Insulin-Like Growth Factor-Binding Protein 3 Induces Caspase-Dependent Apoptosis through a Death Receptor-Mediated Pathway in MCF-7 Human Breast Cancer Cells

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ABSTRACT

Insulin-like growth factor-binding protein (IGFBP)-3 has been shown to potently inhibit cell proliferation in various cell systems. However, the specific mechanisms involved in the antiproliferative action of IGFBP-3 have yet to be elucidated. In the present study, we demonstrate that IGFBP-3 induces apoptosis in an insulin-like growth factor (IGF)-independent manner through the activation of caspases involved in a death receptor-mediated pathway in MCF-7 human breast cancer cells. Induction of IGFBP-3 using an ecdysone-inducible expression system inhibited DNA synthesis in an IGF-IGF receptor axis-independent fashion and resulted in the subsequent induction of apoptosis and an increase in caspase activity. Similar results were obtained when cells were transfected with GGG-IGFBP-3, an IGFBP-3 mutant unable to bind IGFs, corroborating the IGF-independent action of IGFBP-3. Additional caspase activity studies and immunoblot analyses using specific caspase substrates and/or caspase inhibitors revealed that the growth-inhibitory effect of IGFBP-3 results mainly from its induction of apoptosis (in particular, activation of caspase-8 and -7). Analyses of caspase-9 activity and release of cytochrome c into the cytosol confirmed that the mitochondria-mediated pathway is not involved. Taken together, these results show that IGFBP-3 expression leads to the induction of apoptosis through the activation of caspases involved in a death receptor-mediated pathway and that IGFBP-3 functions as a negative regulator of breast cancer cell growth, independent of the IGF-IGF receptor axis.

INTRODUCTION

The insulin-like growth factor-binding proteins (IGFBPs) are components of the insulin-like growth factor (IGF) axis, and their superfamily comprises six high-affinity species (IGFBPs 1–6) and several low-affinity binders [IGFBP-related proteins (1–5)]. The classical role of the IGFBPs involves IGF binding and modulation of IGF signaling; however, recent data suggest that some IGFBPs may play a more active, IGF-independent role in growth regulation of various cell systems (6–19). In particular, IGFBP-3 has been shown to potently inhibit the proliferation of various cell types in an IGF-independent manner. The concept of IGF-independent actions for IGFBP-3 has been supported by the demonstration that (a) exogenous IGFBP-3 binds to specific proteins on the cell surface, and this interaction is strongly correlated with the ability of IGFBP-3 to inhibit cell growth (8, 9); (b) overexpression of human IGFBP-3 cDNA inhibits cell proliferation (10–12); (c) IGFBP-3 mediates transforming growth factor β (13), retinoic acid (14), antiestrogen (15), vitamin D analog (16), and tumor necrosis factor (TNF)-α (17)-induced growth inhibition; (d) regulation of IGFBP-3 gene expression plays a role in signaling by p53, a potent tumor suppressor protein (18); and (e) IGFBP-3 fragments inhibit the stimulation of DNA synthesis induced by either IGF-I or insulin (19). Recently, several reports have demonstrated that IGFBP-3 induces apoptosis in PC-3 prostate cancer (20) and MCF-7 breast cancer cells (21) and enhances ceramide-induced apoptosis in Hs578T breast cancer cells (22). These apoptosis-inducing effects of IGFBP-3 may be due to sequestering of IGFs, thus blocking their antipapoptotic activities (21). Alternatively, IGFBP-3 may act via IGF-independent pathways because IGFBP-3 can induce apoptosis in an IGF receptor-negative mouse fibroblast cell line (20) and can accelerate apoptosis under conditions where IGF-I does not elicit a survival effect (22). More direct evidence comes from the demonstration that IGFBP-3 mutants that do not bind IGFs stimulate apoptosis in human prostate cancer cells (23). Additional studies by Butt et al. (24) have demonstrated that IGFBP-3 can modulate the expression of the Bax:Bcl-2 protein ratio in apoptotic human breast cancer cells in a p53-independent manner. In addition, caspase-3 activity, as well as IGFBP-3 levels, is increased in Hs578T breast cancer cells undergoing apoptosis in response to the chemotherapeutic agent paclitaxel (25). Despite clear evidence of IGFBP-3’s activation of apoptosis in an IGF-independent fashion, the mechanisms by which these effects are achieved have yet to be elucidated.

Programmed cell death plays a crucial role in normal development, defense of homeostasis, and pathological situations (26–28). Apoptotic signals are generally believed to be mediated through a caspase system controlled by two autonomous and interacting pathways that are associated with either cell surface death receptors or mitochondria. Apoptotic stimuli such as DNA-damaging agents trigger the release of mitochondrial cytochrome c into the cytosol, which controls the assembly of an apoptosome composed of Apaf-1 (apoptotic protease-activating factor 1), Bcl-XL, and procaspase-9 (29). Binding of cytochrome c and dATP to Apaf-1 results in the activation of caspase-9 and the subsequent activation of downstream caspases such as caspase-3, which kill cells by cleaving key intracellular proteins, whereas Bcl-XL presumably inhibits this function. In contrast, binding of death ligands to their death receptors leads to the recruitment of a variety of death domain-bearing adapter proteins, which in turn recruit the most upstream caspases, such as caspase-8 and -10, an event that results in the activation of these caspases and downstream effector caspases. In addition, these activated upstream caspases may act on the mitochondria to facilitate cytochrome c release, thereby further amplifying the apoptotic signal. Here we show that IGFBP-3 inhibits cell growth in an IGF-independent manner and that its growth-inhibitory effect results from the caspase-dependent induction of apoptosis in MCF-7 human breast cancer cells. We also show that IGFBP-3 facilitates the induction of biochemical events such as the activation of the initiator caspase-8 and the processing of caspase-7 but minimal release of cytochrome c from mitochondria and processing of caspase-9, suggesting that IGFBP-3-induced apoptosis may be accomplished through the activation of caspases involved in a death receptor-mediated pathway in MCF-7 human breast cancer cells.

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Reagents and Kits. Tissue culture reagents and plastics were purchased from Mediatech (Hemdon, VA), Becton Dickinson (Franklin Lakes, NJ), and Nunc (Naperville, IL). Special reagents and kits were obtained from the following sources: Zeocin, Genetecin sulfate, and ponasterone A (Invitrogen, Carlsbad, CA); IGBP-3 radioimmunoassay kit (Diagnostic Systems Laboratories, Webster, TX); CellTiter 96 cell proliferation assay (Promega, Madison, WI); TNF-α (Calbiochem, San Diego, CA); IGBP-3 (Austral Biologicals, San Ramon, CA); annexin V apoptosis kit (Clontech, Palo Alto, CA); cell death detection ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN); aminomethylcoumarin (AMC) (caspase substrates) and cell-permeable aldehyde derivatives (caspase inhibitors) of tetrapeptide sequences that are recognized by distinct caspases (Ac–DEVD–AMC and Ac–LEHD–AMC (caspase-9), Ac–IETD–AMC (caspase-8), and Z–VAD–fluoromethylketone (FMK)) (broader-spectrum caspase inhibitor), BIOMOL Research Laboratories (Plymouth Meeting, PA); Z–IETD–FMK (caspase-8 inhibitor) and Z–DEV–D–FMK (caspase-3/-7 inhibitor), BD Pharmingen (San Diego, CA; Refs. 30–33). Antibodies against polyclonal (APD-ribone) polymerase (PARP), cleaved PARP, caspase-9, cleaved caspase-9, caspase-7, and caspase-10 were purchased from Cell Signaling Technology (Beverly, MA); antibody against caspase-8 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); and antibodies against cytochrome c, Bid, and caspase-2 were purchased from BD Pharmingen. IGF-I, IGF-I-labeled IGF-I and -II, and a monoclonal antibody against IGBP-3 were generously provided by Diagnostic Systems Laboratories. The ecysone-inducible expression system was purchased from Invitrogen.

Cells. The MCF-7 human breast cancer cell line and its derivatives expressing an IGBP-3 in an inducible manner were maintained in DMEM supplemented with 4.5 g/liter glucose, 110 mg/liter sodium pyruvate expressing an IGFBP-3 in an inducible manner were maintained in DMEM supplemented with 4.5 g/liter glucose, 110 mg/liter sodium pyruvate, and 15 μM ponasterone A (Invitrogen). Cell Cultures. The MCF-7 human breast cancer cell line and its derivatives expressing an IGBP-3 in an inducible manner were maintained in DMEM supplemented with 4.5 g/liter glucose, 110 mg/liter sodium pyruvate, and 15 μM ponasterone A (Invitrogen). DMEM containing 10% FBS in the presence or absence of 15 μM ponasterone A for 72 h. Cells were grown in 6-well plates until 60–70% confluent, when the media were replaced with DMEM containing 10% FBS in the presence or absence of 15 μM ponasterone A for 72 h. Cells were grown in 6-well plates until 60–70% confluent, when the media were replaced with DMEM containing 10% FBS in the presence or absence of 15 μM ponasterone A for 72 h. Cells were grown in 6-well plates until 60–70% confluent, when the media were replaced with DMEM containing 10% FBS in the presence or absence of 15 μM ponasterone A for 72 h. Cells were grown in 6-well plates until 60–70% confluent, when the media were replaced with DMEM containing 10% FBS in the presence or absence of 15 μM ponasterone A for 72 h. Cells were grown in 6-well plates until 60–70% confluent, when the media were replaced with DMEM containing 10% FBS in the presence or absence of 15 μM ponasterone A for 72 h. Cells were grown in 6-well plates until 60–70% confluent, when the media were replaced with DMEM containing 10% FBS in the presence or absence of 15 μM ponasterone A for 72 h. Cells were grown in 6-well plates until 60–70% confluent, when the media were replaced with DMEM containing 10% FBS in the presence or absence of 15 μM ponasterone A for 72 h. Cells were grown in 6-well plates until 60–70% confluent, when the media were replaced with DMEM containing 10% FBS in the presence or absence of 15 μM ponasterone A for 72 h. Cells were grown in 6-well plates until 60–70% confluent, when the media were replaced with DMEM containing 10% FBS in the presence or absence of 15 μM ponasterone A for 72 h. Cells were grown in 6-well plates until 60–70% confluent, when the media were replaced with DMEM containing 10% FBS in the presence or absence of 15 μM ponasterone A for 72 h. Cells were grown in 6-well plates until 60–70% confluent, when the media were replaced with DMEM containing 10% FBS in the presence or absence of 15 μM ponasterone A for 72 h. Cells were grown in 6-well plates until 60–70% confluent, when the media were replaced with DMEM containing 10% FBS in the presence or absence of 15 μM ponasterone A for 72 h. Cells were grown in 6-well plates until 60–70% confluent, when the media were replaced with DMEM containing 10% FBS in the presence or absence of 15 μM ponasterone A for 72 h. Cells were grown in 6-well plates until 60–70% confluent, when the media were replaced with DMEM containing 10% FBS in the presence or absence of 15 μM ponasterone A for 72 h. Cells were grown in 6-well plates until 60–70% confluent, when the media were replaced with DMEM containing 10% FBS in the presence or absence of 15 μM ponasterone A for 72 h. Cells were grown in 6-well plates until 60–70% confluent, when the media were replaced with DMEM containing 10% FBS in the presence or absence of 15 μM ponasterone A for 72 h. Cells were grown in 6-well plates until 60–70% confluent, when the media were replaced with DMEM containing 10% FBS in the presence or absence of 15 μM ponasterone A for 72 h. Cells were grown in 6-well plates until 60–70% confluent, when the media were replaced with DMEM containing 10% FBS in the presence or absence of 15 μM ponasterone A for 72 h. Cells were grown in 6-well plates until 60–70% confluent, when the media were replaced with DMEM containing 10% FBS in the presence or absence of 15 μM ponasterone A for 72 h.

Reagents and Kits. Tissue culture reagents and plastics were purchased from Mediatech (Hemdon, VA), Becton Dickinson (Franklin Lakes, NJ), and Nunc (Naperville, IL). Special reagents and kits were obtained from the following sources: Zeocin, Genetecin sulfate, and ponasterone A (Invitrogen, Carlsbad, CA); IGBP-3 radioimmunoassay kit (Diagnostic Systems Laboratories, Webster, TX); CellTiter 96 cell proliferation assay (Promega, Madison, WI); TNF-α (Calbiochem, San Diego, CA); IGBP-3 (Austral Biologicals, San Ramon, CA); annexin V apoptosis kit (Clontech, Palo Alto, CA); cell death detection ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN); aminomethylcoumarin (AMC) (caspase substrates) and cell-permeable aldehyde derivatives (caspase inhibitors) of tetrapeptide sequences that are recognized by distinct caspases (Ac–DEVD–AMC and Ac–LEHD–AMC (caspase-9), Ac–IETD–AMC (caspase-8), and Z–VAD–fluoromethylketone (FMK)) (broader-spectrum caspase inhibitor), BIOMOL Research Laboratories (Plymouth Meeting, PA); Z–IETD–FMK (caspase-8 inhibitor) and Z–DEV–D–FMK (caspase-3/-7 inhibitor), BD Pharmingen (San Diego, CA; Refs. 30–33). Antibodies against polyclonal (APD-ribone) polymerase (PARP), cleaved PARP, caspase-9, cleaved caspase-9, caspase-7, and caspase-10 were purchased from Cell Signaling Technology (Beverly, MA); antibody against caspase-8 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); and antibodies against cytochrome c, Bid, and caspase-2 were purchased from BD Pharmingen. IGF-I, IGF-I-labeled IGF-I and -II, and a monoclonal antibody against IGBP-3 were generously provided by Diagnostic Systems Laboratories. The ecysone-inducible expression system was purchased from Invitrogen.
Preparation of Cytosolic and Mitochondrial Extracts. For the detection of cytochrome c, cytosolic and mitochondrial extracts were prepared as described previously (37). In brief, cells (2 × 10^6) were harvested by scraping directly in the medium, washed in cold PBS, and permeabilized for 1 min at room temperature in isotonic sucrose buffer [250 mM sucrose, 10 mM HEPES, 1.5 mM MgCl₂, 1 mM EGTA, and 1 mM EDTA (pH 7.1)] containing 0.025% digitonin and Complete Protease Inhibitor mixture. Digitonin was used to selectively permeabilize the plasma membranes of cells to obtain the cytosolic fractions. Cells were centrifuged at 4°C (15,000 × g for 10 min), and the supernatant was designated the cytosolic fraction. The pellet of permeabilized cells was incubated for 10 min on ice in lysis buffer containing 0.5% Triton X-100 and Complete Protease Inhibitor mixture to release membrane- and organelle-bound soluble proteins including mitochondrial cytochrome c. The Triton X-100-soluble (mitochondrial) fraction was separated by centrifugation at 15,000 × g for 10 min, and all fractions were stored at −70°C for Western blot analysis.

Western Blot Analysis. The CM from IGFBP-3-induced cells were size-fractionated by 12–15% SDS-PAGE under nonreducing conditions, whereas cell lysates were size-fractionated under reducing conditions. Size-fractionated proteins were electrotransferred to Hybond-ECL nitrocellulose (Amersham Pharmacia, Arlington, VA). For Western immunoblotting, the membranes were blocked in 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBST) and incubated with primary antibodies diluted in TBST for 2 h at room temperature or overnight at 4°C. Membranes were washed in TBST and then incubated with horseradish peroxidase-conjugated secondary antibodies (Southern Biotechnology Associates, Birmingham, AL), diluted 1:7000, for 1 h at room temperature. Immunoreactive proteins were detected using the Renaissance Western Blot Chemiluminescence reagents (New England Nuclear). For Western ligand blotting, the membranes were incubated overnight with 1.0 × 10^6 cpm of 125I-labeled IGF-I and IGF-II after blocking with normal saline containing 1% BSA at room temperature for 2 h. The filters were washed twice with normal saline containing 0.1% Tween 20 and three times with normal saline for 15 min each, dried, and exposed to film.

Results

Ecdysone-Inducible Expression of IGFBP-3 in the Stably Transfected MCF-7 Cells. Because recent studies suggest that posttranslational modification of IGFBP-3, such as glycosylation, phosphorylation, and proteolysis, influences the biological activity of IGFBP-3 (38–40), an ecdysone-inducible expression system was used to investigate the biological function of intracellularly expressed IGFBP-3 (41). It was first determined whether ponasterone A could induce IGFBP-3 expression in stably transfected MCF-7 cells. Among the clones picked as stable transfectants, IGFBP-3 was expressed in subclones 2, 3, and 6 in an inducible manner and was constitutively expressed in subclones 1 and 16 (Fig. 1A). Expression of IGFBP-3 could be detected by 12 h at concentrations of ponasterone A ranging from 2 to 10 μM without affecting cell viability. Subclone 3 was used for further investigation because physiologically relevant levels of IGFBP-3 were induced by a low concentration of ponasterone A, and noninduced levels were undetectable. The addition of ponasterone A increased the expression of IGFBP-3, but not that of the other IGFBPs, in CM (Fig. 1B). Quantitative analysis of IGFBP-3 concentrations in CM by an IGFBP-3 radioimmunoassay kit indicated that the maximal expression of IGFBP-3 (124 ng/ml) occurs on day 3 at a concentration of 15 μM ponasterone A.

Inhibition of DNA Synthesis by IGFBP-3. Subclone 3 MCF-7 cells were first tested for effects on cell growth by analyzing the effects of IGFBP-3 induction on DNA synthesis. The induced
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To identify the mechanism of the inhibitory effect of IGFBP-3 on monolayer growth, we first determined whether the cellular expression of IGFBP-3 can induce apoptosis using annexin V binding assay and a cell death detection ELISA (42). Induced expression of IGFBP-3 caused an increase in the percentage of cells undergoing apoptosis from 1.5% in the absence of ponasterone A to 21.8% in the presence of ponasterone A ($P < 0.05$ compared with cells without ponasterone A), suggesting that IGFBP-3 can induce apoptosis in this cell system (Fig. 3A). In the same experiments, the parental MCF-7:EcR cells did not show any significant change in the percentage of cells undergoing apoptosis after treatment with ponasterone A (Fig. 2).

**IGFBP-3 Induces Apoptosis in MCF-7 Cells.** To identify the mechanism of the inhibitory effect of IGFBP-3 on cell growth, induction of apoptosis, and caspase activation were observed from day 2, with maximal induction on day 3 (about a 6-fold induction) after the addition of ponasterone A (Fig. 3C).

We next investigated whether the mode of IGFBP-3-induced apoptosis involved the activation of caspase activity specific for apoptosis. Similar to the observations obtained using the cell death detection ELISA, the induced expression of IGFBP-3 increased the level of total caspase activity ($P < 0.05$ compared with cells in SFM) to the levels seen after treatment with 20 ng/ml TNF-α (Fig. 3D). The increase of caspase activity was observed from day 1, with a maximal increase on day 3 (Fig. 3E). A slight increase of caspase activity was also observed over the incubation time in cells in SFM, which might be due to serum deprivation (Fig. 3E).

**Effects of IGFBP-3 Are Mediated through IGF-Independent Action.** We next determined whether the effects of IGFBP-3 on cell growth, induction of apoptosis, and caspase activation were IGF independent. The observed biological effects of IGFBP-3 did not result from blocking the endogenous IGF action because IGFBP-5, which binds both IGF-I and IGF-II with high affinity, had no significant effect on basal DNA synthesis in MCF-7:IGFBP-3 cells without ponasterone A when added at 150 ng/ml but blocked the stimulatory effect of 5 ng/ml IGF-I (Fig. 4A). Furthermore, when MCF-7 cells were transfected with GGG-IGFBP-3, an IGFBP-3 mutant unable to bind IGFs, DNA synthesis was decreased compared with the level shown in cells transfected with empty vector (Fig. 4B). In addition, apoptotic index and total caspase activity were increased in GGG-IGFBP-3-transfected cells (Fig. 4, C and D), which are comparable with those seen in cells overexpressing intact IGFBP-3 (Figs. 2 and 3). Taken together,
these data confirm that effects on cell growth and induction of apoptosis are IGFBP-3 specific and IGF-IGF receptor independent.

Caspases Involved in a Death Receptor-Mediated Pathway Are Activated in IGFBP-3-Induced Apoptosis. To determine which caspases are activated by IGFBP-3, we measured the kinetics of IGFBP-3-induced changes in enzyme activities using substrates that display specificity for each caspase in vitro: Ac-IETD-AMC (caspase-8), Ac-LEHD-AMC (caspase-9), Ac-DEVd-AMC (caspase-3/7), or a mixture of Ac-DEVd-AMC and Ac-LEHD-AMC (total caspase), respectively. It was observed that the induced expression of IGFBP-3 significantly increased total caspase, IETDase, and DEVDase activity (Fig. 5A, *, P < 0.05 compared with cells with SFM) but not LEHDase activity. Because MCF-7 cells do not express a functional caspase-3 due to a deletion within exon 3 of the CASP3 gene (44), the DEVDase activity may come from the activity of caspase-7 or -2 in our cell system. In contrast, increased IETDase activity may come from the activation of caspase-8, -10, or -6 on the basis of Ac-IETD-AMC specificity (32). To identify the caspases involved in IGFBP-3-induced apoptosis, the activation of procaspase-8, -10, -6, -9, -7, and -2 was examined in cells treated with ponasterone A by Western immunoblotting (Fig. 5B). As expected from the caspase activity studies, we found a significant decrease in the level of procaspase-8, and -7, but not procaspase-10, -6, -9, and -2, after the addition of ponasterone A. Maximal decrease of procaspase-8 and -7, accompanied by an increase of cleaved PARP, was observed on day 3 after the addition of ponasterone A, suggesting that IGFBP-3 activates caspase-8 and -7, although the cleaved caspases were not detected in our cell system.

IGFBP-3-Induced Apoptosis Is Mediated by Caspase-Dependent Pathways. Previous studies have demonstrated that exposure of MCF-7-cells to TNF-α (25–50 ng/ml) leads to apoptotic cell death (30–60%) within 24 h (43). In the present study, cells treated with 20 ng/ml TNF-α or 15 μM ponasterone A both showed a marked decrease in proliferation (Fig. 6A) and an increased apoptotic index (Fig. 6B), as well as elevated caspase activity (Fig. 6C) on day 2 after treatment. Furthermore, TNF-α and IGFBP-3-induced cell death was accompanied by an increase in the level of cleaved PARP (Fig. 6D). These changes induced by TNF-α or IGFBP-3 were completely prevented by cotreatment with the broad-spectrum caspase inhibitor Z-VAD-FMK (Fig. 6, A–D), suggesting that both TNF-α- and IGFBP-3-induced apoptosis are mediated by caspase-dependent pathways. At the same time, the caspase-8 inhibitor Z-IETD-FMK also completely abolished the changes in cell proliferation, apoptotic index, caspase activity, and cleaved PARP expression by TNF-α or IGFBP-3 (Fig. 6, A–D), confirming that caspase-8 is a key initiator caspase involved in the induction of apoptosis by IGFBP-3.

IGFBP-3 Does Not Affect the Release of Cytochrome c into the Cytosol in MCF-7 Cells. Mitochondrial release of cytochrome c into the cytosol during apoptosis is thought to be caspase independent, even though in some cases of death receptor-mediated apoptosis, caspase-8 or -10 may act on mitochondria to facilitate cytochrome c release, thereby further amplifying the apoptotic signal (45). To determine whether IGFBP-3 triggers cytochrome c release, we examined the amount of cytochrome c in the cytosolic and mitochondrial fractions of cells after treatment with ponasterone A. Because TNF-α has been reported to elicit mitochondrial cytochrome c release and Bid cleavage in MCF-7 cells (43), cells were treated with 20 ng/ml TNF-α for 48 h as a positive control. There was minimal cytochrome c in the cytosolic fraction in SFM-cultured and ponasterone A-treated cells, whereas detectable cytochrome c was increased in TNF-α-treated cells (Fig. 7, top panel). Simultaneously, the amount of cytochrome c in the mitochondrial fraction was decreased in TNF-α-treated cells, but not in the SFM-cultured and ponasterone A-treated cells (Fig. 7, top panel). Bid is normally cleaved by a caspase-8-dependent process, and cleaved Bid elicits mitochondrial cytochrome c release. The amount of intact Bid in the cytosolic fraction was decreased in TNF-α-treated cells, whereas no change was observed in ponasterone A-treated cells or SFM-cultured cells (Fig. 7, middle panel). These results suggest that IGFBP-3 does not affect the release of cytochrome c from mitochondria.

DISCUSSION

IGFBP-3, which is the most abundant IGFBP in human serum, acts not only as a carrier of IGFs, thereby prolonging their half-
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Fig. 5. Activation of caspases involved in death receptor-mediated pathway in insulin-like growth factor-binding protein 3-induced apoptosis in MCF-7 cells. A, lysates of cells incubated with or without ponasterone A or in 10% (fetal bovine serum or 20 ng/ml tumor necrosis factor α for 72 h were assayed for total caspase (DEVDase and LEHDase), DEVDase (caspase-3-like), IETDase (caspase-8), and LEHDase (caspase-9) activities (mean ± SE; n = 6). *P < 0.05, treated cells versus cells in serum-free media. B, Western immunoblot analysis of caspase-8, -10, -6, -9, -7, and -2 and cleaved PARP in MCF-7:IGFBP-3 cells with or without ponasterone A at the indicated time. Proteins showing a significant change after addition of ponasterone A are indicated by an asterisk.

In the present investigation, we have begun to address specific questions regarding the intracellular mechanisms involved in IGFBP-3-induced apoptosis in MCF-7 breast cancer cells. To determine these mechanisms, we used MCF-7 cells expressing cellular IGFBP-3 in an inducible manner. Because MCF-7 cells do not express detectable IGF-I (48–50), and experiments in this study were performed in phenol red-free SFM, our results suggest that the apoptosis-inducing effect of IGFBP-3 in MCF-7 cells is independent of the IGF-IGF receptor axis, although the model system does not rule out the effects of residual IGFs. Nevertheless, use of a IGFBP-3 mutant and other IGFBPs confirms that IGFBP-3-induced cell growth inhibition is specific for IGFBP-3 and IGF-independent: (a) MCF-7:IGFBP-3 cells used in this study are not inhibited by IGFBP-5, which binds both IGF-I and -II with high affinity; and (b) more definitively, MCF-7 cells transfected with the IGFBP-3 mutant, which is unable to bind IGFs, show inhibition of DNA synthesis, induction of apoptosis, and activation of caspase.

Here we demonstrate for the first time that IGFBP-3 induces apoptosis in MCF-7 cells by activating multiple caspases involved in a death receptor-mediated pathway, such as caspase-8 and -7, but not via the mitochondria-mediated pathway. Furthermore, these findings do not reflect clonal variation of the MCF-7:IGFBP-3 cells used in this study because the expression of IGFBP-3 by adenoviral transduction results in the same biological effects in MCF-7 cells (data not shown). Despite the lack of functional caspase-3 due to a deletion in the CASP3 gene (44), MCF-7 cells underwent apoptosis, suggesting that other executioner caspases, such as caspase-7 or -2, may be involved. We demonstrate that IGFBP-3 activates caspase-7 but not caspase-2, as shown by Western immunoblotting, explaining the elevated caspase-3-like (DEVD-ase) activity, which is consistent with previous studies showing that caspase-7 was processed in doxorubicin-induced apoptosis of MCF-7 cells (51), but caspase-2 was not processed after TNF-α or staurosporine treatment (52). Caspase-8 can activate caspase-3 and -7 directly and/or through triggering the activation of caspase-9 by the release of cytochrome c from mitochondria (26–29). To assess the relative importance of caspase-8 in the cytotoxic signaling of IGFBP-3, we examined the effect of Z-IETD-FMK (caspase-8 inhibitor) during IGFBP-3-induced apoptosis. The caspase-8 inhibitor significantly blocked the actions of IGFBP-3 to increase total caspase activity, to induce apoptosis as measured by the cell death detection ELISA assays, to cleave PARP, and to suppress cell proliferation. Thus, on the basis of this caspase activity assessment and the studies using the caspase-8 inhibitor, IGFBP-3-induced apoptosis in MCF-7 cells involves the caspase-8-mediated activation of executioner caspases such as lives, but also functions as a modulator of the IGF activity by regulating the availability of free IGFs for their interaction with IGF receptors (1–2). Recently, there has been a growing accumulation of data demonstrating that IGFBP-3 may have its own biological actions, inhibiting cell proliferation in an IGF-independent manner (6–19). Conceptually, IGFBP-3 can exert its actions on target cells in several ways: (a) inducing apoptosis; (b) regulating the cell cycle; and (c) possible cross-talk with major signal transduction pathways. Mechanisms for inducing apoptosis, in part, have been demonstrated by studies showing that IGFBP-3 increases the ratio of proapoptotic (Bax and Bad) to antiapoptotic (Bcl-2 and Bcl-2) proteins in apoptotic breast cancer cells (24), and caspase-3 activity and IGFBP-3 levels in CM are increased in paclitaxel-treated Hs578T breast cancer cells (25). As a regulator of the cell cycle, IGFBP-3 has been shown to regulate the induction of the cyclin-dependent kinase inhibitory protein p21/WAF/CIP1 by vitamin D analogs in prostate cancer cells (46). In addition, an active Ras-mitogen-activated protein kinase pathway has been shown to block IGFBP-3-induced cell growth inhibition, whereas inhibition of the mitogen-activated protein kinase pathways restored the biological function of IGFBP-3 (47). Although these early investigations have revealed, in part, suggestive mechanisms for IGFBP-3 action, mechanisms of action for IGFBP-3 remain largely undetermined.
caspase-7, but not the activation of caspase-9. At the same time, the broad-spectrum caspase inhibitor Z-VAD-FMK also abolished the negative effects on cell proliferation, induction of apoptosis, caspase activity, and levels of cleaved PARP by IGFBP-3, suggesting that the antiproliferative effect of IGFBP-3 results mainly from the induction of apoptosis. During the preparation of this paper, Butt et al. (53) also reported that IGFBP-3-mediated apoptosis in T47D human breast cancer cells was significantly blocked by treatment with Z-VAD-FMK, further supporting our findings.

Cytochrome c release from mitochondria during apoptosis can be achieved through two different mechanisms. Direct stimuli such as genotoxic agents or UV light can release cytochrome c in a caspase-independent manner, whereas activated caspase-8 or -10 may also facilitate cytochrome c release from mitochondria.

Fig. 6. Activation of caspases by insulin-like growth factor-binding protein 3 in a caspase-dependent manner. MCF-7:IGFBP-3 cells were incubated with 15 μM ponasterone A or 20 ng/ml tumor necrosis factor α in phenol red-free serum-free media ± 25 μM Z-VAD-fluoromethylketone or 20 μM Z-IETD-fluoromethylketone for 72 h. A, cell proliferation assay by CellTiter 96 assay. B, apoptotic index by cell death detection ELISA. C, total caspase activity. *, *P < 0.05; **, ***, **P < 0.005; ****, ***, ***P < 0.0005 (treated cells versus cells in serum-free media; mean ± SE; n = 6). D, Western immunoblot analysis of cleaved PARP.

Fig. 7. Subcellular distribution of cytochrome c after induction of insulin-like growth factor-binding protein 3 in MCF-7 cells. MCF-7:IGFBP-3 cells were incubated with 15 μM ponasterone A or 20 ng/ml tumor necrosis factor α in phenol red-free serum-free media for 48 h. Cytosolic and mitochondrial fractions prepared as described in “Materials and Methods” were immunoblotted with monoclonal antibody to cytochrome c and Bid. The results are representative of at least three independent experiments.

Fig. 8. Schematic representation of insulin-like growth factor-binding protein (IGFBP)-3-induced apoptosis. IGFBP-3 may inhibit cell growth by induction of apoptosis either indirectly, by sequestrating insulin-like growth factors away from the insulin-like growth factor receptor, or directly via putative IGFBP-3 receptor and/or translocation to the nucleus. IGFBP-3 increases the ratio of proapoptotic (Bax and Bad) to antiapoptotic (Bcl-2 and Bcl-XL) members of the Bcl-2 family (24). Our study demonstrates the existence of an apoptotic cascade, which utilizes caspases involved in the death receptor-mediated pathway such as caspase-8 and -7.
thereby further amplifying the apoptotic signal (48). In our study, the induced expression of IGFBP-3 did not affect the redistribution of cytochrome c, suggesting that IGFBP-3-induced apoptosis does not result from the disruption of mitochondrial function and caspase-9 activation, which is consistent with previous studies on cisplatin- or somatostatin-induced apoptosis in MCF-7 cells (54, 55). Minimal detection of cytochrome c in the cytosol of both control cells and panonasterone A-treated cells may result from serum deprivation as observed previously (56).

In summary, these findings help define the temporal sequence of events that links the initiator and effector caspases in IGFBP-3-induced apoptosis in MCF-7 cells. Fig. 8 depicts the proposed mechanism for IGFBP-3-induced apoptosis in MCF-7 cells, based on the results from the present study and other previous studies (24). The present study supports the existence of an IGFBP-3-regulated apoptotic cascade, which utilizes caspases involved in death receptor-mediated pathways, such as caspase-8 and caspase-7, suggesting that IGFBP-3 functions as a negative regulator of breast cancer cell growth, independent of the IGF axis. These events occur on the cell surface as demonstrated previously in the prostate cancer cell system (20). It is tempting to speculate that IGFBP-3-induced apoptosis may be initiated by interaction of IGFBP-3 with the cognate receptor(s), which has yet to be identified, thereby subsequently activating initiator caspase-8 and downstream effector caspases.

REFERENCES

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