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Advances in Brief

Human Prostate Cancer Expresses the Low Affinity Insulin-like Growth Factor Binding Protein IGFBP-rP1[1]

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Abstract

Many of the alterations in the insulin-like growth factor (IGF) axis in prostatic disease have been associated with changes in the insulin-like growth factor binding proteins (IGFBPs), a multigene family of proteins that are thought to mediate the action of IGFs on target tissues. IGFBP-related protein 1 (rP1), also known as IGFBP-7 or mac25, is a recently described member of the IGFBP family, the biological function of which has yet to be completely ascertained. In this study, we analyzed the localization of IGFBP-rP1 in prostate cancer and benign prostate tissues using immunohistochemistry and a polyclonal antibody, T1A12, that is specific for IGFBP-rP1. The most intense staining was observed in nerves, whereas smooth muscle cells in the prostate stained weakly. Lymphocytes were always negative. When normal prostatic secretory epithelium was present, staining was usually absent. The lining secretory epithelium stained positively in 0 of 12 (0%) cases of benign prostatic hyperplasia, 57 of 63 (90.5%) primary adenocarcinomas, and 7 of 7 (100%) prostate cancer metastases. Prostatic intraepithelial neoplasia showed a similar pattern of staining to that observed for the invasive tumors. Analysis of Northern blots showed that none of the prostate cancer cell lines (LNCaP, C4, C4–2, C4–2B4, 9069E3, DU145, and PC3) expressed IGFBP-rP1 mRNA. This lack of expression was confirmed by immunohistochemistry of s.c.-generated tumor xenografts of LNCaP and C4–2 and by immunoblot on serum-free-conditioned media from all prostatic cell lines. In contrast to these results, tumor xenografts generated by direct intraosseous injection of LNCaP or C4–2 to bone marrow space resulted in tumors that stained positively for IGFBP-rP1. Our results show that IGFBP-rP1 is expressed in both *in situ* and invasive prostate neoplasms, but not typically in normal secretory or BPH epithelium; furthermore, the expression of IGFBP-rP1 can be induced in human prostate cancer cell lines *in vivo* on interaction with an appropriate host environment.

Introduction

IGFBP[3]-rP1 is a recently described member of the IGFBP family. First identified as mac 25 (1, 2), it was renamed IGFBP-7 because of its structural homology to the IGFBP family and its capacity to bind IGFs (3). Because its affinity for IGF is low compared with IGFBP-1 through -6, IGFBP-7 was proposed to be the first member of a new subgroup of low-affinity IGFBPs, IGFBP-rPs of which there are now four members, IGFBP-7 being renamed IGFBP-rP1 (4). IGFBP-rP1 expression is decreased in some tumor cells as compared with normal cells, such as in meningioma (1). Furthermore, its expression is decreased in replicating mammary cells as compared with senescent mammary epithelium (2).

T1A12 was identified by subtractive cDNA cloning using RNAs from a normal breast cell line, Hs578Bst, and the corresponding tumor cell line, Hs578T. Sequence analysis demonstrated that *T1A12* was actually *IGFBP-rP1*, and the protein product was shown to bind IGFs (5). A polyclonal antibody was raised against a decapeptide located in the COOH-terminal sequence of T1A12. The immunoreactivity for T1A12 was found in normal mammary epithelium, but was absent in breast adenocarcinoma (5).

Previously, we have shown that although the mRNA for IGFBP-rP1 is expressed in all normal tissues, the protein localization is compartmentalized to specific cell types.[4] It was of interest that normal breast and prostate epithelium had an opposite pattern of staining. Although normal breast epithelium was intensely immunoreactive, normal prostate secretory epithelium was usually negative. As IGFBP-1 to -6 expression has been shown to be modified in prostate tumors (6–8), we were particularly interested in looking at the expression of this new member of the IGFBP family in prostatic adenocarcinomas. In this study, we summarize the results of immunohistochemical staining on a cohort of human prostate cancers, as well as an examination of the expression of IGFBP-rP1 in several human prostate cancer cell lines both *in vitro* and *in vivo*.

Materials and Methods

Cell lines and Cultures. Bone cell lines (MG-63 and Saos-2) and breast cancer cell lines (MDA-MB-231, MCF-7, and Hs578T) were purchased from American Type Culture Collection and cultured according to the manufacturer's recommendations. The prostate-derived cell lines LNCaP, C4, C4–2, C4–2B4, DU145, PC-3, and 9069E3 and the stromal cells were cultured in T medium supplemented with 5% FBS, as described previously (9). C4, C4–2, and C4–2B4 are sublines derived from LNCaP and constitute an experimental model of prostate tumor progression complete with the acquisition of androgen independence and the capacity to metastasize to bone (10). 9069E3 is an epithelial cell culture derived from near diploid cells from an adenocarcinoma of the prostate (11). Human prostate stromal cells were derived from a prostate adenocarcinoma sample after primary culture in our laboratory. To determine the effect of androgens, cells were plated in T medium supplemented with 5% FBS (9). At 80% confluency, the cells were washed three times in PBS, and then the medium was replaced by serum-free, phenol-free DMEM/F12 (Irvine Scientific, Santa Ana, CA). R1881, a nonmetabolizable synthetic androgen (NEN DuPont, Wilmington DE), was added to a final concentration of 0.1 nM. Controls were performed using the ethanol vehicle. After 48 h, conditioned medium and total RNA were collected as described below.

Tissue Specimens. Sixty-three patients with prostatic adenocarcinoma were treated by prostatectomy with or without pelvic lymph node dissection; two of those patients had positive pelvic nodes, whereas none had evidence of distant metastasis. The age range of these patients was 42–74 years (median, 60). Approximately one-third of the neoplasms were Gleason score 5 or 6, one-third were Gleason score 7, and one-third were Gleason score 8–10. Seven

patients (three at autopsy) had prostate cancer metastatic to lymph nodes; their age range was 67–83 years (median, 76).

Immunohistochemistry. Formalin- or zinc-formalin-fixed, paraffin-embedded tissues of prostate adenocarcinomas (12), benign prostatic hyperplasias, and human prostate cancer cell line xenograft tumors established in athymic or SCID mouse hosts (10, 13) were sectioned at 5-μm thickness, deparaffinized in xylene, rehydrated in a graded ethanol series, rinsed in PBS briefly, and incubated for 15 min at room temperature in 10% normal goat serum (Jackson Immunoresearch Laboratories, West Grove, PA), followed by a treatment with the avidin-biotin blocking kit (Vector Laboratories, Burlingame CA). After a brief rinse, the sections were incubated overnight at 4°C with a dilution of the T1A12 antibody (1:1500 dilution in 5% normal goat serum, 1% BSA, and 0.5% FSG in PBS). The specificity of the T1A12 antibody has been demonstrated by: (*a*) blocking of signal by preincubation with the peptide immunogen; and (*b*) by the recognition of a single ~37-kDa band on Western blotting of positive control cell (Hs578N) protein that could also be blocked by the peptide immunogen.[3] This antibody has also been used, by Burger *et al.* (5), to specifically recognize mac25/IGFBP-rP1 in breast tissue and has been cited by Hwa *et al.* (20) as being specific for mac25/IGFBP-rP1. After several washes in PBS, the tumor slides were incubated for 30 min at room temperature with a 1:100 dilution of a biotinylated goat antirabbit IgG (Jackson Immunoresearch Laboratories) or Multi-link (Multilink; BioGenex, San Ramon, CA). Endogenous peroxidase activity was quenched by treating the tissues for 30 min in 0.3% hydrogen peroxide. The slides were then incubated in a 1:60 dilution of streptavidin-peroxidase for 30 min at room temperature (BioGenex). Peroxidase activity was visualized using diaminobenzidine as the chromagen in a 5-min incubation containing hydrogen peroxide (1.1%). The sections were rinsed in water, counterstained with hematoxylin, dehydrated, and mounted.

Cell lines were stained using the same protocol. Cells were plated at low confluency in their regular culture medium. After 48 h, the cells were washed several times in PBS before replacing the media with serum and phenol red-free DMEM/F12 (Irvine Scientific). The medium was changed at 24 h, and the cells were fixed at 48 h using 2% paraformaldehyde. The staining was then performed as described above.

Northern Blot. Total RNA was extracted from cells using the acid guanidium/isothiocyanate method of Chomczynski and Sacchi (14) using RNA-STAT (BioTecX, Houston, TX). Total RNA (30 μg) was separated by electrophoresis in denaturing conditions (7.4% formaldehyde agarose gel in borate buffer), as described previously (15), and transferred to nitrocellulose membrane (Hybond N; Amersham Corp., Arlington Heights, IL). Hybridization was performed at 65°C in RapidHyb buffer (Amersham Corp.). ^{32}P-labeled probes were generated by random nonamer priming (Megaprime kit; Amersham Corp.) of a 1.1-kbp gel-purified *Eco*RI fragment of *T1A12/mac25*.

Results

IGFBP-rP1 Staining in Human Prostate Tissues. Twelve cases of benign prostatic hyperplasia, 63 prostatic adenocarcinomas, and 7 prostate carcinoma metastases were analyzed by immunohistochemistry. Peripheral nerves were always strongly positive, whereas lymphocytes within the specimens were always negative, constituting good internal controls to survey the quality of staining. Smooth muscle cells were moderately positive in the stroma of both adenocarcinomas and benign prostatic hyperplasias. When normal prostatic secretory lining cells were present in sections of adenocarcinoma and benign hyperplasia, their staining was usually negative; when staining was apparent in normal epithelium it was weak-moderate and localized almost exclusively to cells of basal layer. In all cases of prostatic hyperplasia (12 of 12), the secretory cell layer was usually negative, but there was occasionally weak focal positivity (Fig. 1*A*). The basal cell layer displayed a variable weak to moderate positivity (Fig. 1*A*). In prostatic adenocarcinoma samples, tumor cells were more often positive and showed a similar or higher level of staining intensity when compared with normal basal layer epithelial cells (Fig. 1*B*). Fifty-seven of 63 (90.6%) tumors were positive, showing a cytoplasmic staining, with a positivity ranging from 25–100% of the tumor cells. When high-grade PIN was present, the immunohistochemical pattern was the same as that for the invasive tumor component. There was no correlation between the Gleason score and the level of staining. Metastases of prostate cancer showed positive staining in all specimens examined (seven of seven; Fig. 1*C*). The results are summarized in Table 1.

IGFBP-rP1 Expression in Prostate Tissue and Cancer Cell Lines. The 1.1-kb IGFBP-rP1/mac25 mRNA was found by Northern blot (Fig. 2) in normal prostate tissues, probably originating from either the stromal component of the organ or from the strongly positive neural elements. In support of a stromal source of the transcript, IGFBP-rP1 mRNA was observed in a stromal cell line derived from a prostatic adenocarcinoma. In fact, under normal cell culture conditions of 5% FBS, the highest levels of IGFBP-rP1 expression were observed in a prostate cancer-derived stromal culture. No IGFBP-rP1 mRNA could be detected in a well-differentiated prostatic adenocarcinoma cell line, 9069E3, or in any other androgen-dependent or -independent prostate cancer cell lines tested (LNCaP, C4,
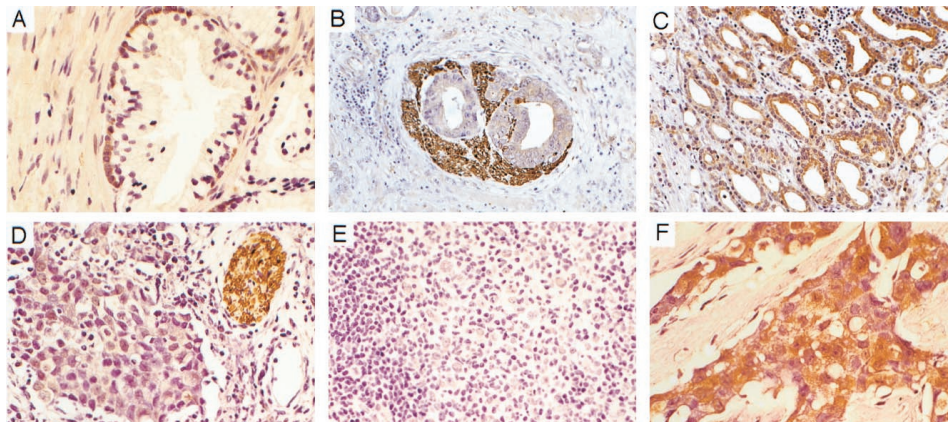

Fig. 1. Representative immunostaining of IGFBP-rP1 in prostate. *A-C*, human specimens were immunostained as described in "Materials and Methods." *A*, benign prostatic hyperplasia. Diffuse positive staining of fibromuscular stroma with occasional positive basal cells. Secretory epithelial cells are negative. There is an intense positive reaction in peripheral nerves. *B*, prostate adenocarcinoma. Intense positive reaction in peripheral nerves. Adenocarcinoma cells are now positive in contrast to the normal secretory epithelial cells. *C*, lymph node metastasis of a prostate adenocarcinoma. Most of the adenocarcinoma cells are now strongly positive. Lymphoid tissue are negative in contrast to prostatic stroma. *D-F*, immunostaining of prostate cancer cell line xenografts was performed, as described in "Materials and Methods." *D*, s.c. tumor of androgen-sensitive LNCaP cells coinjected with MS osteosarcoma fibroblasts. Xenograft is weakly positive in most cells; other s.c. xenografts of LNCaP and C4–2 were negative. Note the strong staining of the peripheral nerves. *E*, C4–2 s.c. tumor. Androgen-independent C4–2 cell xenografts were usually negative by immunostaining for IGFBP-rP1. *F*, LNCaP intraosseous tumor. Intraosseously generated xenografts of either LNCaP or C4–2 (data not shown) were consistently and strongly positive for IGFBP-rP1 staining. *A*, *B*, *D*, and *F*, magnification, ×62.5; *C* and *E*, magnification, ×125.

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Table 1 *IGFBP-rP1 immunoreactivity in prostate tissues*

Tumors	IGFBP-rP1 positivity
Benign prostate hyperplasia	0/12 (0%)
Prostate adenocarcinoma	57/63 (90.5%)
Gleason score 5 and 6	21/21 (100%)
Gleason score 7	19/21 (90.5%)
Gleason score 8–10	17/21 (81%)
Metastasis	7/7 (100%)

C4–2 and C4–2B4 and PC-3 and DU145). Two osteosarcoma cell lines, MG63 and Saos-2, and a colon carcinoma cell line did express low levels of IGFBP-rP1 mRNA under normal cell culture conditions. Treatment with androgens (0.1 nM R1881 for 48 h) under serum-free conditions did not alter the expression pattern of IGFBP-rP1 in any of the prostate cancer cell lines (data not shown).

Immunohistochemistry and protein blotting for IGFBP-rP1 were performed on breast and prostate cancer cell lines under serum-free conditions (data not shown). The immunostaining was weak or absent in cell lines and difficult to improve because of a cross-reactivity with the FBS. However, although the Hs578T cell line showed very weak immunostaining for IGFBP-rP1, the secreted protein was detected easily in the conditioned medium by Western blotting (data not shown).[3] There was no detectable IGFBP-rP1 protein in the conditioned medium of LNCaP, C4–2, DU145, and PC-3 treated or not by R1881 (data not shown), a finding that is consistent with the lack of any detectable mRNA in the prostate cancer cell lines (Fig. 2). In contrast to these data, however, were the results of prostate cancer cell xenografts in bone marrow spaces. s.c.-generated LNCaP and C4–2 cell xenografts were mostly negative, but were weakly stained when positive for IGFBP-rP1 (Fig. 1, *D* and *E*), however, intraosseously generated LNCaP and C4–2 cell xenografts routinely stained positively (Fig. 1*F*) with no overt differences in staining pattern or intensity between the two cell lines.

Discussion

IGFBPs have been shown to be modified in prostatic disease, suggesting that they play a role in the onset and development of prostatic hyperplasia and adenocarcinoma. Several prostate cancer cell lines have shown autocrine proliferation *in vitro* that involves IGF-I and/or IGF-II in combination with IGF-I receptors (16, 17). The stromal cell cultures derived from BPH specimens produce higher levels of IGF-II than normal prostate stromal cells, and IGFBP-2 expression is lost in favor of expression of IGFBP-5 (18). On the other hand, studies performed on prostate cancer specimens show an increase in IGFBP-2 expression in tumor cells *versus* normal cells (7, 19), but a more recent study tends to moderate the difference, with an increase in high Gleason score *versus* low Gleason score and benign tissues (8). For IGFBP-3, the role of posttranslational modification seems to be essential; for example, despite no change in the mRNA level as observed by *in situ* hybridization, the level of protein detected by immunohistochemistry is increased in PIN, but decreased in adenocarcinoma (7). This trend was confirmed without correlation with tumor grade or stage by Thrasher *et al.* (19), whereas Figueroa *et al.* (8) showed a significantly higher immunoreactivity in low Gleason score tumors when compared with high Gleason score cancers. Even if the levels are slightly different from one study to another, it seems clear that IGFBP-2 and -3 are modified in prostatic disease. Because IGF-I and IGF-II levels are also altered, there is probably a role for the IGF axis in the regulation of prostate cancer.

In the case of IGFBP-rP1, we found immunoreactivity in prostatic adenocarcinoma and PIN, with no segregation by stage or grade, whereas normal secretory epithelium and epithelium of hyperplastic glands were negative; positivity, when present, was usually restricted to the basal cell layer. The presence of IGFBP-rP1 in the basal cell layer of the prostate is supported by the *in situ* hybridization data of Hwa *et al.* (20); however, our data clearly demonstrate that IGFBP-rP1 expression is not present in the secretory lining of epithelial cells of normal glands, whereas the expression is deregulated and strong in PIN and prostate neoplasms. Regardless of the strong staining in prostate neoplasms, the role of IGFBP-rP1 in prostatic neoplasia is unclear. It can bind IGF, but with a low affinity compared with other IGFBPs (5–6-fold lower for IGF-I and 20–25-fold lower for IGF-II; Ref. 3). Recently, it has been shown that IGFBP-rP1 has an even higher affinity for insulin (21), the significance of which is unknown. In prostate biology, however, the role of insulin remains to be explored in relation to the IGF axis and IGFBP-rP1, in particular.

IGFBP-3 is the first IGFBP shown to trigger its own effects independently of its binding to IGFs. IGFBP-3 directly mediates the effects of TGF-β1 by inducing apoptosis in an IGF:IGF receptor-independent mechanism (22). The type V TGF β receptor is a putative receptor for IGFBP-3 (23). IGFBP-rP1 might act by sequestering IGF-1 in the prostate, resulting in an inhibition of the antiapoptotic effect of IGF-1. It might also act via its own receptor signaling pathway, the existence of which remains to be investigated. No receptor has been demonstrated thus far for any of the low-affinity IGFBP. In prostate epithelial cells immortalized by SV40, Hwa *et al.* (20) demonstrated that the IGFBP-rP1 is regulated by IGF-I, TGF-β and retinoic acid.

Because there is a remarkable difference in the pattern of IGFBP-rP1 expression in breast and prostate cancers, it is possible that there is a differential regulation by male or female sex steroids. It would seem that only ER-negative breast cancer cells express IGFBP-rP1 mRNA, although some ER-negative breast cancer cell lines do not express it. By Northern blot, estrogen was not found to alter IGFBP-rP1 mRNA levels in ER+ cell lines (0.01 nM estradiol for 48 h), thereby eliminating a role for estradiol in the suppression of IGFBP-rP1. Whether the loss of ER is in any way required for the expression of IGFBP-rP1 remains to be determined.

Burger *et al.* (5) showed that the loss of expression of IGFBP-rP1 in breast cancer is associated with a loss of heterozygosity in 50% of the informative cases, perhaps explaining why not all ER-negative tumor cells express IGFBP-rP1. For prostate cancer cell lines, we found no mRNA expression in any of the commonly studied andro-
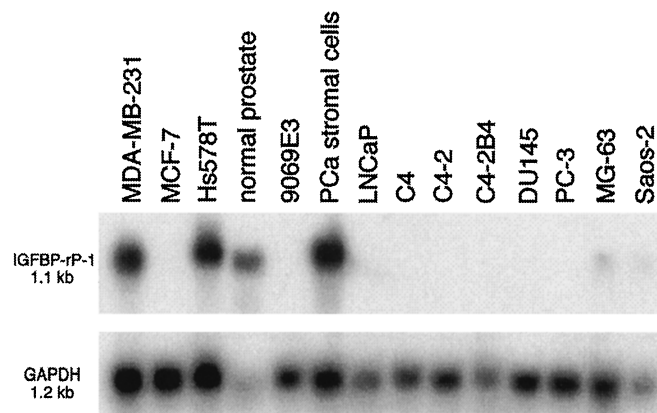

Fig. 2. IGFBP-rP-1 mRNA expression. Total RNA (30 μg) from each cell line was electrophoresed and hybridized to the 1.1-kbp cDNA probe of IGFBP-rP-1. MDA-MB231 and Hs578T are ER negative, whereas MCF-7 is an ER-positive human breast cancer cell line. Normal prostate RNA was from pooled normal human prostate tissues (Clontech). 9069E3 is a well-differentiated prostatic adenocarcinoma cell line. PCa stromal cells are the primary culture of nonepithelial cell population derived from a prostate adenocarcinoma specimen. C4, C4–2, and C4–2B4 are sublines derived from the human prostate cancer cell line LNCaP. DU145 and PC3 are human androgen-independent prostate cancer cell lines. MG-63 and Saos-2 are human osteogenic sarcoma cell lines.

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gen-dependent or -independent cell lines (*e.g.,* LNCaP, C4–2, PC-3, and DU145), and there was no induction of IGFBP-rP1 expression after treatment of the AR-positive cells with androgens (1 nM R1881 for 48 h). Because most prostate cancers do not lose their AR (in fact, under conditions of androgen deprivation, most prostate cancers will amplify their AR and actually express more of it; Ref. 24) and both normal and hyperplastic prostate epithelium are AR-positive but lack IGFBP-rP1 expression, it is unlikely that there is a demonstrable association between AR positivity and IGFBP-rP1 expression. Furthermore, alteration of chromosome 4, where IGFBP-rP1 is located, is not a typical finding in prostate cancer because no loss of heterozygosity of this particular region has been reported. Obviously, this mechanism would favor the loss of IGFBP-rP1 expression, which is in contrast to the increased expression that we have observed in prostate cancer. Finally, amplification of chromosome 4 does not seem to be a common event in prostate cancer because the only study is a trisomy of chromosome 4 in 1 patient among 30 patients studied by Brothman *et al.* (25), also with no evidence of deletion. It is possible that some other mechanism (for example, demethylation; Refs. 26 and 27) is responsible for deregulating the expression of IGFBP-rP1 in prostate cancer. Methylation is essential to genomic imprinting, which has been demonstrated for two genes of the IGF axis: *IGF-II* (26) in prostate tissues and its receptor *Igf2r* (28). Finally, these differences between breast and prostate expression of IGFBP-rP1 may not be due to differences in sex steroid hormone regulation because it does not seem to involve either androgen or estradiol.

Data from human prostate tumor xenografts in immune-compromised mouse hosts has suggested that 3-D architecture alone is insufficient to induce the expression of IGFBP-rP1. s.c.-generated LNCaP and C4–2 tumors do not express IGFBP-rP1, as determined by immunohistochemical staining. In contrast, the bone environment has the capacity to induce IGFBP-rP1 expression. Therefore, bone can supply the appropriate environment for the reexpression of IGFBP-rP1 by prostate cancer cell xenografts, effectively recapitulating the expression observed in primary human prostate cancer tissues and metastases.

In conclusion, prostatic intraepithelial neoplasia, prostatic adenocarcinoma, and prostate cancer metastases express IGFBP-rP1, whereas secretory epithelium in normal prostate and benign prostatic hyperplasia does not. Furthermore, the LNCaP human prostate cancer progression model will express IGFBP-rP1 when grown under the influence of bone, but not when grown s.c. *in vivo*. These results indicate that local microenvironment may mediate the expression of IGFBP-rP1 in prostate cancer.

Acknowledgments

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