Published in final edited form as:

Prostate. 2010 March 1; 70(4): 377–389. doi:10.1002/pros.21071.

Leukocytic Promotion of Prostate Cellular Proliferation

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Abstract

BACKGROUND—Histological evidence of pervasive inflammatory infiltrate has been noted in both benign prostatic hyperplasia/hypertrophy (BPH) and prostate cancer (PCa). Cytokines known to attract particular leukocyte subsets are secreted from prostatic stroma consequent to aging and also from malignant prostate epithelium. Therefore, we hypothesized that leukocytes associated with either acute or chronic inflammation attracted to the prostate consequent to aging or tumorigenesis may promote the abnormal cellular proliferation associated with BPH and PCa.

METHODS—An in vitro system designed to mimic the human prostatic microenvironment incorporating prostatic stroma (primary and immortalized prostate stromal fibroblasts), epithelium (N15C6, BPH-1, LNCaP, and PC3 cells), and inflammatory infiltrate (HL-60 cells, HH, and Molt-3 T-lymphocytes) was developed. Modified Boyden chamber assays were used to test the ability of prostate stromal and epithelial cells to attract leukocytes and to test the effect of leukocytes on prostate cellular proliferation. Antibody arrays were used to identify leukocyte-secreted cytokines mediating prostate cellular proliferation.

RESULTS—Leukocytic cells migrated towards both prostate stromal and epithelial cells. CD4+ T-lymphocytes promoted the proliferation of both transformed and non-transformed prostate epithelial cell lines tested, whereas CD8+ T-lymphocytes as well as dHL-60M macrophagic and dHL-60N neutrophilic cells selectively promoted the proliferation of PCa cells.

CONCLUSIONS—The results of these studies show that inflammatory cells can be attracted to the prostate tissue microenvironment and can selectively promote the proliferation of non-transformed or transformed prostate epithelial cells, and are consistent with differential role(s) for inflammatory infiltrate in the etiologies of benign and malignant proliferative disease in the prostate.

Keywords

BPH; PCa; immune; inflammatory infiltrate; chemokine; microenvironment

INTRODUCTION

Immunohistochemical studies examining the histopathology of benign prostatic hyperplasia/hypertrophy (BPH) and prostate cancer (PCa) have reported the presence of pervasive inflammatory infiltrate comprising leukocytes associated with acute inflammation, chronic

inflammation, or both. Inflammatory cells comprising neutrophilic or lymphocytic infiltrates were identified in 90% of transurethral resection of the prostate (TURP) specimens from 80 patients diagnosed with BPH but no history of prostatitis or prostatic infection [1]. Chronic lymphocytic infiltrate was observed in association with areas of benign hypertrophy and cancer in whole mount radical prostatectomy specimens from a series of 40 consecutive patients with clinically localized PCa [2]. Chronic inflammatory infiltrate was also detected in 30–60% of 1,197 randomly selected men with BPH as part of the Medical Therapy of Prostatic Symptoms (MTOPS) study. Patients with chronic inflammatory infiltrate had larger prostate volumes and demonstrated significantly more clinical progression and acute urinary retention than those with no evidence of inflammation [3]. Theyer et al. [4] reported that the majority of BPH tissues examined demonstrated infiltration of various T-cell lymphocyte populations typically associated with chronic inflammation. Finally, a recent histological study of sextant needle biopsies among men diagnosed as biopsy-negative for cancer found high levels of polymorphonuclear leukocytic infiltrates in all 93 patients examined but similar levels of mononuclear leukocytic infiltrate in only 7 of these same patients [5].

These studies suggest that leukocytes associated with acute (e.g., neutrophilic) or chronic (e.g., monocytic/macrophagic or lymphocytic) inflammation are commonly observed in association with BPH and/or PCa. However, it is not clear why leukocytes are attracted to the prostate or whether they act to promote or inhibit the abnormal proliferation of cells associated with both BPH and PCa. Previous studies from our laboratory have shown that cytokines known to attract particular leukocyte subsets are secreted from prostatic stroma consequent to aging and also from malignant prostate epithelium [6-8]. These observations suggest that the prostatic microenvironment itself may attract and possibly sequester circulating leukocytes. Depending upon the actual cell type, leukocytes associated with either acute or chronic inflammatory responses can contribute to the destruction or the proliferation and repair of tissue. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of granulocytes (primarily neutrophils) from the blood into the injured tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular and immune systems, and various cells within the injured tissue. Chronic inflammation leads to a progressive shift in the type of cells which are present at the site of inflammation towards macrophages, lymphocytes, and plasma cells, and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process [9,10]. Taken together, these observations suggest that leukocytes associated with either acute or chronic inflammation may be attracted to the prostate consequent to aging or tumorigenesis. Moreover, once present, these inflammatory cells could act to either promote or attempt to prevent the abnormal cellular proliferation associated with BPH and PCa.

To begin to address these questions, we have developed an in vitro system to mimic the human prostatic microenvironment that incorporates prostatic stroma, epithelium, and inflammatory infiltrate. The studies presented here show that both aging stroma and malignant prostatic epithelial cells secreted leukoattractants that were sufficient to induce the migration of neutrophils, macrophages, and CD4+ and CD8+ T-lymphocytes towards prostate cells. Moreover, transformed prostate epithelial cells proliferated in response to all leukocytic cell types tested, whereas non-transformed prostate epithelial cells exhibited mixed proliferative responses. The results of these studies are consistent with a role for inflammatory infiltrate in the promotion of both benign and malignant proliferative disease in the prostate.

MATERIALS AND METHODS

Cell Lines and Culture

N15C6 and BPH-1 cells are non-transformed prostate epithelial cells and grow continuously in culture but do not form colonies in soft agar or tumors in immunocompromised mice [11-13]. N15C6 were maintained in 5% HIE media [Ham's F12 (Mediatech, Inc., Herndon, Virginia)] with 5% FBS (Life Technologies, Inc.), 5 µg/ml insulin, 10 ng/ml EGF, 1 µg/ml hydrocortisone (Sigma-Aldrich, St. Louis, MO) or in defined serum-free (SF) media supplemented to 5 mM ethanolamine (Sigma-Aldrich), 10 mM HEPES (Sigma-Aldrich), 5 µg/µl 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), 0.05 mg/ml gentamycin (Invitrogen, Carlsbad, CA), and 0.5 mg/L fungizone, 50 mg/L gentamycin, and 0.52 mg/L plasmocin (Cambrex Bioscience, Walkersville, MD) [11,12]. BPH-1 cells were maintained in 10% RPMI or SF RPMI (0.1% BSA) and antibiotics as described above [13]. The androgen sensitive LNCaP and 22Rv1 and androgen-insensitive PC3 and DU145 transformed prostate epithelial cell lines were maintained in 10% RPMI media or SF RPMI [14-17]. N1 immortalized prostate stromal fibroblasts were maintained in 5% HIE media or SF HIE [8]. PF1, PF2, PF3, PF4, and PF5 primary stromal fibroblast cultures were derived from peri-urethral prostate tissues histologically assessed as free from cancer, and were cultured and maintained in 5% HIE or SF HIE as described previously [6]. HL-60 cells, a promyelocytic leukemic cell line, was acquired from the American Type Culture Collection (ATCC# CCL240) and was maintained in 10% RPMI media with 1% Pen/Strep [18]. HL-60 cells were promoted to terminally differentiate along a granulocytic (neutrophilic) lineage (dHL-60N) through treatment with 1.25% dimethyl sulfoxide (DMSO) for 14 days or along a monocytic/macrophagic lineage (dHL-60M) through treatment with 10 nM phorbol myristic acid (PMA) for 18 hr [19]. T-lymphocytic cell lines HH (CD4+) and Molt-3 (predominantly CD8+) were acquired from the American Type Culture Collection (ATCC# CRL-2105; CRL-1552), respectively and maintained in 10% RPMI media (Invitrogen #A10491) with 1% Pen/Strep [20-22]. All cell lines were cultured in a humidified 5% CO₂ atmosphere at 37°C.

ELISA Assays

Protein levels were measured by sandwich ELISA using SF media conditioned for 24 hr by 1×10^6 prostate cells or complete leukocyte-conditioned media. ELISAs were conducted on media aspirated after centrifugation to pellet cells. For prostate cells, the conditioned SF media was first concentrated using Centriplus centrifugal filters (Millipore, Billerica, MA) with a 3 kDa molecular weight cutoff. ELISA was performed using Human CXCL5/ENA-78 DuoSet DY254, IL-8/CXCL8 DuoSet DY208, CCL2/MCP-1 DuoSet DY279, CCL5/RANTES DuoSet DY278, or CXCL12/SDF-1 alpha capture antibody MAB350, detection antibody BAF310, and standard 350-NS ELISA reagents (R&D Systems, Minneapolis, MN). For all ELISAs, a standard curve was generated with the provided standards and utilized to calculate the quantity of chemokine in the sample tested. All reactions were performed in duplicate, and the resulting values were averaged [6,8].

Proliferation Assay

N15C6, BPH-1, and PC3 cells were plated at 10,000 cells/well and LNCaP cells were plated at 25,000 cells/well in quadruplicate per time point and allowed to adhere for 24 hr. 50,000 dHL-60N, dHL-60M, HH, or Molt-3 cells were added to each well and the cell mixtures incubated for an additional 24 hr. At the end of the incubation period the dHL-60N, dHL-60M, HH, or Molt-3 cells were removed by gentle aspiration and the adhered epithelial cells trypsinized and counted as described previously [6]. Each proliferation assay was

repeated three times, and cell numbers averaged and standard deviations were calculated for each time point to permit statistical analysis.

Migration Assays

For peripheral blood mononuclear cell (PBMC) migration studies, leukocytes were isolated from venous blood from healthy donors and depleted of monocytes by adherence to plastic for 2 hr. Monocyte-depleted white blood cells (1×10^6 cells/ml) were fluorescently labeled with 20 mM of 2,7-bis-(2-carboxyethyl)-5-carboxyfluorescein acetoxymethyl (BCECF AM) (Molecular Probes, Eugene, OR) according to the manufacturers directions. The cells were washed and resuspended at 1×10^6 cells/ml in SF RPMI media, and 1×10^5 labeled cells in 100 μ l were added to the upper chamber of a 24-well Transwell chemotaxis insert with pore size of 5 μ m (Corning, Corning, NY). The lower chambers contained 200 μ l SF RPMI (as control), SF HIE, or SF HIE that was conditioned for 48 hr by four independently derived cultures of primary prostate stromal fibroblasts and concentrated as described above. After 30 and 60 min, the number of fluorescently labeled migrating cells into the lower chamber was determined at 485/535 nm wavelength using a Tecan GENios plate-reader (Phenix, Austria). The results were expressed as the average fold increase of the number of cells migrating toward lower divided by the number of cells migrating toward the control wells (SF RPMI) [23,24].

For all other studies, an in vitro model system was utilized composed of leukocytes (dHL-60N, dHL-60M, HH, or Molt-3 T-lymphocytes), non-transformed (N15C6, BPH-1) and transformed (LNCaP, PC3) prostate epithelial cells, and non-transformed immortalized (N1) or primary (PF1) prostate stromal fibroblasts. The migration assays utilized a Boyden chamber system (BD Biosciences, San Jose, CA) with 3 μ m (dHL-60N, dHL-60M) or 8 μ m (dHL-60M, HH, Molt-3) membranes. For each assay, 375,000 dHL-60N or dHL-60M, or 300,000 HH or Molt-3, cells were seeded into the upper chamber and leukocyte chemotaxis was measured after 4 hr of exposure to chemoattractants (cytokines, plated cells, or fibroblast-conditioned media) or control vehicle using modified Boyden chamber assays (BD Biosciences). All experiments acquired replicate or triplicate measures and were graphed as averaged values across multiple assays.

Cytokine Arrays

1×10^6 dHL-60N, dHL-60M, HH, or Molt-3 cells were incubated in 5 ml of complete media for 24 hr. Cells were removed by centrifugation and 1 ml of the supernatant was incubated with the RayBio® Human Cytokine Antibody Array 5 (Cat# AAH-CYT-5) (Raybiotech, Norcross, GA). Arrays were processed according to manufacturer's protocols and evaluated using software provided by the manufacturer.

Statistical Analysis

Data were averaged and standard deviations calculated for graphical depiction and statistical analysis by *t*-test or analysis of variance with $P < 0.05$ considered statistically significant.

RESULTS

Prostate Cells Secrete Leukotactic Cytokines

Previous studies from our laboratory have demonstrated the secretion of the CXC-type chemokine CXCL12 (SDF-1) by prostate stromal fibroblasts and CXCL5 (ENA-78) by both fibroblasts and prostate epithelial cells [6-8]. CXCL12 and CCL2 (MCP-1) are chemotactic for macrophages whereas CXCL8 (IL-8) and CXCL5 are strongly chemotactic for neutrophils [25,26]. Therefore, we tested whether several commonly available prostate-derived cells secreted measurable levels of these four cytokines. As summarized in Table I,

sandwich ELISA measurements taken from 24 hr conditioned SF media demonstrated similar levels of secreted CXCL5 and CXCL8 protein for all cell lines tested, with the most robust secretion detected for N1 immortalized and PF1 primary prostate stromal fibroblasts as well as BPH-1 non-transformed and PC3 and DU145 transformed prostate epithelial cells. Robust CCL2 secretion was detected for PF1 primary prostate stromal fibroblasts with weaker expression detected for N1 immortalized prostate fibroblasts and for BPH-1 prostate epithelial. Consistent with its role as a stromally derived cytokine, CXCL12 secretion was detected only for N1 and PF1 prostate stromal fibroblasts. Taken together, these data suggested that CXCL5 and CXCL8, both strongly chemotactic for neutrophils, were secreted robustly by both stromally derived and epithelial-derived prostate cells, whereas CCL2 was secreted differentially by prostate epithelial and fibroblast cells, and CXCL12 exclusively by prostate stromal fibroblasts.

In Vitro Model for Leukocyte Chemotaxis

In order to accurately mimic the prostate tissue microenvironment, it was first necessary to test whether the cytokine levels secreted by prostate cells was sufficient to induce leukocytic migration. These studies first focused on examining neutrophil and macrophage chemotaxis towards CXCL5 secreted by both prostate stromal and epithelial cells, and CXCL12 secreted solely by prostate stromal fibroblasts. For these assays, pro-myelocytic HL-60 cells were promoted to terminally differentiate along a granulocytic (neutrophilic) lineage (dHL-60N) through treatment with 1.25% DMSO for 14 days or along a monocytic/macrophagic lineage (dHL-60M) through treatment with 10 nM PMA for 18 hr [19] (Fig. 1A). Incubation of complete media or complete media conditioned by HL-60, dHL-60N, or dHL-60M with the RayBio® Human Cytokine Antibody Array confirmed cellular differentiation, as the proteins secreted by HL-60, dHL-60N, and dHL-60M differed significantly from each other and from the culture media alone (Fig. 1B,C). In particular, dHL-60M secreted high levels of several CCL-type cytokines, including CCL2 (MCP-1), CCL4 (MIP-1beta), CCL5 (RANTES), and CCL24 (Eotaxin-3). dHL-60M also secreted high levels of the CXCL-type cytokines CXCL5 (ENA-78) and CXCL8 (IL-8), as well as TIMP-1 and Osteopontin. dHL-60N were less secretory than dHL-60M and secreted high levels only of CCL2, CCL4, CXCL8, and TIMP-1. None of these proteins were appreciably secreted by undifferentiated HL-60 cells, and were not present in the culture media alone.

To study dHL-60N and dHL-60M chemotaxis, a 24-well Boyden chamber plate was supplemented with complete media containing vehicle, known chemoattractant cytokine (CXCL8 for dHL-60N or CCL2 for dHL-60M), or increasing concentrations of CXCL5 or CXCL12 in complete media. Porous inserts were added to the plate and seeded with 375,000 dHL-60N or dHL-60M in complete media (Fig. 2A–D). After 4 hr incubation, the number of dHL-60N or dHL-60M cells that migrated through the membranes and into the bottom chamber was counted. These assays showed that ~50% more dHL-60N migrated towards CXCL8 and ~25% more towards 100–1,000 pg/ml CXCL5 compared to vehicle (Fig. 2E) but that dHL-60N did not migrate towards CXCL12 (Fig. 2F). Thus, as expected, the dHL-60N cells differentiated along a neutrophilic lineage demonstrated preferential migration towards the neutrophilic chemotactic chemokines, CXCL5 and CXCL8. dHL-60M cells migrated robustly towards CCL2, 100–1,000 pg/ml CXCL5 (Fig. 2E), and 10–100 pg/ml CXCL12 at levels two- to threefold higher than that observed for vehicle (Fig. 2F). Thus, the dHL-60M cells differentiated along a macrophagic lineage demonstrated preferential migration towards the macrophagic chemokine CCL2 and CXCL12. dHL-60M also migrated towards CXCL5 which, though typically considered a neutrophilic chemokine, has been shown to be a chemoattractant for macrophagic leukocytes as well [27,28]. The results of these studies also suggested that dHL-60M were more mobile than dHL-60N, as they migrated more robustly towards the strongly macrophilic cytokine, CCL2, than did

dHL-60N towards the strongly neutrophilic cytokines, CXCL5 and CXCL8 (Fig. 2E,F). Taken together, these studies showed that HL-60 cells differentiated along neutrophilic or macrophagic lineages trafficked towards chemokines at levels similar to those secreted by prostate stromal fibroblasts and both non-transformed and transformed prostate epithelial cells.

Modulation of Leukocyte Chemotaxis by Prostate Stromal and Epithelial Cells

Previous studies from our laboratory demonstrated that prostatic stromal fibroblasts and epithelial cells secrete several leukotactic proteins [6-8]. ELISA demonstrated robust secretion of leukotactic CXCL5, CXCL8, CXCL12, and CCL2 by both N1 immortalized and PF1 primary cultured prostate stromal fibroblasts and of CXCL5, CXCL8, and CCL2 by several non-transformed and transformed prostate epithelial cell lines (Table I). Therefore, we next examined whether prostate epithelial and stromal fibroblast cells secreted these cytokines at levels sufficient for leukocytic chemotaxis. As shown in Figure 3, >2-fold more dHL-60N or dHL-60M cells migrated towards N15C6 or BPH-1 prostate epithelial cells compared to migration towards vehicle-supplemented media alone. The addition of CCL2 did not augment dHL-60M chemotaxis above those levels observed in the presence (Fig. 3B) or absence (Fig. 2E,F) of prostate epithelial cells, suggesting that the prostate epithelial cells alone provided sufficient chemoattractant to induce high levels of dHL-60 migration. In contrast, dHL-60N migrated much more robustly in the presence (Fig. 3A) of prostate epithelial cells than in their absence (Fig. 2E,F). The addition of CXCL8 further augmented dHL-60N chemotaxis (Fig. 3A).

The migration of both dHL-60M and dHL-60N cells was significantly enhanced in the presence of N15C6 or BPH-1 prostate epithelial cells (Fig. 3A,B). These studies showed that prostate epithelial cells secreted sufficient chemoattractants to promote leukocytic chemotaxis.

It is likely that infiltrating leukocytes interact with multiple cell types within the human prostate. Therefore, an initial test of how two common cell types in the prostate, stromal fibroblasts, and glandular epithelial cells, may modulate leukocytic infiltration was conducted. For these experiments, N15C6 or BPH-1 cells were seeded as described above, the media was aspirated, then replaced with media conditioned by a transient culture of PF1 primary prostate stromal fibroblasts. These studies showed that dHL-60N migration towards N15C6 prostate epithelial cells, and dHL-60M migration towards BPH-1 prostate epithelial cells, was negatively modulated by media conditioned by PF1 primary prostate fibroblasts (Fig. 3A,B). These data suggested that interactions between prostate epithelial and stromal fibroblast cells could differentially modulate leukocytic chemotaxis.

The specificity of dHL-60N chemotaxis towards prostate epithelial cells was tested by plating non-transformed N15C6 or transformed PC3 prostate epithelial cells in the bottom chambers as described above, then pre-treating the cells for 1 hr with 1 µg/ml antibodies against the chemokine receptors CXCR2 or CXCR4, or the control IgG. dHL-60N cells were added and the number of cells which migrated through the membrane was counted 4 hr later. As seen in Figure 3C, >2-fold more dHL-60N migrated into the bottom chambers seeded with N15C6 or PC3 cells compared to unseeded wells. However, dHL-60N migration towards N15C6 cells was significantly inhibited by pre-treatment with antibody against CXCR2, though not by pre-treatment with antibody against CXCR4 or IgG. Pre-treatment of PC3 cells with any of the antibodies tested did not inhibit dHL-60N migration. These studies suggested that one or more CXCR2 agonists mediating dHL-60N migration were likely secreted at higher levels by PC3 compared to N15C6 cells.

dHL-60N and dHL-60M Promote the Proliferation of Transformed Prostate Epithelial Cells

Although both dHL-60N and dHL-60M were attracted to N15C6 and BPH-1 cells, it was unclear whether proteins secreted by these leukocytes would promote, repress, or have no effect, on prostate epithelial cell proliferation. To test this, non-transformed N15C6 or BPH-1, or transformed LNCaP or PC3, prostate epithelial cells were co-cultured for 24 hr with media (as control) or with dHL-60N or dHL-60M cells, then counted. These studies showed that dHL-60M promoted the proliferation of both LNCaP and PC3 transformed prostate epithelial cells, whereas dHL-60N cells promoted the proliferation of PC3 cells only. The proliferation of non-transformed N15C6 and BPH-1 was unaffected by the presence or absence of dHL-60N or dHL-60M cells (Fig. 3D). Taken together, this data suggested that myeloid lineage leukocyte dHL-60N and dHL-60M cells selectively promoted the proliferation of transformed, but not non-transformed, prostate epithelial cells.

HH and Molt-3 T-Lymphocytes Promote the Proliferation of Transformed Prostate Epithelial Cells

Several studies have demonstrated the infiltration of human prostate tissues by CD4+ and/or CD8+ T-lymphocytes. The studies described above suggested that myeloid-derived leukocytes can modulate the proliferation of prostate epithelial cells. In order to test whether lymphoid-derived leukocytes might also be capable of modulating prostate epithelial cell proliferation, two additional cell lines, the T lymphocytic CD4+ HH and predominantly CD8+ Molt-3 cells were incorporated into the in vitro model. Incubation of culture media or culture media conditioned by HH or Molt-3 cells with the RayBio® Human Cytokine Antibody Array demonstrated similar secretory profiles for both T-lymphocyte derived cell lines (Fig. 4A,B). As noted above, previous studies from our laboratory demonstrated that prostatic stromal fibroblasts and epithelial cells secrete several leukotactic proteins [6-8] (Table I). Therefore, we sought to determine whether PBMCs comprising predominantly T-lymphocytes would demonstrate chemotaxis towards cultured prostate stromal fibroblasts and/or epithelial cells. The results of these studies showed that media conditioned by primary prostate stromal fibroblasts derived from four different individuals attracted 20–60% more PBMCs than unconditioned media (Fig. 4C). Migration assays similar to those described above using modified Boyden chambers demonstrated >3-fold migration of HH or Molt-3 cells towards PC3 or N15C6 cells (Fig. 4D). To test whether lymphoid-derived leukocytes could modulate prostate epithelial cells proliferation, non-transformed N15C6 or BPH-1, or transformed LNCaP or PC3, prostate epithelial cells were co-cultured for 24 hr with media (as control) or with HH or Molt-3 cells (or no cells, as a control), then counted. These studies showed that both HH and Molt-3 T-lymphocytes promoted the proliferation of PC3 transformed prostate epithelial cells, whereas only HH T-lymphocytes promoted the proliferation of N15C6 and, to a lesser extent, BPH-1 cells (Fig. 5A). Taken together, this data suggested that lymphoid lineage leukocyte HH and Molt-3 cells promoted the proliferation of both non-transformed and transformed prostate epithelial cells.

dHL-60N and dHL-60M cells are terminally differentiated myeloid cells, and therefore proliferate poorly after differentiation. Therefore, the reciprocal effect, if any, of prostate epithelial cells on the proliferation of these myeloid lineage leukocytes could not be assessed. However, this was not the case for the lymphoid-derived HH and Molt-3 leukocytes. The proliferation of these cells was assessed after 24 and 96 hr of growth in the presence or absence of each of the four prostate epithelial cell lines used in these studies. These studies showed that HH T-lymphocytes proliferated significantly better in the presence of non-transformed BPH-1 as well as transformed LNCaP and PC3 cells than in their absence, but were growth-inhibited in the presence of non-transformed N15C6 cells. Molt-3 T-lymphocytes proliferated significantly better in the presence of all four prostate epithelial cell lines tested than in their absence (Fig. 5B). These studies showed that the

proliferation of HH and Molt-3 lymphoid-derived leukocytes was largely promoted by interaction with both non-transformed and transformed prostate epithelial cells.

As noted above, the myeloid-lineage dHL-60N and dHL-60M both promoted the proliferation of transformed PC3 cells (Fig. 3D). In contrast, the HH T-lymphocytes promoted the proliferation of both non-transformed N15C6 and transformed PC3 cells whereas Molt-3 T-lymphocytes promoted only PC3 cell proliferation (Fig. 5A). The secretomes of these four leukocytic cell types differed substantially, suggesting that the secretion of different cytokines by these cells might mediate epithelial proliferative responses (Figs. 1 and 4). To address this, the secreted proteins levels of specific cytokines identified as highly upregulated by the leukocytic cells were assessed by ELISA. As shown in Table II, the high levels of CCL5 expression by dHL-60M and HH T-lymphocytes detected by antibody array was confirmed and quantified at 401 pg/ml/million cells. High levels of CCL2 secretion by dHL-60N and dHL-60M cells was also confirmed and quantified at 272 and 352, respectively, pg/ml/million cells. Both N15C6 and PC3 cells were then tested to determine whether they would respond proliferatively to either CCL2 or CCL5. PC3 cells failed to proliferate in response to the same levels of the same two cytokines (data not shown). However, as shown in Figure 6, treatment of N15C6 cells with both of these cytokines elicited a modest but reproducible proliferative response at levels 20–40% above that achieved in the absence of cytokine. Although N15C6 cells proliferated in response to HH T-lymphocytes (Fig. 5A), which secreted high levels of CCL5 (Table II), they had failed to proliferate in response to dHL-60N (Fig. 3D), which secreted high levels of CCL2 and CCL5 (Table II), or to dHL-60M (Fig. 3D), which secreted high levels of CCL2 (Table II). These data suggested that, although particular cytokines may promote prostate epithelial cell proliferation in isolation in vitro, this effect may be tempered or abrogated by opposing endogenous (epithelial) or exogenous (e.g., leukocytic or stromal) secreted factors in vivo.

DISCUSSION

The studies reported here were intended to test the hypothesis that leukocytes associated with either acute or chronic inflammation potentially attracted to the prostate consequent to aging or tumorigenesis may act to promote the abnormal cellular proliferation associated with BPH and PCa. Previous studies from our laboratory and others revealed that multiple leukoattractant cytokines, including CXCL1, CXCL2, CXCL5, CXCL6, CXCL8, CXCL12, CCL2, and CCL5, were secreted by aging primary prostate stromal fibroblasts [6-8,29]. Such data are consistent with the idea of a “stromal address code” that attracts and trafficks multiple leukocytic cells types to the aging prostate [30]. We have also showed that prostate epithelial cells secrete some of these same chemokines [6-8]. Therefore, cytokine secretion by both prostate stromal and epithelial cells could serve as leukoattractants for various cells involved in both acute and chronic inflammatory responses. Moreover, all of the aforementioned cytokines have been shown to promote the proliferation of both prostate epithelial and stromal cells [6-8,29]. This suggests that the secretion of the cytokines by prostate stromal cells, epithelial cells, or infiltrating leukocytes could serve as growth factors to promote the proliferation of all of these cell types in the prostate.

Data reported in this study showed that non-transformed N15C6 and BPH-1, transformed LNCaP and PC3 cells, primary prostate stromal fibroblasts and dHL-60N secrete the neutrophilic chemokines CXCL5 and CXCL8 at levels sufficient to promote dHL-60N migration and sequestration. Moreover, data presented here suggested that one or more CXCR2 agonists mediating dHL-60N migration were likely secreted at higher levels by PC3 compared to N15C6 cells. Previous studies from our laboratory showed that PC3 cells secreted the neutrophilic CXCR2 agonist CXCL5 at levels an order of magnitude higher

than N15C6 cells [7], while data presented here shows that PC3 cells also secrete higher levels of CXCL8 than N15C6 cells. Previous data from our laboratory also showed that primary and metastatic prostate tumors expressed significantly higher levels of CXCL5 than normal benign or hyperplastic glands, while Murphy et al. reported similar data for CXCL8 expression in the prostate [7,31]. Other studies have reported that the majority of proteins comprising corpora amylacea and calcified amylacea are actually specific to neutrophil granules, and suggests that prostate-infiltrating neutrophils may contribute to the etiology of prostate carcinogenesis [32]. Thus, secretion of neutrophilic chemokines, especially by PCa cells, may account for histological evidence for neutrophilic infiltration associated with malignant glands in the prostate [5,7,32,33]. Further, both neutrophilic chemokines secreted by PCa cells and leukocytes differentiated along a neutrophilic lineage preferentially promote the proliferation of malignant prostate epithelial cells [7,31,34,35]. Taken together, these data suggest that leukocytes associated with acute inflammatory responses are attracted to the prostatic microenvironment and may play a role in prostate carcinogenesis.

ELISA showed that none of the prostate epithelial cells tested appreciably secreted a major macrophilic chemokine, CCL2. However, both dHL-60N and dHL-60M cells terminally differentiated along neutrophilic and macrophagic lineages, respectively, secreted high levels of CCL2, as did the primary prostate fibroblast cells. These data suggest that infiltrating neutrophils and macrophages, as well as the prostatic stroma, may provide a strong macrophilic environment. As recently reviewed by Guruvayoorappan [36], tumor-associated macrophages (TAMs) have been observed as diffusely distributed throughout tumors, within tumor zones and tumor edges, around ductal areas, and in the tumor stroma, yet a role for TAMs in cancer progression is controversial. Nevertheless, immunohistochemical studies have demonstrated that patients with a high TAM volume density had a significantly shorter median PCa specific survival time than patients with lower TAM volume density, suggesting that macrophage infiltration is associated with prostate tumor growth [37]. Moreover, studies using mouse models have shown that systemic administration of anti-CCL2 neutralizing antibodies significantly retarded prostate tumor growth and attenuated CD68+ macrophage infiltration *in vivo*, which was accompanied by a significant decrease in microvascular density, suggesting that CCL2-mediated macrophagic infiltration contributed to PCa [38]. These studies are consistent with data reported here, that both LNCaP and PC3 transformed PCa cell lines responded proliferatively to dHL-60M cells terminally differentiated along a macrophagic lineage. Taken together, these data suggest that monocytic/macrophagic cells may be attracted to the prostatic microenvironment and can promote the proliferation of transformed prostate epithelial cells, thus, may promote prostate tumor growth.

Studies described here showed that primary prostate stromal fibroblasts secrete proteins chemotactic for primary T-lymphocytes (PBMCs) as well as immortalized CD4+ and CD8+ T-lymphocyte cell lines. Both non-transformed and transformed prostate epithelial cells also secrete proteins chemotactic for immortalized T-lymphocytes. Although many chemotactic proteins are secreted in common by prostate stromal fibroblast and epithelial cells, some are exclusively secreted by stromal fibroblasts. In particular, prostate stromal fibroblasts, but not epithelial cells, secrete CXCL12 [6,8] (Table I). CXCL12 is a known leukoattractant for T-lymphocytes, which express the major cognate receptor for this chemokine, CXCR4 [39]. Other leukoattractant protein secreted in common by prostate stromal fibroblasts and epithelial cells include CCL2, CCL5, and CXCL8 (Table I). Using modified Boyden chamber assays similar to those used in the current study, Zang et al. [40] showed that T-lymphocytes migrated in response to CCL2, CCL5, and CXCL8 at levels of 0.1–1.0 ng/ml, which are similar to those shown here secreted by prostate epithelial cells (Table I). The receptors for these chemokines—CCR5 for CCL2 and CCL5, CXCR2 for CXCL8 and CXCR4 for CXCL12—are all robustly expressed by T-lymphocytes [39–41]. Another

observation from the current study is that the proliferation of HH and Molt-3 T-lymphocytes was promoted by the presence of prostate epithelial cells (with the exception that HH cells did not proliferate in the presence of N15C6 cells). Taken together, these studies suggest that several cytokines and chemokines secreted by either or both prostate stromal fibroblasts and epithelial cells may encourage T-lymphocyte infiltration, survival, and proliferation. If so, the prostate, especially the more secretory aging prostate, may disrupt homeostatic mechanisms that normally function to regulate T-lymphocyte localization and population size. T-lymphocyte numbers are tightly regulated in vivo such that clonal expansion of CD8⁺ cells during the course of viral infection is followed by an equivalent contraction in cell number upon resolution of the infection [42,43]. Apoptosis-driven T-cell contraction can be suppressed by CCL2, which has been shown to promote T-cell survival, especially antigen-activated CD8⁺ T cells [44]. Nanomolar levels of CCL5 stimulate T-lymphocyte proliferation and cytokine production, whereas higher micromolar levels results in CCL5 oligomerization and T cell apoptosis [45]. Thus, the expression of low levels of both CCL2 and CCL5 by prostate epithelial and/or aging stromal fibroblast cells may function to promote T-lymphocyte sequestration and proliferation in the prostate.

The studies reported here also showed that predominantly CD8⁺ Molt-3 cells promote the proliferation of malignant PC3 prostate epithelial cells, whereas CD4⁺ HH cells promote the proliferation of PC3 as well as non-transformed N15C6 and, to a lesser extent, BPH-1 prostate epithelial cells in vitro. These observations suggest that T-lymphocytic infiltration in the prostate may contribute to the overly proliferative phenotype characteristic of both BPH and PCa in vivo. Recent work reported by Ebelt et al. [46] showed that lymphocytic infiltrate in human prostate tissues was distributed in a dispersed pattern in hyperplastic epithelium but as peri-tumoral clusters in malignant tissues. The T-lymphocyte subsets most often observed were CD3⁺ and CD4⁺, with few CD8⁺ cells noted [46]. The observation of CD4⁺, rather than CD8⁺, cells reflects a growing, although imperfect, consensus that tumor infiltration by CD8⁺ T cells predicts a positive outcome, while CD4⁺ cells predict a negative outcome [47]. Of note, the CD8⁺ Molt-3 T-lymphocytic cells used in the current study failed to promote prostate epithelial cell proliferation to the extent observed for the CD4⁺ HH T-lymphocytic cells, an observation that is consistent with potential differences in anti-tumor activities for CD8⁺ versus CD4⁺ effector T cells.

It is likely that the tissue microenvironment within the human prostate in vivo is complex and comprises diverse interactions between multiple cell types. Though based on a simple model system, the in vitro studies pursued here illustrate some aspects of this complexity. For example, data described here showed that conditioned media from PF1 primary prostate stromal fibroblasts modulated the migration of neutrophilic dHL-60N and macrophagic dHL-60M cells towards prostate epithelial cells. This modulation was neither entirely agonistic nor antagonistic, but depended upon the identity and, likely, the secretome of the epithelial component of the microenvironment. Other data showed that N15C6 cells failed to proliferate in response to dHL-60N or dHL-60M cells even though both leukocytic cell lines secreted robust levels of CCL2 and CCL5, two cytokines that separately induced proliferative responses from N15C6 cells. As with the stromal/epithelial interactions described above, this data suggests that interactions between leukocytic and prostate epithelial cells may be agonistic or antagonistic, with the exact relationship likely dependent upon the medley of secreted factors that act to stimulate or repress cellular proliferation in the prostate. The data reported here also demonstrated that all of the leukocytic cell types tested—neutrophilic, macrophagic, and lymphocytic—stimulated the proliferation of malignant prostate epithelial cells, but only lymphocytic cells clearly stimulated the proliferation of both transformed and non-transformed prostate epithelial cells. These data suggest that, during the course of tumorigenesis, PCa cells may acquire the ability to respond proliferatively to a host of cytokines secreted by immune cells, an ability that is not

developed in non-transformed cells. Alternatively, this data may suggest that non-transformed cells successfully repress proliferative signals from inflammatory cells, and that this ability may be lost in cancer cells.

CONCLUSIONS

In conclusion, the studies reported here using an in vitro model system designed to partially mimic the prostate tissue microenvironment provide initial information regarding leukocytic trafficking to the prostate and demonstrate that inflammatory cells can selectively promote the proliferation of non-transformed or transformed prostate epithelial cells. These studies are consistent with differential role(s) for inflammatory infiltrate in the etiologies of benign and malignant proliferative disease in the prostate. The results of these studies warrant the development of in vivo models to accurately recapitulate interactions between inflammatory infiltrate, prostatic stroma (including fibroblastic, myofibroblastic, and endothelial cells) and glandular prostatic epithelium that may contribute to the development of benign and malignant proliferative disease in the prostate.

Acknowledgments

This work was supported by awards from the NIDDK/NIH George M. O'Brien Center for Urologic Research at the University of Michigan 1 P50 DK065313 (J.A.M.), NIH/NIDDK 1 R01 DK081841 (J.A.M.), and NIH/NIDDK 2T32DK007758 (J.A.M., K.L.M.).

Grant sponsor: NIDDK/NIH George M. O'Brien Center for Urologic Research; Grant number: 1 P50 DK065313; Grant sponsor: NIH/NIDDK; Grant numbers: 1 R01 DK081841, 2T32DK007758.

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Fig. 1. Terminally differentiated neutrophils (dHL-60N) and macrophages (dHL-60M) secrete chemotactic factors. **A:** Photomicrographs (400x) of HL-60 promyelocytic cell morphology compared to that of the same cells promoted to terminally differentiate along a granulocytic (neutrophilic; dHL-60N) lineage or along a monocytic/macrophagic (dHL-60M) lineage. **B:** Autoradiograph of RayBio® Human Cytokine Antibody Array after incubation with complete media or media conditioned by HL-60, dHL-60N, or dHL-60M cells. Four of the more robustly dHL-60N or dHL-60M secreted cytokines (CCL2, CCL5, CXCL8, TIMP-1) are indicated. “P” specifies positive control and “N” specifies negative control. **C:** Chart displaying cytokines detected in media conditioned by HL-60, dHL-60N, dHL-60M or in unconditioned complete media.

Fig. 2.

CXCL5 and CXCL12 are chemotactic for dHL-60N and dHL-60M. Diagram showing experimental schema for modified Boyden Chamber assays. Leukocytes were seeded into the upper chamber and separated from the bottom chamber by a porous (3 μ m for dHL-60N, 8 μ m for dHL-60M membrane). Vehicle, chemokine, or prostate cells (epithelial or fibroblast) were seeded into the lower chamber. Assays measured movement of leukocytes plated in upper chamber through the membrane towards chemoattractants in the bottom chamber as well as the proliferative response of cells plated in the bottom chamber. **A:** Basal levels of chemotaxis towards vehicle in bottom chamber (negative control); **(B)** chemotaxis towards chemokine in bottom chamber (positive control); **(C)** basal levels of prostate epithelial cell proliferation; **(D)** chemotaxis of leukocytes towards cells in bottom chamber; prostate epithelial cell proliferation in the presence of leukocytes. **E:** Chemotactic migration of dHL-60N or dHL-60M cells towards increasing concentrations of CXCL5. CCL2 is included as a positive control for dHL-60M; CXCL8 as a positive control for dHL-60N. CXCL5 is strongly chemotactic for dHL-60M, less so for dHL-60N. **F:** Chemotactic migration of dHL-60N or dHL-60M cells towards increasing concentrations of CXCL12. CCL2 is included as a positive control for dHL-60M; CXCL8 as a positive control for dHL-60N. CXCL12 is strongly chemotactic for dHL-60M, less so for dHL-60N. * Indicates significantly ($P < 0.05$) higher levels of cellular migration compared to vehicle control.

Fig. 3.

dHL-60N and dHL-60M chemotaxis and proliferation are promoted by prostate stromal and epithelial cells. **A:** Chemotactic migration of dHL-60N cells towards vehicle (set as equal to onefold) or towards N15C6 or BPH-1 prostate epithelial cells seeded into chambers containing complete media with vehicle, 5 ng/ml CXCL8, or complete media conditioned by PF1 primary prostate stromal fibroblasts. PF1 conditioned media negatively modulated the migration of dHL-60N towards N15C6, but not BPH-1, cells. * Indicates significantly higher and # indicates significantly lower levels of cellular migration ($P < 0.05$). **B:** Chemotactic migration of dHL-60M cells towards N15C6 or BPH-1 prostate epithelial cells seeded into chambers containing complete media with vehicle (set as equal to onefold), 2 ng/ml CCL2, or towards complete media conditioned by PF1 primary prostate stromal fibroblasts. PF1 conditioned media negatively modulated the migration of dHL-60M towards BPH-1, but not N15C6, cells. * Indicates significantly higher and # indicates significantly lower levels of cellular migration ($P < 0.05$). **C:** N15C6 or transformed PC3 prostate epithelial cells were seeded into chambers pre-treated with antibody against CXCR2, CXCR4, or an IgG control. Chemotactic migration of dHL-60N cells towards N15C6 cells was inhibited by pre-treatment with antibody against CXCR2, but not by pre-treatment with antibodies against CXCR4 or the IgG control. Chemotactic migration of dHL-60N towards PC3 cells was not inhibited by pre-treatment. * Indicates significantly higher and # indicates significantly lower levels of cellular migration ($P < 0.05$). **D:** The proliferation of transformed PC3 and LNCaP or non-transformed N15C6 or BPH-1 cells was measured after 24 hr exposure to dHL-60N (left) or dHL-60M (right) or vehicle. PC3 cells proliferated significantly in response to the presence of dHL-60N cells, whereas both PC3 and LNCaP cells proliferated significantly in response to the presence of dHL-60M cells ($*P < 0.05$).

Fig. 4.

Prostate stromal fibroblast and epithelial cells promote T-lymphocyte chemotaxis. **A:** Autoradiograph of RayBio® Human Cytokine Antibody Array after incubation with complete media or media conditioned by CD4⁺ HH and CD8⁺ Molt-3 T-lymphocytes. Two of the more robustly secreted cytokines, CCL5 and TIMP-1, are indicated. “P” specifies positive control and “N” specifies negative control. **B:** Chart displaying cytokines detected in media conditioned by CD4⁺ HH and CD8⁺ Molt-3 T-lymphocytes or in unconditioned complete media. **C:** Chemotactic migration of largely T-lymphocytic PBMCs towards SF RPMI (control), serum-free HIE (SF) media, or SF media conditioned by PF2, PF3, PF4, or PF5 primary prostate stromal fibroblast cultures measured at 30 and 60 min was measured using a modified Boyden chamber assay. 20–60% more PBMCs migrated towards the SF media conditioned by primary prostate stromal fibroblast cultures than SF media alone. **D:** Chemotactic migration of CD4⁺ HH (left) or CD8⁺ Molt-3 (right) T-lymphocytes cells towards vehicle (set equal to onefold), towards N15C6 or PC3 prostate epithelial cells seeded into chambers containing complete media with vehicle or with 5 ng/ml CXCL8. Both CD4⁺ HH and CD8⁺ Molt-3 T-lymphocytes migrated robustly towards the prostate epithelial cells. No additive migratory effect was observed for wells containing both prostate epithelial cells and CXCL8 (**P* < 0.05).

Fig. 5.

Prostate epithelial cells and T-lymphocytes promote mutual proliferation. **A:** The proliferation of transformed PC3 (black diamonds) and LNCaP (gray diamonds) or N15C6 (black squares) or BPH-1 (gray squares) non-transformed prostate epithelial cells was measured after 24 hr exposure to CD8+ Molt-3 (left) or CD4+ HH (right) T-lymphocytes, or vehicle. PC3 cells proliferated significantly better in response to the presence of CD8+ Molt-3 cells, whereas PC3, N15C6, and BPH-1 cells proliferated significantly better in response to the presence of CD4+ HH cells (* $P < 0.05$). **B:** CD8+ Molt-3 T-lymphocytes remained viable and proliferated significantly better in the presence of PC3 (black diamonds) and LNCaP (gray diamonds) transformed or N15C6 (black squares) or BPH-1 (gray squares) non-transformed prostate epithelial cells than in the absence of prostate cells (white squares) (left), whereas CD4+ HH T cells remained viable and proliferated significantly better in the presence of PC3 (black diamonds) and LNCaP (gray diamonds) transformed or BPH-1 (gray squares) non-transformed prostate epithelial cells than in the absence of prostate cells (white diamonds) (right) (* $P < 0.05$).

Fig. 6. CCL2 and CCL5 promote the proliferation of N15C6 prostate epithelial cells. N15C6 treated with vehicle (0) or increasing doses of CCL2 (**A**) or CCL5 (**B**) for 24 hr proliferate to levels 20–40% above that achieved with vehicle. PC3 cells did not proliferate in response to the same conditions (not shown).

TABLE I

Prostate Cell Chemokine Secretion

	CXCL5 (ENA-78)	CXCL8 (IL-8)	CXCL12 (SDF-1)	CCL2 (MCP-1)
N15C6	9 (± 1.1)	10 (± 0.8)	0	0
BPH-1	565 (± 70.4)	81 (± 5.5)	0	18 (± 3.2)
22RV1	0	0	0	0
LNCaP	0	0	0	0
PC3	47 (± 3.4)	34 (± 3.3)	0	0
DUI45	28 (± 6.3)	94 (± 6.3)	0	0
N1	1,935 (± 107.3)	311 (± 24.1)	28 (± 5.5)	3 (± 0.1)
PF1	1,090 (± 40)	681 (± 58.7)	32 (± 0.1)	875 (± 177.5)

pg/ml/million cells ($\pm$ standard deviation from the mean).

TABLE II
Leukocytic Chemokine Secretion

	CCL5 (RANTES)	CCL2 (MCP-1)	CXCL8 (IL-8)
dHL-60M	401 (± 7.0)	352 (± 73.5)	775 (± 41.7)
dHL-60N	8.5 (± 0.7)	272 (± 28.3)	151 (± 17.7)
HL-60	68 (± 1.4)	52 (± 4.2)	70 (± 2.8)
Molt-3	0	0	3 (± 0.7)
HH	432 (± 21.2)	0	4 (± 0.0)
Media	0	0	5 (± 0.7)

pg/ml/million cells ($\pm$ standard deviation from the mean).