A B S T R A C T
Insulin-like growth factor I (IGF-I) has well-characterized mitogenic and antiapoptotic effects that are essential for maintenance of the normal prostate and may be important during regression of the normal prostate and/or prostate tumors induced by androgen-targeting therapies for prostate cancer. IGF-I activity is modulated by IGF-binding proteins (IGFBPs). Here we examine IGFBP expression during regression of androgen-dependent Shionogi carcinoma tumors after castration. In this model, we observe a 90% reduction in Shionogi tumors by 10 days postcastration. Northern blotting of RNA from tumors collected at various times after castration indicates a rapid induction of IGFBP-5 concomitant with apoptotic regression of tumors, as detected by Apoptag staining of tumor sections after castration. IGFBP-5 mRNA was not detectable in tumors from control animals, but levels increased 120-fold in tumors 3 days after castration. The mRNAs for IGFBP-3 and -4 were abundant in Shionogi tumors from intact mice and decreased to —33% and —20% of control, respectively. Castration had no significant effect on IGFBP-2 expression. Treatment with calcium channel blockers inhibited castration-induced apoptosis and tumor regression and also significantly inhibited up-regulation of IGFBP-5 after castration. These data provide strong evidence for a functional role of IGFBP-5 expression in mediating the apoptosis induced by androgen deprivation in androgen-dependent neoplasia.

I N T R O D U C T I O N
Standard therapy for prostate cancer involves targeting androgens by blocking androgen production (by castration or the administration of luteinizing hormone-releasing hormone agonists), or activity by administration of antiandrogens. Androgen ablation therapy generally leads to significant involution of prostate tumors, but the molecular mechanisms underlying this regression are incompletely described.

IGF-I has an important influence on the growth and survival of both normal and neoplastic prostate epithelial cells (1, 2). We have recently demonstrated in a prospective study that a strong positive correlation exists between serum IGF-I levels and prostate cancer risk (3). The well-recognized mitogenic and antiapoptotic effects of IGF-I (reviewed in Refs. 4 and 5) are modulated by a family of high-affinity IGFBPs (IGFBPs 1–6; reviewed in Refs. 6 and 7).

IGFBPs have been shown to have apoptotic effects on both PC-3 prostate cancer cells and MCF7 breast cancer cells in vitro (8, 9). We have previously reported that castration-induced apoptosis in the normal rat prostate gland is associated with an increased expression of IGFBPs (10). The Shionogi carcinoma is a well-characterized model of androgen-dependent neoplasia (11). We undertook these experiments to determine whether regression of androgen-dependent Shionogi carcinoma after castration involves changes in local IGF biology.

M A T E R I A L S A N D M E T H O D S

Shionogi Tumor Growth. Animal studies were conducted in accordance with local humane animal care standards. The Toronto subline of the transplantable SC-115 AD mouse mammary carcinoma (12) was used in all experiments. Shionogi tumor cells were maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% heat-inactivated FCS. For in vitro study, approximately 5 x 10^5 cells of the Shionogi carcinoma were injected into the left flank of adult male DBA/2J mice, which were housed within our own facilities (n = 5 animals/group; two tumors/animal). When Shionogi tumors reached 1–2 cm in diameter (usually 2–3 weeks after injection), castration was performed through an abdominal incision under methoxyflurane anesthesia. Details of the maintenance of mice, tumor stock, and operative procedures have been described previously (13). Tumors were measured each day, and tumor volume was calculated as L x W x H x 0.5236 (14). Animals were sacrificed at various times after castration, and tumors were excised and fixed in 10% neutral buffered formalin for paraffin embedding, and the remainder was immediately frozen in liquid nitrogen for isolation of RNA.

Inhibition of Apoptosis by Calcium Channel Blockers. To determine whether changes in IGFBPs after castration are regulated by androgen or associated with apoptosis, calcium channel blockers [600 μg of nifedipine and 100 μg of amlodipine (Norvasc)] were given three times/day by oral administration to mice bearing Shionogi tumors 1 day before castration to inhibit castration-induced apoptosis. Tumor tissues were harvested 3, 8, and 10 days after castration.

Quantification of Apoptosis after Castration. To characterize changes in apoptotic rates after castration, the number of apoptotic bodies/high power field were counted and compared in control tumors before castration, regressing tumors 3, 5, 10, and 15 days after castration. Tumors were harvested at various times postcastration, fixed in 10% neutral buffered formalin, and embedded in paraffin. Fixed sections (5 μm) were cut from each specimen and stained with digoxigenin-dUTP antibodies (Apoptag; Oncor, Inc., Gaithersburg, MD). The number of Apoptag-positive cells/high power field in five random fields was counted and averaged.

Northern Analysis. Total RNA was isolated from tissue using the RNAzol B method (Teltest). Total RNA (40 μg) was fractionated on 1% agarose gels and transferred onto Zeta-Probe membrane (Bio-Rad) in 50 mM NaOH. The cDNAs for rat TRPM-2 (15); IGFBP-2, -3, -4, and -5 (16); IGF-I (American Type Culture Collection); and IGF-IR (American Type Culture Collection) were labeled with [α-32P]dCTP using the T7 Quick-Prime kit (Pharmacia). Prehybridization, hybridization, and washing were performed as described previously (10). Blots were subjected to autoradiography with intensifying screen at ~80°C. Quantitative analysis of gene expression was accomplished by densitometric scanning of autoradiograms, and results were corrected for minor loading differences by normalizing to 28S rRNA.

R E S U L T S
Apoptotic Regression of Shionogi Tumors after Castration. Androgen-dependent Shionogi tumors develop rapidly in intact mice, reaching a volume of ~1000 mm^3 by 4 weeks. In this model, we observe a 90% reduction in tumor volume by 10 days postcastration...
(Fig. 1). The extent of apoptosis in Shionogi tumors was determined by Apoptag staining of tumor sections. We observed minimal apoptosis in tumors from intact mice (Fig. 2, A and C). The number of cells undergoing apoptosis increased 5-fold by 3 days after castration and peaked around 5 days (Fig. 2, B and C). Northern blot analysis was used to confirm the expression of TRPM-2/clusterin (Fig. 2D), an androgen-repressed gene known to be associated with involution of Shionogi tumors (12).

Effects of Castration on IGFBP Expression in Shionogi Tumors. Castration resulted in a dramatic increase in IGFBP-5 expression, decreased expression of IGFBP-3 and -4, and had no effect on IGFBP-2 expression, as determined by Northern blot analysis. IGFBP-2 mRNA is expressed in control tumors and does not undergo a significant change in response to castration (Fig. 3A). IGFBP-3 mRNA is abundant in control tumors, and decreases to ~33% of control 3 and 10 days after castration (Fig. 3B). IGFBP-4 mRNA is also strongly expressed in Shionogi tumors from intact mice and decreases to ~20% of control 3 days after castration (Fig. 3C). By 10 days postcastration, IGFBP-4 levels increase slightly, reaching ~40% of control. IGFBP-5 mRNA is very low in tumors from control mice but increases 120-fold in...
tumors 3 days after castration (Fig. 3D). By 10 days postcastration, IGFBP-5 mRNA levels had declined slightly, but remained 100-fold of control levels. In relation to the time course of tumor regression, this dramatic increase in IGFBP-5 expression occurs early on, before the peak of apoptotic cell death.

Effect of Calcium Channel Blockers on Castration-induced Tumor Regression and IGFBP Expression. Apoptosis in many cell types requires a rise in intracellular \( \text{Ca}^{2+} \) levels. Use of calcium channel blockers in vivo prevents apoptosis in a number of cell types (17–19). In particular, treatment with calcium channel blockers prevents apoptosis of androgen-dependent prostate cells and delays prostate regression after castration (20–22). We confirmed that administration of calcium channel blockers to mice bearing Shionogi tumors effectively prevents tumor regression after castration (Fig. 1). Importantly, the up-regulation of IGFBP-5 in tumors by castration was significantly inhibited by calcium channel blocker treatment (Fig. 3D), whereas the expression of other IGFBPs after calcium channel blocker treatment was not significantly altered (Fig. 3, A–C).

Effects of Castration on IGF-IR and IGF-I Gene Expression. IGF-IR mRNA levels decreased by ~50% 3 days after castration and then partially recovered by 8 days (Fig. 4A). IGF-I gene expression increased slightly as a result of castration, reaching 1.3-fold of control by 8 days (Fig. 4B). Expression of IGF-II was not detected in tumors from either intact or castrated animals (data not shown).

DISCUSSION

We demonstrate that apoptotic regression of androgen-dependent Shionogi tumors is associated with major changes in IGFBP gene expression within the neoplasms. Whereas IGFBP-3 and -4 expression decrease slightly after castration, the magnitude of increase in expression of IGFBP-5 is remarkable. Furthermore, gene expression of IGFBP-2, -3, and -4 is strong in control tumors in contrast to IGFBP-5, which is barely detectable. The effects of castration on gene expression of IGF-I and IGF-IR in Shionogi tumors were minimal compared to the changes in expression of IGFBPs. In view of the large increase in IGFBP-5 gene expression in Shionogi tumors after androgen ablation, it is possible that the overall increase in IGF binding capacity in the tumor microenvironment results in lower IGF bioactivity. Alternatively, there is evidence that IGFBPs have direct effects on cell growth and survival that are independent of IGF-I (23, 24). These mechanisms are not mutually exclusive.

It has been recognized for some time that IGFs have a central role in regulating proliferation and apoptosis of prostate epithelial cells (1, 25). However, more attention has been given recently to the apoptosis-inducing effects of IGFBPs. It has previously been hypothesized that IGFBP-5 serves to trigger apoptosis in the prostate after androgen ablation (26). Our observation that castration is followed by dramatic increases in IGFBP-5 expression in Shionogi tumors that precede the time of maximum apoptosis provides strong support for this hypothesis.

It is well established that during the process of apoptotic execution, the intracellular free calcium concentration increases, and, consequently, endonucleases, which can degrade nucleosomal DNA, are activated. On the basis of these findings, we treated mice bearing Shionogi tumors with calcium channel blockers before castration to prevent castration-induced apoptosis. As expected, calcium channel blocker treatment inhibited regression of Shionogi tumors after castration through the inhibition of apoptotic cell death precipitated by androgen ablation. Furthermore, up-regulation of IGFBP-5 after castration was significantly reduced by calcium channel blocker treatment. Although functional analysis of IGFBP-5 is required to determine the specific role after castration, these findings suggest that...
IGFBP-5 is an apoptosis-related gene rather than an androgen-repressed gene.

We have previously reported that increases in IGFBP gene expression are associated with apoptotic regression of the normal rat prostate induced by either castration (10) or the antiandrogen bicalutamide. IGFBP expression has also been shown to increase during postlactational involution of the rat mammary gland (27) and after treatment of breast cancer cells with antiestrogens (28). Thus, there is accumulating evidence that apoptosis associated with steroid hormone deprivation involves up-regulation of IGFBP expression.

Our results represent the first report documenting up-regulation of IGFBP-5 after castration in a model of androgen-dependent neoplasia. Study of the functional role of IGFBP-5 in castration-induced apoptosis is now justified.

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Castration-induced Apoptosis of Androgen-dependent Shionogi Carcinoma Is Associated with Increased Expression of Genes Encoding Insulin-like Growth Factor-binding Proteins

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