Insulin-like Growth Factor-binding Protein-5 Inhibits the Growth of Human Breast Cancer Cells in Vitro and in Vivo*

Alison J. Butt†, Kristie A. Dickson, Fiona McDougall, and Robert C. Baxter

From the Kolling Institute of Medical Research, University of Sydney, Royal North Shore Hospital, St. Leonards, New South Wales 2065, Australia

The role of insulin-like growth factor-binding protein (IGFBP)-5 in human breast cancer cell growth is unclear. We determined the effects of IGFBP-5 expression on the growth of human breast cancer cell lines in vitro and in vivo. Expression of IGFBP-5, both by stable transfection and adenoviral-mediated infection, was inhibitory to the growth of MDA-MB-231 and Hs578T human breast cancer cells over a 13-day period. IGFBP-5 expression resulted in a G2/M cell cycle arrest and the induction of apoptosis in both cell lines, an effect that was abrogated in the presence of the broad-spectrum caspase inhibitor, z-VAD-fmk. IGFBP-5-induced apoptosis was associated with a transcriptional increase in expression of the proapoptotic regulator bax and decrease in the anti-apoptotic bel-2 compared with vector controls. Secreted IGFBP-5 when added exogenously to breast cancer cells was not internalized and had no effect on cell growth or apoptosis, suggesting that IGFBP-5 may elicit its inhibitory effects via a novel, intracellular mechanism. In athymic nude mice, stable expression of IGFBP-5 significantly inhibited both the formation and growth of tumors derived from MDA-MB-231 cells. IGFBP-5-expressing tumors also had a significantly elevated level of bax mRNA and decreased levels of bel-2 mRNA compared with vector tumors. These data suggest that IGFBP-5 is a potent growth inhibitor and proapoptotic agent in human breast cancer cells via modulation of cell cycle regulation and apoptotic mediators.

IGFBP-5 expression is up-regulated by antiproliferative agents such as retinoic acid (8), vitamin D-related compounds (9), and antiestrogen ICI 182780 (10), with some evidence that it may mediate their growth inhibitory effects (8, 10). Similarly, growth stimulation of human breast cancer cells by estradiol is associated with a down-regulation of IGFBP-5 expression (10), although exogenous IGFBP-5 had no effect on IGF-I-stimulated DNA synthesis in the breast cancer cell line, MCF-7 (11). However, there are some conflicting data in vivo, with Chan et al. (12) reporting a decrease in IGFBP-5 gene expression following growth inhibition of the rat mammary gland with ICI 182780, whereas Huynh et al. (10) observed an ICI-induced increase in IGFBP-5 mRNA in 9,10-dimethyl-1,2-benzanthracene-induced mammary tumors. These results suggest that IGFBP-5 has cell type-specific effects on cellular proliferation.

The role of IGFBP-5 in the apoptotic process is also somewhat ambiguous, with conflicting data from in vivo and in vitro studies. In vivo, increased expression of IGFBP-5 has been observed in tissues undergoing apoptosis such as the involuting prostate (13) and mammary (14, 15) glands, atretic ovarian follicles (16), and in the rat brain following hypoxic-ischemic injury (17). Allan et al. (18) have also reported that IGFBP-5 expression is highly restricted to regions of cell death in the developing mouse limb bud. In vitro, expression of IGFBP-5 mRNA increases following induction of apoptosis in ovine granulosa cells (19). However, Perkins et al. (20) have reported opposing effects of IGFBP-5 in human breast cancer cells in vitro. They demonstrated that addition of exogenous IGFBP-5 to Hs578T cells protects these cells from ceramide-induced apoptosis, suggesting IGFBP-5 may have a survival function in response to apoptotic stimuli (20, 21). A similar conclusion was reached by Roschier et al. (22) following their demonstration that induction of apoptosis in cerebellar granule cells is associated with a down-regulation of IGFBP-5 mRNA and protein expression, an effect that is reversed by IGF-I (22). Furthermore, castration-induced IGFBP-5 expression enhances the mitogenic and antiapoptotic effects of IGFs and accelerates progression to androgen independence in prostate cancer models (23).

IGFBP-5 is expressed in breast cancer tissue (24) and cell lines (25) and is positively correlated with estrogen receptor status. Because of the contradictory data on the role of IGFBP-5 in cancer cell growth, we directly examined the effect of stable expression of IGFBP-5 on the growth of human breast cancer cell lines in vitro and in vivo. Furthermore, using a transient overexpression system, we have characterized the proapoptotic effects of IGFBP-5 in human breast cancer cells. We show that IGFBP-5 significantly inhibits the growth of human breast cancer cells both in vitro and in vivo, and induces a caspase-dependent, intrinsic apoptotic pathway. Interestingly, these growth effects appear to be mediated by intracellular IGFBP-5, independent of signaling through a cell-surface receptor.

* This work was supported by National Health and Medical Research Council, Australia, Grant 107242 (to A. J. B. and R. C. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed. Tel.: 61-2-9926-8486; Fax: 61-2-9926-8484; E-mail: abutt@med.usyd.edu.au.
‡ The abbreviations used are: IGFBP, insulin-like growth factor-binding protein; IGFRI, type I IGF receptor; IR, ionizing radiation; FCS, fetal calf serum; SF, serum-free/insulin-free; rh, recombinant human; CM, conditioned media.
IGFBP-5-induced Growth Inhibition and Apoptosis

EXPERIMENTAL PROCEDURES

Cell Culture of Breast Cancer Cells—The human breast cancer cell lines MDA-MB-321 and Hs578T were routinely maintained in RPMI 1640 media containing 10% FCS, 10 μg/ml insulin, and 2.92 μg/ml gentamicin under standard conditions. Stably Transfectants—A 0.9-kb IGFBP-5 cDNA, cloned from U2-OS osteosarcoma cells, was kindly provided by Dr. Sue Firth, Kolling Institute (26). MDA-MB-231 cells were stably transfected with the IGFBP-5 cDNA in the expression vector pcDNA3 (Invitrogen), using LipofectAMINE (Invitrogen) according to the manufacturer’s protocol. IGFBP-5 transfectedants (MDA/BP-5) and vector controls (MDA/VEC) were selected in media containing 800 μg/ml genetin for 21 days post-transfection, then mixed populations of transfectedants were grown up for subsequent experiments. Adenoviral-mediated Expression of IGFBP-5—The human IGFBP-5 cDNA was subcloned into the adenoviral shuttle vector, pAdTrack-CMV, and replication-defective IGFBP-5-expressing adenovirus was produced essentially as previously described (27). Proliferating cultures of MDA-MB-231 and Hs578T were incubated with varying concentrations of adenovirus stock (approximately 4.5 × 10² plaque forming units/μl) for 6 h in serum-free, insulin-free (SF) media. Virus was then replaced and refreshed with growth media. As the pAd-Track-CMV vector contains a green fluorescent protein marker, levels of infection were assessed by the percentage of green fluorescent protein-positive cells using flow cytometry. For the cell proliferation assays, cells were infected with 0.1 μl of adenovirus stock. For analysis of apoptosis induction, Hs578T cells were infected with 2 μl of adenovirus stock, and MDA-MB-231 cells were infected with 1 μl of adenovirus stock. These concentrations were shown to give >90% infection efficiency and low toxicity as assessed by flow cytometry (data not shown). Measurement of Cell Proliferation—Cells were seeded at 1 × 10⁴ cells/well in 12-well plates in the presence of 10% FCS media. On days 3, 6, 9, and 13 post-seeding, cells were trypsinized and the viable cell numbers were assessed by trypan blue exclusion. Analysis of Cell Cycle Distribution—Cells were plated at 5 × 10⁴ per well in 6-well plates for 24 h, then incubated in SF media for 48 h. Cells were trypsinized and resuspended in 70% ethanol and kept at −20 °C for at least 24 h. Fixed cells were then washed and suspended in 1 ml of propidium iodide (10 μg/ml propidium iodide, 1 mg/ml RNase A) for at least 1 h in the dark at 4 °C. Cell cycle analysis was performed using a Coulter ELITE flow cytometer (Coulter, Hialeah, FL). 20,000 cells were analyzed for each sample, and quantitation of cell cycle distribution was performed using a Muilticycle software (Phoenix Flow Systems, San Diego, CA). Clonogenic Survival Assays—The long term survival of MDA-MB-231 transfectedants following doses of irradiation was assessed by a clonogenic survival assay. 2 × 10³ cells were seeded into 6-well plates in triplicate for 24 h. Cells were then washed with fresh media and irradiated with various doses of x-rays (2.5, 5, or 7.5 gray), then incubated for 14 days in 10% FCS. At this time, cells were counted and the percentage survival determined by the proportion of attached cells surviving (assayed by trypan blue exclusion) relative to unirradiated controls. Measurement of DNA Fragmentation by Flow Cytometry—Both floating and adherent cells were analyzed for induction of apoptosis by flow cytometry. 24 h post-adenoviral infection, cells were rinsed with fresh SF media, then incubated for 48 h with or without the caspase-inhibitor z-VAD-fmk (100 μM; Bachem AG, Switzerland). Floating and attached cell populations were combined and 1 × 10⁵ cells were prepared and analyzed as described above. Labeled nuclei were gated on light scatter to remove debris and the percentage of nuclei with a sub-G1 content was determined. Apoptosis Assay—Cells were plated at 5 × 10⁵ per well on sterile glass coverslips in 6-well plates for 24 h then infected with IGFBP-5 or vector adenovirus. 24 h post-adenoviral infection, cells were washed with SF media and incubated for 48 h. Cell monolayers were rinsed with phosphate-buffered saline, fixed in ice-cold methanol for 10 min, and then stained with 0.8 μg/ml 4,6-diamidino-2-phenylindole (Sigma). The percentage of apoptotic cells was determined microscopically as cells with visible nuclear fragmentation. In situ DNA Fragmentation Assay—For analysis of DNA synthesis, MDA-MB-231 and Hs578T cells were plated in 48-well plates at 5 × 10⁵/well and infected with adenovirus as described above. DNA synthesis was determined using incorporation of [³H]thymidine essentially as previously described (28) 24 h post-infection, or following addition of 20 or 100 ng/ml recombinant human (rh) IGF-II or 10 μg/ml IGFR neutralizing antibody (dB3, Calbiochem-Novabiochem, Alexandria, New South Wales, Australia) as indicated for 24 h in SF media. Immunoblot Analysis—Total cell lysates were prepared from cells 48 h post-seeding for stable transfectedants or post-infection with adenovirus following an incubation in SF media and 50 μg were resolved under reducing conditions on 12% SDS-polyacrylamide gels using standard methods. For analysis of Bax expression post-irradiation, cells were irradiated with various doses of x-rays (2.5, 5, or 7.5 gray), then incubated for 4 days in 10% FCS before preparation of total cell lysates as described above. For IGFBP-5 immunoblots, 50 μg of conditioned media (CM) samples were electrophoresed under non-reducing conditions. Resolved proteins were transferred to nitrocellulose membranes and probed with anti-Bax polyclonal (1:1000 dilution; BD Pharmingen), anti-Bcl-2 monoclonal (0.8 μg/ml; Dako, Carpinteria, CA), or anti-human IGFBP-5 (51;100; Ref. 29) antibodies overnight at 4 °C. This was followed by incubation with anti-rabbit (for Bax, 1:10,000 dilution) or anti-mouse (for Bcl-2, 1:2000 dilution) IgG conjugated with horse-radish peroxidase. Immunoreactive protein bands were visualized with ECL (Pierce). Blots were checked for equal loading by reprobing with anti-α-tubulin antibody (1:10,000, Sigma). Protein bands were quantitated by densitometry (Video Densitometer model 620, Bio-Rad). For IGFBP-5 immunoblots, membranes were incubated with anti-chicken IgY secondary antibody (1:5000 dilution; Promega, Madison, WI) conjugated with alkaline phosphatase and visualized with colorimetric detection. Purity of cytoplasmic and nuclear extracts (Fig. 6) was confirmed by immunoblot with CPP32 (1:1000 dilution; Transduction Laboratories, Lexington, KY, Ref. 30) and pol(AdP-ribose) polymerase (1:2000 dilution; Roche Molecular Biochemicals; Ref. 31) antibodies, respectively. Analysis of IGFBP-5 in Conditioned Media—MDA-MB-231 and Hs578T cells were infected with adenovirus as described above, then incubated in SF media for 48 h. CM was collected and IGFBP-5 was purified by binding to, and elution from, an affinity column of IGF-I agarose, followed by reverse-phase high performance liquid chromatography on a Jupiter 5μ, 300-Ä C18, 250 × 4.6-mm column (Phenomenex, Torrance, CA). IGFBP-5 was eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid, 15–24% over 10 min, then 24–33% over 20 min, and 33–48% over 12 min, at 24 °C. Purified IGFBP-5 fragments (24% over 10 min, then 24 °C for 30–32 min. Concentrations of IGFBP-5 in CM from IGFBP-5 expressing cells were quantitated by IGFBP-5-specific radioimmunoassay as previously described (29). Ligand Blot—To examine intracellular IGFBP-5, cells were infected with adenovirus or treated with exogenous IGFBP-5, then incubated for 48 h in SF media. Cytoplasmic and nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) following the manufacturer’s instructions. Protein content was estimated and samples were electrophoresed (30 μg for nuclear samples from stable transfectedants, 50 μg for all other samples) and transferred to nitrocellulose membranes, then probed with 125I-IGF-I (1 × 10⁴ cpm/ml) overnight. Northern Analysis—RNA extraction and Northern analysis were performed essentially as previously described (32). The IGFBP-5 cDNA probe was kindly provided by Dr. S. Shimasaki (University of California San Diego, La Jolla, CA) and the bca cDNA probe was kindly provided by Dr. G. Facko (Kolling Institute of Medical Research, St. Leonards, NSW, Australia). Probes were labeled using a HexaLabel DNA labeling kit (MBI Fermentas, Vilnius, Lithuania) and [α-³²P]deoxy-CTP. Filters were hybridized at 42 °C overnight, then washed in 1× SSC (standard saline citrate) at 42 °C, followed by a wash in 0.1× SSC heated to 42 °C. The ribosomal phosphoprotein, 36B4, was used as a loading control (33). Filters were quantitated using a phosphorimaging scanner (Molecular Dynamics, Pujol, Japan). Densitometric data were normalized by expressing the ratio of target mRNA to 36B4 mRNA. Real Time Quantitative PCR—Total RNA was isolated and reverse transcribed using oligo(dT)₁₇ and SuperScript II reverse transcriptase polymerase as previously described (32). Real time quantitative PCR was performed using the Bio-Rad iCycler in combination with SYBRgreen (Molecular Probes). A final concentration of 400 μM gene-specific primers at 10 μM each. 10 μl of first strand reaction followed by 50 °C for 2 min, 95 °C for 10 s, 55 °C for 10 s, and 72 °C for 30 s, for 40 cycles. The efficiency of the primers was determined at 100% and all experiments were performed at similar efficiency. Data were analyzed using the 2⁻¹°Cq method for data analysis.
ment, seven mice were injected with MDA/BP-5 cells. Tumor growth was monitored weekly by measuring tumor length and width using calipers, and tumor volume (cm³) was calculated using the formula: length × width² × 0.5. Experiments were terminated when tumors reached ~1 cm³. Blood samples were taken from all animals at termination and tumors were frozen in liquid nitrogen for RNA analysis. Results were pooled from two separate experiments and all experiments were carried out with the approval of the Institutional Animal Care and Ethics Committee.

**Results**

Growth Inhibitory to Human Breast Cancer Cells—The effects of IGFBP-5 on the growth of human breast cancer cells were examined using both stable transfection of human IGFBP-5 cDNA as well as transient adenoviral-mediated expression. Cell growth was assessed by cell counting over a 13-day period, and concentrations of IGFBP-5 secreted into the media were also determined at each time point. Secretion of IGFBP-5 remained high throughout the 13-day growth period and was low or undetectable in vector control cells (data not shown). Fig. 1A shows the effects of stable expression of IGFBP-5 on the growth of MDA-MB-231. MDA/BP-5 cells were significantly growth inhibited over the 13-day growth period compared with vector controls (p < 0.03 by repeated measures analysis of variance). Interestingly, despite much higher levels of IGFBP-5 secretion, a similar inhibitory effect was observed following adenoviral-mediated expression of IGFBP-5 in MDA-MB-231 (Fig. 1B) and Hs578T (Fig. 1C). In both cell lines, transient overexpression of IGFBP-5 significantly inhibited cell growth compared with vector controls (p < 0.005 and p < 0.005, respectively, by repeated measures analysis of variance).

**IGFBP-5 Expression Induces a G1/M Cell Cycle Arrest in Human Breast Cancer Cells**—We determined the effects of both stable and transient expression of IGFBP-5 on DNA synthesis using incorporation of [³H]thymidine. Fig. 2A demonstrates that both stable and adenoviral-mediated expression of IGFBP-5 results in a significant decrease in DNA synthesis over 24 h compared with vector controls (p < 0.02 stable transfectants; p < 0.003 MDA-MB-231; p < 0.05 Hs578T).

The effects of IGFBP-5 on cell cycle progression were examined by flow cytometric analysis after a 48-h incubation in SF media. Fig. 2B demonstrates that stable expression of IGFBP-5 in MDA-MB-231 cells resulted in a significant accumulation in G2/M phase of the cell cycle compared with vector controls (p < 0.05). Adenoviral-mediated expression of IGFBP-5 also resulted in a significant increase in the percentage of MDA-MB-231 and Hs578T cells in the G2/M phase compared with vector controls (Fig. 2C, p < 0.005 and p < 0.02, respectively). A significant decrease in the percentage of cells in G0/G1 was also observed when compared with vector controls (p < 0.03 and p < 0.01, respectively). Interestingly, no significant differences in the S phase fraction were observed between IGFBP-5-expressing cells and vector controls, which may reflect the relative insensitivity of this method compared with analysis of active DNA synthesis.

**IGFBP-5 Expression Induces Caspase-dependent Apoptosis via Modulation of Bcl-2 Proteins**—The effect of IGFBP-5 on the induction of apoptosis was examined in MDA-MB-231 and Hs578T cells using transient adenoviral-mediated expression. The extent of DNA fragmentation characteristic of apoptotic cell death was assessed using both scoring of 4,6-diamidino-2-phenylindole-stained nuclei for fragmentation and flow cytometric analysis of the hypodiploid (pre-G₁) fraction. Analysis of 4,6-diamidino-2-phenylindole-stained nuclei showed a significant increase in nuclear fragmentation in IGFBP-5-infected MDA-MB-231 (from 4.7 ± 0.5 to 7.6% ± 1.1, p < 0.0001) and Hs578T (from 4.8 ± 0.7 to 11.2% ± 1.6, p < 0.0001) cells compared with vector controls (Fig. 3A).

Fig. 3B illustrates that expression of IGFBP-5 resulted in a significant increase in the percentage of the population in the pre-G₁ fraction compared with vector controls in both MDA-MB-231 (from 2.7 ± 0.5 to 7.3% ± 1.1, p < 0.02) and Hs578T (from 2.9 ± 0.8 to 9.0% ± 3.3, p < 0.006) cells. Incubation of IGFBP-5-expressing MDA-MB-231 and Hs578T cells with the caspase inhibitor z-VAD-fmk resulted in a significant decrease in the level of apoptosis (p < 0.01 and p < 0.02, respectively) to levels that were not significantly different from vector controls (Fig. 3B). Levels of apoptosis in vector-infected cells were not significantly affected by z-VAD-fmk (p = 0.2 for MDA, p = 0.9 for Hs578T).
The effect of stable IGFBP-5 expression on the long term survival of MDA-MB-231 transfectants following exposure to IR was examined. Cells were irradiated with various doses of x-rays, then radiosensitivity was evaluated using clonogenic survival assays. At doses of 2.5 and 5 gray x-rays, MDA/IGFBP-5 cells showed significantly reduced long term (up to 14 days) survival (p < 0.0001 and p < 0.002, respectively) compared with vector controls (Fig. 3C). At the higher dose of 7.5 gray, the surviving fraction was low in both MDA/VEC and MDA/IGFBP-5 cells.

We have previously shown that IGFBP-3-induced apoptosis and radiosensitivity is associated with modulation of members of the Bcl-2 family of apoptotic regulators (2, 3). We investigated whether the apoptotic effects of IGFBP-5 were mediated via a similar pathway. Fig. 3D shows that IGFBP-5 expression in stable transfectants was associated with elevated levels of Bax protein compared with vector controls, both basally and 4 days after exposure to increasing doses of radiation. This suggests that the increased radiosensitivity of the IGFBP-5-expressing cells might result from Bax-mediated apoptosis.

To further investigate these IGFBP-5-mediated effects, both stable transfectants and adenoviral-infected cells were analyzed for expression of the proapoptotic Bax and antiapoptotic Bcl-2 proteins. Levels of bax and bcl-2 mRNA were also determined in these cell populations. Fig. 4A shows that expression of IGFBP-5 in both MDA-MB-231 and Hs578T cells was associated with a significant induction in the expression of Bax protein (p < 0.02 stable transfectants; p < 0.04 adenovirus both cell lines). In addition, stable transfection of IGFBP-5 in MDA-MB-231 cells resulted in a significant down-regulation of Bcl-2 protein compared with vector controls (p < 0.005), although no change in Bcl-2 expression was observed following transient, adenoviral-mediated expression of IGFBP-5.

IGF-independent Effects of IGFBP-5 on Human Breast Cancer Cell Growth—We examined whether the growth inhibitory and proapoptotic effects of IGFBP-5 in human breast cancer cells were mediated via the ability of IGFBP-5 to sequester IGFs and ablate their mitogenic and anti-apoptotic effects. Because IGFs generally act through the IGFRI (34), the response of MDA/VEC to the mitogenic effects of IGFRI activation was determined by examining levels of [3H]thymidine incorporation in the presence or absence of up to 100 ng/ml rhIGF-II. Fig. 5A illustrates that MDA/VEC cells were unre-
responsive to IGF-II. Furthermore, IGF-I and IGF-II levels are undetectable in these cells (data not shown), confirming the results of Sciacca et al. (35). To confirm a lack of autocrine IGF action, we examined the levels of [3H]thymidine incorporation in cells incubated with an IGFRI-blocking antibody (IR-3) compared with untreated cells. Fig. 5A demonstrates that 10 μg/ml IR-3 had no effect on basal DNA synthesis, suggesting that endogenous IGFs are not significantly mitogenic in MDA-MB-231. We have previously demonstrated in IGF-responsive T47D human breast cancer cells, that 10 μg/ml IR-3 can reverse the mitogenic effects of 20 ng/ml IGF-II (3). Fig. 5B illustrates that treatment with 10 μg/ml IR-3 also had no significant effect on the basal level of apoptosis as determined by flow cytometry (p = 0.17), indicating that IGF activation of IGFRI does not elicit an autocrine survival signal in MDA-MB-231. Hs578T cells have been previously demonstrated to be unresponsive to the mitogenic and anti-apoptotic effects of IGFs (36). These data suggest that the inhibitory and proapoptotic effects of IGFBP-5 in MDA-MB-231 and Hs578T cells are unlikely to be mediated via sequestration of IGFs and are independent of IGFRI signaling.

**The Cytotoxic and Cytostatic Effects of IGFBP-5 Are Not Mediated by Secreted IGFBP-5**—MDA-MB-231 or Hs578T cells were infected with IGFBP-5 or vector adenovirus, under SF conditions. Levels of apoptosis were determined in MDA-MB-231 and Hs578T cells 48 h post-infection with IGFBP-5 or vector adenovirus, under SF conditions. Cells were analyzed by flow cytometry, and the percentage of cells in the pre-G1 peak was determined. Values shown are means of triplicate wells from two independent experiments ± S.E. B, the effect of IGFBP-5 on the long term survival of MDA-MB-231 cells in response to apoptotic stimuli was determined. MDA/VEC (white bars) and MDA/BP-5 (black bars) stable transfectants were irradiated with various doses of x-rays as indicated, and survival was measured 14 days post-irradiation by clonogenic assays. Values shown are means of three data sets from two independent experiments ± S.E. C, the effect of IGFBP-5 on the long term survival of MDA-MB-231 cells in response to apoptotic stimuli was determined. MDA/VEC (white bars) and MDA/BP-5 (black bars) stable transfectants were irradiated with various doses of x-rays as indicated, and survival was measured 14 days post-irradiation by clonogenic assays. Values shown are means of three data sets from two independent experiments ± S.E. D, expression of Bax was determined by immunoblot analysis 4 days post-irradiation in MDA/VEC and MDA/BP-5 cells irradiated with 2.5 (lanes 2 and 6), 5 (lanes 3 and 7), or 7.5 (lanes 4 and 8) gray or unirradiated controls (lanes 1 and 4). Expression of α-tubulin was used as a loading control. Immunoblots are representative of two independent experiments.

**Fig. 3. Expression of IGFBP-5 induces caspase-dependent apoptosis.** A, 4,6-diamidino-2-phenylindole-stained cells were scored for nuclear fragmentation, a morphological marker of apoptosis following a 48-h incubation in SF media, and the percentage of apoptotic cells was determined (magnification ×40). Cellular fluorescence from the green fluorescent protein marker is also visible. Values shown are means of triplicate wells from two independent experiments ± S.E. B, levels of apoptosis were determined in MDA-MB-231 and Hs578T cells 48 h post-infection with IGFBP-5 or vector adenovirus, under SF conditions. Cells were analyzed by flow cytometry, and the percentage of cells in the pre-G1 peak was determined. Values shown are means of triplicate wells from at least four independent experiments ± S.E. C, the effect of IGFBP-5 on the long term survival of MDA-MB-231 cells in response to apoptotic stimuli was determined. MDA/VEC (white bars) and MDA/BP-5 (black bars) stable transfectants were irradiated with various doses of x-rays as indicated, and survival was measured 14 days post-irradiation by clonogenic assays. Values shown are means of three data sets from two independent experiments ± S.E. D, expression of Bax was determined by immunoblot analysis 4 days post-irradiation in MDA/VEC and MDA/BP-5 cells irradiated with 2.5 (lanes 2 and 6), 5 (lanes 3 and 7), or 7.5 (lanes 4 and 8) gray or unirradiated controls (lanes 1 and 4). Expression of α-tubulin was used as a loading control. Immunoblots are representative of two independent experiments.
FIG. 4. IGFBP-5 expression is associated with modulation of Bcl-2-like proteins. A, expression of Bax and Bcl-2 was determined by immunoblot analysis in MDA/VEC (lane 1) and MDA/BP-5 (lane 2) and cells were infected with vector (lanes 3 and 5) or IGFBP-5-adenovirus (lanes 4 and 6) following a 48-h incubation in SF media. Expression of α-tubulin was used as a loading control. Protein levels were determined by densitometric analysis and values from two independent experiments ± S.E. are shown in the graph. B, total RNA was extracted from stable transfectants and adenoviral-infected cells following a 48-h incubation in SF media, and analyzed for expression of IGFBP-5 and bax mRNA by Northern blotting. Human IGFBP-5 cDNA is 0.9 kb, also shown is low level expression of endogenous IGFBP-5 mRNA (6 kb). Lanes are as above. The ribosomal phosphoprotein 36B4 was used as a loading control. The graph shows the means of bax mRNA levels from two experiments quantitated densitometrically in relation to the control gene 36B4 and expressed as a percentage of the corresponding vector levels ± S.E. C, real time quantitative PCR was used to quantify bcl-2 mRNA levels in IGFBP-5-expressing cells and vector controls, following 48 h incubation in SF media. Values are corrected using actin expression and expressed relative to VEC controls. Values shown are means of duplicate samples from two experiments ± S.E.
cells for 48 h with 1 μg/ml of each of the IGFBP-5 fractions or rhIGFBP-5 expressed by 911 retinoblastoma cells (27), then analyzed cells for DNA fragmentation by flow cytometry. Fig. 6B demonstrates that none of the forms of IGFBP-5 analyzed had a significant effect on the induction of apoptosis compared with untreated controls. We also determined the effect of 24 h incubation with intact or proteolyzed IGFBP-5 on DNA synthesis using incorporation of [3H]thymidine. Fig. 6C shows that there was no significant change in the rate of DNA synthesis following treatment with any of the fractions compared with untreated controls.

These results indicate that the cytostatic and cytotoxic effects that we have observed in IGFBP-5-expressing cells are not initiated by IGFBP-5 or its fragments from outside the cell, suggesting that they may be mediated by intracellular IGFBP-5. We examined the expression of IGFBP-5 in the cytoplasmic and nuclear compartments of both stable transfectants and adenoviral-infected MDA-MB-231 and Hs578T cells by IGF-II ligand blot. Purity of each fraction was confirmed by immunoblot analysis of CPP32 (cytoplasmic, Ref. 30) and poly(ADP-ribose) polymerase (nuclear, Ref. 31) expression. Fig. 6D illustrates that active IGFBP-5 was expressed in both the nuclear and cytoplasmic fractions of IGFBP-5-expressing cells. Levels of IGFBP-5 expression in Hs578T cells were higher than adenoviral-infected MDA-MB-231 cells, despite these cells being infected with similar efficiency as assessed by green fluorescent protein expression (data not shown). This may explain the more potent proapoptotic effects of IGFBP-5 in these cells. Interestingly, an additional, higher molecular weight band of ~60,000 was observed in Hs578T lysates and to a lesser extent in MDA-MB-231 cells, consistent with an IGFBP-5 dimer. IGFBP-5 bands visible in stable vector-transfected MDA-MB-231 cells, representing low-level endogenous expression, result from the long exposure of the blot. However, interestingly, there did not appear to be a significant increase in the expression of nuclear IGFBP-5 in MDA/BP-5 compared with MDA/VEC, despite the fact that IGFBP-5 expression is proapoptotic in the former cell population. This is consistent with our recent observations that nuclear translocation of IGFBP-3 is not required to mediate its proapoptotic effects (3).

To determine whether the active (i.e. able to bind IGFs) IGFBP-5 detected intracellularly in adenoviral-infected cells had first been secreted and then taken up by the cells, we incubated MDA-MB-231 and Hs578T cells with 30-kDa CM-derived IGFBP-5 (Fig. 6A, lanes 1 and 4, respectively) for 48 h, then examined the levels of intracellular IGFBP-5 by ligand blot analysis. Fig. 6E demonstrates that there was no detectable uptake of exogenous IGFBP-5 after 48 h incubation. Paint bands visible in some of the lanes represent low levels of endogenous IGFBPs because of the long exposure of the blot. Use of immunoblot to detect IGFBP-5 was unsuccessful because of multiple nonspecific bands (data not shown).

IGFBP-5 Is Inhibitory to the Growth of MDA-MB-231 Breast Cancer Cells in Vivo—MDA/VEC or MDA/BP-5 cells were injected into groups of 12 or 13 female nu/nu mice, respectively, and tumor size was measured weekly up to 4 weeks post-injection when some tumors had reached ~1 cm³. The percentage of animals that developed visible tumors was significantly reduced in the MDA/BP-5 group (46.15%) compared with the MDA/VEC group (100%, p = 0.002 by χ² test). Tumors derived from MDA/BP-5 had a significantly decreased growth rate (as determined by tumor volume) compared with MDA/VEC tumors (Fig. 7A; p < 0.0001). In addition, there was a significant decrease in the wet tumor weight of MDA/BP-5 tumors compared with MDA/VEC tumors (Fig. 7B, p < 0.0005).

To confirm that tumors derived from MDA/BP-5 cells maintained expression of IGFBP-5 in vivo, we isolated RNA from the tumors and determined the levels of IGFBP-5 mRNA expression by Northern blot analysis. The transfected human IGFBP-5 cDNA codes for a message of 0.9 kb. A band of this size was detected in all the MDA/BP-5 tumors and not in the vector control tumors (Fig. 7C). We also analyzed RNA from MDA/BP-5 cells as a positive control for IGFBP-5 cDNA expression (Fig. 7C, lane C). Serum samples were analyzed for IGFBP-5 expression using an IGFBP-5-specific radioimmunoassay but levels were too low to be detected by this method.

Inhibitory Effects of IGFBP-5 on the Growth of MDA-MB-231 Cells in Vivo Are Associated with an Induction of bax mRNA and Decrease in bcl-2 mRNA—We investigated the possible mechanism of IGFBP-5-induced growth inhibition in vivo, by examining the mRNA levels of the apoptotic regulators, bax and bcl-2 in MDA/BP-5- and MDA/VEC-derived tumors using real time PCR analysis (Fig. 7D). Levels of mRNA were expressed as a ratio of the control gene actin and as a percentage of the levels in MDA/VEC-derived tumors. There was a significant induction of bax mRNA expression and a significant decrease in bcl-2 mRNA levels in IGFBP-5-expressing tumors compared with vector controls (p < 0.02, p < 0.04, respectively).

DISCUSSION

The role of IGFBP-5 in the growth of human breast cancer cells is not well understood, with some studies suggesting it may be growth inhibitory and associated with induction of apoptosis, whereas others demonstrate a survival role in response to apoptotic stimuli. To address this ambiguity, we have examined and characterized the effects of IGFBP-5 on the growth of human breast cancer cells both in vitro and in vivo.

The human breast cancer cell lines MDA-MB-231 and Hs578T both secrete low levels of IGFBP-5 (<10 ng/ml). Stable transfection of MDA-MB-231 cells with IGFBP-5 cDNA resulted in an increase in secreted levels to ~40 ng/ml. Other breast cancer cell lines secrete similar or higher levels than this (e.g. MCF-7 cells secrete ~30 ng/ml) emphasizing the relevance of this model in studying the effects of IGFBP-5 on breast cancer cell growth. Interestingly, T47D breast cancer cells secrete much higher levels of IGFBP-5 (~200 ng/ml), yet
are tumorigenic and have low basal levels of apoptosis (2). This suggests that some cancer cells may acquire resistance to the cellular effects of IGFBP-5, a phenomenon that we have previously reported to occur for IGFBP-3 (37), and which may involve ras-dependent signaling pathways (28).

Both stable and adenoviral-mediated expression of IGFBP-5 in MDA-MB-231 and Hs578T cells resulted in a significant inhibition of in vitro cell growth, an arrest in G2/M phase of the cell cycle, and a decrease in DNA synthesis. Interestingly, despite much higher levels of IGFBP-5 expression in adenoviral-infected cells compared with stable transfectants, a similar degree of growth inhibition was observed. This suggests that the mechanisms that initiate these inhibitory effects can reach a saturation level beyond which further increases in IGFBP-5 have no additional effect. Indeed, even at the lower levels of IGFBP-5 expression in stable transfectants, breast tumor growth in nude mice was ablated. Antiproliferative effects have been observed in cervical carcinoma cells following addition of up to 200 ng/ml exogenous IGFBP-5 (8) and following stable transfection of IGFBP-5 cDNA in osteosarcoma cells (6). In breast cancer cells, endogenous IGFBP-5 has been shown to mediate the growth inhibitory effects of antiestrogens (10) and...
vitamin D-related compounds (9, 38). However, Perks et al. (20) reported that addition of 100 ng/ml exogenous IGFBP-5 had no direct effects on the growth of Hs578T cells. Interestingly, we have previously reported that the antiproliferative effects of IGFBP-3, which shares much structural and functional homology with IGFBP-5, are associated with a cell cycle arrest in the G1/S phase (3). This suggests that IGFBP-3 and IGFBP-5, while both inhibitory to breast cancer growth, may influence cell cycle progression via distinct pathways.

IGFBP-5 expression resulted in the induction of a caspase-dependent pathway of apoptosis, and reduced the survival of breast cancer cells following the apoptotic stimulus of IR, in association with increased protein levels of the proapoptotic regulator Bax. Furthermore, expression of IGFBP-5 both in vitro and in vivo induced a transcriptional up-regulation of bax and down-regulation of the anti-apoptotic bcl-2, suggesting IGFBP-5 may induce a mitochondrial apoptotic pathway. Surprisingly, changes in Bcl-2 protein were not seen following transient, adenoviral-mediated expression of IGFBP-5, possibly because of the short term nature of this experiment. However, levels of Bax were up-regulated both basally and in response to radiation, altering the critical ratio of these mediators toward apoptosis (39). We have previously demonstrated that IGFBP-3 also has intrinsic antiproliferative and proapoptotic effects in human breast cancer cells and modulates expression of Bcl-2-like proteins (3). These current results suggest that IGFBP-5 may act along a similar intracellular apoptotic pathway, possibly by direct transcriptional modulation of apoptotic genes. These results are in contrast to studies by Perks et al. (20) in Hs578T cells who showed that, whereas exogenous IGFBP-5 had no direct cytotoxic effects, it inhibited apoptosis induced by ceramide or integrin detachment (21). This may reflect post-translational differences in exogenous and endogenously produced IGFBP-5 (for example, glycosylation state), or indicate that intracellular expression of IGFBP-5 interacts with different, cell surface-independent signaling pathways as has been suggested by our recent studies with IGFBP-3 (3).

The inhibitory and proapoptotic effects of IGFBP-5 on breast cancer cell growth appear to be independent of IGF signaling, as neither MDA-MB-231 nor Hs578T cells (36) are responsive to the mitogenic and anti-apoptotic effects of IGFs. Previous
IGFBP-5-induced Growth Inhibition and Apoptosis

Acknowledgments—We thank Joelyn Weiss for assistance with mice injections, Phillip Vial and Regina Phillips (Clinical Oncology, Royal North Shore Hospital, Sydney) for irradiating breast cancer cells, Dr. Malcolm King (Clinical Immunology, Royal North Shore Hospital, Sydney) for assistance with flow cytometry, Dr. Patric Delhanty (Kolling Institute) for help with real time PCR, and Dr. Sue Firth (Kolling Institute) for providing the full-length IGFBP-5 cDNA.

REFERENCES

1. Jones, J. I., and Clemmons, D. R. (1995) Endocr. Rev. 16, 3–34.
2. Butt, A. J., Firth, S., King, M. A., and Baxter, R. C. (2000) J. Biol. Chem. 275, 39174–39181.
3. Butt, A. J., Fraley, K. A., Firth, S. M., and Baxter, R. C. (2002) Endocrinology 143, 2690–2699.
4. Rajah, R., Valentini, B., and Cohen, P. (1997) J. Biol. Chem. 272, 12181–12188.
5. Andres, D. L., and Birnbaum, R. S. (1992) J. Biol. Chem. 267, 22467–22472.
6. Schneider, M. R., Zhou, R., Hoeflich, A., Krebs, O., Schmidt, J., Mohan, S., Wolf, E., and Lahm, H. (2001) Biochem. Biophys. Res. Commun. 288, 445–449.
7. Ewton, D. Z., Coohil, S. A., Mohan, S., Chernausek, S. D., and Florini, J. R. (1998) J. Cell Physiol. 177, 47–57.
8. Higo, H., Duan, C., Clemmons, D. R., and Herman, B. (1997) Biochem. Biophys. Res. Commun. 239, 706–709.
9. Rosen, F., Yang, X. F., Huyhn, H., and Pollak, M. (1997) J. Natl. Cancer Inst. 89, 452–456.
10. Huyhn, H., Yang, X. F., and Pollak, M. (1996) Cell Growth Diff. 7, 1501–1506.
11. Chen, J. C., Shao, Z. M., Shekikh, M. S., Hussain, A., LeRoith, D., Roberts, C. T., and Fontana, J. A. (1994) J. Cell Physiol. 153, 69–78.
12. Cockett, T. W., Pollak, M., and Firth, S. M. (1999) Clin. Cancer Res. 7, 2545–2554.
13. Nickerson, T., Pollak, M., and Huyhn, H. (1998) Endocrinology 138, 807–810.
14. Tenner, E., Barber, M. C., Travers, M. T., Logan, A., and Flint, D. J. (1997) J. Biol. Chem. 272, 20922–20928.
15. Tenner, E., Barber, M. C., Allan, G. J., Beattie, J., Webster, J., Whiteall, C. B. A., and Flint, D. J. (2002) Development 129, 4547–4557.
16. Bernard, N., Pisselet, C., Monniaux, D., Locatelli, A., Benz, P., Gasser, F., Hafez, E., and Monget, P. (1996) Biol. Reprod. 55, 1356–1367.
17. Beilharz, E. J., Kempt, N. D., Kempt, M., Sirimanne, R., Draganon, M., and Gluckman, P. D. (1983) Brain Res. Mol. Brain Res. 18, 209–215.
18. Allan, G. J., Flint, D. J., Darling, S. M., Geh, J., and Patel, K. (2000) Anat. Embryol. 202, 1–11.
19. Monget, P., Pisselet, C., and Monniaux, D. (1998) J. Cell Physiol. 177, 13–25.
20. Perkins, C. M., Bowen, S., Gill, Z. P., Newcomb, P. V., and Holly, J. M. P. (1999) J. Cell Biochem. 75, 652–664.
21. Perks, C. M., McCraig, C., and Holly, J. M. P. (2000) J. Cell Biochem. 80, 245–258.
22. Roscher, M., Kussisto, E., Suuronen, T., Korhonen, P., Kyyrekko, L., and Salminen, A. (2001) J. Neurochem. 76, 11–20.
23. Miyake, H., Polakk, M., and Gleave, M. E. (2000) Cancer Res. 60, 3058–3064.
24. Yee, D. A., Sharma, A., and Hilsenbeck, S. G. (1994) J. Natl. Cancer Inst. 86, 1785–1789.
25. Figueroa, J. A., and Yee, D. (1992) Breast Cancer Res. Treat. 22, 81–90.
26. Firth, S. M., Clemmons, D. R., and Baxter, R. C. (2001) Endocrinology 142, 2147.
27. Firth, S. M., Ganeshrasad, U., Porrorn, P., Cook, D. I., and Baxter, R. C. (1999) Protein Exp. Purif. 16, 202–211.
28. Martin, J. L., and Baxter, R. C. (1999) J. Biol. Chem. 274, 16407–16411.
29. Baxter, R. C., Meka, S., and Firth, S. M. (2002) J. Clin. Endocrinol. Metab. 87, 721–726.
30. Mancini, M., Nicholson, D. W., Roy, S., Thornberry, N. A., Peterson, E. P., Casciola-Rosen, L. A., and Rosen, A. E. (1998) J. Cell Biol. 140, 1485–1495.
31. de Murcia, G., and Menissier de Murcia, J. (1994) Trends Biochem. Sci. 19, 172–176.
32. Delhanty, P. J. D., and Baxter, R. C. (1998) Endocrinology 139, 260–265.
33. Masakowski, P., Breathnach, R., Bloch, J., Gannon, F., Krust, A., and Chambon, P. (1982) Nucleic Acids Res. 10, 7895–7903.
34. Baserga, R., Hong, A., Rubin, M., Prisco, M., and Valentini, B. (1997) Biochem. Biophys. Acta 1332, F103–F126.
35. Sicari, C., Costantino, A., Protagli, M., Wilkes, J., Frasca, S., Scalpi, A., Sbracia, P., Goldfine, I. D., Vigneri, R., and Belfiore, A. (1999) Oncogene 18, 2471–2479.
36. Chb, Y. M., Muller, H. L., Lamson, G., and Rosenfeld, R. G. (1993) J. Biol. Chem. 268, 14964–14971.
37. Firth, S. M., Fanayan, S., Dunn, B., and Baxter, R. C. (1998) Biochem. Biophys. Res. Commun. 246, 325–329.
38. Rosen, F., and Pollak, M. (1999) Int. J. Oncol. 15, 589–594.
39. Sedlak, T. W., Oliva, Z. N., Yang, E., Wang, K., Boise, L. H., Thompson, C. B., and Korsmeyer, S. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7834–7838.
40. Andres, D. L. (1998) Am. J. Physiol. 274, E744–E750.
41. Andres, D. L., Loop, S. M., Zapf, J., and Kiefer, M. C. (1993) Biochem. Biophys. Res. Commun. 195, 25–30.
42. Schedl, L. J., Young, T., Firth, S. M., and Baxter, R. C. (1998) J. Biol. Chem. 273, 18347–18352.
43. Runnebaum, I. B., Nagarajan, M., Bowman, M., Soto, D., and Sukumar, S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10567–10571.
44. Osborne, R., Merlo, G. R., Mitsudomi, T., Venesio, T., Liscia, D. S., Cappa, A. P. M., Chiba, I., Takahashi, T., Nau, M. M., Callahan, R., and Minna, J. (1991) Cancer Res. 51, 6194–6198.
