

Prostate stromal cells produce CXCL-1, CXCL-2, CXCL-3 and IL-8 in response to epithelia-secreted IL-1

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It is well accepted that tumor microenvironment is essential for tumor cells survival, cancer progression and metastasis. However, the mechanisms by which tumor cells interact with their surrounding at early stages of cancer development are largely unidentified. The aim of this study was to identify specific molecules involved in stromal–epithelial interactions that might contribute to early stages of prostate tumor formation. Here, we show that conditioned medium (CM) from immortalized non-transformed prostate epithelial cells stimulated immortalized prostate stromal cells to express cancer-related molecules. CM obtained from epithelial cells triggered stromal cells to express and secrete CXCL-1, CXCL-2, CXCL-3 and interleukin (IL)-8 chemokines. This effect was predominantly mediated by the cytokines of the IL-1 family secreted by the epithelial cells. Thus, prostate epithelial cells induced the secretion of proinflammatory and cancer-promoting chemokines by prostate stromal cells. Such interactions might contribute to prostatic inflammation and progression at early stages of prostate cancer formation.

Introduction

Prostate cancer is the second leading cancer diagnosed in elderly men in the western world (1). It is probably that carcinogenesis is initiated by genetic aberrations in the prostatic epithelium. The perturbation of reciprocal homeostatic smooth muscle–epithelial interactions plays a key role in further malignant progression of epithelial cells harboring initiating mutations (2). The prostatic stroma is comprised of fibroblasts, smooth muscle cells, nerves and lymphatics (3). Aberrant cross talk between cancer cells and adjacent stromal cell populations may lead to the selection of a tumor microenvironment that further promotes tumor progression. However, the mechanisms by which stromal cells influence the initiating events of tumorigenesis are poorly understood. The aim of the present study was to decipher the potential contribution of epithelial–stromal interactions to prostate cancer initiation, focusing on secretion of chemokines that are known to promote inflammation and tumorigenesis.

Chemokines are chemotactic cytokines that attract, activate and regulate cell trafficking of various types of leukocytes (4). In addition to their effect on chemotactic migration of leukocytes, chemokines were shown to play diverse roles in tumor development through their effect on angiogenesis, hematopoiesis, metastasis and tumor rejection (5–8). Moore *et al.* (9) demonstrated that human prostate cancer cell lines can constitutively produce angiogenic CXC chemokines (9). The tumorigenic potential of PC-3 prostate cancer cells was shown to be partially attributable to the production

Abbreviations: CM, conditioned media; ELISA, enzyme-linked immunosorbent assay; hTERT, human telomerase reverse transcriptase; IL, interleukin; IL-1Ra, interleukin-1 receptor antagonist; mRNA, messenger RNA; NF- κ B, nuclear factor-kappa B; QRT-PCR, quantitative real-time polymerase chain reaction.

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of the angiogenic CXC chemokine, interleukin (IL)-8, whereas the Du145 prostate cancer cell line utilizes a different angiogenic CXC chemokine, growth-regulated alpha (also called CXCL-1) to mediate tumorigenicity (9). Recently, IL-8 was proposed to serve as a molecular determinant of androgen-independent prostate cancer growth in LNCaP and LAPC-4 cell lines (10). Apart from tumor cells themselves, adjacent cell types were shown to secrete chemotactic agents. Vascular endothelial cells represent one source of paracrine mediators of prostate cancer progression such as IL-1 and IL-8 (11). Another example was shown by Gallagher *et al.* (12), who have demonstrated a paracrine induction of chemokines expression in fibroblasts cocultivated with melanoma cells. In this study, the authors showed that when melanoma cells are cocultured with primary fibroblast cells, the chemokines CXCL-1, CXCL-2 and IL-8 are significantly upregulated in the cocultured primary fibroblasts, whereas the melanoma cells are almost not affected (12).

Prostate cancer initiation is commonly associated with chronic or recurrent inflammation (13). Among the possible causes of prostate inflammation are infection, chemical and physical trauma due to urine reflux and dietary factors (14). Genetic studies support the causative role of inflammation in prostate cancer (reviewed in ref. 14). However, not much is known as to the mechanisms by which immune cells infiltrate the prostate (15).

IL-1 is a pleiotropic cytokine involved in various immune responses, inflammatory processes and hematopoiesis. Excessive IL-1 levels have been implicated in the pathogenesis of acute or chronic inflammatory diseases and malignancies affecting tumor growth, invasion and metastasis (16). Until today, several members of the IL-1 family of ligands were described and their receptors were characterized (16). Different levels of IL-1 family ligands and their receptors were shown to be present in normal and malignant prostate (17) suggesting a role for IL-1 in the development of prostate cancer as well.

In this study, we demonstrate that immortalized prostate epithelial cells secrete factors that induce the stromal cells to express the chemokines CXCL-1, CXCL-2, CXCL-3 and IL-8. Our data suggest that the effect of the epithelial cells is mediated by the cytokines of the IL-1 family. The paracrine induction of a group of proinflammatory cytokines by the prostate stromal cells might facilitate inflammatory responses, cancer development and progression. Our data suggest a link between stromal–epithelial interactions and inflammation at early stages of prostate cancer development.

Materials and methods

Cell cultures

Prostate stromal culture (PM) was derived from the human telomerase reverse transcriptase (hTERT) immortalized PM151T cells (18). Prostate epithelial cultures (EP) were derived from hTERT-immortalized EP153T cells or from EP156T (18). To produce more rapidly proliferating epithelial cells, EP156T cells were infected with GSE56, a dominant-negative peptide for p53 (19), H-Ras^{V12} and androgen receptor using the procedure detailed in (20). These cells were designated as EP156T^{modified} (EP156T^m). Growth conditions and media components are detailed in (18). Shortly, EP cells were maintained in MCDB-153+ medium and PM cells in MCDB-131+ (both from Beit Haemek, Biological Industries Co. Kibbutz Beit Haemek, 25115, Israel). Cells were maintained in a humidified incubator at 37°C and 5% CO₂.

Conditioned media experiments

Conditioned media (CM) from epithelial cells were collected in the following manner: EP cells (10⁶) seeded in 15 cm plate were grown for 3 days with their regular medium MCDB-153+. To generate EP-CM, the plates were washed with phosphate-buffered saline and incubated with 30 ml basal medium (MCDB-153 not containing fetal calf serum, bovine pituitary extract (BPE) and human epidermal growth factor (hEGF) supplementation) for 48 h. Then, the medium was collected, filtered and transferred to stromal PM151T cells.

Prior to CM treatment, PM151T cells were seeded (2×10^5) in 10 cm plates and grown for 48 h. Then, cells were brought to quiescence in basal MCDB-131 medium for additional 48 h. Next, PM151T cells were treated with the CM or the control medium for 24 h (unless otherwise indicated) after which the cells were collected.

Isolation of total RNA

Total RNA for quantitative real-time polymerase chain reaction (QRT-PCR) was isolated using NucleoSpin RNA extract kit (Macherey-Nagel, Duren, Germany), according to the manufacturer's protocol.

Quantitative real-time polymerase chain reaction

A 2 μ g aliquot of the total RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, USA) and random hexamer primers. QRT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, UK) on an ABI 7300 instrument (Applied Biosystems, Singapore). The expression level for each gene was normalized to that of the GAPDH housekeeping gene in the same sample. The primers were designed using the Primer Express software. Primer sequences were as follows (all sequences are presented 5' to 3'):

Gene	Forward primer	Reverse primer
<i>Gapdh</i>	ACCCACTCCTCCACCTTTGA	CTGTTGCTGTAGCCA-AATTCGT
<i>CXCL-1</i>	AGTCATAGCCACACTCAAG-AATGG	GATGCAGGATTGAGG-CAAGC
<i>CXCL-2</i>	CGCCCAAACCGAAGTCATAG	AGACAAGCTTTCTGCC-CCATCT
<i>CXCL-3</i>	TCCCCCATGGTTCAGAAAATC	GGTGCTCCCTTGTT-CAGTATCT
<i>IL-8</i>	AGCTGGCCGTGGCTCTCT	CTGACATCTAAGTTC-TTTAGCACTCCTT
<i>IL-1α</i>	GTTTAAGCCAATCCATCACT-GATG	GACCTAGGCTTGATG-ATTCTTCCT
<i>IL-1β</i>	GCCTGAAGCCCTTGCTGTAGT	GCGGCATCCAGCTAC-GAAT
<i>IL-24</i>	TGGGCTGTGAAAGACACTATGC	CCGGGCACTCGTGAT-GTTAT
<i>VEGF</i>	TCCTCACACCATTGAAACCA	GATCCTGCCCTGTCT-CTCTG
<i>MMP-3</i>	ACAAAAGGATACAACAGGGAC-CAA	CAATTTTCATGAGCAG-CAACGA

Enzyme-linked immunosorbent assay

To measure CXCL-1 and IL-8 secretion by PM cells, following the incubation with epithelial CM, 1 ml medium was collected from the plates, centrifuged at 4°C, 14 000 r.p.m. for 15 min, and the supernatant was transferred to new tubes and kept at -20°C until the day of the assay. The secretion was measured by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' protocols: R&D systems (Minneapolis, USA) for CXCL-1 and Ebioscience (San Diego, USA) for IL-8. To measure the secretion of IL-1 α and IL-1 β by epithelial cells, EP156Ttm cells were grown in MCDB-153+ medium in a 10 cm plate until they reached confluence. Then, their medium was changed to fresh MCDB-153 for 24 h. Supernatants were collected and an IL-1 α or IL-1 β ELISA was performed according to the manufacturer's instructions (R&D Systems).

Western blotting

To measure secreted CXCL-2 and CXCL-3 protein in stromal PM cells, following the incubation with epithelial CM, supernatants were collected from stromal cells incubated with epithelial CM and cells incubated with control medium for 24 h. Supernatants were also collected from epithelial cells used as a source for the CM. All supernatants were concentrated using the vivaspin 15R column (Sartorius Group, Goettingen, Germany) according to the manufacturer's protocol (centrifuged for 120 min 25°C, 3000g). From the concentrate, 120 μ g proteins were fractionated by gel electrophoresis and proteins were transferred to nitrocellulose membranes. The primary antibodies used in this study were rabbit anti-human GRO-Beta (Serotec, Oxford, UK) and rabbit anti-human GRO-Gamma (Serotec) that recognize the CXCL-2 and the CXCL-3, respectively. The protein–antibody complexes were detected by horseradish peroxidase-conjugated secondary antibodies followed by the enhanced chemiluminescence western blotting detection reagents (Amersham, Buckinghamshire, UK).

Reagents

IL-1Ra: recombinant human IL-1 receptor antagonist (R&D systems). IL-1 β : recombinant human IL-1 β (PeproTech Asia, Rehovot, Israel).

Results

Induction of cytokine expression and secretion in prostate stromal cells treated with epithelial CM

To study the possible involvement of stromal–epithelial interactions as initiating events in prostate cancer development, we utilized hTERT-immortalized prostate stromal and epithelial cultures that were previously established in our lab (18). Stromal smooth muscle PM151T as well as epithelial EP156T and EP153T cell cultures were obtained following telomerase (hTERT)-induced immortalization of cells isolated from benign prostate tissues of elderly men that underwent radical prostatectomy due to prostate cancer as described in (18). Stromal (PM)- and epithelial (EP)- immortalized cultures retained features attributed to normal cells, such as the ability to differentiate (18). However, because of their immortalized status, we refer to them as an *in vitro* alternative for very early stages of cancer formation *in vivo*. Importantly, in the absence of pathological processes, both the epithelium and the smooth muscle are essentially quiescent in the adult prostate (2).

Since EP156T-immortalized epithelial cells were proliferating very slowly, we generated a more rapidly proliferating culture by introducing the GSE56 peptide, which inactivates the p53 tumor suppressor gene (19), androgen receptor and constitutively active form of Ras (H-Ras^{V12}). These prostate epithelial cells designated as EP156Ttm were not tumorigenic in mice (I. Kogan-Sakin, S. Madar, N. Goldfinger, unpublished data).

To evaluate possible paracrine effects exerted by prostate epithelial cells on prostate stromal cells, we tested the effect of epithelial CM on prostate stromal culture. First, we examined whether epithelial CM induced any changes in the messenger RNA (mRNA) levels of seven well-known pro-cancerous secreted molecules: CXCL1, CXCL2, CXCL3, IL8, IL-24, matrix metalloproteinase-3 and vascular endothelial growth factor (VEGF), whose function was implicated in tumor progression, angiogenesis or inflammation (6,21–23) by QRT-PCR analysis. Stromal PM cells that were treated with EP156Ttm-derived CM or control media and epithelial EP156Ttm cells were tested. Notably, the mRNA levels of CXCL-1, CXCL-2, CXCL-3 and IL-8 were significantly induced in CM-treated prostate stromal cells as compared with cells treated with control medium (Figure 1). The 2- and 4-fold dilutions of the CM resulted in a corresponding decrease in the levels of the induction, but still induced a significant effect on CXCL-1, CXCL-2, CXCL-3 and IL-8 expression (P -value < 0.05). A kinetic experiment in which stromal cells were incubated with EP156Ttm-CM for 6, 12, 24 and 48 h showed a significant induction of CXCL-1, CXCL-2, CXCL-3 and IL-8 mRNA levels even after 6 h of treatment, reaching a peak at the 24 h time point (supplementary Figure S1 is available at *Carcinogenesis* Online). Expression levels of three additional secreted molecules matrix metalloproteinase-3, VEGF and IL-24 that we tested were not changed following the treatment (supplementary Figure S2 is available at *Carcinogenesis* Online). Importantly, CXCL-1, CXCL-2, CXCL-3 and IL-8 expression was much lower in the EP156Ttm cells that were used as the source for the CM compared with their expression in the PM151T stromal cells following the incubation with CM (supplementary Figure S1 is available at *Carcinogenesis* Online). The latter observation excludes the possibility that the expressed QRT-PCR products measured in the stromal PM cells originated from epithelial cells contamination. To test whether this is a general phenomenon, we repeated the experiment using early passage hTERT-immortalized cells obtained from another donor, EP153T cells as the source for CM. Figure 2 illustrates that CM from EP153T cells also efficiently induced the expression of CXCL-1, CXCL-2, CXCL-3 and IL-8 chemokines in the stromal smooth muscle cells, PM151T. Thus, hTERT-immortalized epithelial cells that represent very early stage of prostate transformation induce the expression of proangiogenic and tumor-promoting chemokines in prostate stromal cells at the mRNA level.

Next, we wished to analyze whether the mRNA induction is also reflected in an increase of the secreted product. To this end, we tested

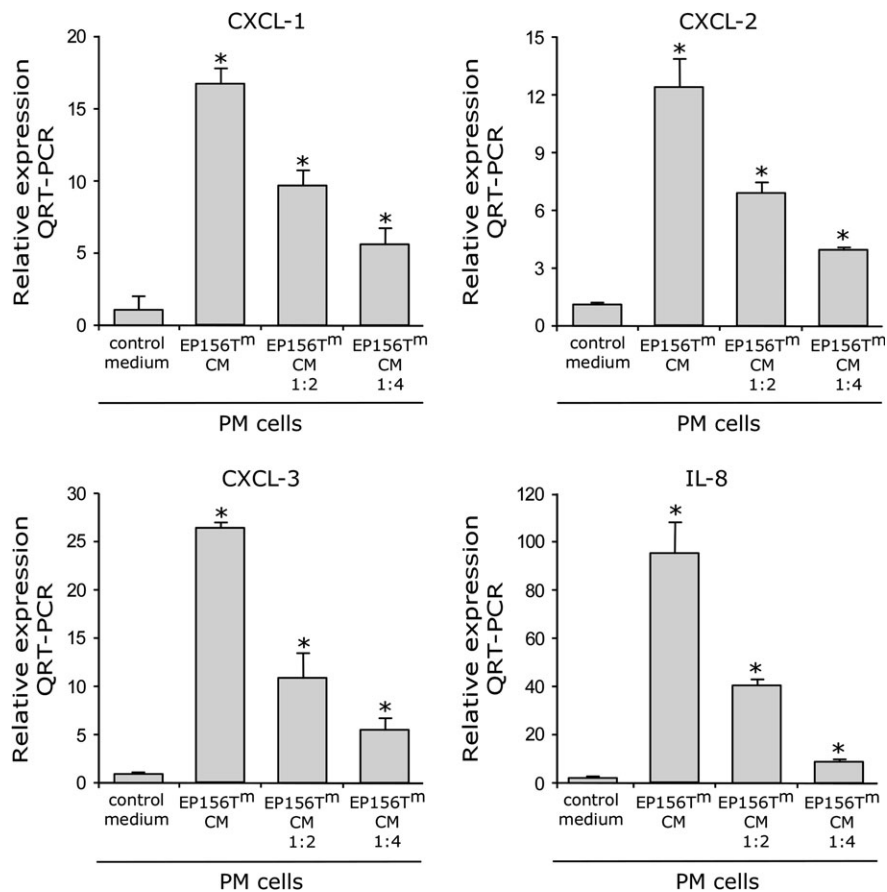


Fig. 1. CXCL-1, CXCL-2, CXCL-3 and IL-8 expression is upregulated in stromal PM151T cells upon EP156T^m-CM treatment. PM151T cells were treated for 24 h with control medium, CM from EP156T^m cells or with the same CM diluted in control medium 1:2 and 1:4. The expression of the chemokines CXCL-1, CXCL-2, CXCL-3 and IL-8 was analyzed by QRT-PCR. Asterisks depict statistically significant differences between PM cells treated with control medium and cells treated with EP156T^m-CM or diluted CM (* $P < 0.05$).

the secretion of CXCL-1 and IL-8 from the stromal cells in response to the EP156T^m-CM using ELISA. As shown in Figure 3A and B, a significant increase was observed in the levels of both secreted chemokines following EP156T^m-CM treatment. EP156T^m cells themselves did not show a significant secretion of CXCL-1 and IL-8 (Figure 3A and B, third bar represents the EP cells as compared with the second that represents the PM cells). Since there were no ELISA assays available for CXCL-2 and CXCL-3, we performed a western blot analysis using supernatants to evaluate whether the secretion of these chemokines from stromal PM cells was elevated following the treatment with EP156T^m-CM. In accordance with the observed elevation in the CXCL-1 and IL-8 protein levels, the levels of CXCL-2 and CXCL-3 were significantly induced in the CM that was further incubated with stromal PM cells than in the CM that was not incubated with stromal cells and in the stromal cells treated with control medium (Figure 3C).

We concluded from these experiments that immortalized prostate stromal cells are triggered to overexpress and secrete CXCL-1, CXCL-2, CXCL-3 and IL-8 chemokines by CM originating from prostate epithelial cells representing very early transformed cells.

IL-1 α and IL-1 β are expressed by EP cells and secreted into the epithelial-derived CM

We next wished to identify the factors that are secreted by the EP cells into the CM that in turn trigger the chemokine production by PM cells. IL-1-dependent activation of the nuclear factor-kappa B (NF- κ B) pathway was shown to induce the secretion of the above chemokines (24–26). Therefore, we were challenged to examine the

possibility that the induction of CXCL-1, CXCL-2, CXCL-3 and IL-8 in the stromal cells upon exposure to epithelial CM is mediated by IL-1 in the medium. Previously, three gene products of the IL-1 family have been studied thoroughly: two agonist proteins, IL-1 α and IL-1 β , and one antagonist protein, interleukin-1 receptor antagonist (IL-1Ra) (16). Thus, we measured IL-1 α and IL-1 β expression in the epithelial cells used as a source for the CM as well as in the stromal cells in the absence or presence of CM. Figure 4A demonstrates that IL-1 α and IL-1 β were virtually not expressed in the stromal cells. However, EP cells expressed high levels of IL-1 α and IL-1 β as indicated by QRT-PCR analysis (Figure 4A). To test whether these factors are also secreted into EP156T^m-derived CM, we performed an ELISA assay. Figure 4B demonstrates that IL-1 α and IL-1 β were secreted by EP156T^m cells into the CM. These results suggest that IL-1 family agonists secreted by prostate epithelial cells could induce a signaling cascade in prostate stromal cells. This signaling might be responsible for the expression and secretion of CXCL-1, CXCL-2, CXCL-3 and IL-8 chemokines in prostate stromal PM cells.

IL-1Ra blocks and human recombinant IL-1 β stimulates cytokine expression by the stromal cells

To test whether the epithelial-produced IL-1 mediated the induction of chemokines by the stromal cells, PM cells were treated with CM in the absence or presence of 10 ng/ml of the recombinant human IL-1Ra antagonist. IL-1Ra binds to the IL-1 receptors without transmitting an activation signal and thereby represents an inhibitor of IL-1 signaling. Figure 5 demonstrates that the addition of the antagonist completely abolished the induction of the four chemokines

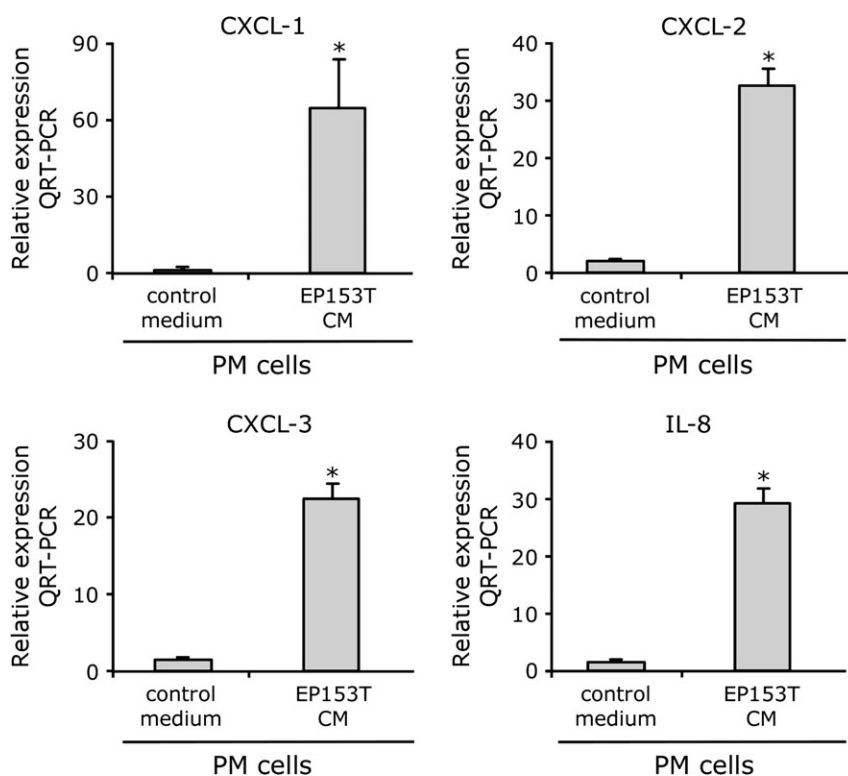


Fig. 2. CXCL-1, CXCL-2, CXCL-3 and IL-8 induction in PM151T cells in response to EP153T cell-derived CM. PM151T cells were incubated with CM from EP153T cells or control medium for 24 h and the expression of the chemokines CXCL-1, CXCL-2, CXCL-3 and IL-8 was analyzed by QRT-PCR. Asterisks depict statistically significant differences between PM cells treated with control medium and cells treated with EP153T-CM ($*P < 0.005$).

examined. It is widely accepted that IL-1 β is active only in its secreted form, whereas IL-1 α is mainly active as an intracellular precursor and as a membrane-bound IL-1 and only rarely as a secreted cytokine (16). In accordance with this notion, we witnessed higher levels of IL-1 β than IL-1 α in the EP156T^m-CM. For this reason, we assumed that the effect of EP-CM on stromal cells is mainly due to IL-1 β secretion from epithelial cells. Thus, we examined the effect of exogenous IL-1 β on stromal cells. Treatment of PM151T cells with human recombinant IL-1 β resulted in a dose-dependent upregulation in the expression of the above chemokines (Figure 6). Importantly, the extent of induction of all four chemokines in stromal cells treated with 50 pg/ml recombinant IL-1 β was very similar to that extent in cells treated with EP-CM that was estimated by ELISA to contain ~40 pg/ml IL-1 β (Figure 4B). Taken together, these results support the notion that IL-1 is the factor in the EP-CM responsible for the induction of CXCL-1, CXCL-2, CXCL-3 and IL-8 in stromal cells.

Based on the observation that IL-1Ra treatment eliminated the effect of the epithelial-derived CM on the stromal cells and that human recombinant IL-1 β induced the expression of the CXCL-1, CXCL-2, CXCL-3 and IL-8, we suggest that this effect is mediated by the secretion of IL-1 family cytokines from the epithelial cells. Activation of the IL-1-signaling pathway in the stromal cells triggers them to secrete the various chemokines.

Discussion

Stromal–epithelial interactions play a key regulatory role in prostate development and in the maintenance of homeostasis in the adult prostate in health and disease (2). It is now well accepted that the tumor microenvironment is an indispensable participant in the neoplastic process, fostering proliferation, survival and migration of the tumor cells. In this study, we explored the molecular mechanisms underlying stromal–epithelial interactions associated with the very

early stages of prostate cancer formation. To approach this issue, we used an *in vitro* system of non-transformed cells. Since normal prostate epithelial cells are not probably to represent the early stages of malignant transformation, we decided to use hTERT-immortalized cells that represent an intermediate stage between a normal and a transformed condition. As we have already characterized the hTERT-immortalized cells (18), both stromal and epithelial cells retained many, but not all, normal features. In the present study, we tested the effect of epithelial CM on the expression of several secreted molecules that are related to inflammation and cancer in prostate stromal smooth muscle cells (PM151T). Our results demonstrated upregulation of the chemokines CXCL-1, CXCL-2, CXCL-3 and IL-8 in PM cells in response to EP-CM treatment. We further showed that cytokines from the IL-1 family expressed and secreted by EP cells are the mediators of stromal response since recombinant IL-1 β induced the CXCL-1, CXCL-2, CXCL-3 and IL-8 expression in PM cells and IL-1Ra completely abolished it. This chemokine signature might favor tumor progression and be indicative of the development of a proinflammatory environment resulting from the effect of the epithelial cells on the neighboring stromal cells. For example, the chemokines CXCL-1, CXCL-2 and CXCL-3 are known to direct migration of leukocytes, to regulate angiogenesis, tumorigenesis and wound healing (27). IL-8, in addition to being a neutrophil chemoattractant, was shown to promote metastasis (27). The concept that inflammation plays a central role in cancer has received increasing attention; supporting data have expanded the concept that inflammation is a critical component of tumor initiation and progression (28). The most important implication of our study is that the chemokines CXCL-1, CXCL-2, CXCL-3 and IL-8 that were originally thought to be produced by inflammatory cells or by tumor cells are now identified as being regulated in the stromal cells in response to epithelial cues, most probably at the initiating stages of tumor formation. This might suggest that prostate stromal cells in their native environment, where their behavior is

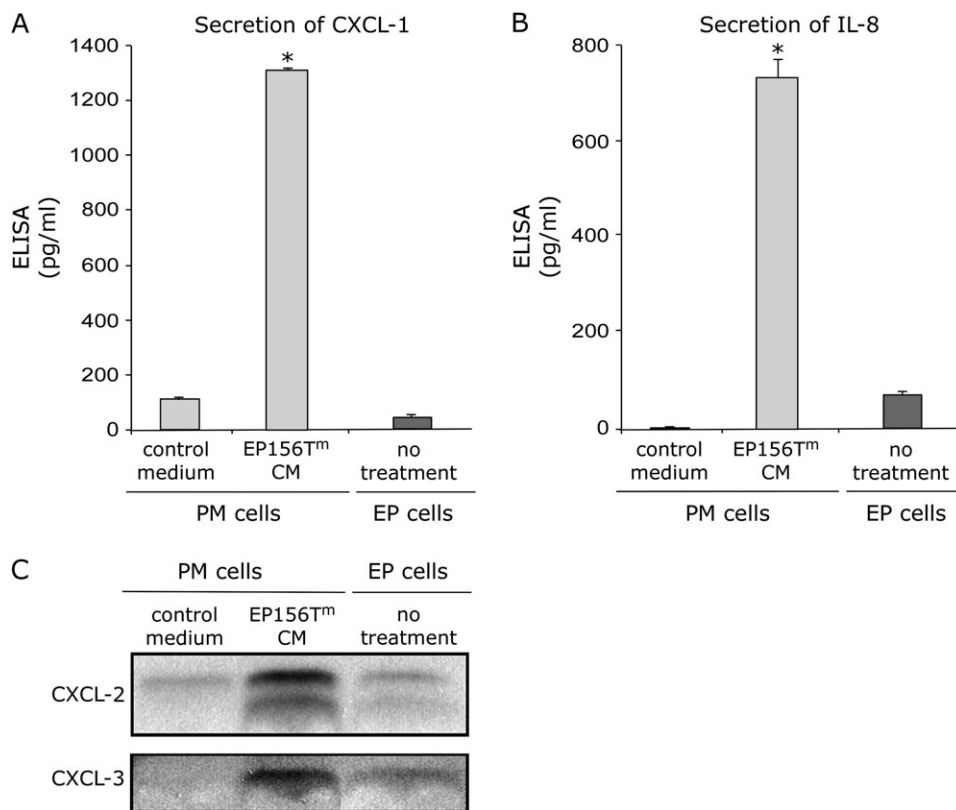


Fig. 3. Prostate stromal PM cells secrete CXCL-1, CXCL-2, CXCL-3 and IL-8 chemokines in response to EP156T^m-CM. Secretion of CXCL-1 (**A**) and of IL-8 (**B**) was examined following 24 h of incubation with EP156T^m-CM using ELISA assay. CXCL-1 and IL-8 levels were also tested in MCDB-153 medium that was not conditioned (control medium) and in EP156T^m-CM that was not incubated with stromal cells as controls. Asterisks depict statistically significant differences between PM cells treated with control medium and cells treated with EP156T^m-CM (* $P < 0.001$). (**C**) Secretion of CXCL-2 and CXCL-3 was examined using western blot analysis. Supernatants of stromal PM cells treated with EP-CM or with control medium and supernatant of EP156T^m were concentrated using Vivaspin R15 columns and 120 μ g proteins were analyzed by western blot analysis using anti-human GRO-Beta (for CXCL-2 detection) and anti-human GRO-Gamma (for CXCL-3 detection) primary antibodies.

accurately controlled by epithelial cells, contribute to the inflammatory reaction and tumor progression at the very initial stages of tumor formation. In agreement with this idea, it is known that precursor lesions of prostate cancer are often associated with inflammation. Thus, a prostate stromal compartment is viewed as a mediator between the epithelial compartment and the inflammatory response in the premalignant lesion.

Our data are supported by previous findings of Gallagher *et al.* (12), showing that the chemokines CXCL-1, CXCL-2 and IL-8 were significantly upregulated in primary fibroblasts following cocultivation with melanoma cells (12).

IL-1 is a key pleiotropic inflammatory cytokine that promotes angiogenesis, tumor growth and metastasis, and its presence in some human cancers is associated with aggressive tumor biology (29). IL-1Ra is often coexpressed with IL-1, and depending on the balance between the cytokine and its antagonist, there is diminution of the biological effects of IL-1 (30). Since treatment with the IL-1-signaling inhibitor, IL-1Ra, completely abolished the effect of the epithelial-derived CM on the stromal cells, we suggest that this effect is mediated by the secretion of IL-1 family cytokines by the epithelial cells and activation of the IL-1-signaling pathway in the stromal cells, thereby triggering them to secrete the above chemokines. Thus, the proinflammatory cytokine IL-1 secreted from prostate epithelial cells might serve as an inducer of prostatic inflammation. Previous findings demonstrated that the most common haplotype of IL-1Ra significantly correlates with an increased risk for prostate cancer (31). Together with these data, our results support the notion that IL-1 cytokine is an important player in the complex process of malignant transformation of the prostate gland.

IL-1 α produced by epithelial cells was shown to induce fibroblast growth factor-7 in prostate stromal cells in a condition called benign prostatic hyperplasia in which there is benign growth in the prostate (32). We show here that addition of recombinant IL-1 β to stromal PM151T cells at the concentration similar to its amount in the CM (50 pg/ml) could induce the expression of CXCL-1, CXCL-2, CXCL-3 and IL-8 to a similar extent. We also show that IL-1 α is found in the CM at much lower concentrations (~ 6 pg/ml). However, we cannot exclude the possibility that these amounts of IL-1 α together with IL-1 β are responsible for the induction of CXCL-1, CXCL-2, CXCL-3 and IL-8 in stromal cells. In this case, our immortalized prostate cell coculture model may also reflect early stages of benign prostatic hyperplasia.

In previous studies, IL-1 was shown to stimulate the NF- κ B-signaling pathway and transcriptionally activate the expression of CXCL-1, CXCL-2, CXCL3 (24,25) and IL-8 (26). CXCL-1, CXCL-2 and CXCL-3 were shown to contain a conserved NF- κ B-responsive element in their promoter. Work is in progress to test whether CXCL-1, CXCL-2, CXCL-3 and IL-8 are upregulated in stromal cells due to NF- κ B signaling in response to epithelial IL-1.

In conclusion, our study provides an insight into molecular mechanisms associated with epithelial-stromal interactions in the premalignant prostate gland. We have shown that due to signals originating from the epithelial cells, the stromal cells are triggered to secrete factors that induce the generation of a proinflammatory microenvironment that may contribute to cancer development and progression. These data further support the notion that targeting the microenvironment or the cross talk between the tumor and the microenvironment may represent a promising therapeutic approach for prostate cancer.

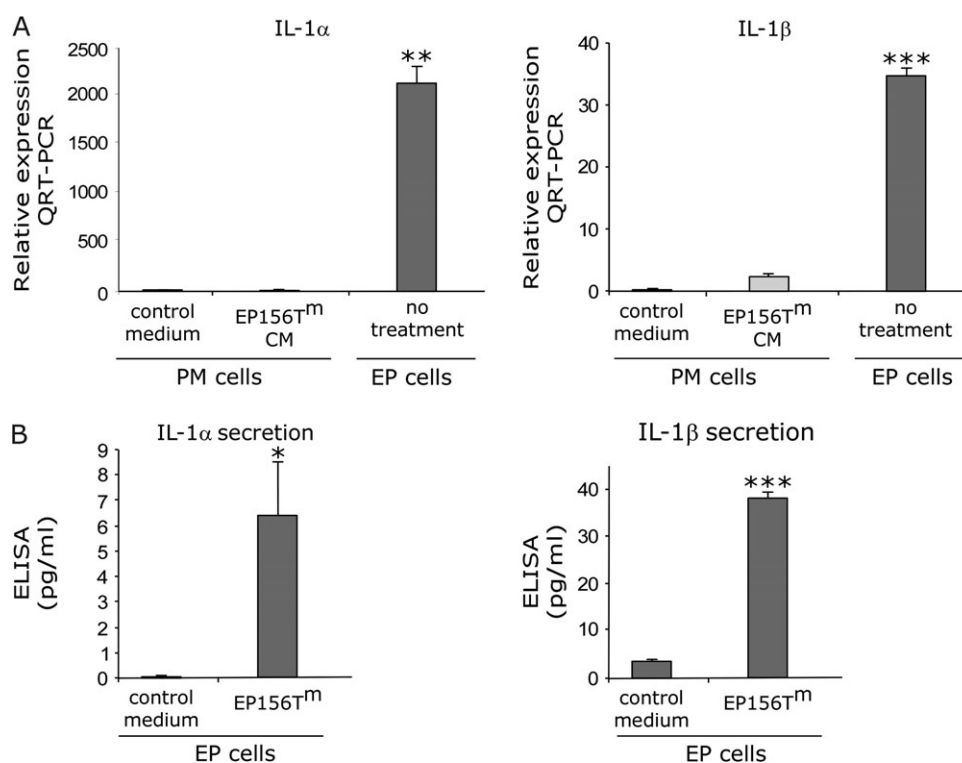


Fig. 4. IL-1 α and IL-1 β are expressed by EP cells and secreted into the epithelial CM. (A) IL-1 α and IL-1 β mRNA expression levels in the PM cells in response to the EP156T^m-CM and in the EP156T^m cells that served to generate this CM were tested by QRT-PCR analysis. (B) Examination of IL-1 α and IL-1 β levels in control medium and in EP156T^m derived CM by ELISA. Asterisks depict statistically significant differences between PM cells treated with control medium and EP156T^m cells in (A) and between control medium and EP156T^m CM in (B) (* P = 0.026, ** P = 0.0017, *** P = 0.0003).

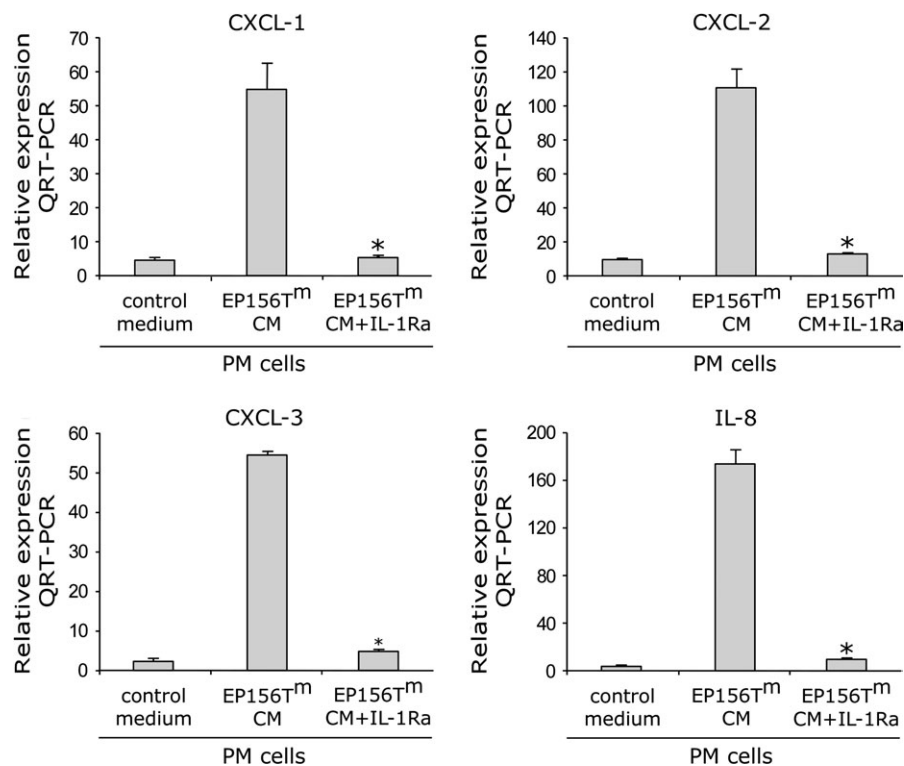


Fig. 5. IL-1Ra blocks EP-CM-dependent activation of cytokine expression by the stromal cells. PM cells were treated for 24 h with control medium or EP156T^m-CM. CM was supplemented with IL-1Ra (10 ng/ml). The expression of the chemokines CXCL-1, CXCL-2, CXCL-3 and IL-8 was analyzed by QRT-PCR. Asterisks depict statistically significant differences between PM cells treated with EP156T^m-CM and PM cells treated with EP156T^m-CM supplemented with IL-1Ra (* P < 0.01).

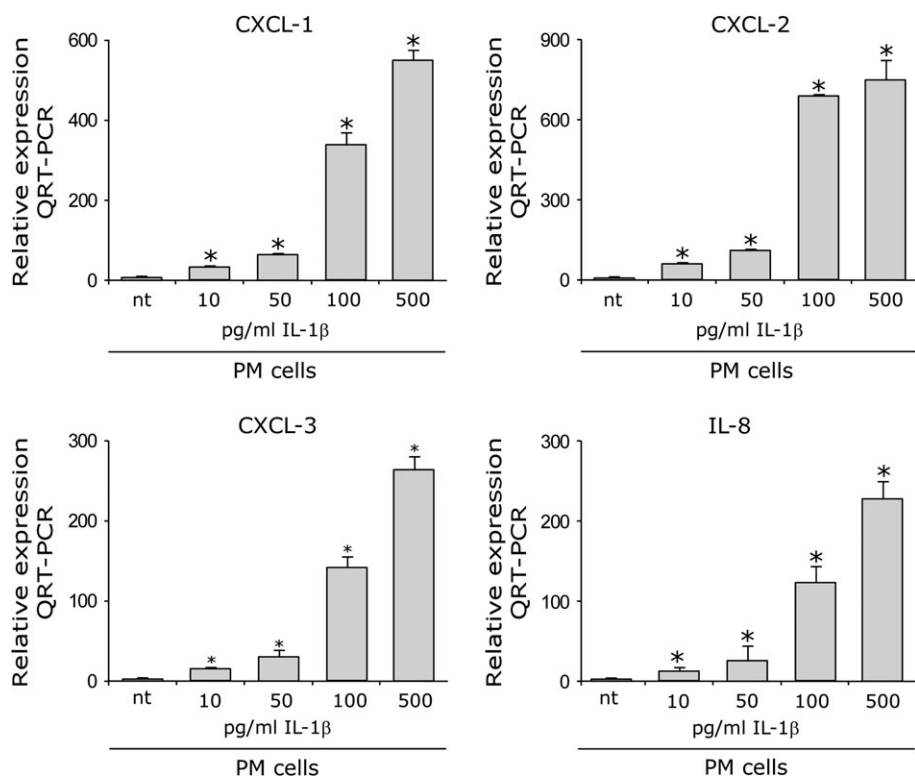


Fig. 6. IL-1 β induces the expression of CXCL-1, CXCL-2, CXCL-3 and IL-8 in prostate stromal PM cells. PM cells were treated for 24 h with increasing amounts of human recombinant IL-1 β and the expression of the chemokines CXCL-1, CXCL-2, CXCL-3 and IL-8 was analyzed by QRT-PCR. Asterisks depict statistically significant differences between non-treated (nt) PM cells and cells treated with IL-1 β (* $P < 0.03$).

Supplementary materials

Supplementary Figures S1 and S2 can be found at <http://carcin.oxfordjournals.org/>

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Conflict of Interest Statement: None declared.

References

- Morales, C.P. *et al.* (1999) Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. *Nat. Genet.*, **21**, 115–118.
- Cunha, G.R. *et al.* (2003) Role of the stromal microenvironment in carcinogenesis of the prostate. *Int. J. Cancer*, **107**, 1–10.
- Kufe, D.W.P. *et al.* (eds). (2003). *Cancer Medicine*. 6th edn. *Biology of Prostate Cancer*. BC Decker, Hamilton, Canada.
- Jang, T.L. *et al.* (2003) The role of cytokines in prostatitis. *World J. Urol.*, **21**, 95–99.
- Cyster, J.G. (1999) Chemokines and cell migration in secondary lymphoid organs. *Science*, **286**, 2098–2102.
- Strieter, R.M. *et al.* (2006) Cancer CXC chemokine networks and tumour angiogenesis. *Eur. J. Cancer*, **42**, 768–778.
- Yang, A. *et al.* (1998) p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol. Cell.*, **2**, 305–316.
- Sallusto, F. *et al.* (2000) The role of chemokine receptors in primary, effector, and memory immune responses. *Annu. Rev. Immunol.*, **18**, 593–620.
- Moore, B.B. *et al.* (1999) Distinct CXC chemokines mediate tumorigenicity of prostate cancer cells. *Am. J. Pathol.*, **154**, 1503–1512.
- Araki, S. *et al.* (2007) Interleukin-8 is a molecular determinant of androgen independence and progression in prostate cancer. *Cancer Res.*, **67**, 6854–6862.
- Pirtskhalaishvili, G. *et al.* (2000) Endothelium-derived factors as paracrine mediators of prostate cancer progression. *Prostate*, **44**, 77–87.
- Gallagher, P.G. *et al.* (2005) Gene expression profiling reveals cross-talk between melanoma and fibroblasts: implications for host-tumor interactions in metastasis. *Cancer Res.*, **65**, 4134–4146.
- Nelson, W.G. *et al.* (2003) Prostate cancer. *N. Engl. J. Med.*, **349**, 366–381.
- De Marzo, A.M. *et al.* (2007) Inflammation in prostate carcinogenesis. *Nat. Rev. Cancer*, **7**, 256–269.
- Haverkamp, J. *et al.* (2007) Prostate inflammation and its potential impact on prostate cancer: a current review. *J. Cell. Biochem.*, **103**, 51344–53.
- Apte, R.N. *et al.* (2008) Is interleukin-1 a good or bad 'guy' in tumor immunobiology and immunotherapy? *Immunol. Rev.*, **222**, 222–241.
- Ricote, M. *et al.* (2004) Interleukin-1 (IL-1 α and IL-1 β) and its receptors (IL-1RI, IL-1RII, and IL-1Ra) in prostate carcinoma. *Cancer*, **100**, 1388–1396.
- Kogan, I. *et al.* (2006) hTERT-immortalized prostate epithelial and stromal-derived cells: an authentic *in vitro* model for differentiation and carcinogenesis. *Cancer Res.*, **66**, 3531–3540.
- Osssovskaya, V.S. *et al.* (1996) Use of genetic suppressor elements to dissect distinct biological effects of separate p53 domains. *Proc. Natl Acad. Sci. USA.*, **93**, 10309–10314.
- Milyavsky, M. *et al.* (2005) Transcriptional programs following genetic alterations in p53, INK4A, and H-Ras genes along defined stages of malignant transformation. *Cancer Res.*, **65**, 4530–4543.
- Wang, J.M. *et al.* (1998) Chemokines and their role in tumor growth and metastasis. *J. Immunol. Methods.*, **220**, 1–17.

22. Przybylo, J.A. *et al.* (2007) Matrix metalloproteinase-induced epithelial-mesenchymal transition: tumor progression at Snail's pace. *Int. J. Biochem. Cell Biol.*, **39**, 1082–1088.
23. Carmeliet, P. (2005) VEGF as a key mediator of angiogenesis in cancer. *Oncology*, **69** (suppl. 3), 4–10.
24. Issa, R. *et al.* (2006) GRO-alpha regulation in airway smooth muscle by IL-1beta and TNF-alpha: role of NF-kappaB and MAP kinases. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **291**, L66–L74.
25. Anisowicz, A. *et al.* (1991) An NF-kappa B-like transcription factor mediates IL-1/TNF-alpha induction of gro in human fibroblasts. *J. Immunol.*, **147**, 520–527.
26. Wolf, J.S. *et al.* (2001) IL (interleukin)-1alpha promotes nuclear factor-kappaB and AP-1-induced IL-8 expression, cell survival, and proliferation in head and neck squamous cell carcinomas. *Clin. Cancer Res.*, **7**, 1812–1820.
27. Balkwill, F. (2004) Cancer and the chemokine network. *Nat. Rev. Cancer.*, **4**, 540–550.
28. Coussens, L.M. *et al.* (2002) Inflammation and cancer. *Nature*, **420**, 860–867.
29. Patschenko, A.G. *et al.* (2003) The interleukin-1 family of cytokines and receptors in human breast cancer: implications for tumor progression. *Int. J. Oncol.*, **23**, 269–284.
30. Weinreich, D.M. *et al.* (2003) Effect of interleukin 1 receptor antagonist gene transduction on human melanoma xenografts in nude mice. *Cancer Res.*, **63**, 5957–5961.
31. Lindmark, F. *et al.* (2005) Interleukin-1 receptor antagonist haplotype associated with prostate cancer risk. *Br. J. Cancer.*, **93**, 493–497.
32. Giri, D. *et al.* (2000) Interleukin-1alpha is a paracrine inducer of FGF7, a key epithelial growth factor in benign prostatic hyperplasia. *Am. J. Pathol.*, **157**, 249–255.

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