Insulin-Like Growth Factor (IGF) Binding Protein-3 Attenuates Prostate Tumor Growth by IGF-Dependent and IGF-Independent Mechanisms

Josef V. Silha, Patricia C. Sheppard, Suresh Mishra, Yaoting Gui, Jacquie Schwartz, Janice G. Dodd, and Liam J. Murphy

Departments of Physiology (J.V.S., P.C.S., S.M., Y.G., J.S., J.G.D., L.J.M.) and Internal Medicine (L.J.M.), University of Manitoba, Winnipeg, Canada R3E 3P4

IGF binding protein (IGFBP-3) inhibits cell growth and promotes apoptosis by sequestering free IGFs. In addition IGFBP-3 has IGF-independent, proapoptotic, antiproliferative effects on prostate cancer cells in vitro. Expression of the large T-antigen (Tag) under the long probasin promoter (LPB) in LPB-Tag mice results in prostate tumorigenesis. To investigate the IGF-dependent and IGF-independent effects of IGFBP-3 on prostate tumor growth, we crossed LPB-Tag mice with cytomegalovirus (CMVBP-3) and phosphoglycerate kinase (PGKBP-3) mice under the cytomegalovirus promoter and the phosphoglycerate kinase promoter, respectively, and also I56G/L80G/L81G-mutant IGFBP-3 (PGKmBP-3) mice that express I56G/L80G/L81G-IGFBP-3, a mutant that does not bind IGF-I but retains IGF-independent proapoptotic effects. Expression of the CMVBP-3 promoter, respectively, and also I56G/L80G/L81G-mutant IGFBP-3 (PGKmBP-3) mice that express I56G/L80G/L81G-IGFBP-3, a mutant that does not bind IGF-I but retains IGF-independent proapoptotic effects.

Epidemiological studies have demonstrated that high plasma levels of IGF-I and low IGF binding protein (IGFBP-3) concentrations are associated with increased risk of prostate cancer (1, 2). Such a combination in cell culture would likely favor cell proliferation. IGFBP-3 functions both to inhibit the actions of IGF-I and -II and also in an IGF-independent manner to promote apoptosis and inhibit cellular proliferation of variety of cell lines including human prostate cancer cells (3–5). In addition, in a recent report, IGFBP-3 in combination with retinoid X receptor ligand has been shown to inhibit prostate cancer cell xenografts (6). High plasma IGF-I levels are also a risk factor for benign prostatic hyperplasia, a condition associated with increase prostate cancer risk (7). IGF-I is mitogenic for prostate cancer cells in vitro. Prostate tumor size and the steady-state level of p53 were attenuated in LPB-Tag/CMVBP-3 and LPB-Tag/PGKBP-3 mice, compared with LPB-Tag/wild-type (Wt) mice. A more marked effect was observed in LPB-Tag/CMVBP-3, compared with LPB-Tag/PGKBP-3, reflecting increased levels of transgene expression in CMVBP-3 prostate tissue. No attenuation of tumor growth was observed in LPB-Tag/PgKmBP-3 mice during the early tumor development, indicating that the inhibitory effects of IGFBP-3 were most likely IGF-dependent during the initiation of tumorigenesis. At 15 wk of age, epidermal growth factor receptor expression was increased in LPB-Tag/Wt and LPB-Tag/PgKmBP-3 tissue, compared with LPB-Tag/PGKBP-3. IGF receptor was increased in all transgenic mice, but pAkt expression, a marker of downstream IGF-I action, was increased only in LPB-Tag/Wt and LPB-Tag/PgKmBP-3. After 15 wk of age, a marked reduction in tumor growth was apparent in LPB-Tag/PgKmBP-3 mice, indicating that the IGF-independent effects of IGFBP-3 may be important in inhibiting tumor progression. (Endocrinology 147: 2112–2121, 2006)
use the simian virus 40 (SV40) large T-antigen (Tag) as the oncogenic transgene, the cellular tumor suppressor p53 is inactivated. In the case of the long probasin promoter (LPB)-Tag mouse model of prostate cancer, transgene expression is targeted specifically to the prostate (14, 15).

The N-terminal domain of IGFBP-3 appears to be most important for binding to IGF-I and -II with high affinity. Mutations in the N terminal of IGFBP-3 result in molecules that do not bind IGF-I or -II (16). Six residues, Ile66, Tyr23, Arg27, Leu77, Leu80, and Leu81, have been identified as important in high-affinity binding of IGF to IGFBP-3. Of these, Ile66, Tyr23, and Leu81 are most important and substitution with glycine or alanine results in a mutant IGFBP-3 that lacks the ability to bind IGF-I or IGF-II but retains the ability to bind plasma membranes (5) and promote apoptosis and inhibit proliferation in prostate and breast cancer cell lines (5, 17, 18).

We previously generated transgenic mice that overexpress human IGFBP-3 using the phosphoglycerate kinase (PGKBP-3) and cytomegalovirus (CMVB-3) promoters (19). These mice demonstrate fetal and postnatal growth retardation. More recently we generated transgenic mice that overexpress the I56G/L80G/L81G-mutant IGFBP-3 (PGKmBP-3) (20). The PGKmBP-3 transgenic mice do not have a growth-retarded phenotype.

In this report we tested the hypothesis that IGFBP-3 inhibits prostate cancer growth by examining the progression from epithelial hyperplasia to dysplasia and carcinoma in F1 crosses of PGKBP-3, CMVB-3, and PGKmBP-3 transgenic mice that ubiquitously overexpress IGFBP-3 or PGKmBP-3 with LPB-Tag mice (14).

Materials and Methods

Transgenic mice

The generation and characterization of PGKBP-3 and CMVB-3 mice have been previously reported (19). The I56G/L80G/L81G-mutant IGFBP-3 plasmid was generated by site-directed mutagenesis and cloned downstream of the phosphoglycerate promoter in the same plasmid used for the generation of PGKBP-3 mice. Characterization of the PGKBP-3 transgenic mice has been reported elsewhere (20). The 12T-5 strain of LPB-Tag transgenic mice that express the SV40 Tag oncogene are prostate-specific probasin promoter was used for these studies (14). The 12T-5 strain of LPB-Tag mice develop palpable prostate tumors starting approximately 2 months of age. The Prostate Pathology Committee of the Mouse Models of Human Cancer Consortium (National Cancer Institute, National Institutes of Health) has identified the strain as manifesting mouse prostate intraepithelial neoplasia (PIN) that progresses to adenocarcinoma (15). All transgenic mice were generated in the same CD-1 genetic background. Homozygous male PGKBP-3, CMVB-3, or PGKmBP-3 mice and normal wild-type male CD-1 mice were bred with heterozygous LPB-Tag female mice. Male F1 offspring were genotyped and approximately 25% of all the offspring were double-transgenic male animals. These were killed at various ages for determination of prostate size and histology. The presence of the various transgenes was detected using Southern blot analysis (IGFBP-3) or PCR using tail DNA as previously described (14, 19, 20). All experiments were performed in accordance with protocols approved by the Animal Care Committee of the Faculty of Medicine (University of Manitoba).

IGFBP-3 and IGF-I assays

Human IGFBP-3 was measured using an immunoradiometric assay from Diagnostic Systems Laboratories (Webster, TX). Total plasma IGF-I was measured by a sensitive rat IGF-I RIA kit (Linco Research, Inc., St. Charles, MO).
Apoptosis

Apoptosis in comparable areas of high-grade PIN in prostate tumors from LPB-Tag/wild-type (Wt), LPB-Tag/PGKB-3, LPB-Tag/CMVBP-3, and LPB-Tag/PGKmBP-3 mice was quantified using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling assay (ApopTag peroxidase in situ apoptosis detection kit, Chemicon, Temecula, CA), at 9, 13, and 17 wk of age. Three to five sections from three or four mice per transgenic strain were analyzed. Approximately 360 cells per section, or approximately 4300 cells per Tg strain, were scored.

Statistical analysis

All data are expressed as the mean ± sem. Statistical analysis was initially performed using an ANOVA and followed by the either Tukey honestly significant difference test, in which multiple comparisons were made between groups, or Dunnett’s t test in which comparisons were made between multiple groups and a single control group, using online statistical software (http://faculty.vassar.edu/lowry/VassarStats.html). Comparison was made between LPB-Tag/Wt mice and the other groups of mice for both the whole data set and data from each time point. Prostate weight was log transformed, and least squares regression analysis was used to determine the lines of best fit and their confidence limits. The statistical significance of the difference in the slope and intercept was then determined.

Results

The increase in prostate weight with age in Wt/Wt, LPB-Tag/Wt, LPB-Tag/CMVBP-3, and LPB-Tag/PGKB-3 mice is shown in Fig. 1. There was no significant difference in prostate weights in Wt/Wt, CMVBP-3/Wt, and PGKB-3/Wt mice. For the purpose of clarity, only a single curve is shown for Wt/Wt, CMVBP-3/Wt, and PGKB-3/Wt mice in Fig. 1. Prostate tumorigenesis, as assessed by prostate weight, was markedly attenuated by overexpression of IGFBP-3 under either the CMV or PGK promoters (P < 0.001 by ANOVA). This attenuation was more marked in LPB-Tag/CMVBP-3 mice, compared with LPB-Tag/PGKB-3 mice (P < 0.05). Once initiated, prostate tumors grew at a slightly slower rate in LPB-Tag/CMVBP-3 and LPB-Tag/PGKB-3 mice than LPB-Tag/Wt mice (Fig. 1). Prostate tumor weight was log transformed, and least squares regression analysis was used to obtain the line of best fit for the relationship between prostate weight and time. The slope of this relationship was significantly less in LPB-Tag/CMVBP-3, compared with LPB-Tag/Wt mice (P < 0.001), and similar trend was apparent in LPB-Tag/PGKB-3 mice (Table 1). In LPB-Tag/Wt mice, a prostate weight of 10 g was achieved at approximately 17 wk of age. This weight was achieved after a further delay of approximately 2.5 and approximately 5 wk in LPB-Tag/PGKB-3 and LPB-Tag/CMVBP-3 mice, respectively. Although the PGKB-3 and CMVBP-3 mice were approximately 10% smaller than Wt mice (19), differences in body weight did not account for the apparent reduction in prostate tumor growth. A significant reduction in relative weight of the prostate gland was still apparent when expressed as a percentage of total body weight. Examination of the different lobes of the prostate gland in various transgenic strains gave similar results to that seen when the whole prostate gland was considered (data not shown).

In an attempt to understand the differences in prostate tumor growth in LPB-Tag/CMVBP-3 and LPB-Tag/PGKB-3 mice, we examined plasma levels of IGF-I and human IGFBP-3 transgene and the abundance of the transgene-derived mRNA in prostate tissue from CMVBP-3 and PGKB-3 mice. Plasma levels of the transgene-derived IGFBP-3 were similar in CMVBP-3/Wt and PGKB-3/Wt mice and were also similar to that seen in LPB-Tag/PGKB-3 mice that did not differ significantly from each other. * and **, P < 0.05 and P < 0.01, respectively, for the difference between the double-transgenic mice and LPB-Tag/Wt mice as determined by ANOVA and Tukey honestly significant difference test.
However, transgene expression was markedly increased in prostate tissue from CMVBP-3/Wt, compared with PGKBP-3/Wt mice (Fig. 2B). The RNase protection assay is specific for the transgene and does not detect murine IGFBP-3 (20), hence the absence of signal in the lanes containing prostate RNA from Wt/Wt mice. The abundance of human (h)IGFBP-3 mRNA in prostate tissue from CMVBP-3/Wt mice was increased 5.6 ± 0.9-fold, compared with PGKBP-3/Wt mice (P < 0.001).

The phenotypic manifestations of overexpression of mutant IGFBP-3 in PGKmBP-3 mice have been previously reported (20). These mice do not demonstrate growth retardation and have slightly higher levels of IGF-I and murine IGFBP-3 than Wt mice, possibly reflecting compensation for the IGF-independent growth-inhibiting effects of mutant IGFBP-3 (20). There was no significant difference in prostate tumor growth in LPB-Tag/Wt and LPB-Tag/PGKmBP-3 mice for the first 15 wk of life (Fig. 3). However, a marked reduction in tumor growth was observed in LPB-Tag/PGKmBP-3 mice after 15 wk of age, and at subsequent time points, there was no significant difference in prostate tumor size in LPB-Tag/PGKmBP-3 and double-transgenic mice expressing the intact IGFBP-3 driven by the same promoter. A total of 43 LPB-Tag/PGKmBP-3 mice were examined from three different PGKmBP-3 stud male mice. Because the different stud males contributed different numbers of offspring to

### TABLE 1. Regression analysis of prostate weight vs. age in transgenic mouse strains

<table>
<thead>
<tr>
<th></th>
<th>LPB-Tag/Wt</th>
<th>LPB-Tag/PGKBP-3</th>
<th>LPB-Tag/CMVBP-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope</td>
<td>0.131 ± 0.004</td>
<td>0.126 ± 0.006</td>
<td>0.099 ± 0.007a</td>
</tr>
<tr>
<td>Y-intercept</td>
<td>-1.269 ± 0.061</td>
<td>-1.499 ± 0.082b</td>
<td>-1.274 ± 0.099</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>R = 0.971</td>
<td>R = 0.945</td>
<td>R = 0.896</td>
</tr>
<tr>
<td>Doubling time at 10 g, wk</td>
<td>2.29 ± 0.07</td>
<td>2.39 ± 0.12</td>
<td>3.07 ± 0.22b</td>
</tr>
</tbody>
</table>

* P < 0.001 for the difference between double-transgenic and LPB-Tag/Wt mice.

b P < 0.05 for the difference between double-ransgenic and LPB-Tag/Wt mice.

![Fig. 2. Serum IGF-I and hIGFBP-3 levels and prostate transgene-derived mRNA levels in PGKBP-3/Wt and CMVBP-3 mice.](image)

A. Serum levels - μg/ml

- IGF-I
- hIGFBP-3

Wild-type
CMVBP-3
PGKBP-3
PGKBP-3/LPB Tag

B. Prostate mRNA

WT
CMVBP-3
PGKBP-3

hIGFBP3 transgene
Cyclophilin

Fig. 2. Serum IGF-I and hIGFBP-3 levels and prostate transgene-derived mRNA levels in PGKBP-3/Wt and CMVBP-3 mice. A. Data represent the mean ± SEM levels for n = 5 or more mice per group at approximately 4 months of age. * and **, P < 0.01 and P < 0.001, respectively, for the difference between the transgenic and wild-type mice. B. RNase protection assay using a hIGFBP-3-specific probe. Cyclophilin is included as an internal control.

![Fig. 3. Mutant IGFBP-3 overexpression attenuates prostate tumor development at the later time points. Prostate weight was assessed in the various strains of mice at different ages. The data represent the mean ± SEM. The number of mice killed at each time point is shown above. For simplicity only a single line has been used to depict data for Wt/Wt, PGKBP-3/Wt, and PGKmBP-3/Wt mice that did not differ significantly from each other. * and **, P < 0.05 and P < 0.01, respectively, for the difference between the prostate weight in LPB-Tag/PGKmBP-3 and LPB-Tag/PGBKBP-3 mice as determined by ANOVA followed by the Tukey honestly significant difference test. N.S., No significant difference between LPB-Tag/PGKmBP-3 and LPB-Tag/PGBKBP-3 mice. #, P < 0.001 for the difference between LPB-Tag/PGKmBP-3 and LPB-Tag/Wt for the data from 17, 19, and 21 wk combined.](image)
each point, we investigated whether there was any difference in prostate weight in offspring of different stud males at 15 and 17 wk in which there was adequate representation of offspring from all three. When prostate weight for individual mice was expressed as a percentage of mean prostate weight for the whole group at each time point, there was no significant difference in prostate weight of the offspring of different PGKmBP-3 stud males.

Immunoblotting and Western ligand blotting were used to investigate the presence of the transgene-derived protein product in prostate tissue from the various transgenic strains. Using antibody specific for hIGFBP-3, an intense signal was apparent in lanes containing prostate extract from LPB-Tag/CMVBP-3 mice (Fig. 4A). Both intact hIGFBP-3 of approximately 40 kDa and a less abundant approximately 19 kDa IGFBP-3 proteolytic fragment, previously reported in other tissue extracts (22), were apparent. hIGFBP-3 was also detected, but less abundant, in prostate extracts from LPB-Tag/PGKBP-3 mice. As anticipated, no signal was observed in extracts from LPB-Tag/Wt or Wt/Wt mice. Weak immunoreactivity was apparent in lanes containing extracts from LPB-Tag/PGKmBP-3 mice (Figs. 4A and 5A). In extracts from these mice, the approximately 40-kDa hIGFBP-3 immunoreactivity was present as a smear and the approximately 19-kDa fragment was not seen, suggesting the possibility of extensive degradation of the non-IGF binding mutant IGFBP-3 consistent with our previous report (20).

Western ligand blotting with 125I-IGF-I confirmed the higher level of expression of the transgene in prostate tissue of LPB-Tag/CMVBP-3 mice, compared with LPB-Tag/PGKBP-3 mice (Fig. 4B). No binding was observed in lanes containing prostate extract from LPB-Tag/PGKmBP-3 mice because this mutant IGFBP-3 does not bind IGF-I (20). Radioactivity was also not detected in lanes containing extracts from LPB-Tag/Wt and Wt/Wt mice, probably because of the low sensitivity of this technique in detecting endogenous murine IGFBP's in prostate tissue extracts under these conditions. The data shown in Fig. 4B are from prostate tissue obtained at 15 wk of age. To ensure that the LPB-Tag/PGKmBP-3 continued to carry a mutant IGFBP-3 at the later time points, we used Western ligand blotting to analyze samples collected at 21 wk of age. Similar results were obtained with these tissues (Fig. 4C). 125I-IGF-I binding was observed in lanes containing extracts from LPB-Tag/PGKmBP-3 mice (Figs. 4A and 5A). In extracts from these mice, the approximately 40-kDa hIGFBP-3 immunoreactivity was present as a smear and the approximately 19-kDa fragment was not seen, suggesting the possibility of extensive degradation of the non-IGF binding mutant IGFBP-3 consistent with our previous report (20).

Western ligand blotting with 125I-IGF-I confirmed the higher level of expression of the transgene in prostate tissue of LPB-Tag/CMVBP-3 mice, compared with LPB-Tag/PGKBP-3 mice (Fig. 4B). No binding was observed in lanes containing prostate extract from LPB-Tag/PGKmBP-3 mice because this mutant IGFBP-3 does not bind IGF-I (20). Radioactivity was also not detected in lanes containing extracts from LPB-Tag/Wt and Wt/Wt mice, probably because of the low sensitivity of this technique in detecting endogenous murine IGFBP's in prostate tissue extracts under these conditions. The data shown in Fig. 4B are from prostate tissue obtained at 15 wk of age. To ensure that the LPB-Tag/PGKmBP-3 continued to carry a mutant IGFBP-3 at the later time points, we used Western ligand blotting to analyze samples collected at 21 wk of age. Similar results were obtained with these tissues (Fig. 4C). 125I-IGF-I binding was observed in lanes containing extracts from LPB-Tag/PGKmBP-3 mice (Figs. 4A and 5A). In extracts from these mice, the approximately 40-kDa hIGFBP-3 immunoreactivity was present as a smear and the approximately 19-kDa fragment was not seen, suggesting the possibility of extensive degradation of the non-IGF binding mutant IGFBP-3 consistent with our previous report (20).
CMVBP-3 and LPB-Tag/PGKB-3 mice but not in lanes with extracts from LPB-Tag/PGKmBP-3 mice. The difference in signal intensity between Fig. 4B and Fig. 4C results from differences in decay in radiolabel and autoradiography exposure time, and no meaningful conclusions can be drawn concerning the abundance of transgene expression at the two time points.

The presence of immunoreactive p53 was assessed in prostate tissue in various mouse strains at 15 wk of age. In LPB-Tag/Wt mice, the SV40 large Tag stabilizes p53, and p53 immunoreactivity was easily detected in prostate extracts from these mice (Fig. 5). A lower level of p53 protein was also detected in extracts from LPB-Tag/PGKmBP-3 mice. No p53 was apparent in lanes containing extracts from Wt/Wt, LPB-Tag/PGKBP-3 or LPB-Tag/CMVBP-3 mice. Expression of dorsolateral proteins, a marker of differentiated function (21), was lost in prostate tissue from LPB-Tag/Wt and LPB-Tag/PGKmBP-3 mice but relatively preserved in LPB-Tag/CMVBP-3 and LPB-Tag/PGKBP-3 mice (Fig. 5C).

Expression of both EGF-R and IGF-IR were up-regulated in the prostate tumors (Fig. 6). EGF-R was most abundant in LPB-Tag/Wt and LPB-Tag/PGKmBP-3 mice, and the lowest levels of expression were apparent in tissue from LPB-Tag/CMVBP-3 mice. In contrast, IGF-IR was significantly elevated in all transgenic mice carrying the LPB-Tag transgene, compared with Wt mice, and there was no significant difference between those expressing intact or mutant IGFBP-3. Despite increased levels of IGF-IR in LPB-Tag/CMVBP-3 and LPB-Tag/PGKBP-3 mice, the abundance of phospho pAkt(Ser 473) was reduced in these mice, compared with LPB-Tag/Wt and LPB-Tag/PGKmBP-3 mice (Fig. 6, lower panel), suggesting that signaling at the IGF-IR was attenuated in the double-transgenic mice expressing intact IGFBP-3.

Prostate tissue from all four groups of tumor bearing mice showed a similar histological appearance. In LPB-Tag/Wt mice and LPB-Tag/PGKmBP-3, by 9 wk of age, virtually all acina demonstrated hypercellularity typical of high-grade PIN, whereas prostate tissue from LPB-Tag/PGKBP-3 mice at the same stage showed a more heterogeneous pattern with many acina appearing normal or demonstrating low-grade PIN (Fig. 7, upper panel). Tissue from LPB-Tag/CMVBP-3 at 9 wk was similar to that from LPB-Tag/PGKBP-3 mice (data not shown). At later time points, prostate tissue from LPB-Tag/CMVBP-3 and LPB-Tag/PGKBP-3 mice resembled early time points in LPB-Tag/Wt mice with high-grade PIN throughout the entire gland. In general, high-grade PIN was associated with loss of DLP staining, increased nuclear localization of androgen receptor staining, and positive staining for PCNA and Tag (Fig. 7, lower panel). There was no obvious difference in immunostaining or histology between tissues from LPB-Tag/PGKBP-3 and LPB-Tag/PGKmBP-3 mice.

The percentage of apoptotic cells in areas of high-grade PIN was assessed in each of the strains of tumor-bearing mice. Apoptotic cells were most abundant in LPB-Tag/CMVBP-3 mice and least abundant in LPB-Tag/Wt mice (Fig. 8). In LPB-Tag/Wt, LPB-Tag/PGKBP-3, and LPB-Tag/CMVBP-3 mice, the percentage of apoptotic cells was relatively constant from 9 through 17 wk. In contrast, in LPB-Tag/PGKmBP-3 mice, the percentage of apoptotic cells was low at 9 and 13 wk but increased significantly at 17 wk (Fig. 8).

**Discussion**

Heterozygous LPB-Tag mice carry a genetic predisposition to neoplasia restricted to the prostate because of the tissue specificity of the long probasin promoter (14). The
Fig. 7. Immunohistochemical analysis of prostate tumors in LPB-Tag/Wt, LPB-Tag/PGKBP-3, and LPB-Tag/PGKmBP-3 mice. Tissue from mice at 9 and 17 wk of age were analyzed using antibodies to androgen receptor (AR), DLP, Tag, and PCNA. The magnification is ×200.
SV40 large T oncoprotein interferes with the cellular tumor suppressor protein p53 mimicking molecular alterations that occur in human prostate cancer (12, 14). The LPB-Tag mice are an appropriate transgenic model of prostate cancer to study the effect of IGFBP-3 because many features of human prostate cancer are reproduced including androgen dependence of tumor formation and growth; tumor formation in normal hormone- and immune-competent environment; and the expression of biomarkers associated with human PIN that predict progression to invasive carcinoma such as increased PCNA, a marker of proliferation, decreased apoptosis, enhanced growth factor receptor expression (erbB family), increased expression, and nuclear localization of the androgen receptor. Most importantly, the histological feature of the putative precursor lesions, mouse PIN, and progression to adenocarcinoma are also apparent in this mouse model (15).

In this manuscript, we report the attenuation of prostate tumor growth in mice that overexpress IGFBP-3. These experiments were performed by generating double-transgenic mice. Both the IGFBP-3 and the LPB-Tag mice were generated in the same CD1 strain, thereby eliminating the potential confounding effects of different genetic backgrounds. These observations represent the first demonstration of attenuated prostate tumor growth in vivo by IGFBP-3 overexpression.

The marked reduction of tumor growth seen in both LPB-Tag/PGKBP-3 and LPB-Tag/CMVBP-3 mice was predominantly due to paracrine/autocrine effects in the prostate rather than the result of systemic IGFBP-3 because the effect was more marked in LPB-Tag/CMVBP-3 mice that have higher levels of transgene expression in the prostate but similar levels of circulating IGFBP-3 to LPB-Tag/PGKBP-3 mice. Furthermore, attenuation of prostate tumorigenesis was apparent despite significantly increased levels of IGF-I in the circulation in both these strains of double-transgenic mice (19).

After 15 wk of age, the tumors in LPB-Tag/PGKBP-3 and LPB-Tag/CMVBP-3 grew rapidly, although at a slightly slower rate than that seen in LPB-Tag/Wt mice, suggesting that the predominant effect of overexpression of IGFBP-3 was at the early stages of tumor development. The mechanisms involved in tumor development in LPB-Tag mice are not fully understood, but tumor development is delayed until after sexual maturation in this model and is clearly androgen dependent (14). Both PGKBP-3 and CMVBP-3 male mice are fertile and testosterone levels are not markedly different in these mice and Wt mice (Ref. 19; and Silha, J., and L. J. Murphy, unpublished observations). Although not studied in this LPB-Tag model, prostate cancer development is thought to be IGF-I dependent in the early stages, whereas the tumor progression may be less dependent on IGF-I as the disease progresses (23), possibly as a result of amplification of the IGF-IR and/or downstream signal transduction pathways (24). Our observations in LPB-Tag/PGKBP-3 and LPB-Tag/CMVBP-3 mice would be consistent with this notion. In these mice tumor development was delayed, but once well established, the tumor appeared to grow at a rate approaching that seen in LPB-Tag/Wt mice. Furthermore, prostate histology in LPB-Tag/PGKBP-3, LPB-Tag/CMVBP-3, and LPB-Tag/Wt mice appeared similar.

Not unexpectedly prostate tumor development and growth in LPB-Tag/PGKmBP-3 was similar to LPB-Tag/Wt mice during the first 15 wk. Because mutant IGFBP-3 does not bind IGF-I (20), it would be unable to inhibit IGF-I action during the critical early stages of prostate tumorigenesis. We have previously shown that PGKmBP-3 transgenic mice have low levels of human IGFBP-3 in the circulation (~0.5 µg/ml), compared with PGKBP-3 transgenic mice (~5 µg/ml), despite identical transgene promoters and similar levels of tissue transgene mRNA (19, 20). We speculated that the mutant IGFBP-3 was more rapidly cleared from the circu-

**FIG. 8.** Apoptosis in prostate tumors in LPB-Tag/Wt, LPB-Tag/PGKBP-3, LPB-Tag/CMVBP-3, and LPB-Tag/PGKmBP-3 mice. Upper panel, Representative sections from tumors from 17-wk-old mice. The magnification is ×200. Lower panel, The percentage of apoptotic cells in similar high-grade PIN lesions in tumors from the four groups of mice were enumerated at each of the time points. The data represent the mean ± SEM. *, P < 0.05 for the difference LPB-Tag/Wt mice and other mouse strains at each time point; #, P < 0.05 for the difference from the 9-wk time point within the one mouse strain.
lation and degraded because mutant IGFBP-3 is unable to bind IGF-I, which appears to be necessary for the formation of stable ternary complexes with the acid-labile subunit (25). Western blotting of prostate extracts confirmed that mutant IGFBP-3 was more degraded than native IGFBP-3. Human prostate tissue contains prostate-specific antigen that can proteolyze IGFBP-3 (26). It is likely that mouse prostate tissue contains similar kallikreins that can degrade IGFBP-3. Immunohistochemical analysis indicated that regions of the prostate demonstrating high-grade PIN had low levels of expression of dorsolateral proteins. We speculate that in these regions IGFBP-3 proteolysis may be reduced.

The unexpected finding in this report was the decline in tumor growth in LPB-Tag/PGKmBP-3 after 15 wk of age. Whereas this most likely represents an IGF-independent effect of IGFBP-3 similar to those reported by other investigators using in vitro cultures of prostate and breast cancer cells (5, 11, 17, 18, 22), it is unclear why these IGF-independent effects were not manifested earlier. We carefully excluded the possibility that this was artifactual by reviewing the parentage of each of the mice and also analyzing the tumor extracts from 21-wk-old mice by Western ligand blotting (Fig. 4). The LPB-Tag/PGKmBP-3 offspring used for the age 19- and 21-wk data points were from the same stud PGKmBP-3 male that contributed offspring to earlier time points, and thus, the data from the later time points are not the result of a specific stud male. Consistent with this apparent delayed effect of mutant IGFBP-3 expression, we demonstrate a significant increase in the percentage of apoptotic cells in the high-grade PIN lesion with time in LPB-Tag/PGKmBP-3 mice.

Under in vitro conditions, it is possible to demonstrate multiple and opposing effects of IGFBP-3 on cell proliferation and apoptosis. IGFBP-3 has IGF-dependent antiproliferative, proapoptotic effects related to binding IGF-I and restriction of access of IGF-I to the IGF-IR. Under certain conditions, IGF-dependent effects of enhancing cell survival and proliferation, possibly by enhancing delivery of IGF-I to the cell membrane receptor, can also be demonstrated with IGFBP-3 (27). In addition, as discussed above, IGFBP-3 has IGF-independent antiproliferative, proapoptotic effects actions that have been reported in vitro in the past. Various potential mechanisms have been proposed to explain these IGF-independent effects of IGFBP-3. These include the interaction of IGFBP-3 with various cell membrane-associated proteins such as the type V TGFβ receptor (28) and autocrine motility factor (29) as well as other as-yet-undefined proteins (30, 31). In addition, IGFBP-3 has been shown to interact with nuclear transcription factors such as the retinoid X receptor-α and stimulate apoptosis via this interaction (32).

This report represents the first demonstration of the IGF-independent antiproliferative effects of IGFBP-3 in vivo. IGF-independent effects of IGFBP-3 demonstrated in vitro are apparent only under conditions in which IGF-I is absent (5, 11, 16) or in cell lines that are not dependent on IGF-I for growth because they lack IGF-IR (3, 4). It is possible that the IGF-independent effects of IGFBP-3 are inhibited by IGF-I or not apparent in cells in which the IGF-I signal transduction pathway is activated. Thus, early in prostate tumorigenesis in LPB-Tag/PGKmBP-3 mice in which the tumors are growing in response to IGF-I, these IGF-independent effects of IGFBP-3 may be blocked or masked by IGF-I stimulated mitogenesis.

An alternative explanation for the apparent lack of effect of mutant IGFBP-3 during early prostate cancer growth in this model may be related to the enhanced degradation of mutant IGFBP-3 in prostate tissue. With the loss of markers of differentiation, such as dorsolateral protein as the tumors progress, there may also be a loss of IGFBP-3 protease activity and consequently enhanced levels of mutant IGFBP-3 that could exert a progressively more marked effect with increasing tumor mass. Lack of sufficient quantities of recombinant mutant IGFBP-3 precluded us from directly testing this hypothesis.

Whereas the exact mechanism whereby overexpression of mutant IGFBP-3 exerts its antiproliferative effect requires further investigation, our data clearly demonstrate that local overexpression of IGFBP-3 attenuates prostate tumorigenesis. Recently Majeed et al. (33) reported that development and progression of the transgenic adenocarcinoma of mouse prostate (TRAMP) model of prostate cancer is delayed in the lit/lit mouse, which has low IGF-I and GH levels as a result of an inactivating mutation in the GH releasing factor receptor. Their data suggest that IGF-I, GH, or both are important in prostate cancer development and progression. Wang et al. (34) used a slightly different model in which the GH receptor gene was disrupted to demonstrate the importance of GH/IGF-I signaling for prostate carcinogenesis in the C3(1)/T-antigen mice. Our observations, using a slightly different model of prostate cancer, support an important role of local IGF-I levels in prostate tumor progression. Furthermore, our data also suggest the use of IGFBP-3 and its mutant may be a useful therapeutic strategy in the treatment of prostate cancer.

Acknowledgments

Received October 6, 2005. Accepted February 2, 2006.

Address all correspondence and requests for reprints to: Liam J. Murphy, Departments of Physiology and Internal Medicine, University of Manitoba, Winnipeg, Canada R3T 2N2.

This work was supported by the Prostate Cancer Research Foundation of Canada, the Canadian Institutes for Health Research, and the Prostate Cancer Research Program of the U.S. Army Medical Research and Materiel Command (Award W81XWH-04-1-0907). The U.S. Army Medical Research Acquisition Activity (820 Chandler Street, Fort Detrick, MD 21702-5014) is the awarding and administering acquisition office. L.J.M. is a recipient of the Henry G. Friesen Chair in Endocrine and Metabolic Research.

Present address for Y.G.: Center of Reproductive Medicine, Shenzhen Hospital of Peking University, Shenzhen 518036, People’s Republic of China.

The content of this manuscript does not necessarily reflect the position or the policy of the Government, and no official endorsement should be inferred. The paper is part of United States Provisional Patent US60/710,893, filed on August 25, 2005, “Methods of attenuating prostate tumour growth by insulin-like growth factor binding protein 3.” Applicants were Josef V. Silha, Janice G. Dodd, and Liam J. Murphy. There is no commercial involvement in the research or the patent prosecution at this time. P.C.S., S.M., Y.G., and J.Sc. have nothing to declare.

References


12. Chokkalingam AP, Atoy, D, Stanczyk FZ, Sesterhenn IA, Mostofi


Silha et al. • Prostate Tumors in IGFBP-3 Transgenic Mice


8. Angelloz-Nicoud P, Lalou C, Binoux M 1998 Prostate carcinoma (PC-3) cell proliferation is stimulated by the 22-25 kDa proteolytic fragment (11-169) and inhibited by the 16-kDa fragment (1-95) of recombinant human-insulin-like growth factor binding protein-3. Growth Horm IGF Res 8:71–75


