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Elevated Epithelial Expression of Interleukin-8 Correlates with a Myofibroblast Reactive Stroma in Benign Prostatic Hyperplasia

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Abstract

Objectives—Numerous inflammatory diseases display elevated IL-8, and most are associated with a reactive stroma. IL-8 expression is also elevated in benign prostatic hyperplasia (BPH) yet little is known about reactive stroma in BPH. Whether a reactive stroma response exists in BPH, whether this correlates with elevated IL-8, and whether IL-8 can induce a reactive stroma phenotype has not been determined. This study was designed to specifically address these issues.

Methods—Normal prostate transition zone (NPTZ) tissue and BPH specimens, as identified by BCM Pathology, were examined by quantitative immunohistochemistry to correlate IL-8, smooth muscle α -actin (α -SMA), vimentin, calponin, and tenascin-C. In addition, human prostate stromal cell cultures were used to evaluate the effect of IL-8 on expression of reactive stroma biomarkers.

Results—BPH nodules exhibited elevated epithelial IL-8 immunoreactivity, and this correlated with elevated α -SMA, reduced calponin and altered deposition of tenascin-C, relative to NPTZ tissue ($p < 0.05$). Multiple vimentin-positive prostate stromal fibroblast cultures were induced by IL-8 to also co-express α -SMA and tenascin-C, typical of a reactive stroma myofibroblast phenotype.

Conclusions—These data show that BPH reactive stroma is fundamentally different from normal prostate fibromuscular stroma, and is typified by the emergence of a reactive stroma myofibroblast phenotype. This reactive stroma pattern correlated spatially with elevation of IL-8 in adjacent epithelium. Additionally, IL-8 induced expression of myofibroblast markers in human prostate fibroblasts *in vitro*. These studies suggest that IL-8 acts as a regulator of BPH reactive stroma and is therefore a potential therapeutic target.

MeSH Keywords

Benign prostatic hyperplasia; Stromal; alpha-Actin; Calponin; Extracellular Matrix; Interleukin-8; Prostate

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Introduction

BPH affects the periurethral region, inducing glandular and stromal nodules within the transition zone,^{1,2} resulting in urinary obstruction^{3,4}. This may involve a co-evolution of reactive stroma, similar to wound repair stroma⁵⁻⁸. In wound repair, stromal cells effect matrix remodeling, ECM deposition, and growth factor expression^{5,6}. Co-localization of reactive stroma biomarkers and whether this is associated with altered chemokine expression has not been studied in BPH. Similar to other fibroses⁹⁻¹², BPH exhibits elevated interleukins, including IL-8, released by epithelial cells among others¹³⁻¹⁷. IL-8 message is elevated in prostate cancer and BPH¹⁸ and is expressed in epithelial cells *in vivo*¹⁴, and *in vitro*¹⁵. IL-8 is a neutrophil chemoattractant that stimulates proliferation and migration¹⁹. IL-8 is therefore a candidate for coordination of reactive stroma biology. The present study addresses whether BPH is associated with a specific reactive stroma phenotype, whether this correlates with elevated IL-8 and whether IL-8 can induce prostate stromal cells to a reactive phenotype.

We show that IL-8 immunoreactivity is significantly elevated in BPH epithelium, and is spatially correlated with a reactive stroma composed primarily of myofibroblasts. Moreover, IL-8 induced human prostate stromal cells *in vitro* to a myofibroblast phenotype with elevated reactive stroma-associated biomarkers.

Materials and Methods

Tissues

Flash-frozen transition zone specimens from untreated cancer patients who underwent RRP, identified as NPTZ (N=30,28 evaluable) or BPH (N=50,all evaluable) by BCM Pathology (courtesy: Dr. Thomas Wheeler), were thawed in 4% sucrose and processed for immunohistochemistry⁶. NPTZ tissue was selected based on anatomical position, normal glandular histology, and no pathological evidence of nodular hyperplasia or carcinoma. Selection criteria for BPH nodules followed previously published histopathological criteria²¹. E1 nodules: infolded acini with thickened, columnar epithelium and an equivalent contribution of stroma. E2 nodules: large, non-infolded acini with narrow, cuboidal epithelium and less stromal contribution. E1/E2 nodules: an inseparable mix of E1 and E2 types. S nodules: spherical fields of stromal cells with thickened vessel intimae and no identifiable acini. S/E nodules: S nodules containing 5-10% epithelial acini.

Immunohistochemistry

Antibodies specific for m-SMA, calponin, vimentin and tenascin-C were used as published previously⁶. Antibodies to IL-8 (ab16223) and r-SMA (ab32575) were from Abcam (Cambridge,MA). Secondary antibodies included: biotinylated-goat-anti-mouse (D-20691) and biotinylated-goat-anti-rabbit (D-20695;Molecular-Probes,Eugene,OR); FITC-anti-mouse, TexasRed-anti-goat⁶ and RRX-donkey-anti-rabbit (711-295-152;Jackson-ImmunoResearch-Laboratories,West Grove,PA). No significant staining occurred with secondary alone.

Immunohistochemistry utilized the MicroProbe Staining System⁶ (FisherBiotech, Pittsburgh,PA), and Open BioSystems reagents (Huntsville,AL). Antigen retrieval was performed as follows: vimentin⁶; tenascin-C, 0.1% Pronase (3min,50°C); IL-8, steamed in Citrate buffer 20min and cooled 20min. Sections were blocked and incubated with primary antibodies to: m-SMA, calponin, vimentin, tenascin-C (as described previously⁶), r-SMA 1:500 and IL-8 1:50 (both;1hr,37°C). Sections for quantitative IHC were incubated with secondary antibodies: biotinylated-goat-anti-mouse 1:500, 45min at 37°C

(vimentin&tenascin-C) and 4min at 50°C (m-SMA&calponin), biotinylated-goat-anti-rabbit 1:500, 1hr at 37°C and processed for DAB-based detection ⁶.

Dual-Label Immunofluorescence

Sections were blocked (5% serum, 15min, RT) and incubated with mouse primary as described above, except for calponin (1:1000), followed by FITC-anti-mouse (1:50, 45min, 37°C). Sections were incubated with rabbit primaries as described above, then with RRX-anti-rabbit (1:50, 45min, 37°C) and imaged as published previously ⁶.

Histological and Statistical Analysis

Quantitative IHC followed procedures we published previously ^{6,20}. For vimentin, α -SMA, calponin, and tenascin-C, the percentage of positive-staining cells was scored on a scale of 0-3 at X100: 0=0%; 1=5-25%; 2=26-50%; and 3=51-100% positive cells. To evaluate IL-8, individual staining intensity scores (0-2) were assigned at two magnifications (X100&X400), and a summed score (0-4) was determined as follows: 0=no specific staining at either magnification; 1=specific staining above background but of low intensity at either X100 or X400, but not both; 2=low intensity staining above background at both X100 and X400; 3=high intensity staining at either X100 or X400, but not both; 4=high intensity staining at both X100 and X400. For NPTZ, mean marker scores were determined on 5 serial sections using 3 fields/slide containing epithelial acini. Each BPH nodule type was scored from 5 serial sections/patient.

Spearman correlation coefficients were calculated to determine whether correlations existed between mean marker scores in NPTZ or BPH specimens. To exclude the possibility that mean marker scores were affected by inevaluable fields, Spearman coefficients between each marker mean and the number of fields evaluated, and the percentage of total fields tested, were also calculated. Mann-Whitney Tests were used to evaluate differences in marker means between NPTZ and BPH specimens. Multivariate regression with interaction was used to verify whether the number or percentage of fields affected the observed significant differences. Distributions are illustrated using box-and-whisker plots. P values <0.05 were considered statistically significant. All analyses were performed using SPSS 12.0 (SPSS Inc., Chicago, IL).

Cell Lines and Immunocytochemistry

Human prostate stromal cells (HPS-19I; HTS-2T) were prepared and passaged as published previously ⁶. PrSC (prostate stromal cells), Lonza, Inc. (Basel, Switzerland), were maintained according to manufacturer's guidelines. To examine the effects of IL-8, cells (passage 6-12) were seeded (15×10^3 cells/well) onto coverslips, incubated overnight and switched to M_0 ⁶. Cells were incubated in M_0 +4nMIL-8 or M_0 +vehicle (PBS + 1 % BSA) for 72hr, fixed and processed for ICC ⁶. After blocking, primary antibodies were applied (1hr, 37°C): vimentin, 1:50 (sc-7557; Santa Cruz); FITC-conjugated- α -SMA, 1:200 (F3777; Sigma); calponin, 1:10,000 and tenascin-C, 1:4000 (as above). Coverslips were washed, incubated with secondary antibodies (1:200, 45min, 37°C), and images acquired.

Western Analysis

Cells were seeded (HPS-19I= 7.3×10^4 cells/25cm² flask; HTS-2T= 6.5×10^4 cells/flask 25cm²; PrSC= 7.1×10^4 cells/25cm² flask), incubated overnight and switched to M_0 ⁶. Cells were incubated in M_0 +4nMIL-8, or M_0 +vehicle (PBS + 1 % BSA) and cultured for 48hr or 72hr, then lysed (500 μ l RIPA) as previously described²². Lowry analysis (Bio-Rad, Hercules, CA), electrophoresis (10% gel) of control-adjusted protein (0.5-2 μ g) and transfer (PVDF) were performed. Immuno-blotting using m-SMA (1:3000, see above) or GAPDH (1:3000, Calbiochem, clone 6C5) for 2hr, biotinylated-donkey-anti-mouse (1:1000, see above)

for 1hr, and Streptavidin-HRP (1:5000, Amersham) for 30min was followed by detection/exposure, as previously described²². Quantitative analysis utilized NIH-ImageJ.

Results

Immunolocalization of IL-8 and Stromal Markers

Figure 1 shows representative dual-label immunoreactivity patterns for combinations of α -SMA, calponin, tenascin-C and IL-8 in serial sections of BPH and NPTZ specimens. NPTZ exhibited diffuse distribution of tenascin-C (Fig. 1A, green), and no obvious IL-8 immunofluorescence (Fig. 1B&C, red). In contrast, BPH nodules displayed altered and more focal tenascin-C deposition (Fig. 1D, green) in the periacinar stroma associated with intense epithelial IL-8 immunofluorescence (Fig. 1E&F, red). Tenascin-C is an ECM glycoprotein associated with reactive stroma²³. The immediate periacinar stroma in NPTZ exhibited co-localized calponin (Fig. 1G, green) and α -SMA (Fig. 1H, red), typical of smooth muscle (Fig. 1I, overlap-orange). In contrast, calponin was undetectable (Fig. 1J, green) in BPH stroma that was α -SMA-positive (Fig. 1K, red), typical of wound repair myofibroblasts (Fig. 1L)^{6,24}.

Quantitative Analysis

Serial sections (N=50 BPH & N=28 NPTZ) were evaluated using quantitative IHC. NPTZ epithelium exhibited background IL-8 immunoreactivity (Fig. 2A), and periacinar stroma stained intensely for calponin (Fig. 2B). In contrast, high IL-8 immunoreactivity was observed in BPH epithelium (Fig. 2C), with undetectable (background) stromal calponin (Fig. 2D).

To quantitate and establish significance, immunoreactivity was scored as described in *Materials and Methods*. Initially, Spearman correlations were calculated to analyze fluctuations within (not between) each tissue type. No statistically significant correlations occurred within inter-marker fluctuations in NPTZ, nor within S nodules (*data-not-shown*). However, within E1, E2 and E1/E2 BPH nodules, fluctuations in IL-8 positively-correlated with tenascin-C fluctuations, and in S/E nodules fluctuations in α -SMA inversely-correlated with vimentin fluctuations (*data-not-shown*).

Mann-Whitney analysis was used next to compare immunoreactivity scores between BPH and NPTZ (Fig. 2E-H). A significant increase in epithelial IL-8 summed score was observed in all epithelial BPH nodule types when compared to NPTZ (Fig. 2E, $p < 0.05$). A significant decrease in calponin-positive stromal cells was observed in all BPH nodule types when compared to NPTZ (Fig. 2F, $p < 0.05$). A significant increase in α -SMA-positive stromal cells was observed in all epithelial nodule types, except S/E, when compared to NPTZ (Fig. 2G, $p < 0.05$). Tenascin-C and vimentin immunoreactivity exhibited no significant quantitative differences between NPTZ and epithelial BPH nodule types (Fig. 2H), although tenascin-C distribution adjacent to acini was noticeably different in BPH (*compare*: Fig. 1D&F&Fig. 1A&C). There was, however, a significant tenascin-C decrease in S nodules (Fig. 2H, $p < 0.05$) where IL-8 levels were undetectable (Fig. 2E).

Since some fields within BPH and NPTZ specimens were inevaluable for some markers, we wanted to exclude the possibility that neither the number of fields evaluated per slide, nor the percentage of fields that stained, affected the reliability of statistical analysis. By Spearman analysis, no such correlations resulted in NPTZ specimens. In BPH nodule types where a significant Spearman correlation did occur between the marker mean and the number or percentage of nodules analyzed, multivariate analysis with interaction was performed. All Mann-Whitney differences in marker scores between NPTZ and BPH specimens remained significant on multivariate analysis ($p < 0.01$; *data-not-shown*).

IL-8 is associated with general inflammatory responses in nearly every disorder examined.^{12,19} Accordingly, we evaluated leukocytic infiltration using a representative set of 10 BPH and NPTZ tissue sections and observed no association between IL-8 overexpression and infiltration (*data-not-shown*).

IL-8 Induces a Myofibroblast Phenotype

Under vehicle conditions, PrSC were positive for vimentin (Fig.3A) and negative for α -SMA (Fig.3B&C), indicating a fibroblast phenotype. IL-8 treatment induced a myofibroblast phenotype in PrSC, with maintenance of vimentin (Fig.3D) and upregulation of filamentous α -SMA (Fig.3E&F). Interestingly, IL-8-treated PrSC exhibited a change in cell-morphology when filamentous α -SMA was induced (Fig.3E&F). Similarly, vehicle-treated HPS-19I were vimentin-positive (Fig.3G) and α -SMA-negative (Fig.3H) fibroblasts, while IL-8-treated HPS-19I maintained vimentin (Fig.3J) with α -SMA upregulation (Fig.3K&L). Conversely, vehicle-treated HTS-2T were positive for vimentin (red, Fig.3M) and α -SMA (green, Fig. 3N&O), indicating a myofibroblast phenotype. IL-8-treated HTS-2T showed no change in vimentin (Fig.3P) or α -SMA immunoreactivity (green, Fig.3Q,R).

By Western analysis, IL-8 treated PrSC and HPS-19I displayed an apparent increase in α -SMA immunoreactivity at 72hr, relative to vehicle, while HTS-2T showed equivalent α -SMA immunoreactivity whether vehicle or IL-8 was applied (Fig.3S). Under vehicle conditions, PrSC displayed low immunoreactivity for tenascin-C deposition (Fig.4A). IL-8 treatment induced a more focal and intense tenascin-C deposition in PrSC (Fig.4B), indicating a myofibroblast phenotype. Similarly, vehicle-treated HPS-19I were tenascin-C-negative (Fig. 4C), while IL-8-treated HPS-19I displayed tenascin-C deposition (Fig.4D). Conversely, HTS-2T displayed a diffuse and basal tenascin-C immunoreactivity whether treated with vehicle (Fig.4E) or IL-8 (Fig.4F). Tenascin-C ELISA using PrSC or HPS-19I cell-extracts yielded inconsistent tenascin-C upregulation (*data-not-shown*), similar to altered tenascin-C deposition in human BPH (Fig. 1D&F) that lacks any significant pattern upon quantitation (Fig. 2H).

Comment and Conclusions

Our data indicate that overexpression of IL-8 in BPH epithelium correlates with a periacinar reactive stroma phenotype. NPTZ is predominantly smooth muscle, evidenced by co-expression of α -SMA, an early-lineage marker, and calponin, a late-stage marker. Conversely, BPH stroma, periacinar to IL-8-positive epithelium, is characterized by a significant reduction in calponin immunoreactivity, a significant increase in α -SMA immunoreactivity, and by altered patterns of tenascin-C deposition. Of interest, a periacinar myofibroblast phenotype (with focal deposits of tenascin-C) correlated exclusively with BPH epithelial nodules that overexpress IL-8. Pure stromal nodules were negative for IL-8, consisted of fibroblasts, and displayed a significant decrease in tenascin-C and α -SMA immunoreactivity. These data suggest that differentiated smooth muscle is essentially replaced by an expansion of myofibroblasts in BPH periacinar stroma, most likely induced by elevated IL-8 expression in adjacent epithelial cells. These changes are consistent with a phenotypic shift to reactive stroma in prostate disorders^{5,6,8,25}, and in most fibroses associated with an inflammatory stimuli⁹⁻¹². We have reported that a myofibroblast-based reactive stroma co-evolves with PIN and well-differentiated prostate cancer foci^{6,20}. This suggests that a myofibroblast-based reactive stroma is common to both benign transition zone disease and neoplastic peripheral zone disease within the prostate.

Furthermore, this is the first report indicating IL-8 can induce human prostate fibroblast cultures to adopt a myofibroblast phenotype. Responsiveness to IL-8 was restricted to stromal cells with a fibroblast phenotype (vimentin-only). Notably, differentiated smooth muscle cells

(α -SMA-positive, calponin-positive) were refractory to the myofibroblast-inducing effect of IL-8. Moreover, IL-8 did not alter tenascin-C deposition in cells that were α -SMA-positive and calponin-positive (*data-not-shown*) prior to treatment.

Importantly, our data suggest an alternative role for IL-8 in the induction of reactive stroma within the paradigm of inflammatory modulation. IL-8 was recently identified as the most reliable, predictive surrogate marker in diagnosing BPH¹⁷. Accordingly, if IL-8 is both a key regulator of BPH reactive stroma and of inflammatory disorder, it may be a common target for the treatment of chronic inflammation and the symptoms of BPH.

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Abbreviations Key

BCM	Baylor College of Medicine
BPH	benign prostatic hyperplasia
DAPI	4',6 diamidino-2-phenylindole
ECM	extracellular matrix
FITC	fluorescein isothiocyanate
ICC	immunocytochemistry
IF	immunofluorescence
IHC	immunohistochemistry
IL-8	Interleukin-8

M₀	chemically defined culture medium (MCDB110 basal medium, 5 g/ml insulin, 5 g/ml transferrin, and 5 ng/ml sodium selenite)
NPTZ	normal prostate transition zone
PIN	prostatic intraepithelial neoplasia
RIPA	cell lysis buffer (1X PBS, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS)
RRP	radical retropubic prostatectomy; surgical resection of the prostate gland
RRX	Rhodamine Red-X succinimidyl ester
RT	room temperature
α-SMA	smooth muscle α -actin
m-SMA	mouse monoclonal antibody against α -SMA
r-SMA	rabbit monoclonal antibody against α -SMA

Fig. 1. Elevated IL-8 immunoreactivity in the epithelium associates with reactive stroma in BPH, but not with smooth muscle stroma in normal transition zone tissue

Serial NPTZ (A-C & G-I) and BPH (D-F & J-L) tissue sections were IF dual-labeled for: IL-8 (B,C,E,F; red) and tenascin-C (A,C,D,F; green), or α -SMA (H,I,K,L; red) and calponin (G,I,J,L; green) with overlap-expression in orange. Nuclei are labeled with DAPI (blue). Images are representative of typical staining patterns seen in BPH (N=5) and NPTZ (N=5) tissues. D-F & J-L at X400 in order to capture entire periacinar stroma; A-C & G-I at X600. All scale bars = 50 μ m.

Fig. 2. Quantitative IHC for IL-8 and reactive stroma biomarkers

Serial NPTZ (A,B) and BPH (C,D) tissue sections were IHC stained for: IL-8 (A&C) or calponin (B&D) and sections were counterstained with hematoxylin. Images are representative of typical IHC staining patterns seen in all BPH (N=50) and NPTZ (N=28) specimens. Images acquired at X600, all scale bars = 50 μ m. For quantitation (E-H), boxes in gray represent significant differences ($p < 0.05$) between tissue types. Data are represented as a distribution illustrated using a box-and-whisker plot, where the whiskers represent all data analyzed, excluding outliers (represented by black dots), the black line indicates the median, and the box represents 25% of data above and below the median. E, IL-8 is significantly elevated in all BPH nodules, except S nodules, when compared to NPTZ ($p < 0.05$). The summed score for

quantitation of IL-8 is shown for each tissue type listed. Data represent N = 28 NPTZ, 47 E1, 13 E2, 22 E1/E2, 12 S and 5 S/E specimens, respectively. **F**, Calponin is significantly lower in stromal cells in all BPH nodule types when compared to NPTZ ($p < 0.05$). Data represent N = 28 NPTZ, 45 E1, 14 E2, 23 E1/E2, 12 S and 5 S/E specimens, respectively. **G**, α -SMA is significantly different in stromal cells, when compared to NPTZ, in all BPH nodules except S/E nodules ($p < 0.05$). Data represent N = 28 NPTZ, 48 E1, 14 E2, 24 E1/E2, 11 S and 5 S/E specimens, respectively. **H**, Tenascin-C is significantly lower in S BPH nodules when compared to NPTZ ($p < 0.05$). Data represent N = 28 NPTZ, 47 E1, 14 E2, 23 E1/E2, 9 S and 5 S/E specimens, respectively. Vimentin exhibited no significant fluctuation between NPTZ and all epithelial BPH nodules (data not shown).

Fig. 3. IL-8 induces a myofibroblast phenotype in human prostate fibroblasts

PrSC cells (A-F), HPS-19I cells (G-L) and HTS-2T cells (M-R), human prostate stromal cells, were treated with IL-8 (38pM, 4nM & 28nM; 38pM shown) or vehicle (PBS + 1 % BSA) in M₀ and analyzed by IF ICC: vimentin (red) A,D,G,J,M&P and α-SMA (green) B,E,H,K,N&Q. Vehicle treated PrSC and HPS-19I cells displayed a fibroblast phenotype, as evidenced by expression of vimentin (A & G, respectively), and no expression of α-SMA (B & H, respectively). In contrast, IL-8 induced the myofibroblast phenotype, as evidenced by expression of α-SMA in PrSC (E, green) and HPS-19I (K, green) cells. HTS-2T cells, whether treated with vehicle (M-O) or IL-8 (P-R) were strongly positive for vimentin (red, M&P) and α-SMA (green, Q&R). Nuclei are stained with DAPI (blue). Data are representative of N = 9

experiments ($N = 3$ coverslips/condition, per independent experiment, with $N = 3$ independent experiments). *A-L at X200, M-R at X400; Scale bars = 50 μm .* Western blot (*S*) for changes in α -SMA levels after 48hr or 72hr of treatment with IL-8 (38 pM, 4 nM, 28 nM) or vehicle (PBS + 1 % BSA) in M_0 . For all 3 cell cultures, α -SMA immunoreactive bands are shown above the same lanes re-probed for GAPDH. Each pair of bands, at 48hr and 72hr, displays vehicle-treated sample on the left and IL8-treated sample on the right. Band intensity (normalized to GAPDH) was quantitated using NIH Image J software.

Fig. 4. IL-8 induces tenascin-C, a marker of reactive stroma, in human prostate fibroblasts PrSC cells (**A-B**), HPS-19I cells (**C-D**) and HTS-2T cells (**E-F**), human prostate stromal cells, were treated with vehicle (PBS + 1 % BSA) or IL-8 (38pM, 4nM & 28nM; 38pM is shown) in M_0 and analyzed by IF ICC for tenascin-C (**green**). Vehicle treated PrSC and HPS-19I cells displayed a non-reactive phenotype, as evidenced by lack of tenascin-C expression (**A & C, respectively**). In contrast, IL-8 induced the reactive stroma phenotype, as evidenced by altered and more focal immunoreactivity for tenascin-C deposition in PrSC (**B, green**) and HPS-19I (**D, green**) cells. HTS-2T cells, whether treated with vehicle (**E**) or IL-8 (**E**), displayed a similar, more diffuse deposition of tenascin-C. Nuclei are stained with DAPI (**blue**). Data are representative of N = 9 experiments (N = 3 coverslips/condition, per independent experiment, with N = 3 independent experiments). Images acquired at X200; Scale bars = 50 μ m.