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CXCL12 overexpression and secretion by aging fibroblasts enhance human prostate epithelial proliferation *in vitro*

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Summary

The direct relationship between the aging process and the incidence and prevalence of both benign prostatic hyperplasia (BPH) and prostate cancer (PCa) implies that certain risk factors associated with the development of both diseases increase with the aging process. In particular, both diseases share an overly proliferative phenotype, suggesting that mechanisms that normally act to suppress cellular proliferation are disrupted or rendered dysfunctional as a consequence of the aging process. We propose that one such mechanism involves changes in the prostate microenvironment, which 'evolves' during the aging process and disrupts paracrine interactions between epithelial and associated stromal fibroblasts. We show that stromal fibroblasts isolated from the prostates of men 63–81 years of age at the time of surgery express and secrete higher levels of the CXCL12 chemokine compared with those isolated from younger men, and stimulate CXCR4-mediated signaling pathways that induce cellular proliferation. These studies represent an important first step towards a mechanistic elucidation of the role of aging in the etiology of benign and malignant prostatic diseases.

Key words: Aging; CXCL12; paracrine; proliferation; prostate.

Introduction

Benign prostatic hyperplasia (BPH), or noncancerous enlargement of the prostate, is a common condition associated with aging in men (Meigs *et al*., 2001; Verhamme *et al*., 2002; Neuhouser *et al*., 2004). Studies both *in vivo* and *in vitro* have reported higher proliferative and lower apoptotic rates for epithelial cells from hyperplastic compared with normal prostates, suggesting that some proportion of increased prostate volume with age is attributable to increased epithelial cell densities (Berges *et al*., 1995; Colombel *et al*., 1998; Arenas *et al*., 2001). However, the molecular mechanisms responsible for increased epithelial cell proliferation in hyperplastic human prostates are not well described. Work accomplished using rodent and rodent/human *in vivo* and *in vitro* models have suggested that paracrine interactions between glandular epithelial cells and adjacent fibroblastic stromal cells play an important role in the development of benign prostatic proliferative diseases (Thompson *et al*., 1989; Merz *et al*., 1991; Cunha *et al*., 1996; Foster *et al*., 1998; Hayward *et al*., 1998; Song *et al*., 2002). Although useful for the study of various aspects of epithelial–stromal interactions, it is unclear whether rodent-based models accurately recapitulate such interactions in the human prostate. In order to create a human-based system for the study of paracrine interactions during prostate tumorigenesis, some attempts have been made to mimic the *in vivo* system using human *in vitro* models. The most successful efforts to date have utilized cocultured normal human prostate-derived fibroblasts and the LNCaP prostate metastasis-derived cell line (Olumi *et al*., 1998). These studies clearly demonstrated that epithelial–stromal paracrine interactions were required for xenografted LNCaP cells to fully express the malignant phenotype and grow as tumors in athymic mice (Olumi *et al*., 1998).

The *in vivo* and *in vitro* studies described above show that epithelial–stromal interactions are crucial for the regulation of epithelial cell growth. However, because the etiologies of BPH and prostate cancer (PCa) are clearly associated with aging, it is important to determine whether epithelial–stromal interactions in the human prostate change over time and how such changes contribute to the development of hyperplasia and cancer. The studies reported here are the first to examine the effects of an aging microenvironment on paracrine interactions and prostatic epithelial cell proliferation. For these experiments, we utilized a novel *in vitro* model system for benign prostatic hyperplasia developed in our laboratory. The epithelial component of this model, N15C6 cells, was developed from immortalized primary normal prostate epithelial cells. The stromal component consisted of stromal fibroblastic cell populations cultured from the prostates of patients aged 40–52 years (younger) or 63–81 years (older) at the time of surgery. Experiments accomplished using this model show that stromal fibroblast cells cultured from the prostates of older men were less able to suppress epithelial cell proliferation than those cultured from the prostates of younger men. Moreover, older fibroblasts expressed and secreted higher levels of the chemokine CXCL12 compared with younger fibroblasts, and the CXCL12 protein was mechanistically associated with

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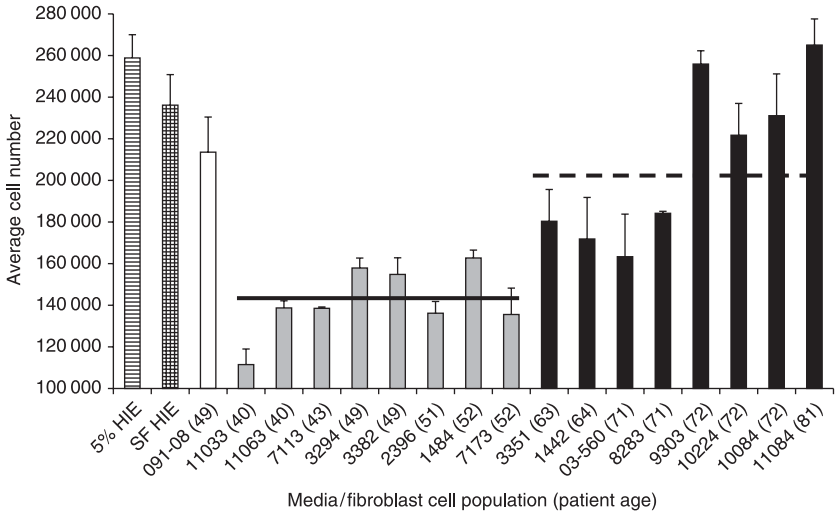

Fig. 1 Conditioned media from aged prostate stromal fibroblasts induce growth of N15C6 prostate epithelial cells. N15C6 prostate epithelial cells were grown for 96 h in 5% HIE, defined serum-free media (SF HIE) or SF HIE media grown for 48 h in the presence of fibroblast cell populations indicated by case number and patient age (in parentheses). Fibroblast population 091-08 was derived from cancer-associated stroma; the rest were derived from histologically benign tissues. Average cell growth in media from younger patients (aged 40–52; gray bars) is shown by solid line; average growth in media from older patients (aged 63–81; black bars) is shown by dashed line. Epithelial cell growth was significantly facilitated by media from older fibroblasts but inhibited by media from younger fibroblasts ($P = 0.002$).

enhanced epithelial cell proliferation. This is the first report to demonstrate that an aging microenvironment directly contributes to the disruption of normal human prostatic stromal–epithelial interactions and that this disruption causes enhanced epithelial cell proliferation similar to that observed in human BPH.

Results

Older fibroblasts are less able to repress epithelial cell proliferation than younger fibroblasts *in vitro*

N15C6 human prostate epithelial cells demonstrated differential growth in conditioned media acquired from fibroblast cell populations originating from younger (40–52 years old) compared with older (63–72 years old) patients (Fig. 1). The average cell number for N15C6 cells grown in defined serum-free HIE conditioned media acquired from younger fibroblasts, 142 000 (± 16 400) cells, was significantly less than that for the same cells grown in defined serum-free HIE media alone, 235 990 (± 14 680) ($P < 0.001$) (Fig. 1). This suggested that conditioned media from younger fibroblast cells exerted a growth-suppressive effect on N15C6 prostate epithelial cells. In contrast, the average cell number for N15C6 cells grown in the presence of defined serum-free HIE conditioned media acquired from older fibroblasts, 209 000 (± 40 000) cells, was significantly higher than that for the same cells grown in the presence of defined serum-free HIE media acquired from younger fibroblasts ($P < 0.001$) and was close to that obtained using defined serum-free HIE conditioned media alone (Fig. 1). Conditioned media acquired from the fibroblasts of two older men, case no. 9303 (72 years old) and case no. 11084 (81 years old), elicited a proliferative response from N15C6 cells that actually exceeded the response observed using defined serum-free HIE media alone (Fig. 1). Taken together, these results demonstrate that conditioned media from older prostate fibroblasts were less able to suppress N15C6 prostate epithelial cell growth, and in two instances actually induced N15C6 cell growth.

The fibroblasts used in the experiments described above were grown in culture from histologically verified benign tissues with one exception, case no. 091-08, which were grown from tissue containing malignant prostate glands. Interestingly, conditioned media from these cancer-associated fibroblasts were not growth suppressive, even though the tissue was acquired from a patient 49 years of age at the time of surgery and therefore defined in our study as a member of the 'younger' patient group.

Differential gene expression patterns distinguish older fibroblasts from younger prostate fibroblasts

The gene expression profiles of four fibroblast cell populations derived from younger patients, case nos 11033 (40 years old), 10063 (40 years old), 2396 (51 years old) and 1484 (52 years old) and of two derived from older patients, case nos 1442 (64 years old) and 8283 (71 years old), was obtained using Affymetrix U133A GeneChips and compared. These experiments identified 54 unique, differentially expressed, transcripts, with 41/54 (76%) up-regulated and 13/54 (24%) down-regulated in older compared with younger stromal fibroblasts. Nine of the transcripts that were up-regulated in RNA derived from older compared with younger prostate stromal fibroblasts encoded secreted proteins. Of these transcripts, the most highly up-regulated was for the gene encoding the secreted chemokine CXCL12, also known as SDF-1 (stromal derived factor 1), which was present at 3.4-fold higher levels in RNA prepared from older compared with younger stromal fibroblasts. Quantitative RT–PCR using a highly sensitive TaqMan assay measured 36-fold higher levels of CXCL12 transcript in older compared with younger fibroblasts ($P < 0.001$), confirming that CXCL12 transcripts were more abundant in older prostate stromal fibroblasts.

CXCL12 is secreted by prostate stromal fibroblasts and induces prostate epithelial cell proliferation

An ELISA specific for CXCL12 confirmed that older stromal fibroblasts (populations 1442, 12154, 8283 and 10224) secreted

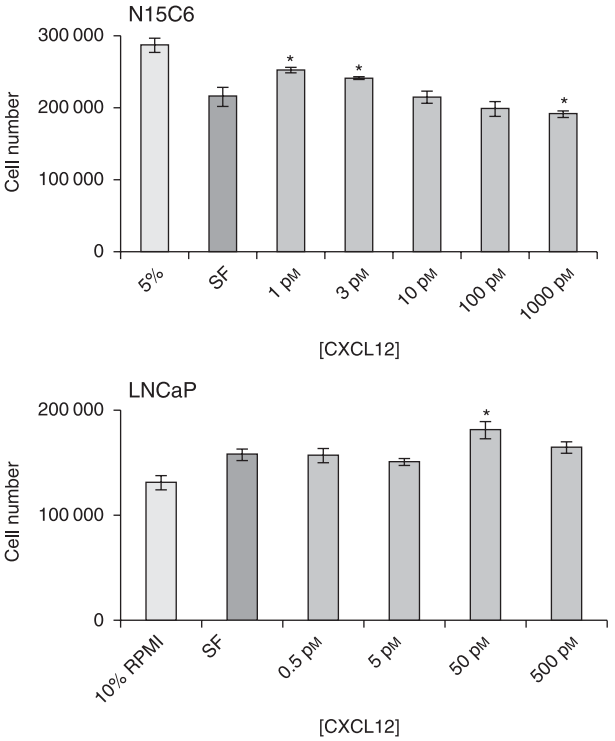

Fig. 2 Low [CXCL12] induces the proliferation of N15C6 and LNCaP prostate epithelial cells. N15C6 or LNCaP prostate epithelial cells were grown for 72 h in complete media (5% HIE for N15C6, 10% RPMI for LNCaP), defined serum-free HIE (SF) or SF supplemented to the indicated concentration of CXCL12. N15C6 cellular proliferation was induced above SF levels at 1–3 pM CXCL12 but was suppressed at 1000 pM CXCL12, whereas LNCaP proliferation was induced at 50 pM CXCL12 ($P < 0.001$).

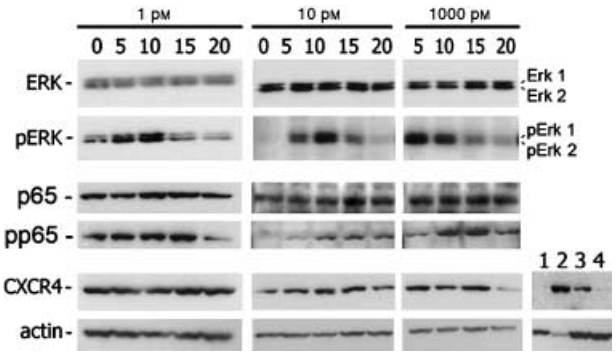

Fig. 3 Low [CXCL12] induces ERK and NF-kappaB phosphorylation. N15C6 cells were grown in 5% HIE media supplemented to 1, 10, or 1000 pM CXCL12 for 0, 5, 10, 15 or 20 min, then harvested, lysed, and examined by Western blot analysis. The treatment did not affect total levels of the ERK or NF-kappaB (p65) proteins, but did rapidly and transiently induce ERK phosphorylation (ppERK) and NF-kappaB (pp65). Actin is shown as a loading control (bottom panel). CXCR4 and actin expression are also shown for N15C6 cells in serum-free media (lane 1), LNCaP cells (lane 2) and two primary cultures of explanted prostate epithelial tissues (lanes 3 and 4) as controls.

higher levels of the CXCL12 protein into the culture media (3.70–17.47 pg mL^{-1}, or 0.47–2.24 pM) compared with younger fibroblasts (populations 11033 and 3382; 3.71–0.65 pg mL^{-1}, or 0.47–0.83 pM). The same assay did not detect CXCL12 in culture media from N15C6 cells and detected very low levels of CXCL12 in culture media from LNCaP cells (3.9 ± 0.035 pg mL^{-1}, or 0.50 pM). These data demonstrated that older prostate fibroblasts secreted higher levels of CXCL12 than younger prostate fibroblasts or epithelial cells.

We next examined whether the addition of CXCL12 to defined serum-free media in the concentration range secreted by older prostate fibroblasts induced N15C6 or LNCaP prostate epithelial cell proliferation. As seen in Fig. 2, the addition of CXCL12 at 1–3 pM for N15C6 cells, or 50 pM for LNCaP cells, was growth stimulatory and facilitated growth significantly above levels observed in defined serum-free media alone ($P < 0.001$). Although the proliferative effect of low levels of CXCL12 was modest, the addition of higher concentrations of CXCL12 in both cases was clearly growth inhibitory. Together with the conditioned media experiments reported above, these results show that CXCL12 is secreted by human prostate stromal fibroblasts and directly stimulates human prostate epithelial cell proliferation at very low, picomolar concentrations.

CXCL12 stimulates phosphorylation of ERK 1/2 and NF-kappaB

Of the three major downstream signaling pathways stimulated by CXCR4/CXCL12 binding – PI3K/FAK, Ras/ERK and PI3K/NF-kappaB – both the Ras-mediated ERK pathway and the PI3K-mediated NF-kappaB pathway are associated with cellular proliferation. We therefore tested whether CXCL12, which was observed to induce N15C6 and LNCaP cellular proliferation, also induced ERK and/or NF-kappaB activation. As seen in Fig. 3, exposure of N15C6 cells to 1 pM, 10 pM or 1000 pM CXCL12 induced rapid, transient ERK phosphorylation and activation. ERK phosphorylation was maximal after 10 min of exposure to 1 or 10 pM CXCL12 and after just 5 min of exposure at 1000 pM CXCL12. Phosphorylation of the p65 subunit of NK-kappaB was not evident after exposure of N15C6 cells to 1 pM CXCL12 but was evident after 10 min exposure to 10 pM or 1000 pM CXCL12. CXCR4 levels were not detectably altered in cells exposed to CXCL12 over the same time period (Fig. 3). These data show that even the very low levels of CXCL12 secreted by older prostate fibroblasts and associated with N15C6 and LNCaP cellular proliferation are sufficient for phosphorylation and activation of ERK 1/2 and NF-kappaB.

CXCL12 and CXCR4 proteins are expressed by human prostate tissues

Immunohistochemical analysis of a tissue microarray (TMA) was used to assess CXCL12 and CXCR4 protein expression in human prostate tissues (Fig. 4). Benign glands (24 tissue cores), epithelial BPH (BPH-E, 65 cores), and malignant glands (77 cores) demonstrated both CXCL12 and CXCR4 protein expression. Benign glands demonstrated the highest levels of expression for both proteins (Fig. 5). Malignant glands and BPH-E demonstrated equivalent CXCR4 expression, and CXCL12 levels were

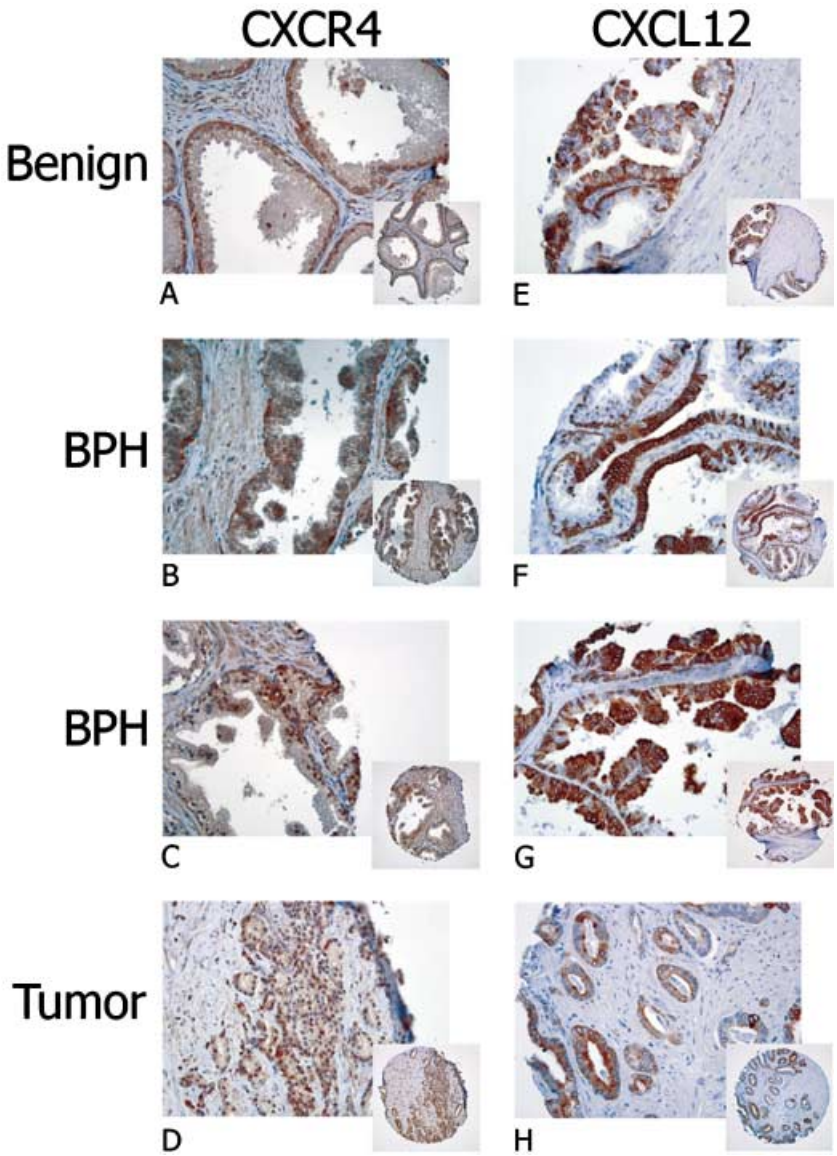

Fig. 4 Benign, hyperplastic and malignant human prostate glands express CXCR4 and CXCL12. Representative portions of identical tissue microarrays are shown of human benign prostate tissues (A, E), benign prostatic hyperplasia, epithelial tissues (B, C, F, G), and malignant prostate tissues (D, H) after incubation with primary antibodies against CXCR4 (1 : 100) (A–D) or CXCL12 (1 : 100) (E–H) and visualization using immunoperoxidase staining.

approximately half that of CXCR4 levels in the same tissues (Fig. 5). The expression pattern for CXCR4 was cytoplasmic and/or membraneous (Fig. 4A,B,D) with the exception of 14/65 (21%) samples of BPH-E, which demonstrated strong nuclear staining (Fig. 4C). The expression pattern for CXCL12 appeared to be exclusively membrane-bound, and likely reflected detection of the protein bound to the CXCR4 receptor (Fig. 4E–G).

Discussion

Several studies have shown that epithelial–stromal interactions are crucial for the regulation of epithelial cell growth. We have hypothesized that epithelial–stromal interactions in the human prostate change over time and that these changes may contribute to the development of hyperplasia and cancer. To test this hypothesis, we have examined the effects of an aging microenvironment on paracrine interactions and prostatic epithelial cell proliferation using a novel *in vitro* model system for benign prostatic hyperplasia developed in our laboratory. The results of these experiments show that the prostate stroma overall exerts a repressive effect on prostate epithelial cell growth. This finding is consistent with the observation that paracrine interactions between human prostate epithelial cells and rat urogenital sinus mesenchyme act homeostatically to maintain both cell types in a nonproliferative, differentiated state (Cunha *et al*., 1996; Hayward *et al*., 1998). Even so, the results of our studies also show that growth of the N15C6 human prostate epithelial cells in media conditioned by older (patients aged 63–81 years) stromal fibroblasts was clearly enhanced compared with growth in media conditioned by younger (patients aged 40–52 years) fibroblasts. In two instances (cases nos 9303 and 11084), N15C6 epithelial cell growth in media conditioned by older prostate fibroblasts actually exceeded levels obtained in complete media (5% HIE). This implies that suppression of

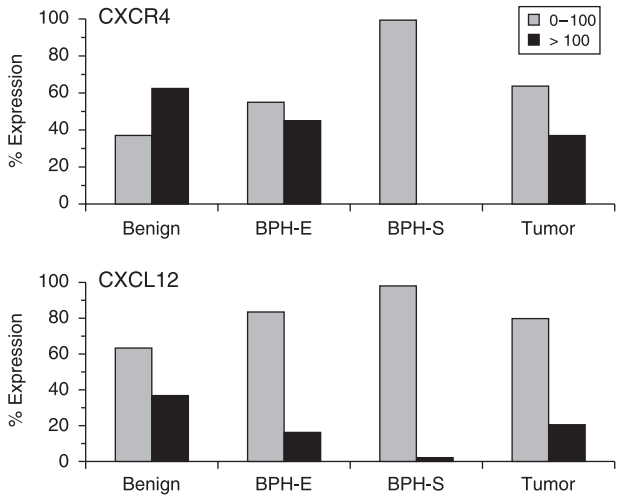

Fig. 5 Quantitation of tissue microarray immunohistochemical analysis. The percent expression of CXCR4 and CXCL12 in Benign, BPH-E, BPH-S and Tumor prostate tissues is shown. Gray bars, staining score 0–100; black bars, score > 100.

epithelial cell proliferation is less efficient in the aging prostate, and that this reduction in efficiency can be directly attributable to paracrine interactions with aging stromal fibroblasts. Interestingly, conditioned media from the single cancer-associated fibroblast population were not growth suppressive, even though the tissue was acquired from a patient 49 years of age at the time of surgery and therefore defined in our study as a member of the 'younger' patient group. This observation is consistent with other studies suggesting that cancer-associated fibroblasts may facilitate prostate epithelial cell proliferation and transformation (Olumi *et al*., 1996, 1998).

Our studies also showed that older prostate stromal fibroblasts transcribe the CXCL12 gene and secrete higher levels of the CXCL12 protein compared with younger stromal fibroblasts. Moreover, 'add back' assays demonstrated that low, picomolar concentrations of CXCL12 directly stimulate human prostate epithelial cell proliferation, and that this effect is likely mediated through phosphorylation and activation of ERK 1/2 and NF-kappaB. The chemokine CXCL12 and its receptor, CXCR4, are known to stimulate lymphocyte proliferation and to mediate lymphocyte trafficking (Nagasawa *et al*., 1994; Moser & Loetscher, 2001). These molecules have also been recently implicated in cancer cell migration and invasion. Cultured prostate cancer cells have been shown to migrate and invade through extracellular matrix and to migrate across bone marrow endothelial cell monolayers in response to CXCL12, activities that can be blocked by pretreatment with antibody against the receptor, CXCR4 (Taichman *et al*., 2002; Mochizuki *et al*., 2004; Singh *et al*., 2004). Cultured breast cancer cells demonstrate enhanced chemotaxis, chemoinvasion and adhesive properties in response to CXCL12, and cells 'knocked down' for CXCR4 transcripts demonstrate significantly reduced ability to invade through Matrigel *in vitro* (Chen *et al*., 2003; Fernandis *et al*., 2004; Lapteva *et al*., 2005). Taken together, these studies indicate a role for CXCL12/CXCR4 interactions in breast and prostate cancer cell migration and invasion. Several of these reports also noted that breast and prostate cancer cells did not proliferate in response to nanomolar levels of CXCL12, although these levels were associated with motility and invasion (Taichman *et al*., 2002; Fernandis *et al*., 2004; Mochizuki *et al*., 2004; Singh *et al*., 2004). We now report that CXCL12 levels as low as 1–5 pM for N15C6 and 50–500 pM for LNCaP prostate epithelial cells stimulate cellular proliferation and that higher levels are clearly growth inhibitory. Together with the studies cited above, this finding suggests that CXCL12 exerts a concentration-dependent dual effect on prostate epithelial cell biology, i.e. lower, picomolar levels stimulate proliferation whereas higher, nanomolar levels repress proliferation but induce motility and invasiveness. These results are consistent with roles for CXCL12/CXCR4 interactions in the etiologies of both benign proliferative disease (BPH) and malignant disease (PCa) in the prostate. Moreover, our observations that low, picomolar levels of CXCL12 equivalent to those secreted by older prostate fibroblasts are associated with prostate epithelial cell proliferation, as well as with activation of ERK 1/2 and NF-kappaB, mechanistically links CXCL12/CXCR4 binding, epithelial/stromal fibroblast paracrine interactions, and epithelial cellular proliferation in the context of an aging microenvironment.

Although the proliferative effects observed for prostate epithelial cells after growth in picomolar levels of CXCL12 were modest, our studies identified several transcripts corresponding to growth-stimulatory secreted proteins. Therefore, the incremental induction of cellular proliferation by CXCL12 likely reflects the effects of just one of several growth-stimulatory factors secreted by aging prostate fibroblasts. As these factors are validated and tested using our *in vitro* system, it is likely that, together, they will recapitulate the significant growth-stimulatory effects observed after exposure of prostate epithelial cells to media conditioned by aging fibroblasts.

The immunohistochemical analyses reported here demonstrate CXCR4 protein expression and less abundant CXCL12 expression in epithelial cells associated with benign glands, BPH nodules characterized by epithelial hyperplasia (BPH-E) and malignant glands, but not in BPH nodules characterized by stromal hyperplasia (BPH-S). CXCR4 demonstrated cytoplasmic and membraneous staining whereas CXCL12 appeared to be exclusively membrane-bound, and likely reflected detection of the protein bound to the CXCR4 receptor. The lack of expression of both CXCR4 and CXCL12 in BPH-S tissues indicates that CXCR4 protein is not expressed in these tissues and that CXCL12 is virtually undetectable in the absence of its receptor. Interestingly, glandular epithelial cells within BPH-E tissue sections demonstrated the most diverse patterns of CXCR4 expression, with 14/65 (21%) of samples exhibiting exclusively nuclear staining, 20/65 (31%) exhibiting both cytoplasmic and nuclear staining, and 25/65 (38%) exhibiting exclusively cytoplasmic staining. A recent retrospective study of tissues resected from stage I non-small-cell lung cancer patients described strong nuclear staining in 29.8% of tumors, and showed that nuclear localization for CXCR4 was significantly associated with longer patient survival (Spano *et al*.,

2004). It is known that CXCR4 undergoes endocytosis mediated by clathrin-coated pits, that this mechanism permits recycling of CXCR4 between the cell membrane and endosomal compartments in hematopoetic progenitor cells and T-cells, and that this process is constitutive in some cells but can be accelerated by CXCL12 (Signoret *et al*., 1997; Zhang *et al*., 2004). Whether the nuclear localization of CXCR4 observed here and by others is due to a disruption or evasion of normal CXCR4 recycling, or to an independent and alternate mechanism, remains to be elucidated.

In summary, these studies show that stromal fibroblasts from the prostates of older men are less able to suppress epithelial cell proliferation than those from younger men, that this effect is at least partially mediated by oversecretion of the CXCL12 chemokine, and that low, picomolar levels of CXCL12 equivalent to those secreted by older prostate stromal fibroblasts initiates a signaling cascade that triggers epithelial cell proliferation. This work represents an important first step towards a mechanistic elucidation of the role of aging in the etiology of benign and malignant prostatic diseases. Future studies focusing on how the balance of growth factors and growth repressors secreted by stromal fibroblasts becomes altered within the context of an aging microenvironment will further elucidate how the aging process directly contributes to the development of hyperplasia and neoplasia in the human prostate.

Experimental procedures

Establishment of cell cultures

All tissue acquisition and processing protocols conformed to those reviewed and approved by the University of Michigan Institutional Review Board. Normal prostate tissue samples were obtained aseptically from patients undergoing radical prostatectomy or cystoprostatectomy after cancer diagnosis. Tissue was taken exclusively from the transitional zone of the prostate surrounding the urethra to facilitate the isolation of fibroblasts associated with benign prostatic hyperplasia (BPH). A small slice of the tissue piece (~25%) was fixed in 10% formalin for immunohistochemical examination. The remaining tissue was minced into pieces of < 1 mm in size, digested overnight in 1 mg mL^{-1} collagenase III (Worthington Biochemicals, Lakewood, NJ, USA) at 37 °C with gentle shaking, spun down, resuspended and plated in 5% HIE media [Ham's F12 (Mediatech Inc., Herndon, VA, USA) with 5% FBS (Invitrogen Life Technologies Inc., Carlsbad, CA, USA), 5 μg mL^{-1} insulin, 10 ng mL^{-1} EGF, 1 μg mL^{-1} hydrocortisone (Sigma Chemical Co., St. Louis, MO, USA). Subcultured fibroblast cell populations were assessed by immunohistochemistry using monoclonal antibodies to vimentin or alpha-actin (Sigma-Aldrich, St. Louis, MO, USA) to distinguish stromal fibroblastic cells from smooth muscle cells, respectively. The fibroblast cell populations used in these studies were stained uniformly for vimentin but not alpha-actin and were maintained in 5% HIE.

The N15C6 cell line was developed as described previously (Macoska *et al*., 2004; Begley *et al*., 2005). For conditioned media experiments, N15C6 cells were grown either in defined serum-free HIE media supplemented to 5 mM ethanolamine (Sigma-Aldrich), 10 mM HEPES (Sigma-Aldrich), 5 μg $μL^{-1}$ transferrin (Sigma-Aldrich), 10 μM 3,3',5-triiodo-L-thyronine (Sigma-Aldrich), 50 μM sodium selenite (Sigma-Aldrich), 0.1% BSA (JRH Biosciences Lenexa, KS, USA), 0.05 mg mL^{-1} gentamycin (Gibco, Carlsbad, CA, USA), and 0.5 μg mL^{-1} fungizone (Cambrex Bioscience, Walkersville, MD, USA), or in defined serum-free HIE conditioned media produced through the incubation of fibroblast cell populations in supplemented serum-free HIE media over a 48-h period. Conditioned media were harvested from all fibroblast cell populations at passages 1–3 with the exception of no. 2396 (passage 4), no. 3294 and no. 10063 (passage 5), and no. 11033 and no. 3382 (passage 11).

Proliferation assays

Cellular proliferation was assessed after plating cells at 50 000 cell/well in triplicate in six-well plates and counting cells after 24 and 96 h of incubation in complete media, serum-free media or serum-free prostate stromal fibroblast conditioned media, as described previously (Macoska *et al*., 2004). Averages and standard deviations of cell number were calculated for each time point under each media condition. To assess the effects of exogenous CXCL12 on cellular proliferation, recombinant human SDF1 alpha/CXCL12 (R & D Systems, 350-NS) was added at the desired concentration in 1 mL SF HIE (or 1 mL SF HIE alone for control) to each well. The cells were counted at 24 and 96 h growth and re-fed at 48 and 72 h growth. Cell counts were normalized to 50 000 cells at 24 h to account for any plating discrepancies.

Affymetrix U133A GeneChip data acquisition

RNA was purified from trypsinized cultured cells by homogenization in Trizol (Invitrogen, Carlsbad, CA, USA) and additional processing using the RNeasy (QIAGEN, Valencia, CA, USA) cleanup procedure. Ten micrograms of RNA obtained from tissues or cell line was used to obtain labeled cRNA following the Affymetrix standard protocol. Expression intensity values for each gene were estimated using a method called Robust Multi-array Average (RMA) using tools available through Bioconductor (www.bioconductor.org). GeneChip gene expression values were normalized using a quantile normalization procedure.

Quantitative real-time PCR

All quantitative real-time assays were conducted with an Applied Biosystems 7900HT instrument and reagents. One microgram of RNA was reverse transcribed by use of Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The resulting cDNA was diluted 1 : 100. Real-time PCR was performed by use of Assays on Demand (Applied BioSystems, Foster City, CA, USA) according to the manufacturer's instructions, except that Real Time Ready with Rox passive dye (QBioGene, Carlsbad, CA, USA)

was used in place of TaqMan Universal PCR Master Mix. Reactions were performed in triplicate, including no-template controls and an endogenous control probe to assess template concentration (ribosomal protein, large, PO). Cycle numbers to threshold were calculated by subtracting averaged control from averaged experimental values, and Fold Gene Expression was calculated by raising these values to the log 2. FAM conjugated, gene-specific assays were Hs00171022_m1 for CXCL12, and Hs99999902_m1 for the control, RPLPO.

ELISA assays

Fibroblasts were grown in 0.01% BSA defined HIE for 3 days. Fibroblast-conditioned media were collected, serially concentrated using Centriplus Centrifugal Filters (Millipore, Billerica, MA, USA) with a 3 kDa molecular weight cutoff, and assessed using the CXCL12/SDF1-alpha ELISA system (R & D Systems). All reactions were performed in duplicate and the resulting values averaged.

Western blot analysis

Cells were lysed, proteins resolved by electrophoresis and electroblotting was carried out as described previously (Chaib *et al*., 2001). Proteins were detected using antibodies against phospho-ERK1/2 (Cell Signaling no. 9101), total ERK1/2 (Cell Signaling, no. 9102), CXCR4 (Abcam no. ab2074), phospho- p65 (Rockland no. 100-401-264), total p65 (Rockland no. 100-4165), or beta-actin (Santa Cruz no. sc-1615), in conjunction with an ECL detection system.

Construction of tissue microarray (TMA) and immunohistochemistry

A TMA was constructed from 31 radical prostatectomy specimens to represent benign prostate tissues [number of cores (N) = 24]; benign prostatic hyperplasia epithelial tissues (BPH-E) (N = 65); benign prostatic hyperplasia stromal tissue (BPH-S) (N = 102) and prostate cancer (N = 77). Antigen retrieval was performed in citrate buffer (10 mM, pH 6.0) using a microwave pressure cooker. The CXCR4 antibody (Abcam, ab2074) was used at 1 : 50 and CXCL12 (R & D Systems, MAB350) at 1 : 100. Digital images were acquired with the BLISS Imaging System (Bacus Laboratory, Lombard, IL, USA). Immunostaining intensity was recorded as absent (1), weak (2), moderate (3), or strong (4) and as the percentage of cells staining on a scale of 1–100%. The product of each (intensity × percentage) was used to calculate a staining score for each tissue. Scoring was performed in a blinded fashion using the Profiler Web-based telepathology system (http://pvdb.path.med.umich.edu/htma/profiler/index.jsp).

Statistical analysis

GeneChip expression values were analyzed using a t-statistic test and by calculation of fold change between data sets. Genes that exhibited both a large t-statistic (> 10.0) and a large fold change (> 2.0) were considered to be differentially expressed. All other data were assessed by t-test or analysis of variance with $P < 0.05$ considered statistically significant.

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References

Arenas MI, Romo E, Royuela M, Ruiz A, Fraile B, Sanchez-Chapado M, Paniagua R (2001) Morphometric evaluation of the human prostate. *Int. J. Andrology* **24**, 37–47.

Begley L, Keeney D, Beheshti B, Squire JA, Kant R, Chaib H, MacDonald JW, Rhim J, Macoska JA (2005) Concordant copy number and transcriptional activity of genes mapping to derivative chromosomes 8 during cellular immortalization in vitro. *Genes Chromosomes Cancer* in press, Epub Oct 18, 2005.

Berges RR, Vukanovic J, Epstein JI, CarMichel M, Cisek L, Johnson DE, Veltri RW, Walsh PC, Isaacs JT (1995) Implication of cell kinetic changes during the progression of human prostatic cancer. *Clin. Cancer Res.* **1**, 473–480.

Chaib H, Rubin MA, Mucci NR, Li L, Taylor JMG, Day M, Rhim JS, Macoska JA (2001) Activated in prostate cancer: a PDZ domain-containing protein highly expressed in human primary prostate tumors. *Cancer Res.* **61**, 2390–2394.

Chen Y, Stamatoyannopoulos G, Song CZ (2003) Down-regulation of CXCR4 by inducible small interfering RNA inhibits breast cancer cell invasion in vitro. *Cancer Res.* **63**, 4801–4804.

Colombel M, Vacherot F, Diez SG, Fontaine E, Buttyan R, Chopin D (1998) Zonal variation of apoptosis and proliferation in the normal prostate and in benign prostatic hyperplasia. *Br. J. Urol.* **82**, 380–385.

Cunha GR, Hayward SW, Dahiya R, Foster BA (1996) Smooth muscle–epithelial interactions in normal and neoplastic prostatic development. *Acta Anat.* **155**, 63–72.

Fernandis AZ, Prasad A, Band H, Klosel R, Ganju RK (2004) Regulation of CXCR4-mediated chemotaxis and chemoinvasion of breast cancer cells. *Oncogene* **23**, 157–167.

Foster BA, Kaplan PJ, Greenberg NM (1998–99) Peptide growth factors and prostate cancer: new models, new opportunities. *Cancer Metastasis Rev* **17**, 317–324.

Hayward SW, Haughney PC, Rosen MA, Greulich KM, Weier HU, Dahiya R, Cunha GR (1998) Interactions between adult human prostatic epithelium and rat urogenital sinus mesenchyme in a tissue recombination model. *Differentiation* **63**, 131–140.

Lapteva N, Yang AG, Sanders DE, Strube RW, Chen SY (2005) CXCR4 knockdown by small interfering RNA abrogates breast tumor growth in vivo. *Cancer Gene Ther.* **12**, 84–89.

Macoska JA, Paris P, Collins C, Andaya A, Beheshti B, Chaib H, Kant R, Begley L, MacDonald JW, Squire JA (2004) Evolution of 8p loss in transformed human prostate epithelial cells. *Cancer Genet. Cytogenet.* **154**, 36–43.

Meigs JB, Mohr B, Barry MJ, Collins MM, McKinlay JB (2001) Risk factors for clinical benign prostatic hyperplasia in a community-based population of healthy aging men. *J. Clin. Epidemiol.* **54**, 935–944.

Merz VW, Miller GJ, Krebs T, Timme TL, Kadmon D, Park SH, Egawa S, Scardino PT, Thompson TL (1991) Elevated transforming growth factor-beta 1 and beta 3 mRNA levels are associated with ras + myc-induced carcinomas in reconstituted mouse prostate: evidence for a paracrine role during progression. *Mol. Endocrinol.* **5**, 503–513.

Mochizuki H, Matsubara A, Teishima J, Mutaguchi K, Yasumoto H, Dahiya R, Usui T, Kamiya K (2004) Interaction of ligand-receptor system between stromal-cell-derived factor-1 and CXC chemokine receptor 4 in human prostate cancer: a possible predictor of metastasis. *Biochem. Biophys. Res. Commun.* **320**, 656–663.

Moser B, Loetscher P (2001) Lymphocyte traffic control by chemokines. *Nature Immunol.* **2**, 123–128.

Nagasawa T, Kikutani H, Kishimoto T (1994) Molecular cloning and structure of a pre-B-cell growth-stimulating factor. *Proc. Natl Acad. Sci. USA* **91**, 2305–2309.

Neuhouser ML, Kristal AR, Penson DF (2004) Steroid hormones and hormone-related genetic and lifestyle characteristics as risk factors for benign prostatic hyperplasia: review of epidemiologic literature. *Urology* **64**, 201–211.

Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR (1996) Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res.* **59**, 5002–5011.

Olumi AF, Dazin P, Tlsty TD (1998) A novel coculture technique demonstrates that normal human prostatic fibroblasts contribute to tumor formation of LNCaP cells by retarding cell death. *Cancer Res.* **58**, 4525–4530.

Signoret N, Oldridge J, Pelchen-Matthews A, Klasse PJ, Tran T, Brass LF, Rosenkilde MM, Schwartz TW, Holmes W, Dallas W, Luther MA, Wells TN, Hoxie JA, Marsh M (1997) Phorbol esters and SDF-1 induce rapid endocytosis and down modulation of the chemokine receptor CXCR4. *J. Cell Biol.* **139**, 651–664.

Singh S, Singh UP, Grizzle WE, Lillard JW Jr (2004) CXCL12–CXCR4 interactions modulate prostate cancer cell migration, metalloproteinase expression and invasion. *Lab. Invest* **84**, 1666–1676.

Song Z, Wu X, Powell WC, Cardiff RD, Cohen MB, Tin RT, Matusik RJ, Miller GJ, Roy-Burman P (2002) Fibroblast growth factor 8 isoform B overexpression in prostate epithelium: a new mouse model for prostatic intraepithelial neoplasia. *Cancer Res.* **62**, 5096–5105.

Spano JP, Andre F, Morat L, Sabatier L, Besse B, Combadiere C, Deterre P, Martin A, Azorin J, Valeyre D, Khayat D, Le Chevalier T, Soria JC (2004) Chemokine receptor CXCR4 and early-stage non-small cell lung cancer: pattern of expression and correlation with outcome. *Ann. Oncol.* **15**, 613–617.

Taichman RS, Cooper C, Keller ET, Pienta KJ, Taichman NS, McCauley LK (2002) Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. *Cancer Res.* **62**, 1832–1837.

Thompson TC, Southgate J, Kitchener G, Land H (1989) Multistage carcinogenesis induced by ras and myc oncogenes in a reconstituted organ. *Cell* **56**, 917–930.

Verhamme KM, Dieleman JP, Bleumink GS, van der Lei J, Sturkenboom MC, Artibani W, Begaud B, Berges R, Borkowski A, Chappel CR, Costello A, Dobronski P, Farmer RD, Jimenez Cruz F, Jonas U, MacRae K, Pientka L, Rutten FF, van Schayck CP, Speakman MJ, Sturkenboom MC, Tiellac P, Tubaro A, Vallencien G, Vela Navarrete R, Triumph Pan European Expert Panel (2002) Incidence and prevalence of lower urinary tract symptoms suggestive of benign prostatic hyperplasia in primary care – the Triumph project. *Eur. Urol.* **42**, 323–328.

Zhang Y, Foudi A, Geay JF, Berthebaud M, Buet D, Jarrier P, Jalil A, Vainchenker W, Louache F (2004) Intracellular localization and constitutive endocytosis of CXCR4 in human CD34+ hematopoietic progenitor cells. *Stem Cells* **22**, 1015–1029.