Reactive Stroma in Human Prostate Cancer: Induction of Myofibroblast Phenotype and Extracellular Matrix Remodeling[1]

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ABSTRACT

Purpose: **Generation of a reactive stroma environment occurs in many human cancers and is likely to promote tumorigenesis. However, reactive stroma in human prostate cancer has not been defined. We examined stromal cell phenotype and expression of extracellular matrix components in an effort to define the reactive stroma environment and to determine its ontogeny during prostate cancer progression.**

Experimental Design: **Normal prostate, prostatic intraepithelial neoplasia (PIN), and prostate cancer were examined by immunohistochemistry. Tissue samples included radical prostatectomy specimens, frozen biopsy specimens, and a prostate cancer tissue microarray. A human prostate stromal cell line was used to determine whether transforming growth factor β1 (TGF-β1) regulates reactive stroma.**

Results: **Compared with normal prostate tissue, reactive stroma in Gleason 3 prostate cancer showed increased vimentin staining and decreased calponin staining ($P < 0.001$). Double-label immunohistochemistry revealed that reactive stromal cells were vimentin and smooth muscle α-actin positive, indicating the myofibroblast phenotype. In addition, reactive stroma cells exhibited elevated collagen I synthesis and expression of tenascin and fibroblast activation protein. Increased vimentin expression and collagen I synthesis were first observed in activated periacinar fibroblasts adjacent to PIN. Similar to previous observations in prostate cancer, TGF-β1-staining intensity was elevated in PIN. *In vitro*, TGF-β1 stimulated human prostatic fibroblasts to switch to the myofibroblast phenotype and to express tenascin.**

Conclusions: **The stromal microenvironment in human prostate cancer is altered compared with normal stroma and exhibits features of a wound repair stroma. Reactive stroma is composed of myofibroblasts and fibroblasts stimulated to express extracellular matrix components. Reactive stroma appears to be initiated during PIN and evolve with cancer progression to effectively displace the normal fibromuscular stroma. These studies and others suggest that TGF-β1 is a candidate regulator of reactive stroma during prostate cancer progression.**

INTRODUCTION

Activation of the host stromal microenvironment is predicted to be a critical step in adenocarcinoma growth and progression (1–5). Several human cancers have been shown to induce a stromal reaction or desmoplasia as a component of carcinoma progression. However, the specific mechanisms of stromal cell activation are not known, and the extent to which stroma regulates the biology of tumorigenesis is not fully understood. In cancers where a stromal reaction has been observed, it seems that the response is similar, if not identical, to a generic wound repair response (6). In wound repair, stromal cells exhibit elevated production of ECM[3] components, growth factors, and matrix remodeling enzymes to create a growth-promoting microenvironment (7). Similarly, reactive stroma in cancer would be predicted to enhance tumor progression by stimulating angiogenesis and by promoting cancer cell survival, proliferation, and invasion (5, 8–12). Indeed, two recent studies have shown that prostate stromal cells stimulate the development and rate of human prostate tumorigenesis in mouse xenograft models (13, 14). Accordingly, it is important to identify and characterize reactive stroma in cancer to establish key regulators of reactive stroma and to delineate the specific mechanisms through which reactive stroma affects carcinoma progression.

An initial step in understanding mechanisms of the stromal reaction in tumor progression is to fully define the reactive stroma phenotype and its formation. Although poorly understood in most cancers, reactive stroma has been described in breast and colon carcinoma (9–12). In these cancers, reactive stroma is a mix of fibroblasts, myofibroblasts, endothelial cells, and immune cells. Although all of these cells potentially affect tumorigenesis, myofibroblasts are of particular interest. Myofi-

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[3] The abbreviations used are: ECM, extracellular matrix; FAP, fibroblast activation protein; MMP, matrix metalloproteinase; TGF, transforming growth factor; PIN, prostatic intraepithelial neoplasia; SPORE, specialized program of research excellence; sm α-actin, smooth muscle α-actin; DAPI, 4′,6 diamidino-2-phenylindole; CAF, carcinoma-associated fibroblast.

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broblasts are activated stromal cells typically found at sites of pathologic tissue remodeling (15). In wound repair, myofibroblasts are derived from granulation tissue fibroblasts (15). In cancer, it has been shown that carcinoma cells have the capacity to induce normal fibroblasts to the reactive myofibroblast phenotype (16, 17). Myofibroblasts in reactive stroma synthesize ECM components such as collagen I, collagen III, fibronectin isoforms, tenascin, and versican (18–22). In addition, myofibroblasts express proteases, including urokinase plasminogen activator, FAP, and MMPs (23–25). Production of these components results in ECM remodeling that could stimulate cancer cell growth and migration. Moreover, myofibroblasts have been reported to secrete growth factors that promote angiogenesis (26, 27). Therefore, myofibroblasts appear to be a key cell type involved in creation of the tumor-promoting reactive stroma environment.

The inductive mechanisms involved in the development of reactive stroma in cancer are poorly understood. Predictably, experimental evidence suggests that TGF-β1, a key mediator of the stromal response in wound repair (28), is likely to play an important role (5, 11, 29). *In vitro* studies have shown that TGF-β1 stimulates phenotypic switching of fibroblasts to myofibroblasts (16) and regulates expression of ECM components (28). In addition, s.c. injection of TGF-β1 was sufficient to induce a stromal reaction characterized by differentiation of stromal cells to myofibroblasts, enhanced collagen production, and stimulated angiogenesis (30, 31). Elevated levels of TGF-β1 have also been shown to enhance tumor growth *in vivo* (32–35). Because TGF-β is overexpressed in many human cancers, including prostate cancer (36–38), it seems likely that TGF-β promotes formation of reactive stroma.

We have suggested previously that reactive stroma in human prostate cancer is likely to function as a mediator of tumorigenesis (5, 39). However, a detailed analysis of whether a stromal reaction occurs as a component of prostate cancer progression has not been reported. Furthermore, the putative reactive stroma in prostate cancer may differ from that described in breast and colon cancer because of fundamental differences in the normal stroma of these tissues. The purpose of this study was to determine whether reactive stroma is a component of prostate carcinoma, to specifically define cell and matrix alterations in this reactive stroma, to characterize the ontogeny of such a reactive stroma relative to the development of prostate cancer, and to identify potential regulators. We show here that a wound repair type of reactive stroma is associated with prostate cancer and was composed of myofibroblasts and fibroblasts rather than normal prostate smooth muscle, which appeared to be displaced by the reactive stroma. Our results show that reactive stroma exhibits fundamental alterations in ECM and expression of markers commonly associated with wound repair stroma. Moreover, we show that genesis of the reactive stroma phenotype occurs in precancerous PIN lesions and may be associated with elevated TGF-β1 expression by PIN epithelial cells. In addition, TGF-β1 induced a human prostate stromal cell line to switch to the myofibroblast phenotype. This study represents the first report to identify and specifically define reactive stroma in human prostate cancer and sets the stage for additional assessing of the specific mechanisms and role of the stromal reaction in prostate tumorigenesis.

MATERIALS AND METHODS

Tissues. Radical prostatectomy specimens from 20 patients were obtained from the Baylor College of Medicine Prostate SPORE tissue bank located at Methodist Hospital. Specimens were processed as described previously (40). Briefly, the tissues were cut into 5-mm thick slices, fixed in 10% neutral buffered formalin, and embedded in paraffin as whole mounts. Whole mount thin sections (5 μm) were stained with H&E and evaluated for histological differentiation. Tissues were selected by a pathologist (G. E. A.) based on the presence of normal prostate, PIN, and Gleason 3 prostate cancer. For initial examination of stromal components, whole mount thin sections were stained with Masson's Trichrome following the standard procedure (Sigma Diagnostics, St. Louis, MO). For immunostaining, the whole mounts were cut in half, and thin sections were mounted on standard microscope slides.

The prostate cancer tissue microarrays were assembled from specimens in the Baylor SPORE tissue bank ($n = 89$ Gleason 3 and $n = 17$ Gleason 4 cancers). For each specimen, the largest and/or highest Gleason cancer focus was identified and mapped on the whole mount sections. Accordingly, 2-mm cores were punched out of the tissue slices and transferred to a recipient block. Samples from normal prostate tissue ($n = 64$) and PIN lesions ($n = 27$) were also included in the array. The tissue microarrays were built using a manual tissue arrayer (Beecher Instruments, Silver Spring, MD). Internal controls were placed at a pre-established pattern throughout the blocks to assess adequacy of staining.

Prostate cancer tissues for frozen sections were obtained through the Baylor SPORE and Methodist Hospital. Tissue cores harvested in the operating room were frozen immediately in liquid nitrogen, embedded in Tissue-Tek Optimal Cutting Temperature compound (Sakura, Torrance, CA), and sectioned.

Immunohistochemistry. The following primary antibodies were used: sm α-actin (mouse monoclonal 1A4), calponin (mouse monoclonal hCP), and tenascin (mouse monoclonal BC-24) were purchased from Sigma Diagnostics; vimentin (goat polyclonal AB1620) and procollagen I (rat monoclonal MAB1912) were purchased from Chemicon (Temecula, CA); vimentin (mouse monoclonal VIM 3B4; Boehringer Mannheim, Indianapolis, IN); FAP (mouse monoclonal 3D11, kindly provided by Dr. John Park, Boehringer Ingelheim); and TGF-β1 (rabbit polyclonal sc-146; Santa Cruz Biotechnology, Santa Cruz, CA). Specificity of each primary antibody has been evaluated and published previously. The following secondary antibodies were used: biotin-conjugated Universal Secondary Antibody was used for mouse monoclonals (Research Genetics, Huntsville, AL); biotin-conjugated goat antirat IgG (554014; BD PharMingen, San Diego, CA); and Texas Red-conjugated donkey antigoat IgG and fluorescein-conjugated donkey antimouse IgG (705-075-147 and 715-095-150; Jackson ImmunoResearch, West Grove, PA). No significant staining was observed if sections were incubated with secondary antibody only.

All immunostaining was performed with the MicroProbe Staining System (FisherBiotech, Pittsburgh, PA), which uses capillary gap technology. Standard slides mounted with tissue were paired with BioTek Solutions POP130 capillary gap plus

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slides (Ventana Medical Systems, Tucson, AZ). Reagents formulated for use with capillary action systems were purchased from Research Genetics and used according to the manufacturer's protocol.

Paraffin sections were deparaffinized using Auto Dewaxer and cleared with Auto Alcohol. Brigati's Iodine and Auto Prep were used to improve tissue antigenicity. After washing with Universal Buffer, antigen retrieval was performed if required (VIM 3B4, incubated in pepsin solution for 3 min at 50°C; procollagen, incubated in PBS + 1% trypsin for 20 min at room temperature). Tissues were washed in Universal Buffer, incubated in Protein Blocker, and washed again. Antibodies were diluted in Primary Antibody Diluent and used at the following conditions: sm α-actin 1:200, VIM 3B4 1:50, and calponin 1:2,000 for 8 min at 50°C; and pro-collagen 1:500 for 1 h at 37°C. Tissues were washed with Universal Buffer and incubated in secondary antibody: Universal Secondary undiluted for 4 min at 50°C; and antirat IgG 1:100 for 45 min at 37°C. Tissues were washed in Universal Buffer, treated with Auto Blocker to squelch endogenous peroxidase activity, and washed again. For detection, sections were incubated in streptavidin horseradish peroxidase, washed in Universal Buffer, and then incubated in stable 3,3′-diaminobenzidine twice at 3 min each at 50°C. Tissues were counterstained with Auto Hematoxylin for 30 s.

For double-label immunofluorescent staining, sections were deparaffinized and treated as described above, except tissues were blocked in Universal Buffer + 5% donkey serum (Sigma Diagnostics) for 15 min at room temperature. Vimentin staining was done first using the goat polyclonal antibody diluted 1:100 for 1 h at 37°C. The antigoat IgG secondary was used at 1:200 for 45 min at 37°C. Then, sections were incubated with sm α-actin, and the antimouse IgG secondary was applied as above. After staining, tissues were dehydrated and mounted with coverslips using VectaShield with DAPI (Vector Laboratories, Burlingame, CA).

Frozen sections were fixed in acetone for 10 min at 4°C, then washed in PBS and Universal Buffer. Tissues were incubated in Protein Blocker at 37°C and then washed in Universal Buffer. Primary antibodies were used at the following dilutions: procollagen 1:20,000; tenascin 1:4,000; and FAP 1:10 for 1 h at 37°C. Secondary antibodies were diluted as before and used for 45 min at 37°C. For detection, Ready-To-Use VectaStain Elite Avidin: Biotinylated enzyme complex reagent (Vector Laboratories) was used according to manufacturer protocol, and stable 3,3′-diaminobenzidine was used twice at 3 min each at 37°C. Tissues were counterstained with Auto Hematoxylin.

Histological and Statistical Analysis. To evaluate the level of vimentin, sm α-actin, and calponin expression, the percentage of positive-staining stromal cells and the staining intensity were graded on a scale of 0–3. Staining percentage: 0 = 0% positive cells; 1 = 1–33% positive cells; 2 = 34–66% positive cells; and 3 = 67–100% positive cells. Staining intensity: 0 = no staining; 1 = staining obvious only at ×400; 2 = staining obvious at ×100 but not ×40; and 3 = staining obvious at ×40. For each sample, the staining percentage and staining intensity scores were multiplied to give the staining index. Staining index: 0 = zero; 1–2 = low; 3–4 = moderate; and 6–9 = high. For each marker, Fisher's exact test was used to compare zero/low staining to moderate/high staining in normal and Gleason 3 stroma (19). $P < 0.05$ was considered statistically significant. Analysis was performed with GraphPad Prism for Macintosh v3.0 (GraphPad Software, San Diego, CA).

To examine colocalization of fluorescent staining, separate vimentin (red), sm α-actin (green), and DAPI (blue) images were captured using a Spot Real Time color digital camera (Diagnostic Instruments, Sterling Heights, MI) attached to a Nikon Eclipse TE300 microscope equipped for epifluorescence (Nikon, Melville, NY). The images were merged using Spot Real Time Software v3.2.6 for Mac OS. The percentage of yellow stromal cells (resulting from colocalization of red and green staining) was graded on a scale of 0–3. Fisher's exact test was used to compare the proportion of grade 3 cases in Gleason 3 and Gleason 4 cancer. Statistical analysis was performed as described above.

Cell Culture. To establish the HPS-TZ1A human prostate stromal cell line, a fresh tissue core from the transition zone was obtained through the Baylor SPORE at Methodist Hospital. The core was cut into discs and placed in a 6-well tissue plate containing Bfs media [DMEM (Life Technologies, Inc., Rockville, MD) supplemented with 5% fetal bovine serum (Hyclone, Logan, UT), 5% Nu Serum (Collaborative Research, Bedford, MA), 0.5 μg/ml testosterone, 5 μg/ml insulin, 100 units/ml penicillin, and 100 μg/ml streptomycin (Sigma Diagnostics)]. The explants were incubated at 37°C with 5% CO_2, and media were changed every 48 h or as necessary. Stromal cells migrated out of the explant and attached to the tissue culture dish. After the cells reached confluence, the explant was removed, and the cells were passaged. The resulting stromal cell line showed vimentin expression in 100% of cells, but cytokeratin expression was not observed with AE1/AE3 pooled monoclonal antibody (Boehringer Mannheim). Cultures at passage 6–14 were used for experiments.

To examine the effect of TGF-β1, HPS-TZ1A cells were trypsinized, diluted to 1.5×10^4 cells/ml in Bfs media, and seeded onto coverslips in 6-well plates (2 ml/well). The cells were allowed to attach overnight, then they were washed with HBSS (Life Technologies, Inc.) and switched to experimental conditions. MCDB 110 supplemented with insulin, transferrin, and sodium selenite (Sigma Diagnostics) was used for basal media (M_0). Cells were incubated in M_0, M_0 + 25 pM (0.625 ng/ml) TGF-β1 (R&D Systems, Minneapolis, MN), or M_0 + 25 pM TGF-β1 + 150 ng/ml TGF-β1 neutralizing antibody (AF-101-NA; R&D Systems) for 72 h. Coverslips were fixed with cold 4% paraformaldehyde according to standard technique and stored at 4°C in PBS. This experiment was repeated three times with multiple coverslips of each condition.

For immunocytochemistry, standard staining procedures were followed. Briefly, coverslips were incubated in PBS + 0.1% Triton X-100 for 5 min, then washed with PBS. Coverslips were blocked with PBS + 1% donkey serum for 30 min at room temperature. Primary antibodies were diluted in PBS + 1% BSA and applied to coverslips for 1 h at 37°C. A goat polyclonal antibody against vimentin (sc-7557; Santa Cruz Biotechnology) was used at 1:50; sm α-actin and tenascin antibodies were diluted as described above. Coverslips were washed with PBS, then incubated in fluorescent-labeled secondary antibodies diluted 1:200 for 45 min at 37°C. Coverslips were washed in PBS, dehydrated, and mounted using VectaShield with DAPI.

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RESULTS

Histology of Prostate Stroma. For initial examination of prostate stroma, radical prostatectomy specimens containing normal tissue and moderately differentiated prostate cancer (Gleason 3) were stained with Masson's Trichrome. This technique differentially stains stromal components and, hence, is useful in distinguishing prostate smooth muscle cells from collagen fibers and other stromal cell types. Analysis of normal prostate stroma showed a mixture of red-staining smooth muscle cells and blue-staining collagen fibers and fibroblasts (Fig. 1*A*). In contrast, the stroma in moderately differentiated prostate cancer stained predominantly blue with very little red staining (Fig. 1*B*). Although isolated smooth muscle cells were present in some cases, this blue staining pattern was consistently associated with regions of cancer and suggested a loss of typical smooth muscle cells plus elevated collagen in prostate cancer stroma. These observations suggest that the stromal microenvironment in cancer is altered and led us to further examine specific reactive stroma markers in human prostate cancer.

Identification of Stromal Cell Phenotypes in Prostate Cancer. To better define reactive stroma in human prostate cancer, stromal cell phenotype was assessed by immunohistochemistry. Fibroblast, myofibroblast, and smooth muscle cell phenotypes were classified according to the immunostaining profiles of stromal cell differentiation markers. Expression of vimentin (mesenchymal cell intermediate filament) without additional smooth muscle markers indicated the fibroblast phenotype. Coexpression of sm α-actin microfilaments and microfilament-associated calponin (early and late stage smooth muscle markers, respectively; Ref. 41) identified prostate smooth muscle cells. The myofibroblast phenotype is considered to be intermediate between fibroblasts and smooth muscle (15). Hence, myofibroblasts were distinguished by coexpression of vimentin and sm α-actin without expression of calponin (15).

Immunostaining was initially performed on radical prostatectomy specimens from 20 patients. Stroma from normal prostate gland showed high levels of sm α-actin and calponin expression in prostate smooth muscle cells (Fig. 1, *E* and *G*), which was consistent with the red staining pattern observed with Masson's Trichrome (Fig. 1*A*). Vimentin staining was observed in interstitial fibroblasts and in vascular smooth muscle but not in prostate smooth muscle (Fig. 1*C*). Fibroblasts were mixed among the prostate smooth muscle cells typical of the fibromuscular stroma reported in human prostate. Of interest, a single layer of periacinar fibroblasts was differentially observed immediately peripheral and adjacent to epithelial acini (Fig. 1*C*, *arrow*). This layer may be discontinuous because periacinar fibroblasts were not detected around all acini. Alternatively, visualization of these cells may depend on the plane of section. As suggested by the initial trichrome stain, there was a fundamental change in expression of stromal cell differentiation markers throughout prostate cancer reactive stroma. Stroma in Gleason 3 cancer nodules exhibited a dramatic increase in vimentin staining (Fig. 1*D*) that directly correlated with blue staining regions observed with Masson's Trichrome (Fig. 1*B*). Similarly, expression of sm α-actin (Fig. 1*F*) was observed in the stroma in Gleason 3 cancer. However, these regions of cancer-associated stroma were negative or exhibited low staining intensity for calponin (Fig. 1*H*).

To confirm these observations, tissue microarrays containing normal prostate tissue ($n = 64$) and moderately differentiated prostate cancer ($n = 89$) were stained for vimentin, sm α-actin, and calponin. Subsequently, each tissue sample was evaluated for the percentage of positive-staining stromal cells and for the staining intensity as described in "Materials and Methods." These parameters were graded on a 0–3 scale and then multiplied to yield the staining index. The stroma from normal regions of radical prostatectomy specimens ($n = 20$) was also evaluated for the staining index of each marker. Quantifications of the results are shown in Table 1. Vimentin expression in normal prostate stroma was typically low (staining index, 1–2). In contrast, 57% of Gleason 3 cancer cases had moderate to high vimentin staining in the reactive stroma (staining index, 3–4 and 6–9). Analysis with Fisher's exact test revealed that the increase in vimentin staining was statistically significant ($P < 0.001$). The level of sm α-actin staining in Gleason 3 reactive stroma relative to normal stroma was slightly reduced; however, the staining index remained moderate to high in 71% of Gleason 3 patients. Although calponin expression was moderate to high in normal prostate stroma, 78% of Gleason 3 cancers showed zero or low calponin staining in the reactive stroma. The decrease in calponin expression was also significant ($P < 0.001$). Consistent with Masson's Trichrome staining and qualitative analysis of marker expression in radical prostatectomy specimens, the tissue array data suggest that human prostate cancer reactive stroma is typified by the myofibroblast and fibroblast phenotypes with a nearly complete reduction in differentiated smooth muscle.

To determine whether reactive stroma in cancer was composed of a homogeneous population of myofibroblasts or a mixed myofibroblast/fibroblast population, double-label immunohistochemistry for vimentin and sm α-actin was evaluated in the radical prostatectomy specimens. Normal prostate stroma was composed of sm α-actin-positive smooth muscle cells (green) and vimentin-positive interstitial and periacinar fibroblasts (red; Fig. 2*A*). Colocalization (yellow) was only observed in vascular smooth muscle of blood vessel walls. Analysis of moderately differentiated cancer (Gleason 3) in these specimens showed colocalization of sm α-actin and vimentin in a majority of reactive stromal cells (Fig. 2, *B–D*). These data indicate that the myofibroblast phenotype predominates in prostate cancer reactive stroma, whereas the remaining cells are fibroblasts.

To quantify the proportion of myofibroblasts in reactive stroma, double-label immunohistochemistry was performed on a prostate cancer tissue microarray. Gleason 3 ($n = 36$) and Gleason 4 ($n = 17$) cancers were graded for the percentage of reactive stromal cells coexpressing vimentin and sm α-actin. As shown in Table 2, colocalization was observed in all cases examined. The majority of Gleason 3 cancers showed coexpression in ~50% of reactive stromal cells. Interestingly, an even higher percentage of myofibroblasts was observed in the reactive stroma of poorly differentiated Gleason 4 cancers. With Fisher's exact test, the increased proportion of myofibroblasts in Gleason 4 cancers as compared with Gleason 3 cancers was statistically significant ($P = 0.03$). Taken together, these data show that while normal prostate gland stroma is primarily

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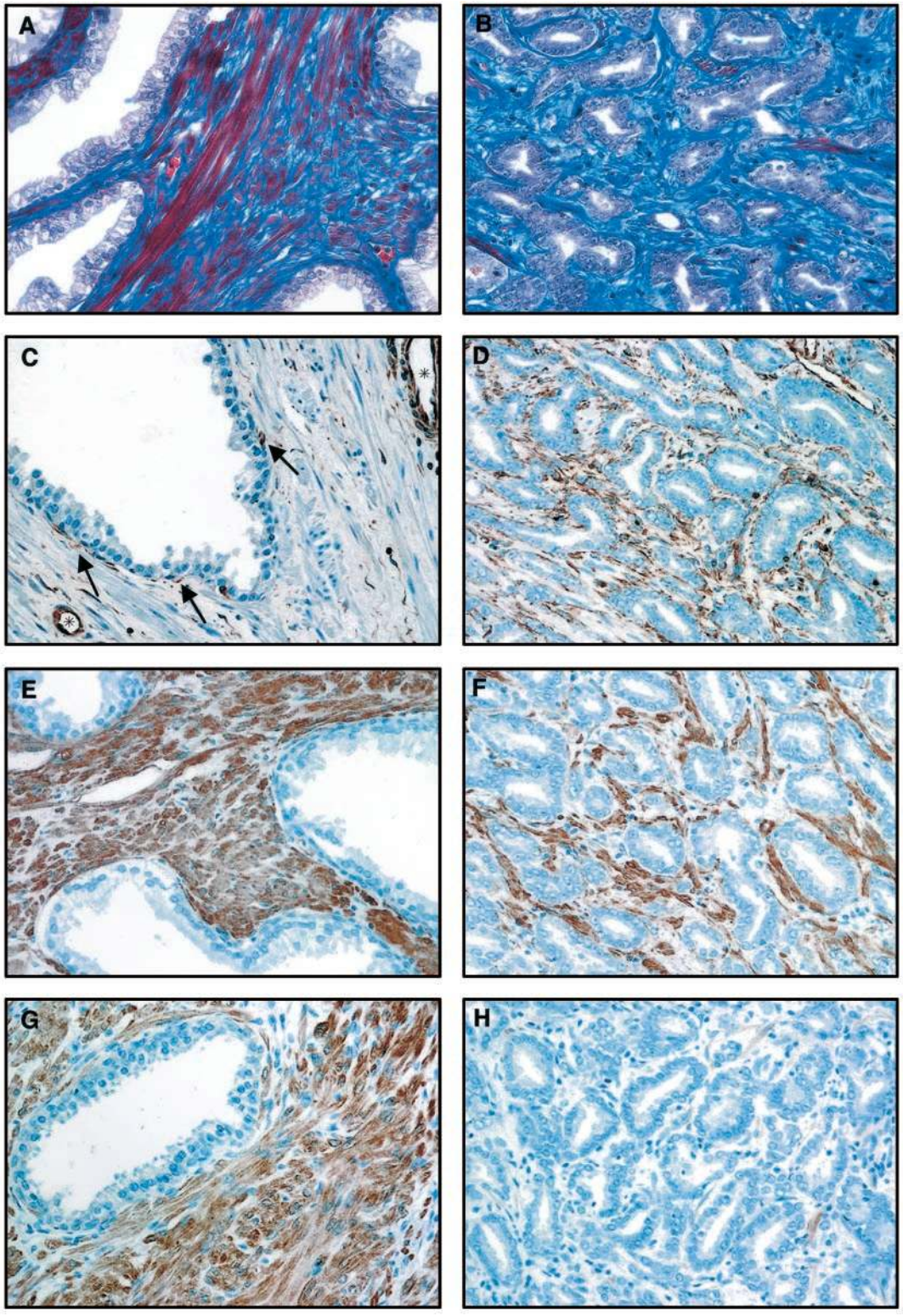

Fig. 1 Stromal cell phenotype in normal prostate tissue and moderately differentiated prostate cancer. Staining with Masson's Trichrome identified smooth muscle cells (*red*) and collagen fibers (*blue*) in normal prostate stroma (*A*), whereas reactive stroma in Gleason 3 prostate cancer showed a loss of smooth muscle cells (*B*). Vimentin immunostaining in normal prostate stroma was low (*C*), but vimentin-positive fibroblasts were distributed throughout the stroma and adjacent to epithelial acini (*arrows*). Vimentin was also expressed in blood vessel walls (*asterisks*). sm α-actin (*E*) and calponin (*G*) staining was high in the smooth muscle cells of normal prostate stroma. Reactive stroma in human prostate cancer showed elevated vimentin staining (*D*), whereas sm α-actin staining was maintained (*F*) and calponin staining was reduced (*H*). The expression pattern of these markers suggests that reactive stroma is composed of fibroblasts and myofibroblasts rather than smooth muscle cells. ×400.

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Table 1 Immunohistochemistry staining index for markers of stromal cell differentiation

	Normal prostate stroma (n = 84)				Gleason 3 cancer reactive stroma (n = 89)				
Marker	Zero	Low	Moderate	High	Zero	Low	Moderate	High	P^a
Vimentin	0	74	9	1	2	36	34	17	<0.001
α-Actin	0	0	4	80	1	25	30	33	<0.001
Calponin	0	2	26	56	14	55	14	6	<0.001

[a] Fisher exact test comparing normal stroma to Gleason 3 reactive stroma (zero/low staining *versus* moderate/high staining).

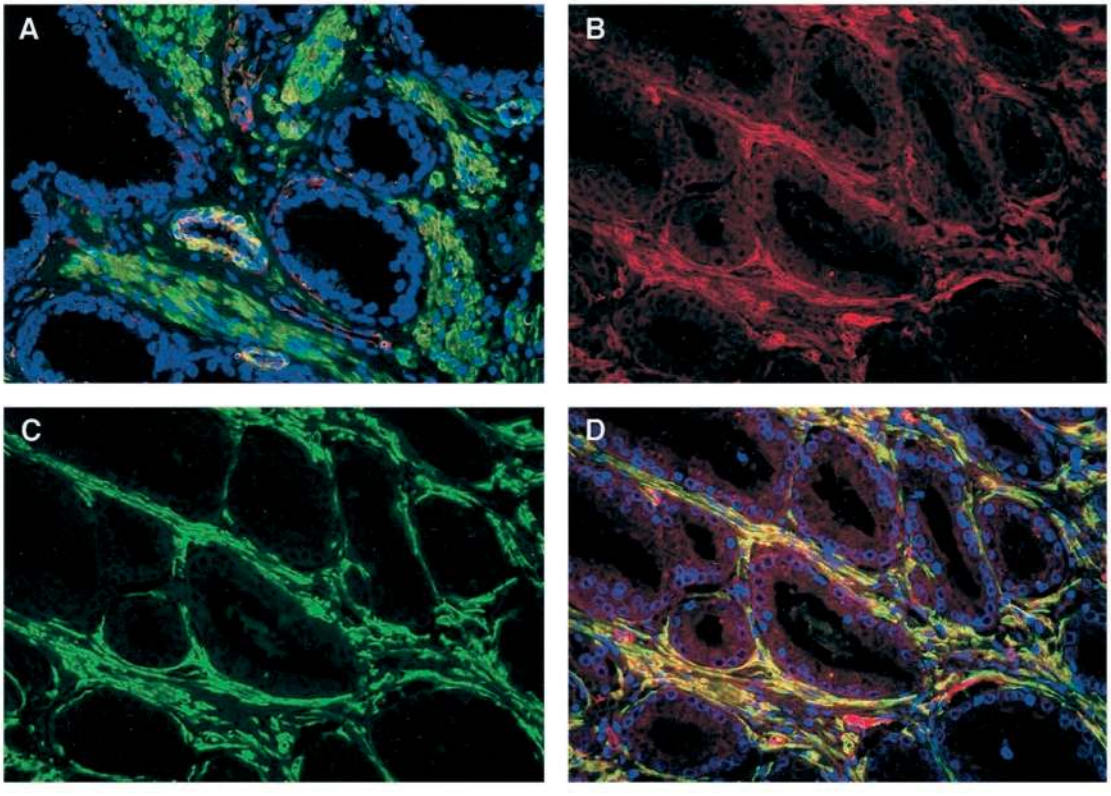

Fig. 2 Double-label fluorescent immunohistochemistry identifies myofibroblasts in human prostate cancer reactive stroma. *A*, merged image showing expression of vimentin (*red*) and sm α-actin (*green*) in normal prostate stroma. Colocalization (*yellow*) was only observed in blood vessel walls. Nuclei are stained with DAPI (*blue*). *B*, elevated expression of vimentin in reactive stromal cells. *C*, expression of sm α-actin in reactive stromal cells. *D*, the images shown in *B* and *C* were merged as described in "Materials and Methods." Coexpression (*yellow*) of vimentin and sm α-actin in prostate cancer reactive stromal cells indicates the myofibroblast phenotype. ×400.

composed of smooth muscle cells, reactive stroma in human prostate cancer is enriched with myofibroblasts and fibroblasts.

Expression of ECM Components in Prostate Cancer. Myofibroblasts synthesize ECM components and remodeling enzymes, thus identification of myofibroblasts in human prostate cancer reactive stroma suggests that the ECM may be altered. The strong blue staining observed with Masson's Trichrome (Fig. 1*B*) indicated a possible increase in collagen fibers, therefore, we examined collagen type I synthesis by immunohistochemistry. The antibody used specifically recognizes the NH_2 terminus of procollagen I peptides in cells synthesizing collagen type I but not mature extracellular collagen fibers in the tissue. Although procollagen I staining was not detected in the stroma of normal prostate tissue (Fig. 3*A*), it was observed in the reactive stroma of 16 of 20 patients with moderately differentiated prostate cancer (Fig. 3*B*). We also examined frozen tissue sections from seven additional cases of Gleason 3 prostate cancer. Intense procollagen I staining was observed in the reactive stroma of all patients. These data

Table 2 Percentage of myofibroblasts in reactive stroma

% positive cells	Gleason 3	Gleason 4	P^a
0 (0% cells)	0	0	
1 (1–33%)	7	3	
2 (34–66%)	20	4	
3 (67–100%)	9	10	0.03

[a] Fisher exact test comparing the proportion of Gleason 3 and Gleason 4 cases with 67–100% myofibroblasts.

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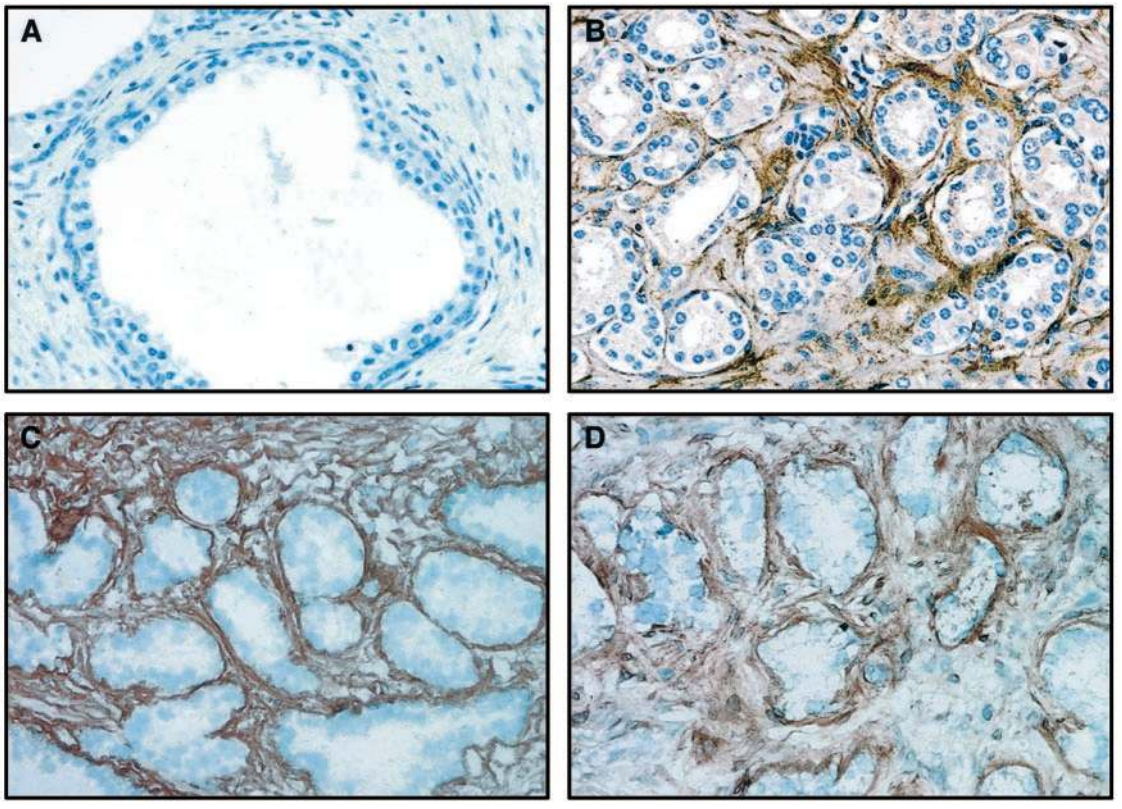

Fig. 3 Immunostaining for markers of ECM remodeling. Synthesis of procollagen I was observed in reactive stoma cells in moderately differentiated human prostate cancer (*B*) but not in normal prostate stroma (*A*). Tenascin was detected throughout the reactive stroma of Gleason 3 prostate cancer (*C*). FAP was expressed by reactive stromal cells adjacent to prostate carcinoma cells (*D*). ×400.

suggest that collagen type I synthesis is associated with reactive stroma in cancer but is essentially quiescent in normal prostate gland stroma.

Studies of human cancers have identified several additional ECM proteins and remodeling enzymes that are produced by reactive stromal cells. We examined the reactive stroma associated with moderately differentiated prostate cancer for expression of two of these factors. Tenascin is an ECM glycoprotein that modulates cell adhesion and migration. It is typically expressed during development and at sites of tissue remodeling (42). Previous reports revealed that tenascin expression was weak in normal tissues, whereas prostate carcinoma showed strong staining in the peritumoral stroma (43, 44). Our results confirmed these observations; stromal expression of tenascin was identified in 7 of 7 Gleason 3 prostate cancers examined. As shown in Fig. 3*C*, tenascin was present throughout the reactive stroma.

FAP was originally identified as a cell surface glycoprotein expressed by reactive stromal cells in colorectal, breast, and other human carcinomas (45). FAP is also expressed in fibroblasts during wound repair but is not observed in normal stroma. Recent studies have shown that FAP exhibits protease activity and may contribute to ECM remodeling (24). Accordingly, we wanted to determine whether the reactive stromal cells in human prostate cancer also express this factor. FAP was observed in 7 of 7 cases examined, with the most intense staining in stromal cells adjacent to carcinoma cells (Fig. 3*D*). To our knowledge, this is the first study to examine FAP expression in human prostate cancer stroma. Production of ECM components and proteases by reactive stromal cells indicates that the ECM in human prostate cancer is being actively remodeled and further suggests a modified stromal microenvironment.

Reactive Stroma in PIN. The reactive stroma microenvironment is predicted to promote cancer progression, particularly at the early stages of tumorigenesis. Because PIN is considered to be the precursor to prostate cancer (46), we examined PIN lesions for early signs of reactive stroma. PIN was identified in 17 of 20 radical prostatectomy specimens. Elevated vimentin expression was observed in the periacinar fibroblasts immediately adjacent to PIN foci (Fig. 4*A*). Additionally, the periacinar fibroblast layer appeared to be thickened in several areas. To confirm these observations, the vimentin staining index was determined for PIN lesions on the prostate tissue microarray ($n = 27$). Compared with the low staining level observed in normal prostate tissue, vimentin staining was increased to moderate/high levels in 48% of PIN cases ($P < 0.001$; Fisher's exact test). Double-label immunohistochemistry with vimentin and sm α-actin antibodies also revealed an increase in fibroblasts and identified some of the cells as myofibroblasts (Fig. 4*B*). These data suggest a local activation of periacinar fibroblasts in response to PIN.

Similar to reactive stroma in Gleason 3 prostate cancer,

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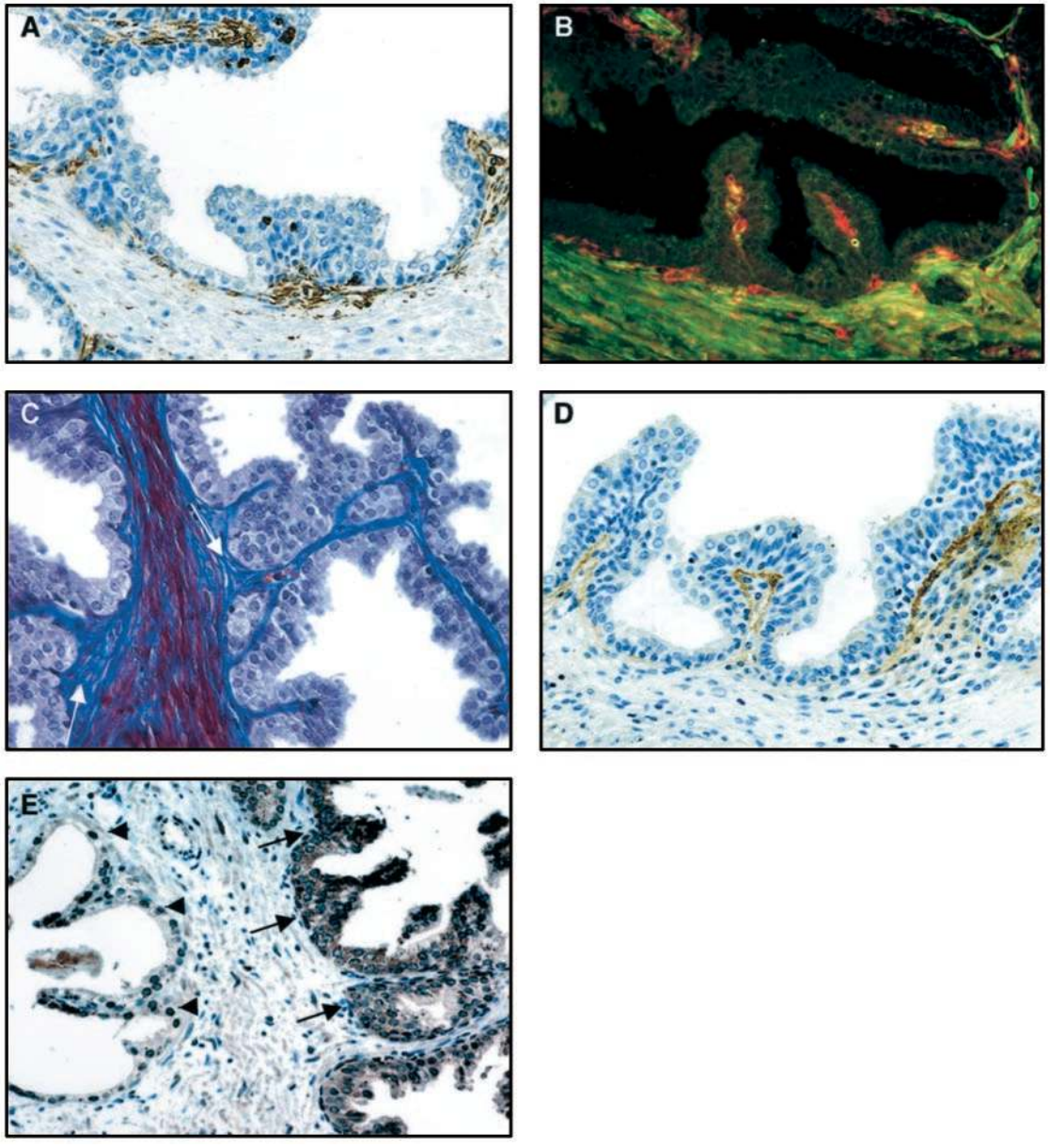

Fig. 4 Reactive stroma in PIN. *A*, elevated vimentin immunostaining was observed in stromal cells immediately adjacent to PIN. *B*, double-label fluorescent immunostaining of vimentin and sm α-actin was used to identify stromal cell phenotypes in PIN. Vimentin-positive fibroblasts (*red*) and myofibroblasts (*yellow*) coexpressing vimentin and sm α-actin were observed adjacent to PIN lesions. In some areas, these reactive stromal cells appear to separate PIN cells from sm α-actin-positive smooth muscle cells (*green*) in the normal prostate stroma. *C*, collagen fibers in reactive stroma adjacent to PIN lesions were stained blue with Masson's Trichrome (*arrows*) and appear to displace the prostate smooth muscle cells (*red*) from the PIN cells. *D*, immunohistochemistry with a procollagen I antibody detected collagen I synthesis in the stroma immediately adjacent to PIN foci but not in the normal prostate stroma. *E*, TGF-β1 expression was elevated in PIN cells (*arrows*) compared with normal prostate epithelium (*arrowheads*). ×400.

Masson's Trichrome showed intense blue staining of the stroma immediately surrounding and within PIN lesions (Fig. 4*C*). This observation indicates that the ECM in PIN stroma may be remodeled. Similar to the vimentin staining pattern, procollagen I was detected in the periacinar fibroblasts and myofibroblasts immediately adjacent to PIN sites (Fig. 4*D*). These results are consistent with previous studies that have shown tenascin expression in the ECM immediately surrounding high-grade PIN (43, 44). Taken together, these data suggest that stromal cell activation and ECM remodeling are induced at the PIN stage. Moreover, the increase in periacinar fibroblasts/myofibroblasts and elevated collagen synthesis appeared to separate the PIN cells from the smooth muscle cells in the normal prostate stroma. This separation can be visualized with double-label

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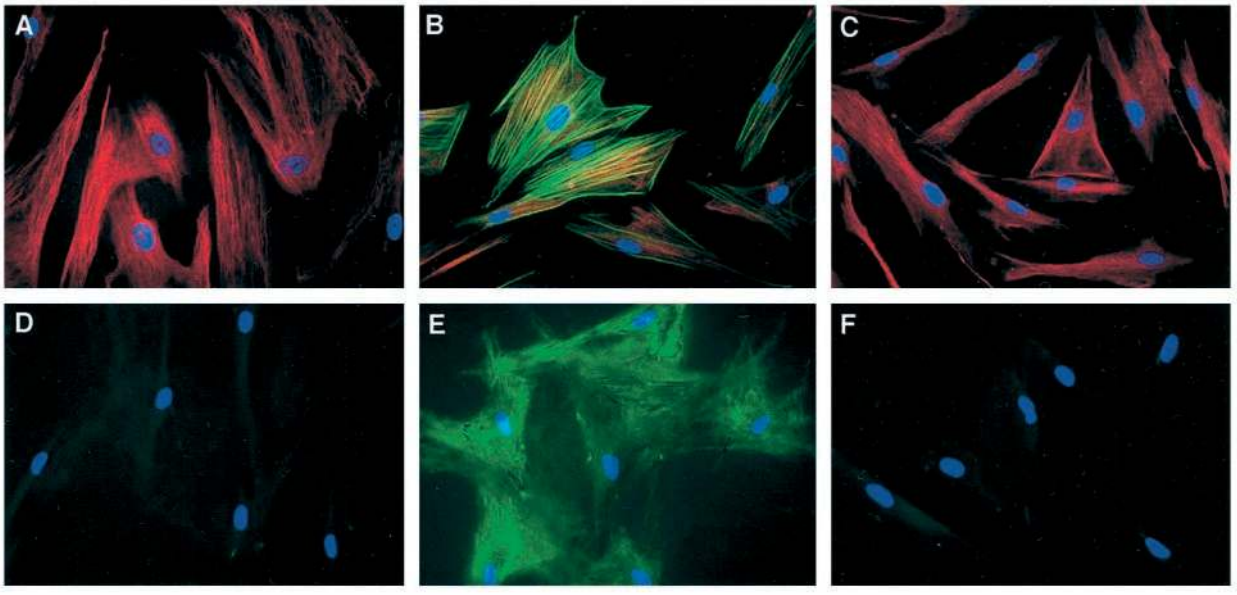

Fig. 5 TGF-β1 induces reactive stroma phenotype *in vitro*. HPS-TZ1A human prostate stromal cells were treated with 25 pM TGF-β1 in defined culture conditions and analyzed for marker proteins by immunocytochemistry. *A–C*, double-label with vimentin (*red*) and sm α-actin (*green*); nuclei are stained with DAPI (*blue*). In basal media (*A*), stromal cells exhibited a fibroblast phenotype, whereas TGF-β1 (*B*) induced expression of sm α-actin and the myofibroblast phenotype. Addition of a TGF-β1 neutralizing antibody (*C*) blocked the phenotypic switch. *D–F*, expression of tenascin (*green*). Stromal cell expression of tenascin was low in basal media (*D*) but elevated in the presence of TGF-β1 (*E*). The response was blocked by a TGF-β1 neutralizing antibody (*F*). ×400.

immunohistochemistry and Masson's Trichrome (Fig. 4, *B* and *C*, *arrows*). Therefore, reactive stroma appears to be induced at the earliest stages of tumorigenesis and may evolve with prostate cancer progression to effectively displace the normal fibromuscular stroma.

TGF-β1 Induction of the Myofibroblast Phenotype. Elevated expression of TGF-β1 has been reported in prostate cancer and was associated with disease progression (38, 47–49). TGF-β1 is thought to act directly on the stroma because TGF-β receptors are down-regulated by carcinoma cells but maintained in stromal cells (48, 50–52). Furthermore, TGF-β1 regulates the stromal response in wound repair (28). Accordingly, TGF-β1 is considered to be a candidate regulator for the genesis of reactive stroma at PIN sites. In support of this concept, a preliminary immunohistochemistry study showed that TGF-β1 staining was elevated in high grade PIN lesions in 4 of 5 cases examined (Fig. 4*E*).

To directly assess the ability of TGF-β1 to regulate the reactive stroma phenotype, we used a human prostate stromal cell line initiated in our laboratory. HPS-TZ1A cells cultured in fully defined media expressed vimentin, but not sm α-actin, indicating a fibroblast phenotype (Fig. 5*A*). Addition of 25 pM TGF-β1 to serum-free, fully defined culture media induced expression of sm α-actin in >50% of the culture, whereas vimentin expression was maintained. Coexpression of vimentin and sm α-actin by these cells indicates induction of a myofibroblast phenotype (Fig. 5*B*). Neutralizing antibodies to TGF-β1 blocked the phenotypic switch (Fig. 5*C*). We also examined expression of the reactive stroma ECM marker tenascin in response to TGF-β1. Under basal conditions, HPS-TZ1A cells expressed very low levels of tenascin (Fig. 5*D*). TGF-β1 (25 pmol) induced elevated expression of tenascin by these cells (Fig. 5*E*), whereas neutralizing antibodies to TGF-β1 blocked the response (Fig. 5*F*). These studies demonstrate that TGF-β1 induces human prostate fibroblasts to switch to the myofibroblast phenotype *in vitro* and stimulates expression of an ECM component found in human prostate cancer reactive stroma. Together, these observations suggest that TGF-β1 may be a regulator of prostate cancer reactive stroma *in vivo*.

DISCUSSION

Before this report, a comprehensive analysis of the reactive stroma environment in human prostate cancer had not been performed. In this study, we report that a stromal reaction occurs during human prostate cancer progression, creating a stroma microenvironment that is fundamentally different from normal prostate gland stroma. Although smooth muscle cells were predominant in normal prostate stroma, reactive stroma in prostate cancer was enriched with myofibroblasts and fibroblasts and showed a significant decrease in differentiated smooth muscle cell content. Indeed, we show here that the reactive stroma compartment is composed of >50% myofibroblasts, an activated stromal cell phenotype that was not observed in normal prostate stroma. The activated stromal cells exhibited increased production of specific ECM components and matrix remodeling enzymes, including collagen type I, tenascin, and FAP. The expression of these proteins in the adult are normally restricted to sites of tissue remodeling, including wound repair granulation tissue and reactive stroma in cancer (7, 18–22, 42, 45). Similar

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to the results presented here, previous studies have pointed to alterations in the ECM of prostate cancer. These studies have shown elevated expression of versican (53), hyaluronic acid (54), tenascin (43, 44), MMP-2, and MMP-9 (55) in prostate cancer stroma, which is consistent with our classification of this stroma as reactive.

Alterations in stromal cell phenotype and increased ECM synthesis were initiated early in prostate cancer progression at the precancerous PIN stage. Moreover, our observations suggest that the normal prostate fibromuscular stroma is displaced by the developing reactive stroma. Alternatively, it has been suggested that abnormal epithelial-smooth muscle signaling during prostate carcinogenesis may lead to smooth muscle dedifferentiation (56). However, our data suggest that myofibroblasts in prostate cancer evolved from a periacinar ring of fibroblasts immediately adjacent to PIN foci, not from normal prostate differentiated smooth muscle. Elevated vimentin staining and collagen I synthesis in stromal cells immediately adjacent to PIN foci suggest that PIN epithelial cells induce a local stromal reaction. In addition, previous studies have reported an increase in tenascin expression in the ECM adjacent to PIN (43, 44). These data suggest that local activation of stromal cells immediately adjacent to PIN is the origin of reactive stroma. Our data also suggest that reactive stroma progresses with the development of prostate cancer. The proportion of myofibroblasts in reactive stroma appears to increase from PIN to moderately differentiated to poorly differentiated carcinoma. Furthermore, we suggest the development of this reactive stroma in PIN acts to displace the normal prostate smooth muscle as PIN progresses to Gleason 3 and 4 cancer. These data suggest that rather than invading into the normal stromal compartment, precarcinoma PIN cells induce a reactive stroma, which coevolves with the carcinoma cells. Together, the carcinoma cells and the reactive stroma displace the normal tissue to comprise the cancer foci, which ultimately forms the tumor.

The regulatory factors that direct a stromal reaction in human prostate cancer are not known. TGF-β1 emerges as a strong candidate because of its key regulatory role in wound repair and stromal cell biology (5, 28). Elevated expression of TGF-β1 has been reported in prostate carcinoma cells (38, 47, 48). Moreover, TGF-β1 action in prostate cancer appears to be directed toward the stromal compartment. Several previous studies have shown that TGF-β receptors are typically expressed in reactive stromal cells and are down-regulated in prostate carcinoma cells (48, 50–52). In this study, we have shown that TGF-β1 is overexpressed in PIN epithelial cells and is capable of inducing HPS-TZ1A human prostate fibroblasts to differentiate to the myofibroblast phenotype and express tenascin *in vitro*. Similarly, TGF-β1 induction of the myofibroblast phenotype has been observed in mammary gland fibroblasts (16). Furthermore, it is well established that TGF-β regulates expression of several ECM components and regulatory proteases/protease inhibitors, including factors expressed specifically at sites of tissue remodeling (28). Previous studies have demonstrated elevated FAP expression in response to TGF-β1 (57). Additionally, it has been reported that TGF-β1 increases collagen type I synthesis (58) and versican expression (59) in human prostate stromal cells. Together, these studies suggest that TGF-β1 is capable of regulating many aspects of periacinar fibroblast activation. In support of this hypothesis, s.c. injection of TGF-β1 was shown to be sufficient to induce a stromal reaction characterized by myofibroblast differentiation, collagen production, and angiogenesis (30, 31). Therefore, TGF-β1 is a likely candidate for the regulation of the stromal reaction in PIN and development of reactive stroma during prostate cancer progression.

Once reactive stroma has formed, a central question emerges as to how reactive stroma may affect the rate of prostate cancer tumorigenesis. It is clear that stromal-epithelial interactions are important to prostate gland development and maintenance of the adult phenotype (56). Accordingly, replacement of normal stroma with a reactive stroma is likely to alter such stromal-epithelial interactions and affect cancer progression. Work by Olumi *et al*. (13) has shown that prostate CAFs promoted tumorigenesis of a SV40 immortalized prostate epithelial cell line (TAg-HPE, derived from a benign prostatic hyperplasia) when cografted in the renal capsule. TAg-HPE cells alone were nontumorigenic, and cografts with normal prostatic stromal cells, termed fibroblasts in this report, did not affect TAg-HPE growth and malignant characteristics. Tag-HPE cell proliferation was increased, and apoptosis was decreased when these cells were cocultured with CAFs, compared with coculture with normal fibroblasts. Because CAFs were isolated from regions of frank carcinoma in radical prostatectomy specimens, it is likely that these cells were derived from the fibroblasts and myofibroblasts of reactive stroma. Additionally, our own studies have shown that the incidence and rate of LNCaP tumorigenesis in nude mice depends on the source of human prostate stromal cells coinoculated with the cancer cells (14). These studies went on to show that the effect of reactive stroma on LNCaP tumorigenesis was due, in part, to initiation of rapid angiogenesis in early tumor development, relative to control tumors lacking human prostate stromal cells.

The altered ECM makeup of reactive stroma is likely to be key in the overall effects of reactive stroma on angiogenesis and tumorigenesis. During wound repair, fibroblasts and myofibroblasts create a matrix that stimulates angiogenesis and promotes epithelial cell growth and migration (7, 42). In support of these findings, a recent study has shown that an inhibitor of collagen type I synthesis and ECM deposition (halofuginone) suppressed tumor progression and angiogenesis in both transplantable and chemically induced mouse bladder carcinomas (60). In halofuginone-treated animals, collagen α1(I) gene expression was decreased in the tumor stromal compartment, which resulted in a 60–70% reduction in tumor volume and a significant decrease in microvessel density (60). Interestingly, collagen type I has been shown to direct the migration and assembly of endothelial cells into new blood vessels (61). These data, in combination with data presented here, suggest that collagen type I expression is likely to be a key feature of reactive stroma in tumorigenesis. Together, these studies point to the central role reactive stroma plays in stimulating tumorigenesis of prostate cancer cells and suggest that possible mechanisms include altered proliferation and apoptotic rates in carcinoma cells, as well as increased angiogenesis.

This study and others indicate that a reactive stroma, distinct from normal stroma, occurs in adenocarcinoma progression. Data reported here represent an initial step in defining

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reactive stroma in prostate cancer progression and in identifying potential regulators of this response. It is possible that specific markers of reactive stroma can be used to better predict the rate of cancer progression or the possibility of recurrence. Future studies will be directed toward identifying specific markers of reactive stroma, which may fulfill the criteria of a differential prognostic indicator. In addition, because reactive stroma biology is likely to affect cancer progression, it may be possible to target specific components of reactive stroma in novel therapeutic approaches to prostate cancer.

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