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Advances in Brief

Regulation of Apoptosis Induced by Transforming Growth Factor-β1 in Nontumorigenic and Tumorigenic Rat Prostatic Epithelial Cell Lines

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Abstract

Transforming growth factor-β1 (TGF-β1), which is induced in the prostate following castration, has been speculated to mediate apoptosis of epithelial cells during prostatic involution. Here, we report the first evidence of a direct effect of TGF-β on induction of apoptosis in prostatic epithelial cells *in vitro*, using NRP-152 nontumorigenic and NRP-154 tumorigenic rat prostatic epithelial cell lines. TGF-β1 induces apoptosis of both cell lines within 24 h, as shown by a decrease in cell viability, *in situ* DNA nick-end labeling, and internucleosomal DNA fragmentation. Moreover, the ability of TGF-β to induce apoptosis of NRP-152 is strictly dependent on culture conditions, because dexamethasone enhances while insulin and insulin-like growth factor-I specifically block apoptosis induced by TGF-β. We suggest that TGF-βs are direct physiological regulators of apoptosis of prostatic epithelial cells.

Introduction

Apoptosis or programmed cell death is now recognized to be of fundamental importance during normal prostatic growth and development, as well as in the carcinogenesis of this highly cancer-prone organ (1–3). In particular, loss or aberration of normal apoptotic mechanisms may be critical or sufficient for the initiation, promotion, or progression of prostatic cancer. The normal homeostatic mechanisms regulating apoptosis in the prostate are poorly understood and thus under intense investigation.

TGF-βs[6] are a group of M_r 25,000 dimeric peptides that modulate numerous cellular functions, including extracellular matrix protein expression, differentiation, cell proliferation (4), and apoptosis in a variety of cells (5–8). A putative role for TGF-βs in apoptosis of prostatic epithelial cells following castration has been implicated by Kyprianou and Isaacs (9) in 1989. They showed that TGF-β1 mRNA was induced in the prostate following castration, and that exogenous TGF-β1 caused partial prostatic regression in intact rats and in organ culture (9, 10). However, these studies leave unanswered the questions of whether active TGF-β protein is induced during castration in levels sufficient to promote programmed cell death and whether physiological levels of active TGF-β are able to induce apoptosis of prostatic epithelial cells directly. We explored the possibility that TGF-β causes apoptosis on prostatic epithelial cells directly using cell lines (NRP-152 and NRP-154) that were derived from the nonneoplastic dorsal-lateral prostate of carcinogen-treated Lobund-Wistar rats (11). Although NRP-154 cells are tumorigenic, NRP-152 cells are nontumorigenic and have retained many of the properties of normal prostatic epithelial cells. NRP-152 cells have the unique property of organizing into a prostatic organoid *in vivo*.[7] Moreover, they are exquisitely responsive to many hormones and growth factors, including retinoids, androgens, and TGF-βs (11), and their growth is regulated by autocrine TGF-βs (12). Furthermore, we have shown that retinoids (12) and androgens[8] effect growth of these cells largely by their ability to regulate autocrine TGF-β expression. In this report, we demonstrate that TGF-β is an important regulator of apoptosis of NRP-152 cells, and that the ability of TGF-β1 to induce apoptosis is strictly dependent on culture conditions, conditions that may influence response patterns of TGF-β *in vivo*.

Materials and Methods

Materials. Sources were as follows: recombinant human TGF-β1, porcine TGF-β2, and recombinant human TGF-β3 from R&D Systems, Inc. (Minneapolis, MN); DMEM/F12, fetal bovine serum, CS, and trypsin-EDTA from Life Technologies, Inc. (Grand Island, NY); bovine insulin and mouse epidermal growth factor from Biofluids, Inc. (Rockville, MD); IGF-I and LR^3-IGF-I from GroPep (Adelaide, SA, Australia); cholera toxin, Dex, and Taxol from Sigma Chemical Co. (St. Louis, MO); and genistein, staurosporine, tumor necrosis factor-α, and okadaic acid from UBI (Lake Placid, NY).

Cell Culture. The rat prostatic cell lines used in this study (NRP-152 and NRP-154) were derived from the dorsal-lateral prostate of carcinogen-treated Lobund Wistar rats without transfection or infection of an immortalizing gene, as described (11). These cells were grown in GM2 (DMEM/F12, 5% fetal bovine serum, 0.1 μM Dex, 5 μg/ml insulin, 20 ng/ml EGF, and 10 ng/ml cholera toxin), and passaged as described (11, 12). Unless indicated, cell growth assays were done as follows: 2×10^4 cells in 0.5 ml of medium (DMEM/F12, 15 mM HEPES, and 1% CS) were dispensed into 24-well Costar dishes. All additions were made following 2 h of plating. Cells were enumerated using a Coulter Counter following detachment with typsin (0.5 ml trypsin-EDTA for 10 min at 37°C).

***In Situ* Detection of Apoptosis.** We used the TUNEL method (terminal deoxytransferase-mediated dUTP-biotin nick end-labeling method) with some modification (13). Cells were cultured and treated on Lab-tech chamber slides and fixed with 10% formalin for 10 min at room temperature. The slides were rinsed four times with distilled water for 2 min, briefly submerged in TdT buffer, and incubated in 100 μM biotin-14-ATP (Life Technologies, Inc., Bethesda, MD), 1× TdT buffer, 0.3 unit/μl TdT at 37°C for 2 h. Following three rinses with distilled water, biotin-labeled DNA was detected by a streptavidin-alkaline phosphatase detection system (Life Technologies, Inc., Rockville, MD). For prostatic tissue embedded in paraffin block, 6-μm sections were prepared, and endogenous phosphatases were heat inactivated for 1 h at 65°C. Following deparaffinization and hydration, tissues were treated with 20 μg/ml proteinase K at room temperature for 15 min, rinsed four times with distilled water, and then processed as above for cells.

Detection of Internucleosomal DNA Ladder. Unless indicated, $1.5–2 \times 10^6$ cells were plated in 10-cm Falcon tissue culture dishes with 10 ml

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[6] The abbreviations used are: TGF-β, transforming growth factor-β; IGF-I, insulin-like growth factor I; CS, calf serum; Dex, dexamethasone; TUNEL, terminal deoxytransferase-mediated dUTP-biotin nick end-labeling; TdT, terminal deoxytransferase.

[7] S. Hayward and G. Cunha, personal communication.

[8] M. S. Lucia, M. B. Sporn, A. B. Roberts, and D. Danielpour. Role of transforming growth factor-β1, β2, and β3 in androgen responsive growth of NRP-152 rat dorsal prostatic epithelial cells, submitted for publication.

DMEM/F12 containing 15 mM HEPES and 1% CS. All factors were added 24 h after plating, and cells were detached by trypsinization following 24 h of treatment (3 ml trypsin-EDTA for 7 min at 37°C). Internucleosomal DNA ladders (14, 15) were detected with a modification of TACS apoptotic DNA ladder kit (Trevigen, Gaithersburg, MD). Cell pellets resuspended in 50 μl PBS were lysed by the addition of 50 μl of lysis buffer and purified as described by the kit. The nicked ends of 1 μg of DNA were ^{32}P-labeled with 2.5 units of Klenow fragment of DNA *Pol*I in the presence of 0.5 μCi of [α-^{32}P]dCTP (3 Ci/μmol, Dupont NEN, Boston, MA) for 30 min at room temperature in a 10-μl reaction volume of 5 mM $MgCl_2$ and 10 mM Tris-HCl (pH 7.5). One-third of this labeled DNA was electrophoresed through to 1.8% agarose-1× TAE at 70 V for 2 h. Gels were then dried directly and exposed to X-Omat AR (Kodak, Rochester, NY) for about 1 h.

Results

NRP-152 are nontumorigenic cells whose continuous growth in culture, similar to primary cultures of prostatic epithelial cells, requires a medium rich in mitogens (*i.e.*, GM2). When NRP-152 cells are cultured in DMEM/F12 medium supplemented with 1% CS, they rapidly approach growth arrest. Under these conditions, treatment with 10 ng/ml TGF-β1 for 4 days causes loss of >80% of the cells (Fig. 1*A*) with an ED_{50} of 0.5 ng/ml (data not shown). Under these conditions, TGF-β1 (10 ng/ml) also causes a dramatic morphological change (*i.e.*, smaller and less adherent) in the remaining cells (Fig. 1*B*).

Two types of assays (TUNEL and DNA laddering; see "Materials and Methods") were used to determine if the death of NRP-152 cells induced by TGF-β1 occurred by an apoptotic mechanism. TUNEL is an *in situ* assay for internucleosomal DNA fragmentation in apoptotic cells that can be visualized by light microscopy (13). NRP-152 cells treated with TGF-β1 were analyzed for apoptosis by TUNEL, along with normal and involuting rat prostate as negative and positive controls, respectively. Greater than 50% of the NRP-152 cells treated with 10 ng/ml TGF-β1 for 48 h showed nuclear staining only (no cytosolic staining), in contrast to almost no staining in the untreated cells (Fig. 2*A*). Similar results were obtained with NRP-154 cells (data not shown). The induction of a DNA ladder between nucleosomal units, which is a hallmark of apoptosis (14), is visible by 24 h of treatment with TGF-β1 in both NRP-152 and NRP-154 cells (Fig. 2, *B* and *C*), with ED_{50}s (quantified by PhosphorImager scanning) of 0.5 and 3 ng/ml of TGF-β1, respectively.

TGF-β1 is unable to induce death of NRP-152 cells in normal growth medium (GM2), suggesting that certain growth factors or hormones in this medium may block cell death by TGF-β1. To determine what factors are responsible for this block, we tested the individual components of this medium and found Dex and insulin to be the major regulators of apoptosis induced by TGF-β1. The individual and combined effects of 0.1 μM Dex and 5 μg/ml of insulin on the ability of TGF-β1 to induce cell death were examined after 4 days of treatment. Data are expressed as cell population doublings (nega-

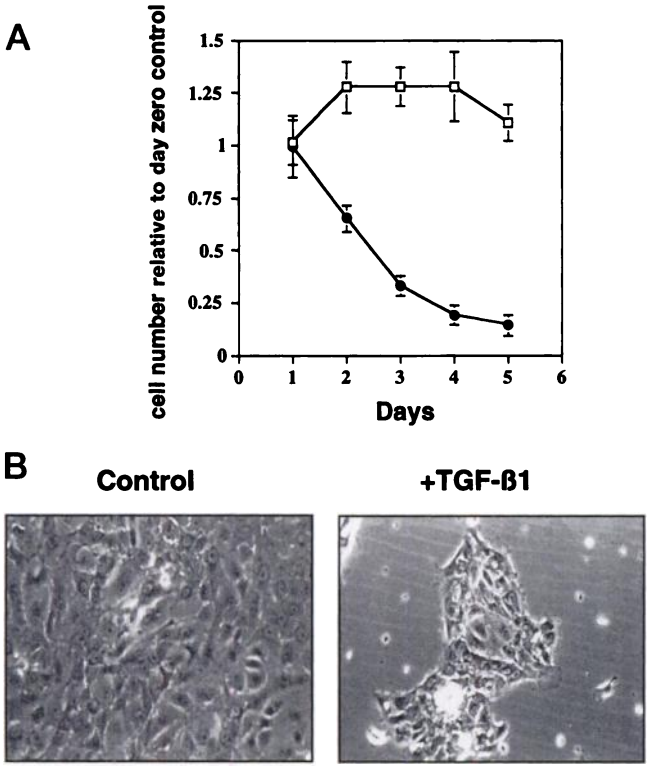

Fig. 1. TGF-β induces death of NRP-152 cells. *A*, changes in NRP-152 cell number in DMEM/F12 + 1% CS were examined as a function of time without TGF-β1 (□) or with 10 ng/ml of TGF-β1 (●). Bars, SD. *B*, the morphologies of the control and TGF-β1-treated cells in *A* were examined by phase contrast microscopy at day 4.

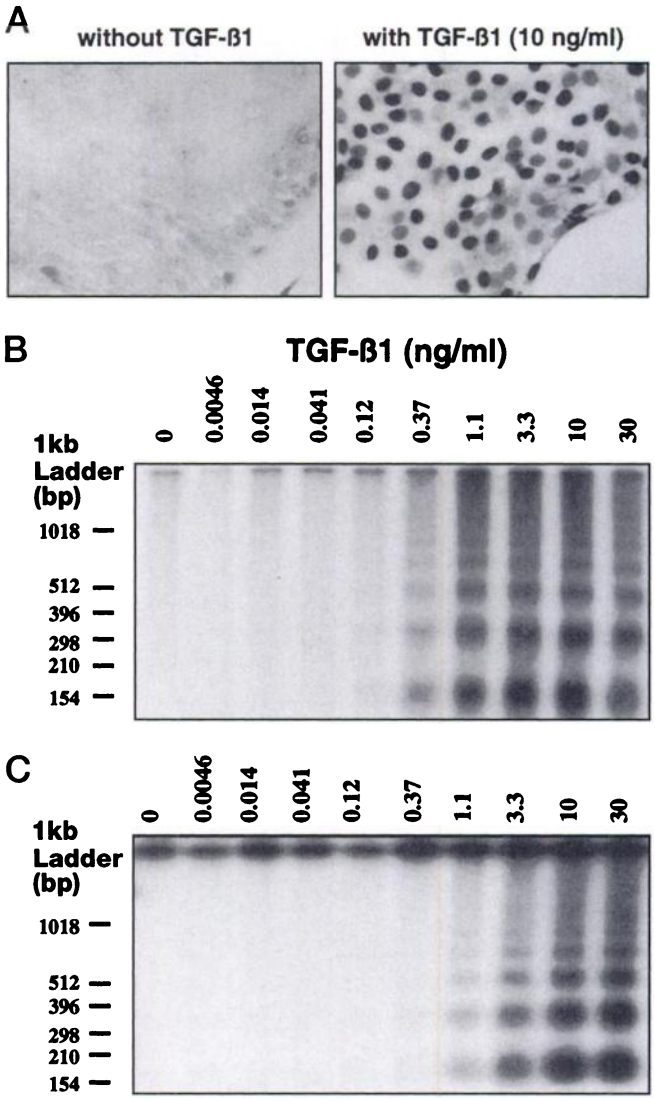

Fig. 2. Demonstration that TGF-β induces apoptosis in NRP-152 and NRP-154 cells. NRP-152 cells after 48-h treatment with vehicle or 10 ng/ml of TGF-β1 (*A*) were analyzed for apoptosis cells by the TUNEL method. Ventral prostate from intact and castrated (24-h) rats were used as negative and positive controls. The effects of various concentrations of TGF-β1 on apoptosis of NRP-152 (*B*) and NRP-154 (*C*) cells were examined by DNA laddering after 24 h of TGF-β treatment.

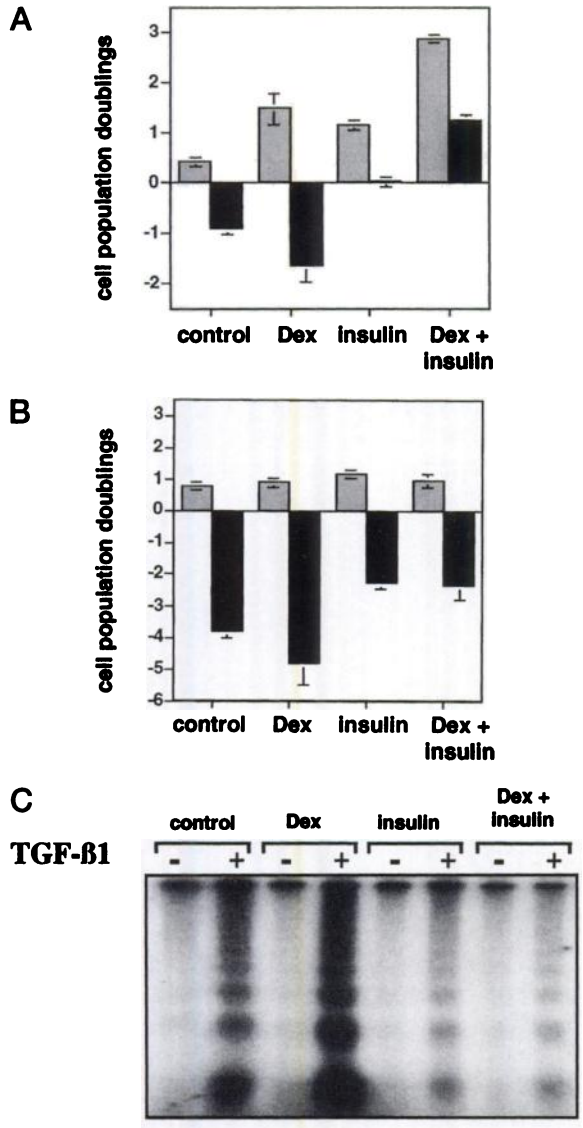

Fig. 3. Effects of Dex and insulin on apoptosis induced by TGF-β1 in NRP-152 and NRP-154 cells. Effects of 0.1 μM Dex and 5 μg/ml of insulin either alone (▨) or with 10 ng/ml of TGF-β1 (■) on cell population doublings of NRP-152 (*A*) and NRP-154 (*B*) cells after 4 days. Two × 10^4 cells/well/0.5 ml (in DMEM/F12 + 1% CS) were dispensed in 24-well dishes, factors were added 2 h later, and cell numbers were determined with a Coulter Counter. *Bars*, SD. In *C*, effects of Dex and insulin either alone or together on DNA laddering in NRP-152 cells induced by TGF-β1 were determined after 24 h of treatment (all factors were added 2 h after cell plating), as described in "Materials and Methods." All determinations (in *A* and *B*) are averaged from three cultures; *bars*, SD.

tive cell doubling implies cell death). Although Dex and insulin each stimulated growth of NRP-152 cells, Dex enhanced whereas insulin (even in the presence of Dex) blocked the ability of TGF-β1 to induce death (Fig. 3*A*). Microscopic observation revealed that insulin protected against cell death rather than simply reversing the cytostatic effects of TGF-β. Under identical conditions, the effects of these agents on NRP-154 cells were different. Although Dex enhanced the TGF-β-induced killing of NRP-154 cells, insulin did not completely block death by TGF-β1 (Fig. 3*B*). The effects of these treatments on the induction of a DNA ladder by TGF-β1 were examined after 24 h of treatment in both cell lines. As expected from the cell number assay, TGF-β1 induced a distinct ladder in NRP-152 cells that was enhanced by Dex and inhibited by insulin (even in the presence of Dex) (Fig. 3*C*). Similar analysis of NRP-154 cells showed that although insulin partially blocked apoptosis induced by TGF-β1 after 24 h, such protection was temporary (data not shown).

To address the question whether the apoptosis-blocking effect of these high levels of insulin may be mediated through the IGF-I receptor, we examined the effective concentrations of insulin and IGF-I required to block apoptosis of NRP-152 cells induced by TGF-β1 (Fig. 4*A*). The ability of TGF-β1 to induce a DNA ladder in NRP-152 cells was half-maximally blocked by either 25 nM insulin or 20 nM IGF-I. The relatively high concentrations of IGF-I required to block apoptosis suggested that this ligand may be inactivated by binding to IGF-I-binding proteins. This possibility was examined by use of a derivatized form of IGF-I (LR^3-IGF-I) that has low affinity for IGF-I binding proteins but binds the IGF-I receptor with equal affinity to native IGF-I. LR^3-IGF (ED_{50}, 50 pM) blocked apoptosis induced by TGF-β1 with about a 500-fold greater specific activity than IGF-I or insulin (Fig. 4*A*), indicating that the effects of insulin may be mediated, in part, through the IGF-I receptor. It is important to note that the above effects are not limited to the TGF-β1 isoform. TGF-β2 and TGF-β3 each induce apoptosis in a manner that is also blocked by LR^3-IGF, similar to that of TGF-β1 (Fig. 4*B*). Although IGF-I has been shown to be a general inhibitor of apoptosis (16, 17), its ability to block apoptosis in NRP-152 cells seems to be more specific for TGF-βs than other apoptosis inducers (tumor necrosis factor-α, staurosporine, okadaic acid, and Taxol) with the exception of genistein, because, under the conditions used here, IGF-I is unable to prevent the above factors from inducing a DNA ladder (Fig. 4*C*).

Discussion

We report here the first evidence that TGF-βs directly induce apoptosis of prostatic epithelial cells, using two unique dorsal-lateral prostatic cell lines. We further show significant effects of glucocorticoid, insulin, and IGF-I as modulators of apoptosis induced by TGF-β1. Our data suggest that TGF-βs play an important role in relaying decisions of whether cells should die, depending on external signals. Because of the demonstrated importance of apoptosis in the prostate (1–3), the role of TGF-β in regulating this process may be critical to normal prostatic growth and development as well as in the control of prostatic cancer.

Recent studies suggest that the IGF-I signaling system, which may play a critical role in normal prostatic growth and carcinogenesis (18, 19), is a major inhibitor of apoptosis (16, 17). Virtually all prostatic epithelial cells are growth stimulated by this mitogen, and prostatic stromal cells have been shown to produce IGF activity (18, 20). Our data that insulin and IGF-I can block apoptosis induced by TGF-β may be similar to that observed in hepatocyte cultures, where insulin and IGF-I are thought to protect against the ability of TGF-β to kill through activation of IRS-I (21). Although IGF-I is believed to be a general inhibitor of apoptosis (16, 17), we show that IGF-I selectively blocks apoptosis of prostatic epithelial cells induced by TGF-β and is less effective at blocking apoptosis induced by other agents. Moreover, recent evidence from our laboratory indicates that the antagonism by IGF-I of TGF-β effects in these cells is specific for the apoptotic process in that it has no effect on the ability of TGF-β to inhibit cell growth.[9] These data, combined with evidence that IGF-I also blocks certain TGF-β-inducible genes,[9] suggest that IGF-I acts specifically by intercepting steps in the TGF-β signal transduction pathway that relay apoptosis.

NRP-152, which is a unique cell line with properties of normal prostatic epithelium, is thus a useful model for studying the mechanism of apoptosis by TGF-β in the prostate. We have reported recently that these cells are capable of transdifferentiating from their normal basal phenotype toward a luminal phenotype when they are

[9] D. Danielpour and A. Y. Hsing, manuscript in preparation.

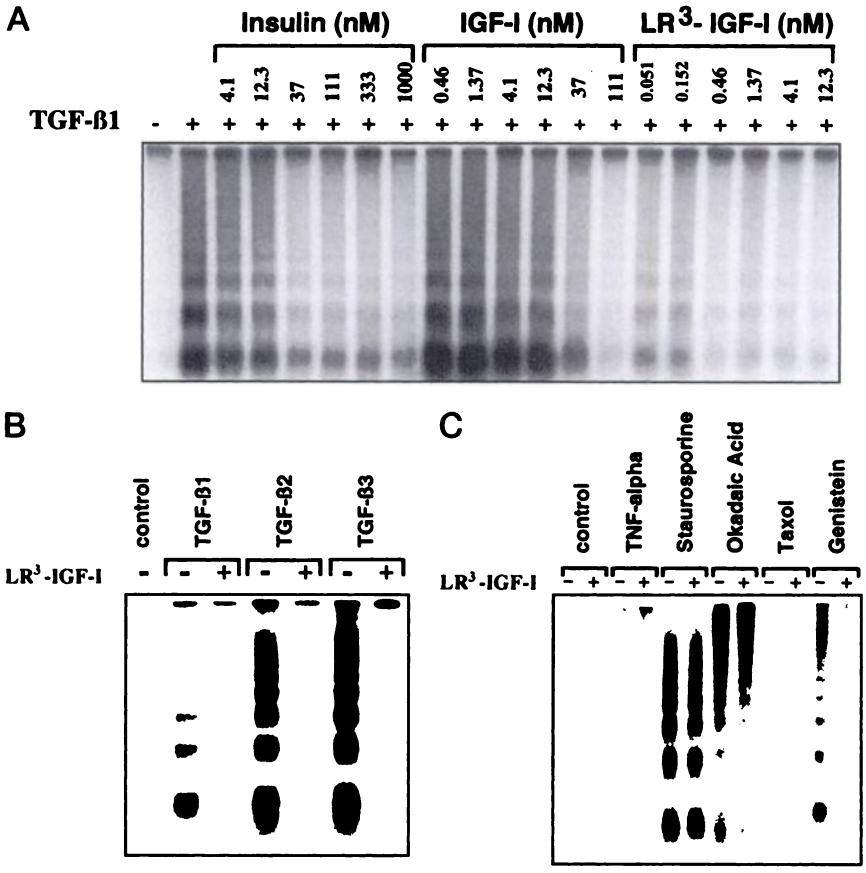

Fig. 4. Effectiveness of insulin, IGF-I, and LR^3-IGF on blocking of apoptosis induced by TGF-β1. In *A*, the ability of 10 ng/ml of TGF-β1 to induce DNA laddering in NRP-152 cells was blocked with various doses of insulin, IGF-I, and LR^3-IGF-I. In *B*, the abilities of TGF-βs 1, 2, and 3 (10 ng/ml each) to induce DNA ladders and the reversal of these ladders by 2 nM LR^3-IGF-I were examined on NRP-152 cells. In *C*, the ability of 2 nM LR^3-IGF-I to block apoptosis induced by 20 ng/ml of TNF-α, 2 nM staurosporine, 20 nM okadaic acid, 20 mM Taxol, and 20 μg/ml of genistein was examined by DNA laddering. The concentrations of the apoptosis inducers used in *C* were twice the minimal concentration required to give an optimal DNA ladder in these cells. Cells were plated in DMEM/F12, 15 mM HEPES, 1% CS, and 0.1 μM Dex. Insulin, IGF-I and LR^3-IGF-I were added about 1.5 h before the addition of the apoptotic inducers. DNA ladders were detected following 24 h treatment with the apoptotic inducers.

cultured in a growth factor-deficient medium (22). Our preliminary data indicate that production of TGF-β and sensitivity to TGF-β-induced apoptosis in these cells is tightly linked to their luminal differentiation. These results correlate directly with the greater sensitivity of prostatic luminal *versus* basal epithelial cells to castration-induced apoptosis and support the potential role of TGF-β as a direct mediator of such apoptosis. Also, the selective production of IGF activity by stroma may be another mechanism by which basal epithelial cells (20), which have greater proximity to stromal cells, resist apoptosis following androgen ablation.

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