Asian Journal of Andrology (2009): 176–182

www.nature.com/aja

Original Article

Tumor formation of prostate cancer cells influenced by stromal cells from the transitional or peripheral zones of the normal prostate

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Abstract

This study was designed to investigate the different involvements of prostatic stromal cells from the normal transitional zone (TZ) or peripheral zone (PZ) in the carcinogenesis of prostate cancer (PCa) epithelial cells (PC-3) *in vitro* and *in vivo* co-culture models. Ultra-structures and gene expression profiles of primary cultures of human prostatic stromal cells from the normal TZ or PZ were analyzed by electron microscopy and microarray analysis. *In vitro* and *in vivo* co-culture models composed of normal TZ or PZ stromal cells and human PCa PC-3 cells were established. We assessed tumor growth and weight in the in vivo nude mice model. There are morphological and ultra-structural differences in stromal cells from TZ and PZ of the normal prostate. In all, 514 differentially expressed genes were selected by microarray analysis; 483 genes were more highly expressed in stromal cells from TZ and 31 were more highly expressed in those from PZ. Co-culture with PZ stromal cells and transforming growth factor-β1 (TGF-β1) increased the tumor growth of PC-3 cells *in vitro* and *in vivo*, as well as Bcl-2 expression. On the other hand, stromal cells of TZ suppressed PC-3 cell tumor growth in the mouse model. We conclude that ultra-structures and gene expression differ between the stromal cells from TZ or PZ of the normal prostate, and stroma–epithelium interactions from TZ or PZ might be responsible for the distinct zonal localization of prostate tumor formation.

Asian Journal of Andrology (2009) 11: 176–182. doi: 10.1038/aja.2008.33; published online 5 January 2009.

Keywords: co-culture, epithelial cells, stromal cells, transitional zone, peripheral zone, prostate cancer

1 Introduction

Prostate cancer (PCa) is the most frequently diagnosed cancer in men in Western countries and the second leading cause of cancer deaths in those parts of the world [1]. Recently, Asian countries, such as Japan and China, have also reported high incidences of PCa and consequent mortality rates. McNeal [2] first proposed the histological division of the prostate into an outer peripheral zone (PZ), a central zone (CZ), the anterior fibromuscular stroma and an inner transition zone (TZ). It has been claimed that 70% of PCa occur in the PZ, approximately 20% are found in the TZ and benign prostatic hyperplasia (BPH) occurs almost exclusively in the TZ. PCa from the TZ are relatively nonaggressive, whereas cancers from the PZ are more aggressive, tend to invade the periprostatic tissues and have higher biochemical recurrence rates [3]. Why do aggressive tumors predominantly occur in the PZ? Most studies have shown that the TZ of the prostate

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Received: 17 October 2008 Accepted: 22 October 2008
Published online: 5 January 2009

is morphologically similar to the PZ. Some authors assume that prostate diseases having a strong preference for a specific zone originate from pre-existing molecular differences in the normal zones. Xia *et al*. [4, 5] found that the apoptosis-related genes, epidermal growth factor (EGF) and transforming growth factor-β_1 (TGF-β_1) are expressed in higher concentrations in the TZ than in the PZ. Leonie *et al*. [6] used oligonucleotide microarray analyses to compare gene expression between the normal PZ and TZ and found that gene expression differs between the PZ and TZ of the prostate. The zones of the prostate gland consist of a complex environment of both stromal and epithelial cells. Cunha [7] showed that stromal cells can modulate the differentiation pattern of normal prostatic epithelial cells and are critical for normal tissue development and maintenance, as well as for disease processes, such as development of PCa. Barclay *et al*. [8] found that, in the co-culture system of stromal cells from PCa and epithelial cells from BPH (BPH-1), the BPH-1 cells showed malignant characteristics; even in co-cultures with normal transitional cells of the bladder there were malignant characteristics, suggesting that the interaction of prostatic epithelium with the stroma plays an important role in the development of prostate disease. Despite the importance of stromal cells in prostate development, function and disease, a comprehensive view of the molecular differences among the stromal cells of different zonal locations is currently lacking [9].

Here, we assume that pre-existing molecular differences in the stromal cells of the PZ and TZ and stroma-epithelium interactions might be responsible for the distinct zonal localization of prostate diseases. Such information may provide not only new insights into the biology of prostate pathogenesis, but also novel therapeutic strategies aimed at preventing the generation of stroma, which is important for disease development and progression. To obtain a comprehensive view of stromal cells from different histological origins, we profiled adult human stromal cell cultures from normal TZs and normal PZs and then developed *in vitro* and *in vivo* co-culture models to examine the different involvements of the stromal cells from the TZs and PZs in the carcinogenesis of the prostate.

2 Materials and methods

2.1 Cell culture

Fresh prostate specimens were obtained from normal organ donors between 20 and 40 years of age at the Shanghai First People's Hospital (Shanghai, China). The experimental protocols were approved by the Shanghai First People's Hospital Medical Ethics Committee. Both pathologists confirmed the zonal-specific tissue. Tissue specimens, cut according to McNeal's zonal anatomy, were mechanically and enzymatically dissociated by treatment with DNase and collagenase. Epithelial cells were separated from stromal cells by discontinuous gradient centrifugation [10]. The stromal cells were cultured with RPMI 1640 (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Melbourne, Australia) and antibiotics (100 mg mL^{-1} streptomycin and 100 IU mL^{-1} penicillin, North China Pharmaceutical Co., Ltd, Shijiazhuang, China) at 37°C under 5% CO_2 and humidified atmosphere. The stromal cells were used at passages 3–5. PCa PC-3 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI 1640 medium with 10% fetal calf serum (FCS) (Gemini, Calabasas, CA, USA).

2.2 Transmission electron microscopy

For the identification and observation of stromal cells from TZs or PZs by a transmission electron microscope (TEM), stromal cells were fixed in 1% glutaraldehyde and 4% paraformaldehyde in phosphate-buffered saline (PBS), postfixed in 1% osmium tetroxide in PBS, dehydrated and subsequently embedded in epoxy resin. Ultrathin sections were stained with uranyl and lead acetates and examined under a CM-120 electron microscope at 80 kV (Philip, Eindhoven, the Netherlands).

2.3 RNA isolation, cDNA labeling and microarray analysis

Total RNA was isolated from cells using TRI reagent (Sigma, Santa Clara, CA, USA) according to the manufacturer's protocol. The RNA was purified using RNAeasy columns (Qiagen GmbH, Hilden, Germany). Complementary DNA (cDNA) for microarray was labeled according to the manufacturer's instructions. RNA from TZ was labeled with Cy3 and RNA from PZ was labeled with Cy5 (Amersham, NJ, USA). A hybridization buffer was added to the probe, incubated at 75°C for 5 min and then added to the SBC human 16 K array (Shanghai Biochip Co. Ltd, Shanghai, China). The hybridization was carried

out at 42ºC for 16 h (UVP, Upland, CA, USA). The slides were then washed, dried and scanned using a scanner (Agilent, Santa Clara, CA, USA). All image analyses were performed using Quantarray software (Perkin-Elmer Life Sciences, USA). A Cy3:Cy5 ratio ≥ 2 or ≤ 0.5 was established and the $-\log_{10}$ (*P* value), a positive value, was calculated to represent significant differences.

2.4 In vitro stromal cell and epithelial cell co-culture models

In vitro co-culture cell models of stromal cells from PZs or TZs with the PCa cell line PC-3 were constructed in six-well plates using a cell insert of 3-μm PET membrane (Falcon, BD, Franklin Lakes, NJ, USA). PC-3 cells were cultured either alone or together with the stromal cells. The stromal cells from PZs or TZs were seeded on six-well plates at densities of 1×10^5 per well; PC-3 cells were seeded onto PET membranes at a density of 1×10^5 per PET. They were all cultured in RPMI 1640 medium supplemented with 10% FBS and antibiotics at 37ºC under 5% CO_2. To determine the effect of TGF-β_1 on the co-culture system of PZ or TZ stromal cells and PC-3 cells, 40 pmol L^{-1} TGF-β_1 was added into the co-culture medium. Each experiment was carried out in triplicate.

2.5 In vivo stromal cell and epithelial cell co-culture models

Male nude mice, 4–6 weeks old, were purchased from the Animal Center of the Chinese Academy of Sciences (Shanghai, China) and maintained in a specific pathogen-free barrier facility according to our institutional guidelines. PC-3 cells (5×10^6) were suspended in 0.1 mLof serum-free RPMI 1640 medium with PZ and TZ stromal cells (5×10^6) and injected at two sites subcutaneously (s.c.) in the hindquarters of male athymic nude mice ($n = 6$). Tumors formed were measured weekly, and the assay was terminated at 4 and 6 weeks after injection. Tumor weight was calculated after being cut from co-culture mice models. Representative portions of all tumors were submitted for routine microscopic examination. In the animal experiment, PZ or TZ stromal cells were inoculated with or without TGF-β_1 (40 pmol L^{-1}) before injection with PC-3 cells.

2.6 Western blot

Expression levels of *PCNA* and *Bcl-2* genes in PC-3 cells after *in vitro* co-culture with stromal cells and TGF-β_1 were investigated using Western blot. As described earlier, PC-3 cells were resuspended in PBS, lysed and sonicated. Proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose. After blocking in 3% skim milk, membranes were probed with a 1:500 dilution of mouse anti-Bcl-2 mAb or a 1:1 000 dilution of mouse anti-PCNA (Santa Cruz Biotech, Santa Cruz, CA, USA), washed and incubated with goat anti-mouse Ig-G secondary antibody (Santa Cruz Biotech). An equal loading of membranes was verified with mouse anti-tubulin (Santa Cruz Biotech).

2.7 Histology

Mice were killed and resected tumors were fixed with 10% buffered formalin and 70% ethanol. Paraffin-embedded specimens were then sectioned at 5 mm thickness and stained with hematoxylin and eosin (H&E) according to standard histopathological techniques.

2.8 Statistical analysis

All data were presented as mean ± SD and analyzed by analysis of variance in combination with paired *t*-test for multiple comparisons.

3 Results

3.1 Morphology and ultrastructure of stromal cells from PZs or TZs of normal prostate

Figure 1 shows the morphology of the confluent monolayer cultures of stromal cells from the TZ or the PZ (Figure 1A). The cellular composition of the stromal cells in culture was further analyzed by immunocytochemistry (IHC) using antibodies specific for smooth muscle cells, fibroblasts and epithelial cells. All stromal cells in culture stained positive for vimentin and negative for actin and prostate-specific antigen (PSA) (Figure 1B). These results suggest that our stromal cultures contained 100% fibroblast cells. Ultrastructural analysis of stromal cells from the PZ and TZ (Figure 1C) clearly showed the presence of an increased number of Golgi complexes and rough endoplasmic reticuli in the TZ compared with the PZ.

3.2 Gene expression profiles in stromal cells from PZs or TZs of normal prostates

We profiled the gene expression of stromal cell

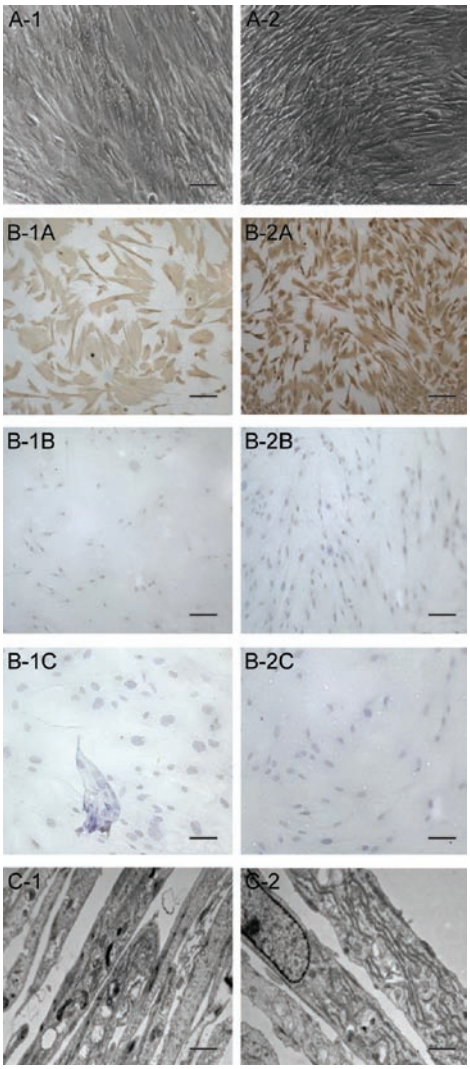

Figure 1. Identification of and differences in morphology and ultrastructure of stromal cells from peripheral zones (PZs) or transitional zones (TZs) of normal prostates by inverted microscope, immunocytochemistry (IHC) and transmission electron microscope (TEM). (A): Primary culture of stromal cells from the TZ or PZ of normal prostate by an inverted microscope (× 200). (A-1) Stromal cells from TZ; (A-2) stromal cells from PZ. (B): Identification of stromal cells from the TZ (B-1) or PZ (B-2) by IHC (× 100). (B-1A) Vimentin expression positive; (B-1B) actin expression negative; (B-1C) PSA expression negative. (B-2A) Vimentin expression positive; (B-2B) actin expression negative; (B-2C) PSA expression negative. Stromal cells from the TZ or PZ are fibroblasts. (C): Identification of morphology and differences in ultrastructure of stromal cells from the TZ or PZ by TEM (× 27 000). (C-1) Stromal cells from the TZ are spindle-shaped fibroblasts. The cell nucleus is long and oval. In the cytoplasm, there are more Golgi complexes and rough endoplasmic reticuli, but a small number of mitochondria; (C-2) Stromal cells from the PZ are slender spindle-shaped fibroblasts. The cell nucleus is oval. In the cytoplasm, there are a large number of rough endoplasmic reticuli, but a small number of mitochondria and Golgi complexes. Bars = 20, 50 and 1 μm in (A), (B) and (C), respecitvely.

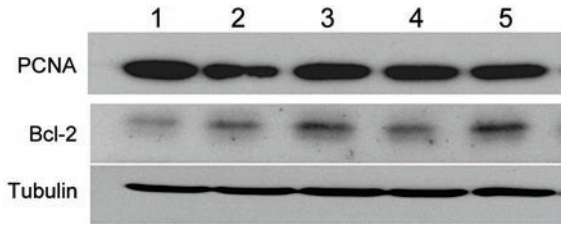

Figure 2. PCNA and Bcl-2 expression of PC-3 from the co-culture system with stromal cells from the peripheral zones (PZ) or transitional zones (TZ) by Western blot analysis. Bcl-2 expression of PC-3 stimulated by TGF-β_1 (lanes 3 and 5) is stronger than that of the control (lanes 2 and 4), and less than that of PC-3 (lane 1). Bcl-2 expression of PC-3 is different in the TZ and the PZ. PCNA expression in PC-3 cells did not change in any of the co-culture models (1: PC-3; 2: PC-3/TZ-TGF-β_1; 3: PC-3/TZ; 4: PC-3/PZ; 5: PC-3/PZ-TGF-β_1).

cultures from the PZ and TZ of normal prostates and found that stromal fibroblast cells from the PZ and TZ have distinct gene expression patterns. In all, 514 differentially expressed genes were selected; 483 genes were more highly expressed in stromal cells from the TZ and 31 were more highly expressed in those from the PZ. According to Gene Ontology (GO) and Dif-Path analysis, many of the genes are involved in the biological processes of prostatic diseases, including regulation of cell proliferation and apoptosis, cell adhesion and immune response.

3.3 Modulation of PC-3 by stromal cells from PZs or TZs in vitro

In the *in vitro* stromal-PC-3 cell co-culture models, we examined whether the stromal fibroblast cells from different zonal locations (PZ or TZ) modulate the PCa PC-3 cells. Using Western blot analysis, we observed a significant increase in Bcl-2 expression in PC-3 cells co-cultured with stromal cells from PZs stimulated by TGF-β_1 compared with TZ stromal cells (Figure 2). Furthermore, PCNA expression in PC-3 cells did not change in either PC-3/PZ or PC-3/TZ co-culture models.

3.4 Modulation of PC-3 tumor growth by stromal cells from PZs or TZs in vivo

To investigate whether different zonal stromal cells (PZ or TZ) could stimulate PC-3 cell tumor growth, we co-injected PC-3 cells (5×10^6) with stromal cells (5×10^6) from either the PZ or the TZ separately and monitored the growth of these mixed tumors. As TGF-β_1 plays

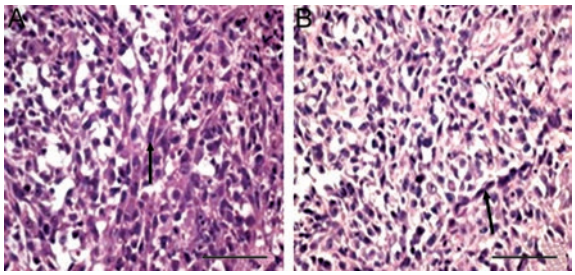

Figure 3. Hematoxylin and eosin staining of tumor tissues from co-cultured mice models (× 400). (A): PC-3/PZ-TGF-β_1 group, dual-nuclear, muscularity and malignant changes of fibroblasts (black arrow). (B): PC-3/TZ-TGF-β_1 group fibroblasts arranged in rows and malignant changes (black arrow). Bars = 20 μm.

a key role in the interaction of the epithelium and the stroma, in this study we stimulated both *in vivo* stromal and epithelial cell co-culture models with or without 40 pmol L^{-1} TGF-β_1. One week after the injection, we observed tumor formation in PC-3, PC-3/PZ, PC-3/TZ, PC-3/PZ-TGF-β_1 and PC-3/TZ-TGF-β_1 co-culture mice models, but tumors of the PC-3/PZ-TGF-β_1 model had grown very fast in all of the co-culture mouse models. On the other hand, tumors of the PC-3/TZ-TGF-β_1 and PC-3/TZ models grew more slowly than those of the PC-3 model. As shown in Table 1, the average tumor weight of the PC-3/PZ-TGF-β_1 model was 1.673 ± 0.320 g, but the average tumor weight of the PC-3/TZ-TGF-β_1 model was significantly lesser, 0.040 ± 0.004 g ($P < 0.05$). Using histological evaluations of the mixed tumors, we found that the stromal cells in the PC-3/PZ-TGF-β_1 tumor showed malignant changes, including large nuclear, dual nuclear and tendency towards muscularity, compared with those of the PC-3/TZ-TGF-β_1 tumor (Figure 3). This result correlates well with the histology of 'reactive stroma' of PCa.

4 Discussion

Stromal-epithelial interactions play an important role in prostate development and PCa [11]. Stroma, as 'soil' for epithelial cells, plays a leading role in the growth, differentiation and malignant change of epithelial cells. Stromal cell fibroblasts are the most important components of the stromal environment, and the dynamic changes in stromal cells lead to the secretion of a large number of active substances, like TGF-β_1, into the stroma as the initiating factors for prostate growth and development [12]. Most analyses identified differences in gene expression between the TZ and PZ of the prostate. As PCa and BPH are preferentially present in the PZ and TZ and interactions between the epithelium and stroma mediate prostate disease development, we hypothesized that there would be differences in gene expression between stromal cells of the TZ and PZ and that stroma-epithelium interactions might be responsible for the distinct zonal localization of prostate diseases. To understand the cellular and molecular basis of stroma-epithelium interactions, we isolated stromal cells from both the TZ and the PZ of normal prostate and employed model cell systems to investigate the interaction between stromal fibroblast and epithelial cells both *in vivo* and *in vitro*. Using TEM, we observed the differences in the ultrastructure between stromal cells from the PZ and TZ, which hinted that their gene expression may be different. By microarray gene chip analysis, Gelmann *et al*. [13] found that the expression of certain genes in the PZ was significantly higher than that in the TZ. We profiled gene expression between stromal cells from the PZ and TZ of the normal prostate and found that there are 514 different genes related to cell growth, differentiation, apoptosis and angiogenesis that are more highly expressed. We compared our data with those of gene expression from PCa and BPH described by Zhao [14] and Leonie [6]. We found that genes (e.g., *CCND1*, *DZIP1* and *LMO2*) preferentially expressed in stromal cells of normal PZs overlap with the differentially expressed genes in stromal cells of PCa, suggesting that PCa predominantly occurs in PZs, leading to zonal-preferentially expressed genes in the stroma.

In the past, research on PCa was focused on epithelial cells and the study of single tumor cell characteristics, and ignored stromal cells. Interactions between prostatic epithelial cells and stromal cells are inseparable. As shown by Singh *et al*. [15], stromal cells of the prostate can impact on the growth of PCa cells when stromal cells co-cultured with PCa LNCaP cells. This culture cannot be independent from the other cell type; therefore, to observe the changes of cells, we used cell insertion (3 μm PET membrane) to build a stromal cell and a PCa cell co-culture system *in vitro*. We used a PET membrane because nutrients can travel through the PET membrane freely while separating the stromal and the epithelial cells from each other, making it easy to

assess the cells' characteristics.

In 1998, most scholars raised the concept of 'reactive stroma' of PCa, which is different from 'stroma' of the normal prostate [16, 17]. Reactive stroma is responsible for the genesis and development of PCa and for promoting invasion, progression and metastasis [18]. Human carcinoma-associated fibroblasts (CAF) caused a dramatic increase in the growth of BPH-1 cells (nontumorigenic, human prostatic epithelial cell line) both *in vivo* and *in vitro* [8, 19, 20]. Experimental evidence suggests that TGF-β_1, a key mediator of the stromal response, is likely to play an important role [21]. In this study, PC-3/PZ and PC-3/TZ co-culture models have different reactions to TGF-β_1, and expression of Bcl-2 in PC-3 cells of both models is different. Bcl-2 expression in the PC-3/PZ-TGF-β_1 co-cultured group is higher than that in PC-3/TZ-TGF-β_1 (Figure 2). Moreover, we found that tumor formation of PC-3 cells with stromal fibroblast cells from the PZ and stimulated by TGF-β_1 occurred very quickly in all of the co-culture mouse models (Table 1). It is interesting to note that tumor formation of the PC-3/TZ-TGF-β_1 and PC-3/TZ models occurred more slowly than that of either the PC-3 model alone or the PC-3/PZ model. This result correlates well with the phenomenon of distinct PZ localization of PCa.

In brief, our data suggested differences in the ultrastructure and gene expression between the stromal cells from normal TZs and PZs of prostates and that tumor formation of PC-3 cells influenced by stromal cells from TZs or PZs of normal prostates might be the leading reason for the distinct localization of PCa. In addition, TGF-β_1 might contribute to the development of prostate tumor growth in the PZ and might play an important role in the development of PCa. Our study has been a foundation for researching the mechanisms of the stroma-epithelium interaction, exploring the pathogenesis of the zonal difference of PCa and discovering the therapeutic targets depending on stroma.

Table 1. Tumor weight of PC-3 co-cultured mice with different stromal cells.

Cells	Tumor weight (g) (mean ± SD)	*P*-value
Control (PC-3)	0.745 ± 0.069	§, †
PC-3/PZ	1.043 ± 0.092	*, †
PC-3/TZ	0.438 ± 0.041	†
PC-3/PZ-TGF-β_1	1.673 ± 0.320	§, *
PC-3/TZ-TGF-β_1	0.040 ± 0.004	§

Abbreviations: PZ, peripheral zone; TGF-β_1, transforming growth factor-β_1; TZ, transitional zone.

Tumor formation of PC-3/PZ-TGF-β_1 or PC-3/PZ groups compared with the control group (PC-3); tumor growth is promoted, whereas tumor growth of the PC-3/TZ-TGF-β_1 or PC-3/TZ groups compared with the PC-3 group is inhibited. The tumor weight of PC-3/PZ-TGF-β_1 group is heavier than those of other groups.

*: $P < 0.05$ PC-3/PZ *vs.* PC-3/PZ-TGF-β_1, $n = 6$;

§: $P < 0.05$ PC-3 *vs.* PC-3/PZ-TGF-β_1 *vs.* PC-3/TZ-TGF-β_1, $n = 6$;

†: $P > 0.05$ PC-3 *vs.* PC-3/PZ *vs.* PC-3/TZ , $n = 6$.

Acknowledgment

This study was supported by grant from the National Natural Science Foundation of China (No. 30672109).

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