Insulin-like Growth Factor (IGF)-binding Protein-3 Induces Apoptosis and Mediates the Effects of Transforming Growth Factor-β1 on Programmed Cell Death through a p53- and IGF-independent Mechanism*

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Insulin-like growth factor (IGF) binding protein-3 (IGFBP-3) is known to block IGF action and inhibit cell growth. IGFBP-3 is thought to act by sequestering free IGFs or, possibly, act via a novel IGF-independent mechanism. Supporting its role as a primary growth inhibitor, IGFBP-3 production has been shown to be increased by cell growth-inhibitory agents, such as transforming growth factor-β (TGF-β), and the tumor suppressor gene p53. In this paper, we demonstrate, for the first time, a novel function of IGFBP-3 as an apoptosis-inducing agent. In the p53 negative prostate cancer cell line, PC-3, the addition of IGFBP-3-neutralizing antibodies or IGFBP-3-specific receptor-negative mouse fibroblast cell line, treatment with recombinant IGFBP-3 as well as transfection of the IGFBP-3 gene induced apoptosis, suggesting that neither IGFs nor IGF receptors are required for this action. Furthermore, treatment with TGF-β1, a known apoptosis-inducing agent, resulted in the induction of IGFBP-3 expression 6–12 h before the onset of apoptosis. This effect of TGF-β1 was prevented by co-treatment with IGFBP-3-neutralizing antibodies or IGFBP-3-specific antisense thiolated oligonucleotides. These findings suggest that IGFBP-3 induces apoptosis through a novel pathway independent of either p53 or the IGF-IGF receptor-mediated cell survival pathway and that IGFBP-3 mediates TGF-β1 induced apoptosis in PC-3 cells.

The insulin-like growth factor (IGF)3-binding protein-3 (IGFBP-3) belongs to a family of high affinity IGFBPs, which bind to IGFs and modulate their actions. In addition to regulating the availability of free IGFs and, therefore, their mitogenic activity (1–3), IGFBPs also play an important role in directly regulating cell growth. These independent cell growth-regulatory effects of IGFBPs have been shown to be either growth-inducing (5–10), or growth-inhibiting (9, 12–17).

We and others have previously demonstrated the effects of IGFBP-3 as a negative regulator of cell proliferation in prostatic and other tissues (8–10, 12, 13). This negative growth regulation by IGFBP-3 has been proposed to involve a separate cellular signaling pathway (18, 19). Further, in support of its role as a negative regulator of cell growth and proliferation, IGFBP-3 gene expression has also been shown to be induced by other growth-inhibitory (and apoptosis-inducing) agents such as transforming growth factor-β1 (TGF-β1) (20–22), retinoic acid (21), tumor necrosis factor-α (TNF-α) (23), and the tumor suppressor gene, p53 (24). However, the direct apoptosis-inducing ability of IGFBP-3 has not previously been demonstrated. In this study, we have investigated a novel role of IGFBP-3 as an apoptosis-inducing agent.

We hypothesized that the growth-inhibitory effect of IGFBP-3 is mediated not only by regulating the availability of free IGFs and by inducing growth arrest, but also by inducing apoptosis. We further considered that this process may involve an IGF-independent mechanism. To test these hypotheses, we investigated the ability of IGFBP-3 to induce apoptosis in a prostate carcinoma cell line (PC-3) and in an IGF receptor-negative (R–/–) mouse fibroblast cell line (13, 19, 25). Furthermore, to determine the importance of IGFBP-3 as a critical cell growth-regulatory factor, we also investigated its role as a mediator of the apoptosis induced by TGF-β1.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture supplies were purchased from Flow Laboratories (McLean, VA), Corning (Corning, NY), and Hyclone (Logan, UT). Recombinant human IGF-I was the kind gift of Fujisawa Pharmaceuticals (Osaka, Japan). Recombinant human IGF-II was generously provided by Eli Lilly (Indianapolis, IN). Recombinant DNA-derived, glycosylated (Chinese hamster ovary) and nonglycosylated (Escherichia coli) human IGFBP-3 were the generous gifts of A. Sommer (Celix Inc., Santa Clara, CA). The IGFBP-3 phosphorothioate oligodeoxynucleotides used in these experiments (originally published by Oh et al.; Ref. 20) were prepared by Oligos Etc., Inc. (Guilford, CT). The IGFBP-3 antisense oligodeoxynucleotides were complementary to the 20 nucleotides that encode the N terminus of human IGFBP-3 as described previously (24) and had the sequence 5′-CAT GAC GCC TGC AAC CGG transferase-mediated dUTP nick end labeling; FACS, fluorescence-activated cell sorting; PBS, phosphate-buffered saline; SFM, serum-free FK-12 media; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis.

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GG-3' (positions 2021–2040); the sequence of the IGFBP-3 sense oligodeoxynucleotides was 5′-CCC CGG TTC CAG GCC GCA TG-3′. IGFBP-3 antibodies were purchased from Diagnostic Systems Laboratories (Webster, TX) and were prepared by affinity purification on an IGFBP-3 column. Control IgG (affinity-purified anti-goat IgG) was purchased from Sigma (St. Louis, MO). Fluorescein isothiocyanate (FITC)-conjugated annexin V, propidium iodide (PI), and binding buffer were purchased from R & D Systems (Minneapolis, MN). The interleukin-1β-converting enzyme (ICE) inhibitor (ICE-I) (Ac-Tyr-Val-Ala-Asp-aldehyde) was purchased from Oncogene Research Products (Cambridge, MA).

**Cell Cultures**—The human fibroblasts from an IGF-I receptor knockout prostatic adenocarcinoma from a 62-year-old male Caucasian. The PC-3 cells were purchased from ATCC (Rockville, MD) and were originally initiated from a grade IV metastatic prostate adenocarcinoma from a 62-year-old male Caucasian. The PC-3 cells were grown in 75-cm2 flasks according to the recommended protocol (FK-12 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin). For each experiment, cells were dissociated, centrifuged, and resuspended in serum-containing FK-12 medium with antibiotics and inoculated at a density of 1 × 105 cells/cm2 in 24-well or 6-well tissue culture dishes and grown to confluence in a humidified atmosphere of 5% CO2 at 37 °C before treatment. After a quick wash with serum-free FK-12 media (SFM), the confluent cells were treated with various concentrations of IGFBP-3, TGF-β1, and/or other specified reagents. SFM with antibiotics was used as the control treatment.

**R eagents**—A minimum of 6,000 cells was maintained for all samples.

**Apoptosis ELISA Assay**—Photometric cell death detection ELISA (Boehringer Mannheim) was performed to quantitate the apoptotic index by detecting the histone-associated DNA fragments (mono- and oligonucleosomes) generated by the apoptotic cells. The assay is based on the quantitative sandwich enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones, respectively, for the specific determination of these nucleosomes in the cytoplasmic fraction of cell lysates. In brief, equal numbers of cells were plated in 24-well culture plates (1 × 105/cm2) in serum-supplemented FK-12 medium and grown to confluency for 72 h. At the time of sample collection, the confluent cells were washed with PBS and treated with various concentrations of IGFBP-3, TGF-β1, or other required agents for the designated time period. The cells were dissociated gently (PBS with 0.1 M EDTA) and pelleted along with the floating cells (mostly apoptotic cells) collected from the conditioned media. The cell pellets were used to prepare the cytosol fractions that contained the smaller fragments of DNA. Equal volumes of these cytosolic fractions were incubated in anti-histone antibody-coated wells (96-well plates), and the histones of the DNA fragments were allowed to bind to the anti-histone antibodies. The polyclonal antibody-labeled monoclonal DNA antibody-tetramethylrhodamine isothiocyanate (TRITC) conjugated antibodies were used to localize and detect the bound fragmented DNA using photometric detection with 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) as the substrate. Valinomycin (1 × 105 M) was used as a positive control (27). SFM-treated conditions were used as negative controls. Each experimental condition was carried out with at least three samples and was repeated at least three times. The reaction protocols were read using a microplate reader (model 3550-UV). Averages of the values ± S.E. from double absorbance measurements of the samples were plotted.

**Preparation of Cell Lysates**—Confluent PC-3 cells were briefly washed with cold PBS and allowed to dissociate in dispersion buffer (1 mM EDTA in PBS, pH 7.4). Free floating cells were collected and centrifuged (2000 rpm for 5 min), resuspended in cold lysis buffer containing 10 mM HEPE, 1.5 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonfyl fluoride, 1 μM aprotinin, and 1 μM pepstatin in PBS (pH 7.4), vortexed, and boiled at 100 °C for 5 min. Aliquots were stored at −70 °C until further use.

**Membrane Preparation**—Samples were collected using the method described above for the preparation of cell lysates. The pellets were resuspended in homogenization buffer (10 mM HEPE, 1.5 mM EDTA, 0.5 mM dithiothreitol, and 1 mM phenylmethylsulfonfyl fluoride, pH 7.4). Cells were homogenized using a Dounce homogenizer, maintaining the uniformity of the homogenization process. Homogenized cells were centrifuged at 3000 rpm for 4 °C for 5 min, and the nuclear pellet was discarded. The supernatant was recentlyrifuged at 12,000 rpm at 4 °C for 30 min, and the pellet containing the debris was discarded. The supernatant containing the plasma membrane was washed in subcellularization buffer (50 mM HEPE, 0.15 mM NaCl, 2 mM MgSO4, 1 mM phenylmethylsulfonfyl fluoride, pH 7.4) and further homogenized using the same technique as mentioned above. Following centrifugation at 33,000 rpm at 4 °C for 1 h using a Sorvall ultracentrifuge (DuPont, OTD B60), the resulting pellet was resuspended in isolation buffer and used for separation on SDS-PAGE.
Western Immunoblots—The Western immunoblot analysis was performed as described previously (31). Serum-free conditioned media from PC-3 cultures treated with various concentrations of TGF-β1 for different time periods were used. SFM incubated with similar culture conditions were used as controls. Samples of 100 μl (from 1 × 10^6 cells) were electrophoresed through 12.5% nonreducing SDS-PAGE overnight at constant voltage, electroblotted onto nitrocellulose, blocked with 5% nonfat dry milk in Tris-buffered saline, probed with specific IGFBP-3 antibodies, and detected using a peroxidase-linked enhanced chemiluminescence detection system (Pierce).

Western Ligand Blot with 125I-IGFBP-3 (Reverse Western Ligand Blot)—PC-3 cell lysates and plasma membrane fractions were electrophoresed through 12.5% nonreducing SDS-PAGE overnight at constant voltage and electroblotted onto nitrocellulose, blocked with 1% bovine serum albumin in Tris-buffered saline, and incubated with 5 × 10^4 cpm of ^125I-IGFBP-3 (DSL, Webster, TX) for 12 h. The membranes were exposed to film for 3 days and visualized by autoradiography.

Densitometric and Statistical Analysis—Densitometric measurement of immunoblots were performed using a Bio-Rad GS-670 Imaging densitometer (Bio-Rad, Melville, NY). Protein levels were estimated by comparing the optical density of each specific protein band from control (SFM) conditions with that from the TGF-β1-treated conditions. All experiments were repeated at least three times. When applicable, means ± S.E. are shown. Student’s t tests were used for statistical analysis.

RESULTS

Induction of Apoptosis in PC-3 Cells by IGFBP-3—We detected IGFBP-3-induced apoptosis in PC-3 cells using both

FIG. 1. Detection and quantification of IGFBP-3-induced apoptosis in PC-3 cells. A, DNA laddering. DNA was extracted from cells treated with IGFBP-3 (500 ng/ml for 72 h; lane 3) and SFM (lane 2), electrophoresed in 1.5% agarose gels, and visualized using ethidium bromide. B, TUNEL. The cytoplasmic DNA fragments were detected in situ in the SFM- (i) and IGFBP-3-treated (ii) PC-3 cells after incubation with biotinylated dNTP mix and terminal deoxynucleotidyltransferase. The free 3’-OH DNA fragments were visualized using a streptavidin-horseradish peroxidase detection system with positive cells appearing dark brown. C, FACS analysis for fragmented DNA. The apoptotic indexes of control and IGFBP-3-treated conditions were compared and quantitated using a flow cytometric analysis. Samples were incubated in a labeling mixture containing digoxigenin-labeled d-UTP, terminal deoxynucleotidyltransferase, and fluorescein-labeled anti-digoxigenin. Fluorescence histograms were evaluated to determine both the percentage positive cells and the relative fluorescence intensity in mean channel fluorescence units. The numbers of apoptotic cells in treated and untreated cells were compared, and the percentages of apoptotic cells in both untreated and treated cells are presented. D, FACS analysis for annexin/PI. Both the apoptotic index and the necrotic index were estimated in a given sample by dual labeling with FITC-labeled annexin and PI. The quadrant statistics show the percentage value of the necrotic cells stained only for PI in quadrant 1 (1), necrotic cells stained for both annexin and PI in quadrant 2 (2), healthy cells that are negative for both annexin and PI in quadrant 3 (3), and apoptotic cells stained only for annexin in quadrant 4 (4). E, the percentages of apoptotic and necrotic cells detected using FACS analysis for annexin/PI are represented in a bar graph. Results are the mean value ± S.E. of three experiments. Cells were treated with IGFBP-3 (500 ng/ml) for 72 h (*, p < 0.001 compared with SFM).
qualitative (DNA laddering and TUNEL) and quantitative (FACS and ELISA) methods. The apoptotic DNA cleavage in cells treated with IGFBP-3 (500 ng/ml for 72 h) was visualized as DNA laddering (Fig. 1A). The DNA extracted from cells treated with SFM showed limited fragmentation (lane 4). However, DNA extracted from IGFBP-3-treated cells demonstrated significant fragmentation, with bands varying in size primarily from 100 to 300 base pairs (lane 3). Cells treated with 10% fetal bovine serum were used as a negative control to demonstrate the absence of any fragmented DNA (lane 2).

As an alternative method of detection, and to localize the apoptotic cells in situ, we detected the fragmented DNA in monolayer cell cultures treated with SFM or IGFBP-3 using TUNEL (Fig. 1B). The DNA fragments bound to the peroxidase-diaminobenzidine reaction product in apoptotic cells were visualized as dark brown cells. Cells in SFM displayed an insignificant number of apoptotic cells (Fig. 1B, i); however, IGFBP-3 treatment revealed numerous apoptotic cells (Fig. 1B, ii). This method was not used to quantitate the number of apoptotic cells in the SFM- and IGFBP-3-treated conditions, since many of the apoptotic cells, after 72 h of incubation, were found floating in the conditioned media. Loss of cells from the culture plate due to the increased apoptotic index was seen as empty spaces in the IGFBP-3-treated condition. However, the control condition showed confluent cells.

FACS analysis of fragmented DNA antibody staining (Fig. 1C) further demonstrated and quantitated the apoptotic index in SFM- and IGFBP-3-treated conditions. The basal apoptotic index due to serum withdrawal was 1.4% of the total number of cells analyzed. The addition of IGFBP-3 to SFM resulted in a significant increase in the apoptotic index (94.7% of the total) as measured by fragmented DNA antibody staining. Using related FACS approaches (Fig. 1D) but labeling the unpermeabilized samples with both FITC-conjugated annexin (which stains only apoptotic cells) and PI (which stains necrotic cells with disrupted membranes), the number of apoptotic cells un-
nder the treated condition and the ratio of apoptotic cells to necrotic cells were calculated. Treatment with IGFBP-3 resulted in a 3-fold increase in apoptotic cells (Fig. 1E) when compared with SFM treatment. The percentage of necrotic cells was low in both treatment conditions and was not increased by IGFBP-3.

Effects of IGFs on IGFBP-3-induced Apoptosis—Similar to the observations obtained using FACS, quantitative analysis with photometric ELISA (Fig. 2) revealed a basal level of apoptosis in SFM-treated cells. In contrast, the serum-treated cultures were completely devoid of apoptotic cells (Fig. 2A). In addition, the addition of IGF-I (200 ng/ml) also prevented the effect of serum starvation on apoptosis. On the other hand, the addition of IGFBP-3 to the SFM induced a further significant increase (p < 0.001 compared with SFM) in the apoptotic index above the basal level caused by serum deprivation (Fig. 2A). This induction of apoptosis by IGFBP-3 was as potent as the apoptosis induced by the ionophore valinomycin, which has previously been demonstrated to be a potent apoptosis-inducing agent (27). A dose response study revealed that IGFBP-3 induced apoptosis at concentrations as low as 50 ng/ml and demonstrated a dose response up to 500 ng/ml (Fig. 2B). This effect was only partially inhibited by exogenous IGF (Fig. 2C) (p < 0.05 compared with IGFBP-3 treatment) and was not inhibited by the IGF analogue (long R3-IGF-1) that does not bind to IGFBPs (Fig. 2C), suggestive of an IGF-independent mechanism for this IGFBP-3 effect. All of these methods confirmed that treatment with IGFBP-3 for 72 h resulted in a significant increase in the apoptotic index in PC-3 cells.

Activation of the ICE Pathway by IGFBP-3—Analyses using the apoptosis ELISA demonstrated that IGFBP-3-induced apoptosis was inhibited by the reversible ICE-I (Ac-Tyr-Val-Ala-Asp-aldehyde) (Fig. 3A). The ICE-I completely suppressed IGFBP-3-induced apoptosis in a dose-dependent fashion at concentrations ranging from 0.4 to 5 μmol/liter (Fig. 3B). These concentrations are known to inhibit ICE and ICE-like proteases and to block apoptosis induced by a variety of stimuli in other cell types (32–34). These data demonstrate the involvement of ICE or ICE-like proteases in the IGFBP-3-induced pathway.

Demonstration of IGFBP-3 Association Proteins/Receptors in PC-3 Cells—Detection of IGFBP-3-binding molecules using reverse Western ligand blots revealed a number of bands varying in size from 18 to 150 kDa that represent proteins with high affinity to 125I-IGFBP-3 (Fig. 4). These molecules were detected both in whole cell lysates (lane 1) and in the purified plasma membrane fraction (lane 2). However, the 150-, 68-, and 18-kDa bands were strongly enriched in the membrane fraction, while some bands (44 and 35 kDa) were seen more prominently in the cell lysates, suggesting a cytoplasmic or nuclear origin. The selective localization of some of these molecules in the membrane fraction suggests the possibility of these proteins serving as IGFBP-3 cell surface receptors that may mediate IGFBP-3 action.

IGF-independent Effects of IGFBP-3—The possibility that IGFBP-3 acts to induce apoptosis independently of IGFs and IGF receptors was investigated by testing the ability of IGFBP-3 to induce apoptosis in the IGF receptor-negative (R−) fibroblast cells derived from an IGF-1R knockout mouse (25). These cells have been shown previously to neither bind nor respond to IGFs. To test the effect of IGFBP-3 on these cells, we used both treatment with exogenous IGFBP-3 protein and transfection with the IGFBP-3 gene (Fig. 5). The R− cell line demonstrated a basal level of apoptosis when cultured in 30% serum. The DNA extracted from R− cells and R− cells transfected with IGFBP-3 (R−/BP-3) grown in 30% serum for 72 h (Fig. 5A) reveals that the DNA fragmentation was far more prevalent in R−/BP-3 (Fig. 5A, lane 3). This observation was also quantitated using photometric ELISA (Fig. 5B). The transfection of the IGFBP-3 gene resulted in a substantial increase in the degree of apoptosis (p < 0.001). The addition of exogenous IGFBP-3 (500 ng/ml) also significantly increased the

![Fig. 4. Detection of IGFBP-3 association molecules.](image)

![Fig. 5. Induction of apoptosis by IGFBP-3 in an IGF receptor negative (R−) cells.](image)
IGFBP-3 Induces Apoptosis

Figure 6. Detection and quantification of TGF-β1-induced apoptosis in PC-3 cells. A, a comparative quantitative analysis of the apoptotic index induced by the SFM, IGF-1, ionophore, IGFBP-3, and TGF-β1 using photometric ELISA. Results are presented as the absorbance (A490-A690). Values are the average ± S.E. for triplicate experiments (*, p < 0.001 relative to SFM). B, TUNEL. The cytoplasmatic DNA fragments were detected in situ in the SFM (i) (serum-free) and TGF-β1-treated (ii) monolayer cultures.

DISCUSSION

The role of IGFBP-3 as a growth-inhibitory protein has been previously demonstrated by us and others in various cell types (9, 12–17). Initially, IGFBP-3 was thought to inhibit growth by binding to IGFs and sequestering them from their receptor. Later, the cell growth-inhibitory effect of IGFBP-3 was suggested to also be IGF-independent and to involve cell growth arrest (14, 15). Recently, this inhibitory effect of IGFBP-3 was suggested to be mediated by interaction with a putative IGFBP-3 receptor. Although the IGF-independent growth-inhibitory role of IGFBP-3 has been recently investigated, an apoptosis-inducing role for IGFBP-3 has not been previously determined. This is the first demonstration of IGFBP-3 as a cell death-promoting agent.

Partial blocking of IGFBP-3-induced apoptosis by IGF suggests two possibilities. First, the presence of IGF may prevent the cells from undergoing apoptotic changes through the IGF receptor-mediated cell survival pathway. Second, some of the IGFBP-3 would not be available to induce apoptosis through its own receptors, since it formed IGF-IGFBP-3 complexes. Furthermore, the inability of IGF to fully block IGFBP-3-induced apoptosis even at a 5-fold higher molar concentrations suggests the notion that the pathway of IGFBP-3-induced apoptosis may not always involve IGF and IGF receptor. In addition, since IGF analogues that do not bind IGFBP-3 did not reverse the IGFBP-3 effect at all, this further suggests that IGFBP-3 induces apoptosis via an IGF-independent pathway through an
IGFBP-3 receptor. This IGFBP-3 cell surface receptor has been first proposed in Hs578T breast cancer cells by affinity cross-linking of 125I-IGFBP-3 to cell membrane and cell lysate extracts (18). In this study, we have shown that PC-3 cells also bind IGFBP-3 and that several potential IGFBP-3 receptors exist in PC-3 cells.

IGFs have been shown to protect cells from undergoing apoptosis through an IGF receptor-mediated cell survival pathway (35–38). Both the effects of decreases in the number of IGF receptors causing massive apoptosis and the overexpression of IGF receptors protecting cells from apoptosis have been demonstrated in vivo (35). The roles of IGFs and the IGF receptors as autocrine survival factors (36) and as protective agents that prevent apoptosis induced by other agents such as etoposide have been shown extensively (37). Mutant versions of p53 protein, commonly associated with malignant states, have been shown to derepress the IGF receptor promoter, with ensuing mitogenic activation by locally produced or circulating IGFs (38). All of the above mentioned studies indicate the important role of IGFs and IGF receptors in preventing cells from undergoing apoptosis through a cell survival pathway. We demonstrated here an alternate pathway for the induction of apoptosis that is independent of these apoptosis-protecting agents. By demonstrating IGFBP-3-induced apoptosis in the IGF receptor-negative (R−) murine fibroblast cell line, we proposed our hypothesis that IGFBP-3 may induce apoptosis independently of the IGF receptor-mediated survival pathway. Therefore, the ratio of free IGFs and IGFBP-3 will regulate cell growth not only by balancing the rate of cell proliferation and cell growth arrest, but also by regulating the rate at which the cells might be induced to undergo apoptosis.

The apoptosis-inducing effect of IGFBP-3 in R− cells provides ample evidence to suggest that similar IGF receptor-independent pathways are present in PC-3 cells and possibly in other cell lines. Treatment with IGF-I partially decreased the incidence of apoptosis in IGFBP-3-overexpressing cells but did not have any effect on R− cells, suggesting that the partial suppression of apoptosis by IGF is through the formation of IGF-IGFBP-3 complexes. Similar to the results found in PC-3 cells, IGFBP-3-neutralizing antibodies partially decreased the degree of apoptosis in IGFBP-3-overexpressing R− cells. In PC-3 cells, IGF-I partially blocked IGFBP-3-induced apoptosis, but the IGF analogue, which binds to the IGF receptor and not to IGFBP-3, was unable to block IGFBP-3-induced apoptosis. These observations not only suggest the involvement of an IGF-independent pathway, but they also demonstrate that IGFBP-3 must be free of IGF to be able to bind to its receptor and initiate its effect on cell death and that the activation of the IGF receptor does not protect cells from IGFBP-3-induced apoptosis.

The expression of the cell growth-inhibitory IGFBP-3 has been shown to be induced by various apoptosis-inducing agents, such as TGF-β1 (20–22), retinoids (21), TNF-α (23), and the tumor suppressor gene p53 (24). IGFBP-3 has been previously shown to mediate the growth-inhibitory effect of both retinoic acid and TGF-β1 (20, 21). However, the mechanism by which the IGFBP-3 reduces the cell number, under these conditions, is not known. In this work, we have demonstrated that IGFBP-3 mediates the growth-inhibitory effect of TGF-β1 by inducing apoptosis. This may apply to other agents that have not yet been investigated.

The PC-3 cells are p53-negative (39) and have the machinery to express low levels of IGFBP-3 (8) under serum-free conditions. TGF-β1 is a potent growth inhibitor of epithelial cells and has been shown to induce apoptosis and down-regulate Bel-2 expression (40, 41). The dramatic elevation of the 44-kDa IGFBP-3 protein within 12 h of TGF-β1 treatment and the significant effect of TGF-β1 on apoptosis that was observed about 18–24 h after treatment suggest that the TGF-β1-induced elevation of IGFBP-3 protein in the conditioned media is the primary signal that activated apoptosis in this cell line. Blocking TGF-β1-induced apoptosis at the IGFBP-3 transcriptional level confirmed the role of IGFBP-3 as the mediator of TGF-β1-induced apoptosis in PC-3 cells. Co-treatment with IGFBP-3 antisense (but not sense) thiolated oligonucleotide and TGF-β1-111 verified the role of IGFBP-3 in the TGF-β1-induced apoptosis. Furthermore, neutralization of IGFBP-3 action in TGF-β1-treated cells with IGFBP-3-neutralizing antibodies (but not control IgG) confirmed that IGFBP-3 must be secreted and allowed to bind to its receptor to initiate apoptosis. The latter observation also confirms that the TGF-β1-mediated increase in IGFBP-3 transcription must pass through steps such as IGFBP-3 secretion and the binding of this protein to its receptor to initiate apoptosis.

Inappropriate expression of genes involved in cell proliferation has been shown to alter regulation of apoptosis. Both Bel-2, which promotes cell survival, and Bax, which promotes cell death, have been implicated as major mediators in the control of apoptotic pathways, and it has been suggested that
the ratio of Bcl-2 to Bax controls the relative susceptibility of cells to death stimuli. TGF-β1, retinoic acid, TNF-α, and p53 are known to induce apoptosis by regulating Bcl-2 and Bax expression (40–47). Since all of these apoptosis-inducing agents also induce IGFBP-3 expression, we anticipate that IGFBP-3-induced apoptosis may also involve regulation of the Bcl-2:Bax ratio. In addition, the expression of ICE or ICE-like proteases that are final mediators of the apoptosis pathway is involved in the mechanism of action of IGFBP-3 as well as the above agents.

The role of IGFBP-3 in mediating p53 effects was proposed when p53 was demonstrated to activate the IGFBP-3 promoter (24). Recently, it has been shown that mutants of p53 that have lost the ability to activate IGFBP-3 and Bax expression but maintained their activation of the cyclin-dependent kinase inhibitor p21 are able to induce cell cycle arrest but are unable to induce apoptosis (48). Furthermore, a p53 mutant that activates Bax expression but only partially activates the IGFBP-3 promoter is only partially effective in inducing apoptosis (49).

Thus, a p53-dependent role of IGFBP-3 has been previously demonstrated. By demonstrating IGFBP-3-induced apoptosis in PC-3 cells that lack the p53 gene, we have demonstrated that IGFBP-3 can also induce apoptosis in a p53-independent fashion.

We present a hypothesis based on the results from this study and other previous reports from this and other groups in the diagrammatic representation shown in Fig. 9. We propose that the independent and interdependent effects of IGFs and IGFBPs on the regulation of cell number involve two pathways that interact at several levels. IGFs mediate survival via the IGF receptor. IGFBP-3 is able to block this pathway by sequestering IGFs away from the IGF receptor. IGFBP-3 mediates apoptosis via its own receptors, while IGFs can prevent this effect by binding to IGFBP-3. Thus, IGFBP-3 can mediate cell death by both IGF-dependent and IGF-independent pathways.

Both normal cell growth (50) and various pathologies associated with neoplastic cell proliferation, such as breast cancer (20, 26, 51, 52), prostate cancer, and benign prostatic hyperplasia (11), are also associated with altered expression of IGFs and IGFBPs. Earlier observations, however, did not directly demonstrate a role for IGFBP-3 in inducing apoptosis but provided ample evidence to suggest that IGFBP-3 is important in regulating cell number in such situations. Our data demonstrate that IGFBP-3 induces apoptosis at physiological concentrations and that IGFBP-3 may act through an IGF-IGF receptor-independent pathway. IGFBP-3 mediates the induction of apoptosis by TGF-β1 and may mediate similar actions of other growth-regulatory factors.

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