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Transforming Growth Factor β1 as a Biomarker for Prostate Cancer

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Abstract Using the mouse prostate reconstitution (MPR) model system, under conditions where the *ras* and *myc* oncogenes are introduced via a recombinant retrovirus into both the mesenchymal and epithelial compartments of the urogenital sinus, poorly differentiated prostate cancer is produced with high frequency (>90%) using inbred C57BL/6 mice. Northern blotting and immunohistochemical analysis showed that the transition from benign prostatic hyperplasia (BPH) to prostate cancer is invariably associated with the induction of elevated transforming growth factor-β1 (TGF-β1) expression. Similar analysis of TGF-β1 in human BPH and prostate cancer is consistent with our MPR results and indicates that the accumulation of extracellular TGF-β1 is significantly more intense in prostate cancer compared to normal or benign prostate tissues. Interestingly, where benign pathologies are observed in the prostatic stroma in the presence of benign prostatic epithelium, extracellular TGF-β1 is seen predominantly in the stromal compartment. Experimental studies clearly demonstrate that mRNA levels of TGF-β1 and other growth related genes are regulated by androgens in prostate cancer cells. Overall, our results suggest that elevated TGF-β1 is involved in the development of prostate cancer. Direct determination of TGF-β1 levels and distribution as well as analysis of localized and systemic effects produced by TGF-β1 may serve as useful biomarkers for prostate cancer.

Key words: biomarkers of prostate cancer, TGF-β1

Prostate cancer is the most common cancer among US males, accounting for 22% of all new cases of cancer and 12% of all cancer deaths (an estimated 32,000) in 1991 [1]. The number of cases will most likely increase steadily over the next decade as the population ages [2]. The prevalence of histologic changes recognizable as cancer (latent cancer) versus the much lower incidence of clinically recognizable disease (clinical cancer) makes cancer of the prostate gland unique among human malignancies. Nearly one-third of all men over 50 have latent or incidental prostate cancer, often revealed at autopsy [reviewed in 3]. Thus, the 100,000 new cases of clinically manifest prostate cancer per year in the USA represent only a small fraction of the latent prostate cancer. Life-threatening prostate cancer results from the low frequency progression of these common, small latent cancers, not from the inevitable progression of most small tumors.

Specific genetic abnormalities are generally assumed to be associated with cancer progression. The early stages of prostate cancer may be defined by a specific set of genetic alterations. The transition from a normal cell to a stable latent cancer cell that is unlikely to progress, or from a stable to an unstable latent cancer cell that is likely to progress, or from unstable latent cancer to more advanced local prostate cancer may be recognizable by specific alterations. Activated oncogenes and elevated proto-oncogene activities have been detected in human prostate cancer [reviewed in 4]. Cytogenetic studies of prostate cancer cells have shown that allelic loss is common and, therefore, link

the loss of suppressor genes with disease progression [5,6]. Loss of retinoblastoma gene expression has been reported in human prostate cancer [7], and expression of the normal retinoblastoma gene in a human prostate cancer cell line suppresses tumorigenicity [8]. Mutated p53 genes have been detected in a limited number of cases of human prostate cancer cells [9]. There is, however, no general consensus about the predominant genetic alterations responsible for the progression of prostate cancer.

Developing objective biomarkers for the progression of prostate cancer is critical for optimal use of current therapies and to evaluate novel, potentially more effective therapies. Currently there are no criteria for differentiating stable latent prostate cancer from unstable latent prostate cancer that is likely to progress. Furthermore, in order to achieve effective medical therapy for prostate cancer, additional objective endpoints for determining localized progression must be established. Although the transition from stable to unstable latent prostate cancer and the subsequent steps in progression are presumed to reflect sequential genetic alterations, it is conceivable that the acquisition of specific, dominantly acting oncogenes or the loss of specific growth-suppressor gene functions is not consistently associated with the development of prostate cancer. Alternatively, diverse molecular pathways may ultimately converge, culminating in common genetic activities such as the expression of specific growth factors.

Using both experimental model systems and human prostate cancer tissues, we have demonstrated that overexpression of transforming growth factor (TGF)-β1 is commonly associated with prostate cancer. Unique patterns of expression as well as the induction of secondary biochemical activities produced by this growth factor may be useful biomarkers for prostate cancer progression.

ELEVATED TGF-β1 EXPRESSION IS ASSOCIATED WITH MALIGNANCY IN MOUSE AND HUMAN PROSTATE

In an expanded MPR model [10], high titer BAGα or Zipras/myc 9 virus [11] was used for infection of enzymatically dissociated total urogenital sinus (UGS) cells or compartment-restricted infection of isolated urogenital sinus epithelium (UGE). BAGα-infected total UGS MPRs demonstrated normal prostate morphology (Fig. 1A), whereas Zipras/myc 9-infected total UGS MPRs produced rapidly growing, poorly differentiated adenocarcinomas (Fig. 1C) in more than 90% of individual experiments. Immunohistochemical staining showed that every carcinoma examined was positive for cytokeratin expression (not shown). Nonmalignant focal epithelial hyperplasias characterized by marked intraluminal bridging and multilayered epithelium (Fig. 1B) were seen in almost 90% of individual Zipras/myc 9-infected UGE MPRs. A noninvasive hypovascular carcinoma and a focal carcinoma were seen in two Zipras/myc 9-infected UGE MPRs, suggesting that oncogene infection restricted to the epithelial compartment can mimic premalignant conditions.

Northern blot analysis (Fig. 1D) was used to determine induction of mRNA levels for specific genes. High levels of v-*gagmyc* RNA were seen in all carcinomas from UGS infections with Zipras/myc 9, but virus-specific RNA was not detected in BAGα-infected UGE or total UGS MPRs. High viral RNA levels were also seen in the Zipras/myc 9-infected UGE MPR which developed a focal carcinoma, but not in those with only focal epithelial hyperplasia. Elevated levels of TGF-β1 were seen only in Zipras/myc 9-infected total UGS carcinomas and the single case of complete carcinoma which developed from a Zipras/myc 9-infected UGE MPR. The pattern of TGF-β3 mRNA expression showed a close correspondence to TGF-β1, supporting a positive correlation of elevated TGF-β1 and -β3 mRNA levels with the transition from a hyperplastic to a malignant phenotype. Additional investigations revealed that elevated levels of TGF-β1 RNA precede the onset of exponential tumor growth (data not shown), prompting studies designed to localize this growth factor by immunohistochemistry.

As previously reported [10] we were able to localize specific TGF-β1 immunoreactivity to the extracellular compartment in C57BL/6 mice with Zipras/myc 9-infected total UGS carcinomas. Subsequent studies using the anti-CC (1–30–1) serum which reacts specifically with extracellular TGF-β1 [12] have confirmed this. All rapidly growing *ras*+*myc*-induced carcinomas studied thus far have demonstrated extra-

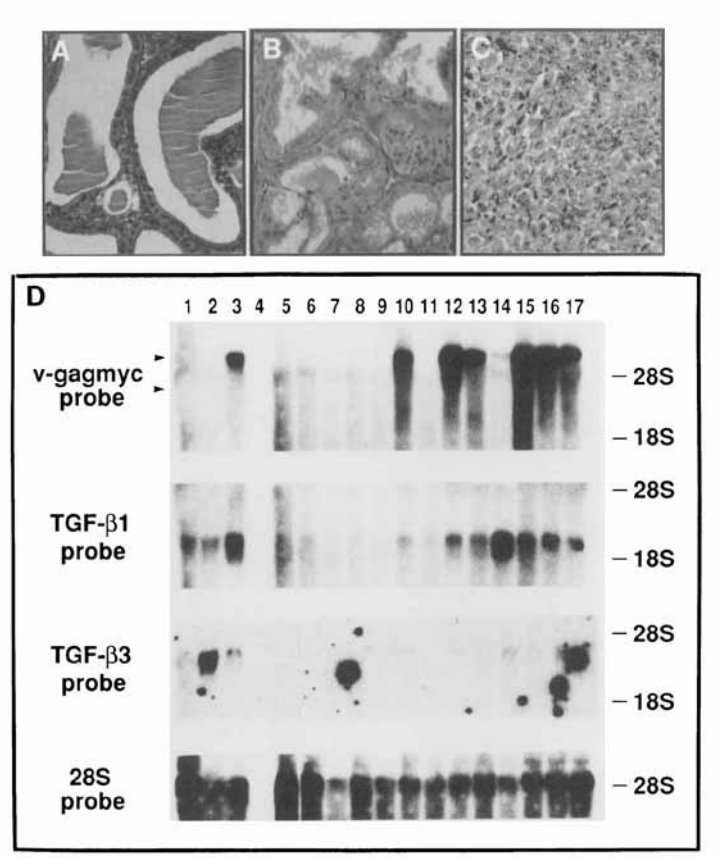

Fig. 1. Morphological characterization and northern analysis of total UGS and restricted UGE infections. (A–C) H&E-stained tissue sections (×250) showing the results of infection of: (A) total UGS with a control (BAGα) retrovirus; (B) UGE only with a retrovirus (Zipras/myc 9) carrying the *myc* and *ras* oncogenes; (C) total UGS with Zipras/myc 9 retrovirus; and (D) northern analysis of total cellular RNAs from MPR tissues. Samples include normal adult ventral prostate (lane 1), uninfected NIH 3T3 cells (lane 2), Zipras/myc 9-infected NIH 3T3 cells (lane 3), BAGα-infected restricted UGE MPRs (lane 5), BAGα-infected total UGS MPRs (lanes 6 and 7), Zipras/myc 9-infected restricted UGE MPRs which produced focal epithelial hyperplasia (lanes 8, 9 and 11), an exceptional case where a malignant carcinoma was produced by Zipras/myc 9 restricted UGE infection (lane 10), and malignant carcinomas produced by total infection with Zipras/myc 9 (lanes 12–17). The blot was sequentially hybridized with ^{32}P-labelled probes for v-*gagmyc* (upper panel), TGF-β1 (second panel from top), TGF-β3 (second panel from bottom) and 28S ribosomal RNA (bottom panel).

cellular staining. Extracellular TGF-β1 was also detected within small malignant foci induced by Zipras/myc 9 in Balb/c mice (see Figure 2B). This strain of mice has a low incidence of *ras*+*myc*-induced carcinoma, but under the influence of the *ras* and *myc* oncogenes develops both benign epithelial hyperplasia and small malignant lesions resembling early stage prostate cancer.

Prostatic tissue samples from patients with prostatic carcinoma and/or BPH were also examined immunohistochemically for expression of TGF-β1 with the CC antiserum. Although BPH tissues demonstrated focal areas of positive extracellular staining, immunoreactive material was significantly more extensive in prostate cancer, where the average positively staining area is 59% versus 26% in BPH [13]. This differential staining pattern was clearly evident in cases where focal areas of prostate cancer were located adjacent to diffuse areas of BPH (see Figure 2C).

In general, where benign pathologies are seen in the prostatic stroma in parallel with benign

prostatic epithelium, extracellular TGF-β1 is seen predominantly in the stromal compartment; but under conditions where prostate cancer is apparent, TGF-β1 accumulates predominantly within areas of cancer. Although there is no direct evidence for functional significance of TGF-β1 in the development of prostate cancer at this time, it is of interest that expression of this specific growth factor is elevated in both human and experimentally induced prostate cancer.

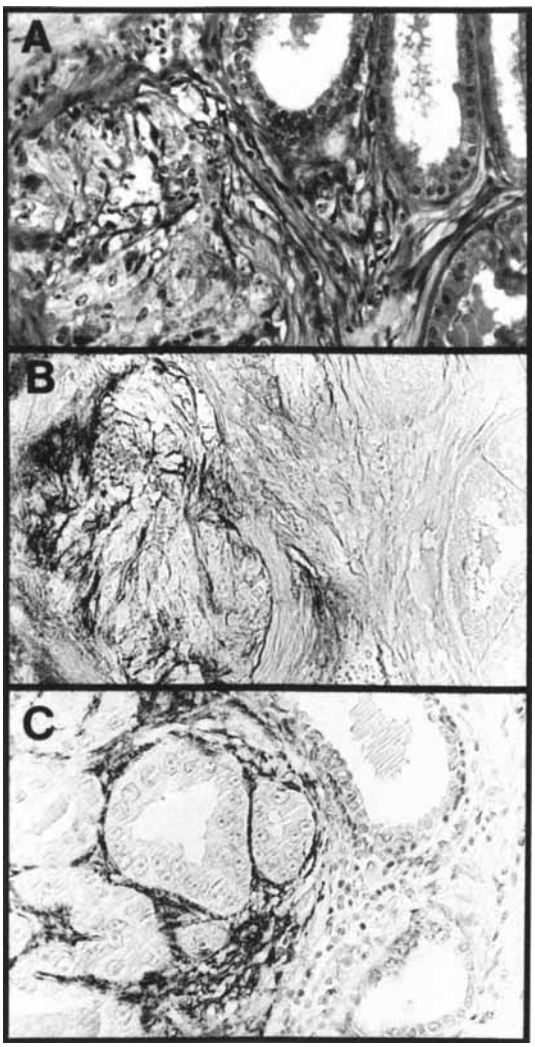

Fig. 2. Immunohistochemical localization of extracellular TGF-β1 with anti-CC serum. (A) H&E stained tissue section (×400) of a *ras*+*myc*-induced carcinoma using the MPR system, showing a poorly differentiated carcinoma on the left and normal-appearing prostate glands on the right. (B) Immunohistochemical staining of the same MPR carcinoma demonstrating TGF-β1 in the extracellular matrix surrounding the malignant epithelial cells with no staining of the area of the benign glands on the right. (C) Human prostate carcinoma, stained for TGF-β1, demonstrating a pattern of staining similar to the mouse tumor. The moderately differentiated carcinoma on the left shows abundant staining in contrast to the benign glands on the right.

DISCUSSION

The natural history of prostate cancer presents unique sets of research and clinical problems. Because only a small percentage of small-sized latent prostate cancer progresses to clinical significance, a major goal of research efforts is the ability to develop objective criteria to discriminate latent cancer that will remain stable and inactive from latent cancer that will progress because of intrinsic instability. In addition, the protracted growth period of many active prostate cancers allows maximal time for therapeutic intervention and/or the objective analysis of progression, possibly via biomarker assays.

In experimental studies using the MPR model, and in the limited number of human specimens examined to date, we have demonstrated that TGF-β1 is commonly associated with prostate cancer. Interestingly, where benign hyperplastic growth of stromal cells is seen together with either normal or benign hyperplastic epithelium elevated TGF-β1 accumulation is seen in the stromal compartment. Alternatively, when prostate cancer is observed, the site of TGF-β1 accumulation is within the epithelial compartment. Additional experimental studies using the MPR model system show that levels of TGF-β1 mRNA increase in mouse prostate cancer following castration, then stabilize at abnormally elevated levels. Therefore, TGF-β1 expression may reflect, to some degree, adaptation to castration and sensitivity to testosterone [14].

TGF-β1 exhibits a broad range of biological properties, some of which are consistent with increasing the invasive potential of a cancer cell. As a fundamental growth factor normally involved in the wound healing response, TGF-β1 can promote angiogenesis [15], enhance cell motility [16], induce stromal cell proliferation [15,17] and increase extracellular matrix turnover [15,18–20]. All of these activities support morphogenesis and differentiation

within the context of normal cell development or, alternatively, may support the invasive behavior and sustained growth of cancer. Support for the hypothesis that TGF-β1 has a role in malignant progression comes from recent studies showing that TGF-β1 provides a permissive environment for the development of tumors in Rous sarcoma virus-infected chickens [21] and that it can directly increase the invasive and metastatic potential of a mammary adenocarcinoma cell line [22].

It is of interest that many TGF-β1 induced biological activities produce extracellular effects including local as well as systemic manifestations. TGF-β1 has also been shown to have significant immunosuppressive activity [23–25]. Such widespread activities may provide the prostate cancer cell that overexpresses this growth factor a selective advantage for the development of malignant properties and further progression. In addition to its extracellular effects, TGF-β1 inhibits epithelial cell growth under normal conditions [17,26]. TGF-β1 can block the entry of normal cells into S phase late in G1 [27,28] through pathways which can involve the retinoblastoma gene product and lead to downregulation of c-*myc* [29,30]. As an inhibitor of epithelial cell growth under normal conditions, TGF-β1 and/or other TGF-β isotypes may serve as objective biomarkers for the activity of potential chemopreventive agents for prostate cancer such as retinoids which have been shown to induce TGF-β2 in cultured keratinocytes and mouse epidermis [31]. Interestingly, in some cancer cells the normal growth inhibitory response to TGF-β1 has been lost [32,33].

Although growth potential is necessary for the development of the complete malignant phenotype, it is not sufficient. Accordingly, this may be the reason for long periods of no or relatively slow growth in latent prostate cancer. Additional permissive biological events external to the cancer cell must occur for rapid progression. These events enable the cancer cell to penetrate the basement membrane; proliferate within matrix material of the stromal compartment; develop tumor vasculature through tumor-mediated angiogenesis; and escape from the host T-lymphocyte mediated immune response. The capacity to breach these physical barriers likely follows the occurrence of initiating genetic alterations, *e.g.*, abnormal expression of nuclear proto-oncogenes or loss of growth suppressor genes. Elevated c-*myc* expression has been demonstrated in prostate cancer compared to BPH [34,35 and reviewed in 4]. Loss of retinoblastoma gene expression [7,8] and mutated p53 genes [9] have also been associated with prostate cancer. Increasingly aberrant nuclear gene activities may impart proliferative potential and possibly underlie progression from stable to unstable latent prostate cancer.

Whereas enhanced growth potential resulting from aberrant nuclear gene expression may establish the transition from stable latent to unstable latent prostate cancer, extracellular changes possibly mediated by TGF-β1 in coordination with other growth factors and cytokines may allow for a second phase of malignant growth. This second phase would be characterized by the development of conditions that are permissive for invasion, allowing the proliferative potential imparted by nuclear genetic alterations to be manifest. During this phase, TGF-β1 produced and secreted by the malignant cancer cell would accumulate in the extracellular matrix material surrounding the cancer. This accumulation could produce localized effects in adjacent stromal cells [36] (see Figure 3). It has been well established that one major response to TGF-β in mesenchyme-derived cells is increased cell proliferation [15,17,37]. This increased proliferation could produce additional increases in TGF-β in the stromal compartment through positive autoregulation [38,39]. As we have reported previously, further paracrine activity involving lymphocytic infiltration may be elicited by such a proliferative response [10]. It is also well established that, in addition to increased proliferation, extracellular matrix production including collagen and fibronectin deposition is induced in stromal cells by TGF-β1 [15,18–20]. Under normal conditions the extracellular matrix may suppress further TGF-β-stimulated stromal proliferation by sequestration of TGF-β and other growth factors known to interact with extracellular matrix components. However, if sufficient matrix is not maintained, enhanced TGF-β1 positive autoregulation and subsequent proliferation may continue. In fact, in some cases mesenchyme-derived TGF-β1 may even elicit increased TGF-β1 production by the

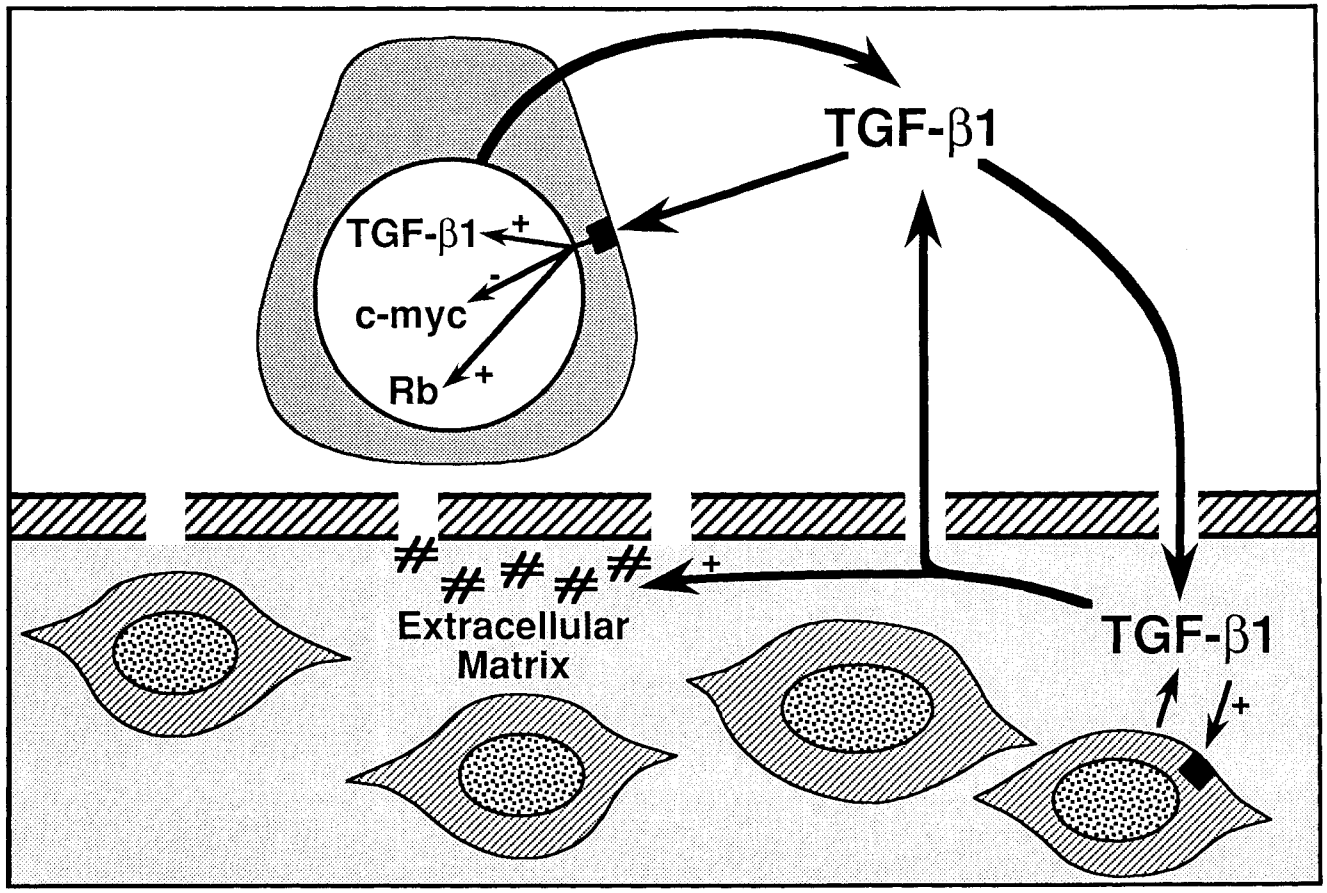

Fig. 3. Schematic representation of the possible role of TGF-β1 in prostate cancer. The prostate epithelial compartment and stromal compartment may be inappropriately brought into communication via TGF-β1. These paracrine effects of TGF-β1 as well as its autocrine activity provide a mechanism to explain the role of TGF-β1 in prostate cancer progression.

cancer cell *per se*. Thus, a "TGF-β1 loop" may be established such that extracellular matrix components would produce negligible effects with respect to growth inhibition. Independent changes within the stromal compartment such as wounding-type responses associated with conditions such as chronic prostatitis may bring about increased cytokine production and provide additional input into this "TGF-β1 loop." By this mechanism, extremely high localized concentrations of TGF-β1 could be achieved, causing immunosuppressive activities and untoward systemic effects.

This proposed mechanism as depicted in Figure 3 can explain how the simple production and secretion of TGF-β1 by the cancer cell is able to elicit widespread biological effects that contribute to progression. A better understanding of this proposed mechanism may lead to the development of specific biomarkers. This would facilitate therapeutic decisions as well as provide objective endpoints for chemoprevention and medical intervention. Greater understanding of prostate cancer's subtle biological complexities will certainly increase our potential to treat the disease.

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