 5 × 10⁴ LNCaP cells and depict mean data from three experiments. Only few PSA was detected in DU-145 culture supernatant (0.137 ng/ml, related to 1 × 10⁵ cells). VPA diminished active PSA by 35.8 ± 16.9%, TSA by 33.3 ± 19.2% (mean ± S.D. from three experiments). Release of free PSA was also found to be reduced.
in LNCaP cultures by VPA (mean: 0.27 ng/ml versus 2.63 ng/ml), or by TSA (mean: 0.97 ng/ml versus 2.63 ng/ml), respectively. Free PSA in the DU-145 culture system was not significantly elevated over background (medium control).

**Discussion**

Comparative analysis of three different prostate tumor cell lines present evidence that alterations of tumor cell growth and adhesion are paralleled by alterations of the CXC expression profile.

Based on our in vitro experiments, PC-3 and DU-145 cells were found to be highly aggressive (PC-3 > DU-145), whereas LNCaP represented the low aggressive phenotype. This is in good accordance with other data, demonstrating PC-3 and DU-145 cells to be more invasive in vitro and in vivo, compared to LNCaP cells (Aalinkeel et al., 2004; Moore et al., 1999).

Remarkably, CXCL1, CXCL3, CXCL5 and CXCL6 mRNA are strongly expressed in the highly aggressive cell lines, whereas they were not detected in LNCaP cells. All these chemokines have in common to be potent inducers of angiogenesis. Presumably, the enhanced synthesis and release of ELR+ chemokines in prostate cancer switches the tumor cell characteristics from low aggressive (defined by a low growth and adhesion potential) to highly aggressive (defined by accelerated tumor growth and adhesion).

Shen and Lentsch (2004) showed that prostate tumors in the TRAMP mouse model have significantly greater expression of macrophage inflammatory protein (MIP)-2, which is an analog to human CXCL1, compared to normal prostate tissue from wild-type controls as well as normal prostate tissue from...
production of CXCL1 and CXCL3 (Moore et al., 1999). In the correlation between PC-3 tumor growth and tumor-derived production of CXCL5 in PC-3 tumor-bearing mice correlated with tumor progression and/or invasion. The in vivo mouse model (Moore et al., 1999).

The tumorigenicity of the prostate tumor cells in the in vivo SCID mouse model (Moore et al., 1999) contrast to LNCaP cells which expressed CXCL1 to a much lower extent. The in vitro data correlated well with the tumorigenicity of the prostate tumor cells in the in vivo SCID mouse model (Moore et al., 1999).

It is not clear, if CXC chemokines directly interfere with the process of tumor growth and/or invasion. The in vivo production of CXCL5 in PC-3 tumor-bearing mice correlated with exponential tumor growth. However, there was no correlation between PC-3 tumor growth and tumor-derived production of CXCL1 and CXCL3 (Moore et al., 1999). In the same model, a positive correlation was calculated between DU-145 tumor growth and tumor-derived CXCL1, but not between tumor growth and CXCL5 and CXCL3. The findings support the theory that individual CXC chemokines might not regulate tumor growth and metastasis. Rather, changes of a cohort of chemokines might be necessary to activate tumor-promoting signals.

Conflicting data are available with respect to CXCR expression. Moore et al. (1999) did not detect CXCR1 and CXCR2 on PC-3 and DU-145 cell surfaces, whereas Konig et al. (2004) reported moderate CXCR1 surface expression on PC-3 and LNCaP cells. Although we measured CXCR1-6 gene activity in PC-3, DU-145 and LNCaP cells, only CXCR3 and CXCR4 were significantly expressed on the cell membrane. Possibly, fixed cells have been used in the above-mentioned experiment (Konig et al., 2004) and therefore, CXCR1 evoked fluorescence might (partially) result from cytoplasmic staining.

No data are available on the relevance of CXCR3 in prostate tumor progression, although this receptor was strongly expressed on the plasma membranes. The most intense fluorescence was measured in the low aggressive LNCaP cells, low fluorescence was seen in the highly aggressive cell lines DU-145 and PC-3. Novel data on MA-10 Leydig tumor cells suggest that activation of CXCR3 by its ligand CXCL10 inhibits tumor cell proliferation (Nagpal et al., 2004). Considering the high CXCL10 mRNA content in our LNCaP cells, compared to DU-145, the CXCR3–CXCL10 axis might be the responsive element for prostate tumor cell growth regulation, with an inverse relationship between CXCR3 surface expression and growth capacity. Indeed, the anti-proliferative drug valproic acid significantly enhanced CXCR3 on DU-145 tumor cells (unpublished observation), which is in line with our hypothesis.

It has been pointed out that CXCR4 surface expression might correlate with tumor progression and malignancy. In corroboration with this hypothesis, the surface protein expression of CXCR4 was demonstrated to be significantly higher on several cancer cell lines than on normal prostatic epithelial cells (Singh et al., 2004). Nevertheless, the relevance of CXCR4 is still not clear and experimental data are conflicting.

Using the flow cytometry technique, significantly high levels of the CXCR4 receptor were detected on the cell surface of DU-145, LNCaP and PC-3 cell lines by Arya et al. (2004), while no or very low CXCR4 surface expression on all three cell lines has been observed by others (Darash-Yahana et al., 2004). FACS analysis of epithelial cells from patients with prostate cancer versus benign prostatic hyperplasia revealed similar CXCR4 expression levels (Hart et al., 2005).

The present results indicate an inverse relationship between CXCR4 surface expression and the malignant behaviour of prostate tumor cells, but a positive relationship between intracellular CXCR4 content and tumor malignancy. In a similar experimental setting, LNCaP cells expressed significantly higher levels of cell surface CXCR4 when compared to PC-3 (Singh et al., 2004), and the amount of CXCR4 positive cells evaluated by flow cytometry has been shown in the order LNCaP > DU-145 > PC-3 (Vaday et al., 2004).

It might be a paradox that CXCR4 surface expression does not correlate with tumor invasiveness/malignancy. Recently, Darash-Yahana et al. (2004) showed that CXCR4, induced by transfection, does not up-regulate chemotactic potential of the tumor cells. A possible explanation for this phenomenon might be that receptor saturation occurs at a very low level and, consequently, quantitative receptor enhancement beyond a specific threshold will not accelerate migratory processes of tumors.

Presumably, the absolute amount of CXCR4 receptors on the cell surface might not account for the tumoral characteristics of prostate cells. Rather, CXCR4 shifting into the cytoplasm may determine the grade of malignancy. Indeed, treatment of LNCaP with the anti-tumoral and differentiation-inducing compound valproic acid induced a CXCR4 down-regulation within the cytoplasm and a concomitant accumulation of CXCR4 on the plasma membrane.

Our hypothesis that CXCR4 translocation might activate tumor signalling events has not been proven yet, and no information is available dealing with this issue. However, analysis of other membrane receptors is in good agreement with our speculation. Nielsen et al. observed an agonist-regulated translocation of the CysLT membrane receptor in the outer nuclear membrane of intestinal cells and increased expression of this receptor in the nuclei of human colon cancer cells. CysLT were not degraded or recycled, rather internalized receptors triggered both ERK1/2 signalling and a proliferative response of tumor cells (Nielsen et al., 2005). Comparative analysis of the CD105 (endoglin) receptor expression of different human tumor cells indicated two protein pools, one of which accounted for receptor surface expression, whereas the other was expressed in cytoplasmic compartments. The authors suggested a complex mechanism of receptor translocation which might account for the modulation of cell proliferation (Postiglione et al., 2005).

Obviously, signalling by a membrane receptor can occur in a novel, intracellular location, providing accession of the receptor by its ligand. However, it should not be ruled out that the weak CXCR4 surface expression might also reflect difficulties in antigen detection caused by tumor-specific antigen glycosylation.

The concept that the receptor localization might vary according to the type of disease is supported by clinical
studies. Immunohistochemical analysis of prostate specimen from normal versus neoplastic tissue revealed strong CXCR4 nuclear staining in both benign prostatic hyperplasia and localized prostate cancer sections while the prostate bone metastases, a poor prognostic indicator, showed strong CXCR4 nuclear and cytoplasmic staining (Hart et al., 2005). Sun et al. (2003) observed predominately weak cytoplasmic staining in normal epithelium, but strong cytoplasmic CXCR4 localization in prostate cancer samples.

We suggest that CXCR4 regulates tumor malignancy at the protein level where rapid turnover of the receptor has been reported (Cheng et al., 2000). One candidate responsible for CXCR4 shifting might be beta-arrestin. Recent discoveries indicate that beta-arrestin can effectively regulate different functions of CXCR4, mediated through its distinct interactions with the C terminus and other regions including the third loop of CXCR4 (Cheng et al., 2000). Beta-arrestin promotes CXCR4 internalization, enhances the CXCR4-mediated activation of ERK and is crucially involved in the CXCR4-induced activation of p38 MAPK. Further, the expression of beta-arrestin in both HeLa and human embryonic kidney 293 cells has been shown to be critically involved in CXCR4-mediated chemotaxis and cell migration (Sun et al., 2002).

Conclusion

Prostate tumor malignancy seems to be accompanied by an enhanced and/or de novo synthesis of angiogenesis stimulating ELR+ CXC chemokines. Further, shifting of CXCR3 and CXCR4 from the cell surface into the cytoplasm might activate pro-tumoral signalling events and indicate progression from a low to a highly aggressive phenotype.

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