Microenvironment and Immunology

Altered TGF-β Signaling in a Subpopulation of Human Stromal Cells Promotes Prostatic Carcinogenesis

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Abstract

Carcinoma-associated fibroblasts (CAF) play a critical role in malignant progression. Loss of TGF-β receptor II (TGFBR2) in the prostate stroma is correlated with prostatic tumorigenesis. To determine the mechanisms by which stromal heterogeneity because of loss of TGFBR2 might contribute to cancer progression, we attenuated transforming growth factor beta (TGF-β) signaling in a subpopulation of immortalized human prostate fibroblasts in a model of tumor progression. In a tissue recombination model, loss of TGFBR2 function in 50% of the stromal cell population resulted in malignant transformation of the nontumorigenic human prostate epithelial cell line BPH1. Mixing fibroblasts expressing the empty vector and dominant negative TGFBR2 increased the expression of markers of myofibroblast differentiation [coexpression of vimentin and alpha smooth muscle actin (αSMA)] through elevation of TGF-β1 and activation of the Akt pathway. In combination, these two populations of stromal cells recapitulated the tumor inductive activity of CAFs. TGFBR2 activity in mixed stromal cell populations cultured in vitro caused secretion of factors that are known to promote tumor progression, including TGF-β1, SDF1/CXCL12, and members of the fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) families. In vivo, tissue recombination of fibroblasts overexpressing TGF-β1 and SDF1/CXCL12 not only induced transformation of BPH1 cells, but also promoted a robust growth of highly invasive cells, similar to effects produced by CAFs. While the precise nature and/or origin of the particular stromal cell populations in vivo remain unknown, these findings strongly link heterogeneity in TGF-β signaling to tumor promotion by tumor stromal cells. Cancer Res; 71(4); 1272–81. ©2011 AACR.

Introduction

The tumor microenvironment includes fibroblasts, smooth muscle, nerves, blood vessels, and components of the inflammatory/immune system. While smooth muscle is the predominant stromal cell type associated with normal prostatic acini, fibroblasts and myofibroblasts are associated with cancer cells and are prominent modifiers of cancer progression (1–3). Cancer-associated fibroblasts (CAF), isolated from within the tumors of cancer patients, are important elements in tumor growth and progression (4). Studies suggest that these fibroblasts are recruited or activated following injury and that this reactive stroma provides the oncogenic signals that promote tumorigenesis (5). The role of CAF in carcinogenesis has been examined by studies in which human prostatic CAF promoted carcinogenesis in the immortalized but nontumorigenic BPH1 prostatic epithelial cell line (6–8).

Molecular markers of fibroblast subpopulations are poorly defined. Generally, fibroblasts are identified by their spindle-shaped morphology and the overlapping expression of various indicators of their fibroblastic phenotype, such as vimentin and fibroblast-specific protein 1 (FSP1). Other markers, which are not expressed in all fibroblasts have been identified (9, 10). This suggests multiple populations of fibroblasts whose specific phenotype may be conditioned by factors, such as developmental stage and status (rest versus activation, as in a wound healing or cancer). Such cells may reflect different lineages or may be the result of site-specific differentiation (3).

Stroma can elicit instructive, permissive, or inductive (reactive) effects on the parenchymal epithelium (11). For example, embryonic mesenchymal cells can instruct epithelial cells to form functional, differentiated glands (12). In contrast, a permissive stroma supports a previously induced epithelial phenotype. Tumor stroma can be considered instructive or reactive because it secretes factors that can stimulate tumor progression in suitably initiated epithelial cells (13).

Transforming growth factor beta (TGF-β) can both induce and suppress differentiation and tumorigenesis in a dose- and
context-dependent manner (14). Loss of the TGF-β type II receptor (TGFβR2) has been observed in the stroma of more than 60% of human prostate cancer patients (15). Ablation of TGFβR2 from approximately 40% of the stromal cells in a mouse resulted in cancer of the forestomach and premalignant prostatic lesions (16). This demonstrated that changes in TGF-β receptor activity in fibroblastic subpopulations can have profound effects on adjacent epithelial morphology.

The present study was designed to test whether stromal heterogeneity has direct consequences on tumor progression in a human prostatic tissue recombination model. Here, we report that heterogeneous expression of TGFβR2 in prostate stromal subpopulations elicits changes consistent with the malignant phenotype induced by CAF.

Materials and Methods

Cells

BPH1 (a non-tumorigenic human prostate epithelial cell) was from our stocks (17). De-identified human prostatic tissue samples were obtained from the Vanderbilt Tissue Acquisition Core via the Department of Pathology consistent with Vanderbilt IRB protocols. Benign human prostate stromal cells (BHPs1) were isolated from a benign prostate surgical sample, also used to establish the BHPsE1 cell line (18). BHPs1 cells were immortalized using human telomerase reverse transcriptase. Carcinoma-associated fibroblasts (CAF) were isolated and bioassayed as previously described (6). Cells were maintained in RPMI-1640 (Gibco) with 1% antibiotic/antimycotic (Life Technologies) and 5% Cosmic Calf Serum (CCS).

ELISA for TGF-β1 and SDF1α

Around 800,000 cells were plated in 10 cm dishes, and after overnight attachment and growth, cells were washed twice with PBS and fed with serum-free RPMI-1640. Conditioned medium (CM) was collected after 48 hours, centrifuged at 13,000 rpm for 15 minutes to pellet debris, and stored at ~80°C for later use. Quantification of TGF-β1 and SDF1α in the CM were assessed by ELISA according to the manufacturer’s protocol [human TGF-β1 (DB100B) and human SDF1α (DAS00) Quantikine from R&D Systems, Inc, Minneapolis, MN]. Each experiment was carried out in triplicate.

Human prostate tissue array

Human prostate tissue samples from 90 patients with diagnosed prostate carcinoma representing different Gleason scores were used to generate a tissue microarray. The microarray contained 0.6 mm core samples, two from the peripheral tumor and one from the peripheral zone (PZ) away from the cancer-involved area. The original diagnostic H&E stained slides as well as the Gleason score were available in all cases. Immunohistochemically stained microarray slides were reviewed by a pathologist (MPR) blinded to the Gleason score, and results were expressed in a semiquantitative manner. w Percentage staining was scored on a scale of 0–4, where 0 = no staining, 1 = less than 25% of cells staining, 2 = 25% to 50% cells staining, 3 = 50% to 75% cells staining, 4 = more than 75% cells staining. w Epithelial and stromal cells were scored separately.

Tissue recombinants and subrenal capsule xenografts

Rat urogenital sinus mesenchyme (rUGM) was prepared from 18-day embryonic Sprague-Dawley rats fetuses (Harlan) as previously described (19). To prepare tissue recombinants, rUGM and CAF cells were mixed in five different ratios (100% rUGM, 75% rUGM: 25% CAF, 50% rUGM : 50% CAF, 25% rUGM: 75% CAF, and 100% CAF) for a total of 250,000 cells and recombined with 100,000 BPH1 cells. After overnight incubation at 37°C, tissue recombinants were grafted under the renal capsule of intact male CB17crl/Hsd-severe combined immune deficient (SCID) mice (Harlan) supplemented with 5 mg testosterone pellets placed in the subcutaneous compartment. For the experiments involving engineered cells, 100,000 BPH cells were recombined with 250,000 stromal cells for BHPs1-rUGM or BHPs1−rUGM. The BHPs1−/rUGM/BHPs1−EV mixtures were composed of 125,000 BHPs1−/rUGM + 125,000 BHPs1−EV cells to obtain a 50/50 ratio. Both BHPs1−/−TGF−β1 and BHPs1−/−SDF1α cells were mixed at a density of 250,000 cells with BPH1 cells. Host mice were sacrificed after 8 weeks. The kidneys were removed, grafts cut into halves, and imaged before processing for histology. Graft dimensions were measured and the resultant tumor volume was calculated using the formula:

\[ V = \text{width} \times \text{length} \times \text{depth} \times \pi/6. \]

This formula underestimated the volume of invasive tumors, and thus tends to understate the invasive characteristics of large invasive tumors.

Results

Cancer-associated fibroblasts counteract the organizational effect of embryonic mesenchymal cells on prostatic remodeling

To date, the study of stromal-epithelial interactions in vivo has assumed each tissue to have a broadly homogeneous nature. A study of stromal heterogeneity requires the presence of at least two different cell types in the stromal compartment. We examined whether rUGM could suppress the epithelial malignant phenotype resulting from exposure to CAF. To test this, we recombined CAF and rUGM cells in five different ratios with BPH1 epithelial cells. These experiments gave broadly predictable results, in that, as the proportion of CAF/rUGM cells increased, the epithelial structures in the recombinants became progressively less organized and the size of the grafts increased. Specifically, the previously described, (20) nonmalignant, well-organized epithelial cords formed under the influence of rUGM became less obvious as the proportion of CAF increased, whereas tumor structures and invasion became more prevalent (Fig. 1A and B). Concurrent with changes in epithelial cell organization, changes were also noted in the stroma. Well-organized smooth muscle surrounding the benign cords became progressively more fibroblastic in the more malignant grafts, with expression of vimentin predominating over markers of prostate stromal differentiation, such as alpha and gamma smooth muscle actin (αSMA and γSMA), desmin, and calponin (Fig. 1A). These results show that the epithelial response is dictated by the overall paracrine signaling environment, and demonstrate that this response can be modified by mixing different populations of stromal cells.
Human prostate cancer stromal cells show heterogeneous TGF-β signaling

Elevated TGF-β expression by CAF are linked to prostatic tumorigenesis (7, 21). Immunostaining for TGFBIR2 has been shown to decrease in prostate stroma with increasing cancer grade (15, 22). However, the importance of TGF-β-responsive versus nonresponsive stromal cells has not been addressed properly. We stained for phosphorylated Smad2, as a
surrogate for TGF-β activity, in tissue recombinants composed of BPH1 with: rUGM, BHPrS1, and CAF. BHPrS1 cells were established from a benign human prostate surgical sample and characterized to express both vimentin (fibroblast) and α-SM-actin (smooth muscle) proteins without epithelial and neuroendocrine markers (Supplementary Fig. 1). Recombinants composed of CAF + BPH1 showed the highest percentage of phospho-Smad2 positive stromal cells (≥50%) compared with either BPH1 + BHPrS1 (<10%) or BPH1 + rUGM (<25%). B, phospho-Smad2 expression in human prostate cancer tissue array. Normal prostate epithelial cells were surrounded by cells expressing low levels of phospho-Smad2. Stromal cells in the tumors as well as those in normal looking areas in the PZ of cancer patients had a phospho-Smad2 SCORE of more than 2.5 representing more than 50% of positive cells (see text for details). C, addition of TGF-β1 ligand to cultured fibroblasts induced the expression of αSMA and phospho-Smad2 in a subpopulation of CAF, whereas the BHPrS1 showed a more homogeneous pattern of expression. Note morphologic changes to CAF with serum compared with BHPrS. This phenomena may be the result of the ability of CAF to remodel collagen matrices.

Figure 2. The prostate tumor stroma shows heterogeneous P-Smad2 expression, a surrogate marker of response to TGF-β. A, Immunohistochemical staining of tissue recombinants for phospho-Smad2. BPH1 + CAF recombinants showed the highest proportion of phospho-Smad2 positive stromal cells (≥50%) compared with either BPH1 + BHPrS1 (<10%) or BPH1 + rUGM (<25%). B, phospho-Smad2 expression in human prostate cancer tissue array. Normal prostate epithelial cells were surrounded by cells expressing low levels of phospho-Smad2. Stromal cells in the tumors as well as those in normal looking areas in the PZ of cancer patients had a phospho-Smad2 SCORE of more than 2.5 representing more than 50% of positive cells (see text for details). C, addition of TGF-β1 ligand to cultured fibroblasts induced the expression of αSMA and phospho-Smad2 in a subpopulation of CAF, whereas the BHPrS1 showed a more homogeneous pattern of expression. Note morphologic changes to CAF with serum compared with BHPrS. This phenomena may be the result of the ability of CAF to remodel collagen matrices.
BPH1 cells, we fed BPH1 cells with CM from CAF and also with previous observations in a similar system, BPH1 cells grew faster in the presence of CAF-CM compared with medium conditioned by the other fibroblasts. BPH1 + BHPs1 + EV/BHPs1 −DN, BHPs1 + EV, or BHPs1 −DN tissue recombinants were grafted into male SCID mice for 8 weeks before harvesting the tissues. BPH1 + BHPs1 + EV/BHPs1 −DN recombinants produced the largest tumors as shown by the gross appearance (arrowhead) and quantification of tumor volume. C, Histological examination revealed malignant transformation of BPH1 only with mixed BHPs1 + EV/BHPs1 −DN stromal cells (i–ii). The adeno-squamous phenotype with invasion into the kidney resembled BPH1−CAF recombinants. The arrow indicates invasion into the host kidney. Immunofluorescence staining of SV40 confirmed the invasion of BPH1 cells into the kidney parenchyma (i) of BPH1 + BHPs1 + EV/BHPs1 −DN grafts, no invasion was seen in the other recombinants (ii–vi). The presence of BHPs1−DN increased the proportion of Ki67 positive cells in the BPH1 + BHPs1 + EV/BHPs1 −DN and BPH1 + BHPs1 −EV recombinants (vi–ix). Malignant transformation of BPH1 cells was accompanied by changes in the stromal compartment toward a myofibroblast phenotype, as noted by the increased expression of vimentin and αSMA (i–ii).

To further define the role of the heterogeneous stroma in prostate cancer, we tested these cells in vivo. Grossly, BPH1 + BHPs1−DNTREB/ BHPs1−EV recombinants formed larger grafts compared with BPH1 + BHPs1−DNTREB and BPH1 + BHPs1−EV recombinants (Fig. 3B). BPH1 + BHPs1−DNTREB/BHPs1−EV recombinants were 2.5 times larger than BPH1 + BHPs1−DNTREB recombinants composed of BPH1 + BHPs1−EV showed minimal growth. The small BPH1 + BHPs1−EV grafts were primarily formed of stromal cells and a few epithelial cords without lumen formation [Fig. 3C, i]. BPH1 + BHPs1−DNTREB grafts exhibited increased epithelium with a few sheets of stromal cells (Fig. 3C, ii). Both BPH1 + BHPs1−EV and BPH1 + BHPs1−DNTREB tissue recombinants had a benign appearance and did not show any sign of invasion into the host kidney (Fig. 3C, iv and v). In contrast, BPH1 + BHPs1−DNTREB/BHPs1−EV grafts (as assessed by H&E staining) resembled a poorly differentiated adenocarcinoma with areas of adenosquamous differentiation. BPH1 cells showed clear invasion into the kidney parenchyma, and there were no clear margins between the kidney and...
showed patterns in which the overall expression in the mixtures of BHPrS1 and as well as markers altered in CAF by Western blot. Cells with a CAF-like phenotype, we focused on several major pathways confirmed the RT-PCR observations that TGF-β1 (Fig. 4A). We used PCR arrays to examine these cells cultured under normal serum conditions. The results revealed that members of the TGF-β superfamily as well as several fibroblast growth factor (FGF), insulin like growth factor (IGF), and interleukins were highly dysregulated in the mixtures compared with the component strains (Supplementary Table 2). The data also confirmed the RT-PCR observations that TGF-β expression was increased in BHPs1-DNT/BR and BHPs1-DNT/BR/BHPrS1-EV cells [Fig. 4B (arrow)]. This again suggests that interactions between stromal cells can modify the overall paracrine signaling environment.

To better understand the molecular mechanisms by which the mixtures of BHPs1-DNT/BR/BHPrS1-EV cells can induce a CAF-like phenotype, we focused on several major pathways and as well as markers altered in CAF by Western blot. Cells were grown under serum free conditions for 48 hours before isolation of protein lysates. As shown in Fig. 4C, expression of phospho-Smad2 was higher in the mixtures of BHPs1-DNT/BR/BHPrS1-EV cells compared with BHPs1-EV and even more than in BHPrS1-EV, suggesting that the high expression of TGF-β1 by BHPrS1-EV in the mixtures has an effect on BHPs1-EV with intact TGFβR2. These data can explain the field effect observed in patient samples in which normal prostate fibroblasts respond to the high levels of TGF-β1 ligand secreted by tumors. Expression of phospho-Akt and p27 were higher in BHPs1-DNT/BR/BHPrS1-EV and BHPs1-DNT/BR compared with BHPs1-EV. There were no significant changes in the activation of the MAPK (mitogen activated protein kinase) pathway as assessed by phospho-p44/p42 expression. However, there was increased expression of the tumor stromal markers vimentin and αSMA, as well as the recently described marker of mesenchymal lineage cells, CD90, frequently found in prostate cancer stroma (23). Collectively, these data suggest that changes in TGF-β signaling may regulate the interactions between stromal cells that might help determine not only the CAF-phenotype, but also the overall paracrine signaling environment responsible for the tumor promoting abilities of the cancer stroma.

**Overexpression of TGF-β1 and SDF1α in benign human prostate fibroblasts induces in vivo malignant transformation of BPH1 cells**

We previously reported that blocking either TGF-β1 or SDF1α in vivo impairs the ability of CAF cells to promote tumorigenicity (7). Since these two factors were elevated in BHPs1-DNT/BR/BHPrS1-EV, we wanted to know whether the overexpression of TGF-β1 or SDF1α alone in normal prostate fibroblasts could elicit changes that mimic CAF behavior. We therefore generated BHPs1-EV, BHPs1-TGFβ1, and BHPs1-SDF1α cells using a retroviral system. Secretion of constitutively active TGF-β1 and SDF1α by these cells were quantitated by ELISA and compared with BHPs1-DNT/BR and BHPs1-EV (Fig. 5A, upper). ELISA for TGF-β secretion corroborated RT-PCR observations that TGF-β1 secreted in mixed populations was clearly induced or suppressed compared with the BHPs1-DNT/BR or BHPs1-EV cells alone. Loss of TGF-β signaling in BHPs1-DNT/BR correlated with increased expression of TGF-β1 (Fig. 4A). We used PCR arrays to examine these cells cultured under normal serum conditions. The results revealed that members of the TGF-β superfamily as well as several fibroblast growth factor (FGF), insulin like growth factor (IGF), and interleukins were highly dysregulated in the mixtures compared with the component strains (Supplementary Table 2). The data also confirmed the RT-PCR observations that TGF-β expression was increased in BHPs1-DNT/BR and BHPs1-DNT/BR/BHPrS1-EV cells [Fig. 4B (arrow)]. This again suggests that interactions between stromal cells can modify the overall paracrine signaling environment.

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suggest that specific stromally secreted factors resulting from partial loss of TGFβR2 can result in paracrine prostate cancer promotion.

**Discussion**

Malignant tumors are complex caricatures of developing organs in which stromal and epithelial cells are engaged in active communication. The tumor stromal cells, and the extracellular matrix which they deposit, play a key role in restraining or promoting tumorigenesis. The specific nature of the human prostate cancer stromal phenotype has been shown to be an independent clinical prognostic marker (24, 25), underlining the importance of paracrine interactions in human disease. In vivo models have been used to demonstrate paracrine mechanisms by which the stromal microenvironment acts to promote tumorigenesis (7, 21, 26). The prostate tumor microenvironment is complex, including cells of many different lineages (27–34). These include, but are not limited to, smooth muscle, various types of fibroblasts, senescent stromal cells, nerves and blood vessels, and a wide variety of immune and inflammatory cell types.

The diversity in the types of cells that compose the tumor stroma makes it difficult to study the contribution of each component. In addition, the lack of stromal cell lines that can retain the tumor-inducing properties shown by CAF cells in vivo represents another important problem. In an attempt to demonstrate the impact of a heterogeneous stromal population in prostate cancer progression, we recreated a controlled tumor microenvironment by impairing TGF-β signaling in about 50% of human benign prostate fibroblasts.

Mesenchyme can restrict the growth of some tumorigenic cells. For example, rat urogenital mesenchyme recombined with Dunning Prostate Adenocarcinoma epithelium resulted in the formation of organized glands and loss of tumorigenicity (35). We have also seen that rUGM decreases the invasive properties of the tumorigenic BPH1(-Cathepsin D) cells (unpublished data).
Figure 5. Induction of CAF phenotype by overexpression of TGF-β1 and SDF1α in normal prostate fibroblasts. A, ELISA was used to test and corroborate the expression of the transgenes in fibroblasts. Fibroblasts expressing constitutively active TGF-β1 ligand showed an increased SDF1α expression. However, overexpressing SDF1α in fibroblasts had no effect on TGF-β1 levels. B, BPH/S1 TGF-β1 and BPH/S1 SDF1α were recombined with BPH1 cells and xenografted under the kidney capsule for about 8 weeks. The volume of the tumors composed of BPH/S1 TGF-β1, BPH1 and BPH/S1 SDF1α, BPH1 were significantly larger than the controls. Note the invasive characteristic of the tumors (arrows). C, Histological examination revealed malignant transformation of the epithelial cells (i–iii). TGF-β1-expressing fibroblasts had a greater impact on the proliferation of BPH1 cells as shown by Ki67 staining as compared with BPH/S1 SDF1α cells. (iv–vi). BPH/S1 SDF1α fibroblasts exhibited light blue stromal Trichrome staining indicative of some collagen in the stroma. In contrast, recombinants composed of BPH/S1 SDF1α stained predominantly red suggesting a more muscular phenotype. Intense blue Trichrome staining in the stroma of BPH/S1 TGF-β1-BPH1 recombinants revealed the extensive collagen deposition in these tumors (vii–x). Intense SDF1α staining corroborated the secretion of the chemokine in grafts composed of SDF1α-expressing compared with TGF-β1-expressing fibroblasts (x–xii).
signaling in 50% of normal prostate stromal cells. This change in the stromal composition resulted in malignant transformation of initiated epithelial cells in a minor but sufficient degree to represent some of the tumor-inductive properties of CAF. PCR array was carried out in normal fibroblasts and compared with those lacking TGF-β responsiveness and a mix of the two populations to screen for molecular changes that might contribute to the CAF phenotype. Our results showed significant alterations in the expression of genes associated with the transition of normal fibroblasts to CAF. Several genes involved in development, cell differentiation, and angiogenesis were altered including TGF-β1, which has been shown in several studies to be involved in prostate cancer progression (38). Interestingly, abrogation of TGF-β signaling in fibroblasts resulted in the increase of TGF-β ligand secretion. Thus, a higher BHPSR1$_{IOTPBR1}$/BHPSR1$_{EV}$ ratio caused more TGF-β1 secretion to support the conversion to a myofibroblast phenotype.

Overexpression of the chemokine SDF1α/CXCL12 can support proliferation and invasion of the prostate cancer cell line, PC3 (39). We have previously shown crosstalk between TGF-β1 and CXCR4, the receptor for SDF1α, which is secreted by CAF in carcinogenesis (7). We found that overexpression of TGF-β1 by normal fibroblasts was sufficient to induce neoplastic changes. Similar observations were described with fibroblasts isolated from reduction mammoplasty (40). Interestingly, SDF1α alone did not induce a robust growth of BPH1 cells, as compared with TGF-β1, but had a dramatic effect in the invasiveness of the cells into the kidney parenchyma. One possible explanation is the absence of complete activation of fibroblasts toward a CAF phenotype, as previously reported (39). It is possible that the high amount of TGF-β1 produced by these fibroblasts is sufficient to result in the basal alterations in the stroma required for tumor promotion, such us conversion to the myofibroblast phenotype and increased secretion of angiogenic factors (two features commonly found in cancer; ref. 41). Once such a conversion occurs, SDF1α might support progression and invasion at a later stage. These observations indicate that an altered heterogeneous stromal environment can promote human prostate cancer formation by changes in the repertoire of secreted factors.

In this study, we demonstrated that heterogeneity of human prostate stroma may be an important contributor to stromally induced carcinogenesis. Abrogation of TGF-β signaling in a subpopulation of prostate stromal cells is consistent with the previously demonstrated loss of stromal TGFβR2 in prostate cancer (22). While the biological situation is unlikely to be this simple, this model represents a step toward modeling stromal complexity. Further exploration of the intrinsic mechanisms responsible for the conversion to the CAF phenotype, and the epithelial responses to such a phenotype, are necessary for the developing of effective cancer therapies that can target both the epithelial and stromal compartments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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