Journal of Cellular Biochemistry 75:652–664 (1999)

Differential IGF-Independent Effects of Insulin-Like Growth Factor Binding Proteins (1–6) on Apoptosis of Breast Epithelial Cells

Claire M. Perks,* Samantha Bowen, Zahidah P. Gill, Paul V. Newcomb, and Jeff M.P. Holly

Division of Surgery, Department of Hospital Medicine, Bristol Royal Infirmary, Bristol BS2 8HW, United Kingdom

Abstract We have demonstrated previously that insulin-like growth factor binding protein (IGFBP)-3 alone has little growth inhibitory effect on Hs578T human breast cancer cells, but that it can dramatically accentuate the apoptotic response to the physiological trigger, ceramide, in an IGF-independent manner. We have now studied the potential of other IGFBPs (1–6) to interact with apoptotic signalling pathways. Hs578T cells were preincubated with a binding protein (100 ng/ml) for 24 h, followed by co-incubation of the binding protein with an apoptotic dose of ceramide or RGD-containing peptide for a further 24 h. Apoptosis was assessed using flow cytometry, MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) assay and morphological assessment. Binding protein profiles were determined using ligand and immunoblotting techniques. Each of the IGFBPs (1–6) alone had no significant ($P > 0.05$) growth inhibitory effects relative to control cells. In contrast to IGFBP-3, which significantly ($P < 0.05$) accentuated C2-induced apoptosis, IGFBP-1, -2, and -6 had no effect, whereas IGFBP-4 and -5 each caused marked ($P < 0.01$) inhibition of ceramide-induced programmed cell death. Apoptosis induced by RGD was also significantly ($P < 0.05$) reduced by IGFBP-5, whereas IGFBP-3 had no effect. These data provide evidence to suggest that individual IGFBPs have specific IGF-independent effects and act differentially on apoptotic signalling pathways. J. Cell. Biochem. 75:652–664, 1999.

Key words: apoptosis; ceramide; IGFBP-1–6; human breast epithelial cells

Tissue homeostasis in a multicellular organism is maintained by a balance between cell proliferation and programmed cell death. Apoptosis is a highly regulated mode of cell death characterised by a number of morphological and biochemical features, including blebbing of the plasma membrane, cell shrinkage, chromatin condensation, and DNA fragmentation into membrane-bound vesicles [Wyllie et al., 1980].

We have been using two physiological inducers of apoptosis: a ceramide analogue, C2, and a synthetic RGD-containing peptide, RGD. The plasma membrane is the site of sphingomyelin hydrolysis, which is now recognised as an important pathway of signal transduction. Ceramide is one product of sphingomyelin hydrolysis and has been implicated as an important mediator of cell death [Obeid et al., 1993]. Tumour growth can therefore be restricted by cytokines, chemotherapy, and radiotherapy, which induce programmed cell death by ceramide-mediated cytoplasmic signalling.

The growth of normal adherent cell types in vitro requires growth factors, but it also requires attachment to the extracellular matrix (ECM) [Ingber et al., 1990]. Cell adhesion to the ECM is mediated by integrin receptors. These bind to matrix proteins outside the cell and associate with cytoskeletal proteins within the cell. These matrix proteins contain the three-amino acid sequence, arginine-glycine-aspartic acid (RGD), which is specifically recognised by their particular integrin receptor [Ruoslahti et al., 1987]. Specifically, integrin-dependent signals have been shown to modulate the control of growth [Giancotti et al., 1990] and cell survival [Frisch et al., 1994]. Maintenance of integrin linkages is essential for cell adhesion. It was previously demonstrated that disruption of these attachments, through the addition of an-

Grant sponsor: Medical Research Council; Grant sponsor: The Wellcome Trust.

*Correspondence to: Claire M. Perks, Division of Surgery, Department of Hospital Medicine, Level 7, Bristol Royal Infirmary, Bristol BS2 8HW, U.K. E-mail: claire.m.perks@bris.ac.uk

Received 31 March 1999; Accepted 19 May 1999

tibodies or peptides, can induce cells to detach from the substratum [Hayman et al., 1985; Knudson et al., 1981], with a resultant induction of programmed cell death. Cells that become transformed or malignant have therefore acquired the ability to undergo anchorage-independent growth [Tucker et al., 1981; Freedman et al., 1974]. Furthermore, IGF-I can protect cells from detachment-induced apoptosis [Valentinis et al., 1998] indicating cross-talk between IGF-I and integrin signalling pathways.

Dysregulation of the rate of cell growth or death can lead to the emergence of many diseases, including neurodegenerative diseases and cancer [Thompson et al., 1995]. The IGF system is composed of ligands, IGF-I and -II, cell receptors, IGFBPs, and IGFBP proteases. The IGFs are the most prevalent growth factor in the body, which, in addition to being general mitogens, are also potent survival factors for many cells. The IGFs can therefore play a significant role in maintaining tissue homeostasis by their ability to both promote cell proliferation and rescue cells from programmed cell death.

To date, the IGFBP family is composed of six distinct IGFBPs that have been described in detail; at least a further three related proteins are thought to exist [Shimasaki et al., 1991; Oh et al., 1996]. It was originally believed that these IGFBPs were simply present to passively modulate the IGF-induced mitogenic and survival signals [Rechler et al., 1993; Jones et al., 1995], being both stimulatory [Blum et al., 1989] and inhibitory [Ritvos et al., 1988] to IGF action.

The major binding protein in adult serum is IGFBP-3, which acts as a carrier of IGF as part of a larger 150 K complex formed by the binding protein bound to IGF, and associated with an acid-labile subunit [Baxter et al., 1993]. Numerous studies on this particular binding protein have implied that it can have more complex effects on cell function than simply passively regulating the IGFs. In addition, IGFBP-3 has been shown to localise to the nucleus [Jaques et al., 1997; Noll et al., 1996].

Data have further demonstrated that IGFBP-3 has an inhibitory role that is in fact independent of IGF-I. Purified mouse IGFBP-3 has been shown to bind to the chick embryo fibroblast cell surface and inhibit cell growth [Lalou et al., 1996]. In addition, human breast cancer cells, Hs578T cells have been shown to possess specific IGFBP-3 binding sites, enabling exogenous IGFBP-3 to inhibit monolayer growth in these cells [Oh et al., 1993]. IGFBP-3 also mediates growth inhibition by transforming growth factor-β TGF-β [Oh et al., 1995], retinoic acid [Gucev et al., 1996], and antiestrogens [Huynh et al., 1996] in breast cancer cells and can specifically induce apoptosis in prostate cancer cells [Rajah et al., 1997]. We have recently demonstrated that IGFBP-3 also has the ability to accentuate ceramide, but not RGD-induced apoptosis in Hs578T, human breast cancer cells [Gill et al., 1997]. In addition, we have shown previously that IGFBP-3 can accentuate both ultraviolet (UV) [Hollowood et al., 1998] and paclitaxel [Fowler et al., 1998] induced apoptosis in an IGF-independent manner.

There are a few examples to illustrate that other binding proteins also have the capacity to act independently of IGF-I. Specifically, IGFBP-1 has been shown to stimulate cell migration in Chinese hamster ovary (CHO) cells [Jones et al., 1993] and IGFBP-5 can stimulate mitogenesis in osteoblasts [Andress et al., 1992]. Despite high structural homology, it is evident that the binding proteins still have the potential to exert a number of differential effects on cell activity, which can be both IGF-dependent or -independent.

The Hs578T cells are an ideal model with which to study the IGF-independent effects of the IGFBPs, since IGFBP-3 has previously been shown to bind to the cell surface and inhibit monolayer growth in these cells in an IGF-independent manner [Oh et al., 1993]. Having previously established that IGFBP-3 was able to accentuate ceramide-induced apoptosis independent of the IGFs in the Hs578T cells [Gill et al., 1997], the aim of this particular study was to compare possible IGF-independent effects of binding proteins -1,-2, -4, -5, or -6 in this model and to establish whether the action of IGFBP-3 was specific to this one binding protein.

MATERIALS AND METHODS

Materials

Human IGFBP-1 peptide was a gift from Dr. J. Cox, Synergen Inc. Human IGFBP-2 was obtained from Sandoz. Recombinant human nonglycosylated IGFBP-3 (ngIGFBP-3) was a kind gift from Dr. C. Maack (Celtrix, CA). IGFBPs -4, -5, and -6 were purchased from Austral Biologicals (CA). The ceramide analogue,

C2, and a synthetic RGD-containing peptide (Gly-Arg-Gly-Asp-Thr-Pro), were obtained from Sigma Chemical Co. (St. Louis, MO) and Calbiochem, respectively. The IGFBP-2 and -4 antibodies were purchased from TCS, whereas IGFBP-3 is an in-house antibody (SCH-2/6). All other chemicals were purchased from Sigma. Tissue culture plastics were obtained from Nunc, Gibco-Life Technologies (Paisley, Scotland).

CELL CULTURES

The human breast cancer cell line Hs578T was purchased from ECACC (Porton Down, Wiltshire) and were grown in a humidified 5% CO_2 atmosphere at 37°C. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with glutamax-1 supplemented with 10% foetal calf serum (FCS), penicillin (5,000 IU/ml), and streptomycin (5 mg/ml)-growth media (GM). Experiments were performed on cells in phenol-red-free serum-free Hepes DMEM and Ham's nutrient mix F-12 (SFM) with sodium bicarbonate (0.12%), bovine serum albumin (BSA) (0.2 mg/ml), transferrin (0.01 mg/ml) and supplemented as before.

Dosing Protocol

Cells were grown in GM for 24 h before switching to SFM for a further 24 h before to dosing. Cells were preincubated with a binding protein (100 ng/ml) for 24 h, followed by co-incubation of the binding protein with an apoptotic dose of ceramide or RGD-containing peptide for a further 24 h.

Flow Cytometry

This technique was used to quantitate the amount of apoptosis present in any given sample. The DNA of apoptotic cells has less capacity to stain than normal cells and appear as a pre-G1 peak on a DNA cell cycle histogram. Cells were seeded at 0.1×10^6 cells/well in six-well plates and dosed as described above. For each experiment, the cells were dosed in triplicate as described under Results. For analysis, both floating cells in the conditioned media and in the phosphate-buffered saline (PBS) wash were collected from each well before trypsinisation. Trypsinised cells were then added to the previous collected solutions. Each sample of cells was fixed in 70% ethanol for a minimum of 24 h before analysis by flow cytometry. The fixed cells were pelleted (6,000 rpm; 5 min) and washed three times with PBS (6,000 rpm; 5 min). The supernatant was removed, and the cells were resuspended in reaction buffer (propidium iodide, 0.05 mg/ml; sodium citrate, 0.1%; RNase A, 0.02 mg/ml; Nonidet-40 [NP-40], 0.3%; pH 8.3) and incubated at 4°C for 30 min before measurement on a FACSCalibur flow cytometer (Becton Dickinson) with an argon laser at 488 nm for excitation. The data were analysed using the Cell Quest software package (Becton Dickinson).

MTT Assay

MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) is converted into a coloured water-insoluble formazan salt by the metabolic activity of viable cells. Cells were seeded at 2.5×10^4 /ml (150 µl growth medium) in 96-well plates and were allowed to grow for 24 h. Growth medium was replaced with SFM 24 h before dosing. MTT reagent (7.5 mg/ml) in PBS was added to the cells (10 µl/well) and the cultures were incubated for 30 min at 37°C. The reaction was stopped by the addition of acidified triton buffer (0.1 M HCl, 10% [v/v] Triton X-100; 50 µl/well); the tetrazolium crystals were dissolved by mixing on a Titertek plate shaker for 20 min at room temperature. The samples were measured on a Bio-Rad 450 plate reader at test wavelength of 595 nm and a reference wavelength of 650 nm.

Western Ligand Blotting Immunoblotting

Hs578T cells ($0.1–0.2 \times 10^6$) were grown to 80% confluency in 6-well plates, then switched to SFM 24 h before dosing. The conditioned media were collected and the cells spun out. The remaining solution was concentrated 10-fold, using Millipore Ultrafree-MC filter units. The pattern of IGF binding proteins secreted by the cells in response to various treatments was assessed by ligand blotting, and the membranes were subsequently immunoblotted for IGFBP-2, -3, and -4 using methods described previously [Clemmons et al., 1990; Sheikh et al., 1992].

Fig. 1. Differential effects of IGFBPs (1–6) on C2-induced apoptosis in Hs578T cells as measured by flow cytometry. Graphic representation of the ratio of cells relative to control of cells in the pre-G1 peak of C2 alone and C2 in combination with IGFBPS (1–6; **A–F**). Graphs show the mean of data from at least three experiments, each performed in triplicate.

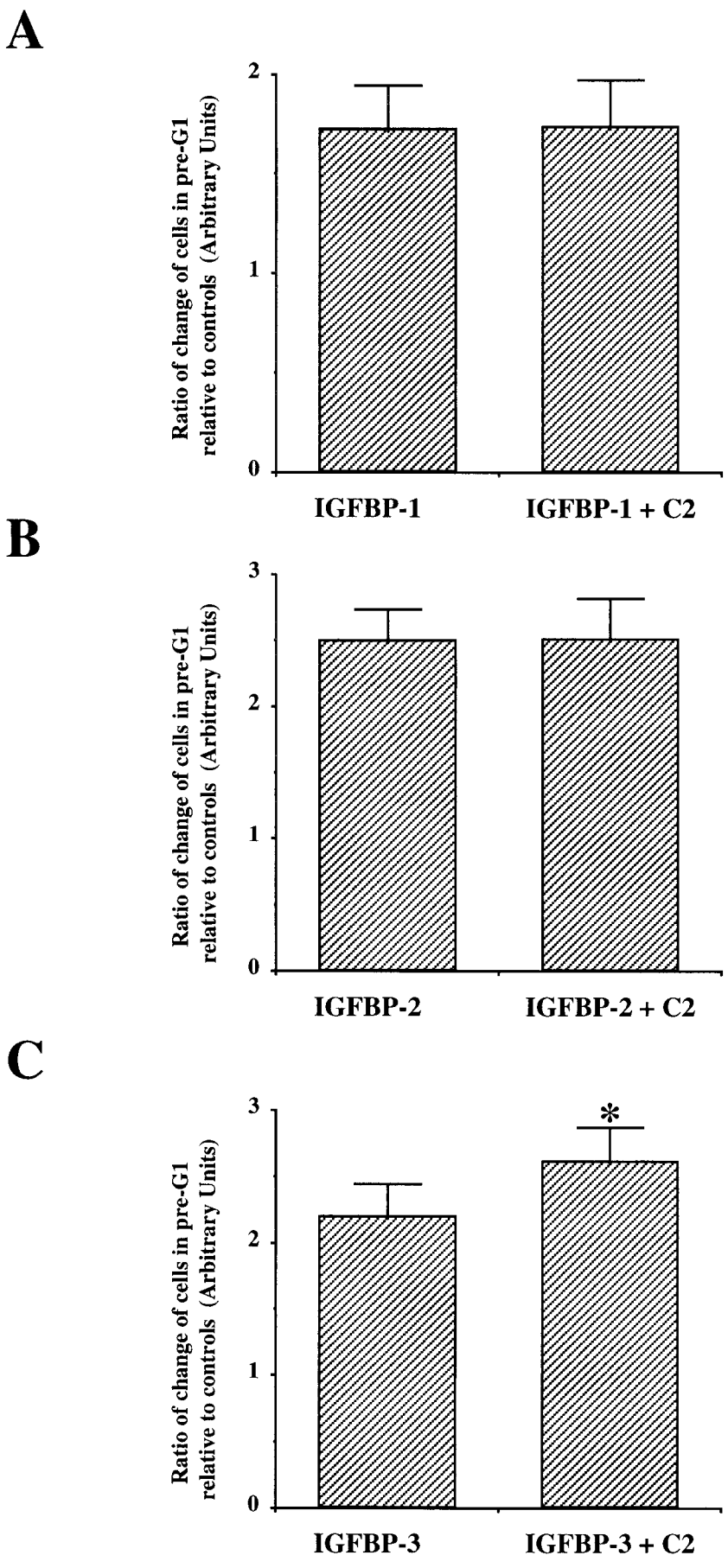
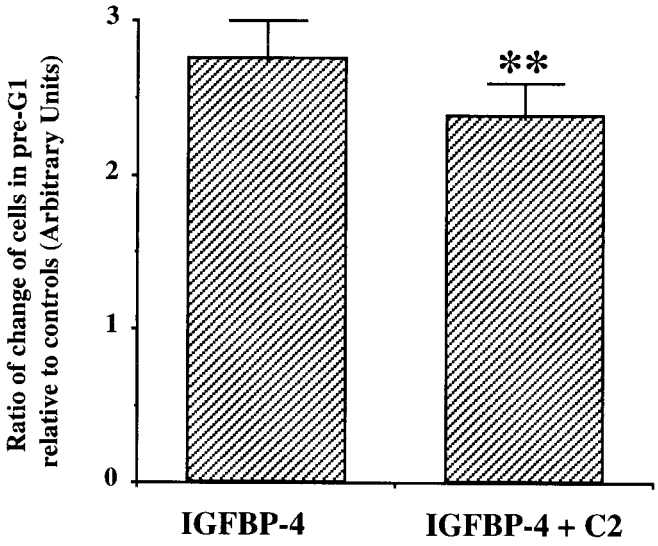
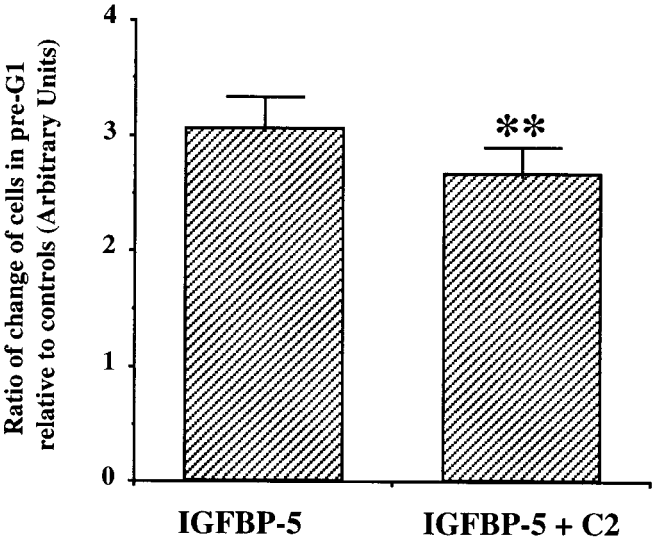

A
Ratio of change of cells in pre-G1 relative to controls (Arbitrary Units)
2
1
0
IGFBP-1
IGFBP-1 + C2
B
Ratio of change of cells in pre-G1 relative to controls (Arbitrary Units)
3
2
1
0
IGFBP-2
IGFBP-2 + C2
C
Ratio of change of cells in pre-G1 relative to controls (Arbitrary Units)
3
2
1
0
*
IGFBP-3
IGFBP-3 + C2

Figure 1.

D



E



F

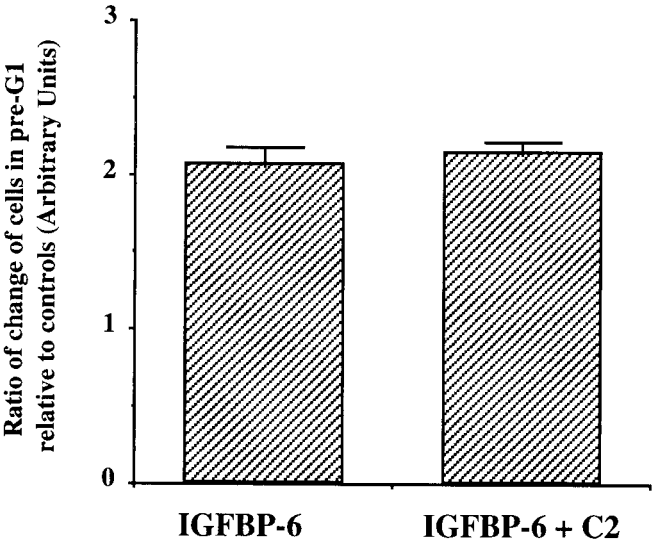


Figure 1. (Continued.)

Briefly, the samples were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); the proteins were then transferred onto a nylon membrane and probed with a mixture of [^{125}I]IGF-I and [^{125}I]IGF-II. The same nylon membrane was then incubated with a specific antibody for either IGFBP-2, -3, or -4 (1:2,000, 1:10,000 and 1:1,000, respectively) overnight at room temperature. After removal of excess unbound antibody, an anti-rabbit antibody (1:10,000) conjugated to peroxidase was added for 1 h. Binding of the peroxidase was visualised using enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham International). The protein content of each sample was determined using a BCA Protein Assay Reagent Kit (Pierce). Optical density measurements were determined using a scanning densitometer (Bio-Rad) and analysed using Molecular Analyst software (Bio-Rad).

Statistical Analysis

The data were analysed using the Microsoft Excel version 5.0a software package. Significant effects were determined using Student's *t*-test. A statistically significant difference was considered to be present at $P < 0.05$.

RESULTS

Flow Cytometry

We have previously established that IGFBP-3 has the capacity to accentuate C2-induced apoptosis in an IGF-independent manner [Gill et al., 1997]. In this study, we initially used flow cytometry to determine whether IGFBPs -1, -2, -4, -5, or -6, like IGFBP-3, had the capacity to interact with the C2-induced apoptotic signalling pathway independently of IGF-I. Figure 1 shows the ratio of change relative to control cells of cells in pre-G1 of samples treated with C2 with or without binding protein (100 ng/ml). Cells were treated with C2 (4–9 μM) alone, IGFBP alone or the combination of C2 and IGFBP. As shown previously [Gill et al., 1997], these data corroborate that C2 alone induced significant ($P < 0.001$) levels of apoptosis, as compared with untreated cells. The dose of C2 was chosen to achieve approximately the same levels of apoptosis in different experiments, as their sensitivity to C2 varied somewhat between experiments. In addition, like IGFBP-3 (100 ng/ml), each of the binding proteins alone (1–6) had no effects on apoptosis relative to the controls. In contrast, to IGFBP-3, which significantly ($P < 0.05$) increased C2-induced apoptosis, we found that IGFBPs -1, -2, and -6 (Fig. 1A,B,F) had no effect on this apoptotic signalling pathway, whereas IGFBP-4 and IGFBP-5 each had the capacity to significantly ($P < 0.01$) inhibit C2-induced programmed cell death in the Hs578T cells.

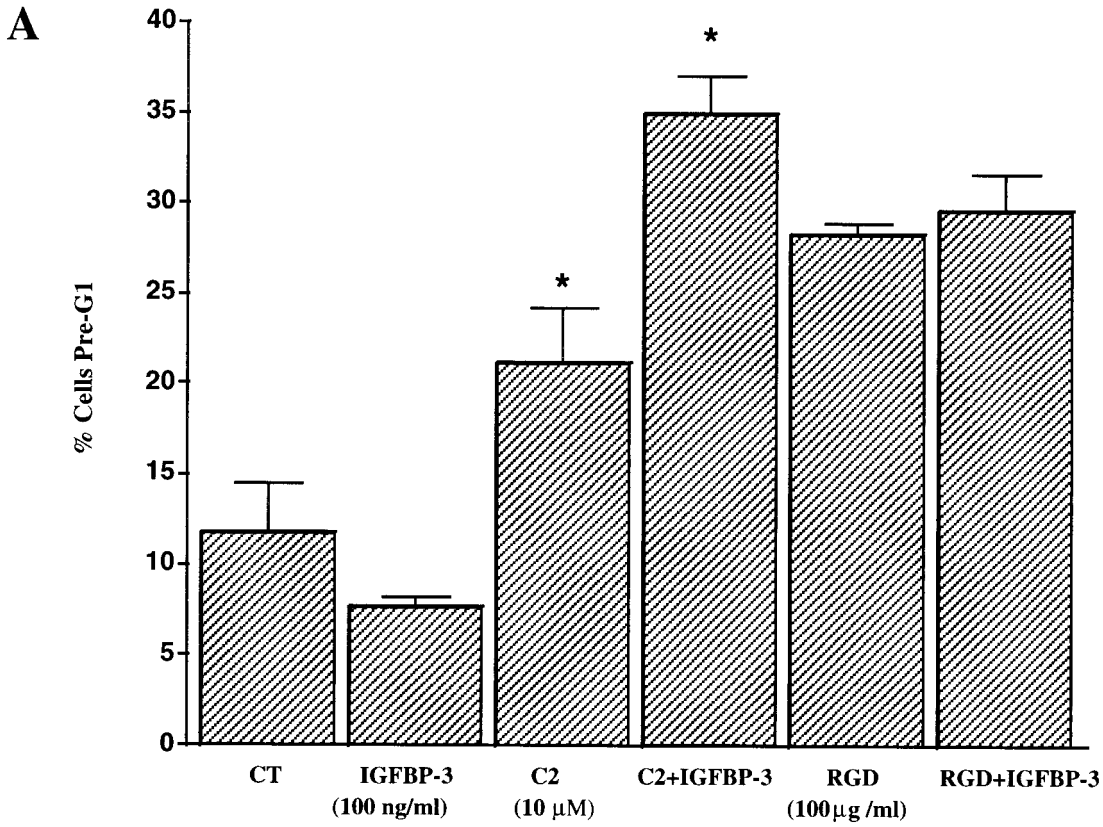
We have also shown previously that IGFBP-3 could accentuate C2, but not RGD-induced apoptosis in this cell line [Gill et al., 1997] (Fig. 2A). We further demonstrated in this study that, in contrast to IGFBP-3, IGFBP-5 had identical effects on both apoptotic pathways in its ability to significantly inhibit RGD or C2-induced programmed cell death (Fig. 2B).

MTT Assay

This technique was used as a crude measurement of cell viability to corroborate the inhibition of C2-induced apoptosis by both IGFBP-4 and -5 seen using flow cytometry. We demonstrated in this assay (Fig. 3A,B) that an apoptotic dose of C2 alone reduced the metabolic activity of the Hs578T cells by approximately 30%. Exogenously added IGFBP-4 or -5 (0–200 ng/ml) alone had no significant ($P > 0.05$) effect on the metabolic activity of the cells. However, it was evident that with increasing doses of IGFBP-4 or -5 the C2-induced reduction in metabolic activity was dramatically attenuated. Specifically, IGFBP-4 significantly ($P < 0.05$) attenuated the C2-induced reduction in metabolic activity by 56% at a dose of 100 ng/ml and by 74% at 200 ng/ml (Fig. 3A). IGFBP-5 significantly ($P < 0.05$) began to attenuate the C2-induced reduction in metabolic activity by 21.6% at a lower dose of 50 ng/ml, 42% at 100 ng/ml, and by 60% at 200 ng/ml.

Morphological Evidence to Support IGFBP-4 and -5 Attenuation of C2-Induced Apoptosis

Figure 4A shows normal, untreated control Hs578T cells. Figure 4B,C shows the addition of 100 ng/ml IGFBP-4 and -5 alone, respectively, indicating no effect on the cells relative to controls. Figure 4D represents cells 24 h after treatment with an apoptotic dose of C2 (5 μM). This illustrates distinct rounding of the cells and a reduction in overall number due to cell detachment. Figure 4E,F shows co-incubation of C2 with IGFBP-4 and -5, respectively. The number of apoptotic cells, as compared


A
% Cells Pre-G1
40
35
30
25
20
15
10
5
0
*
*
CT
IGFBP-3
(100 ng/ml)
C2
(10 μM)
C2+IGFBP-3
RGD
(100μg /ml)
RGD+IGFBP-3

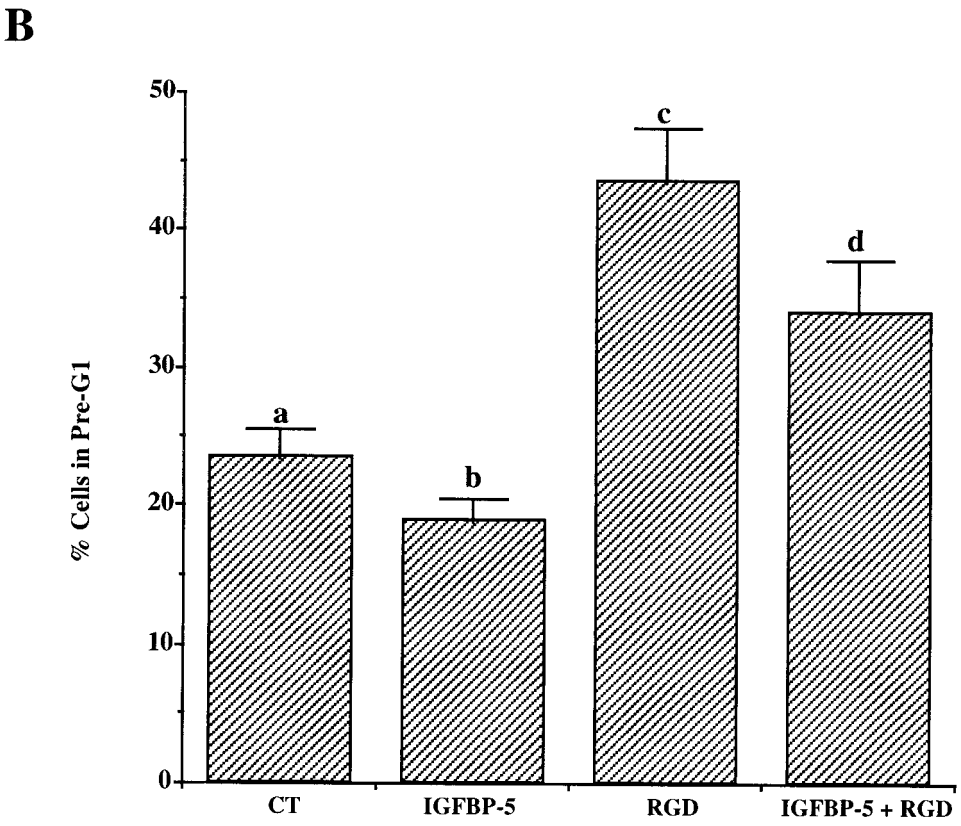

B
% Cells in Pre-G1
50
40
30
20
10
0
a
b
c
d
CT
IGFBP-5
RGD
IGFBP-5 + RGD

Figure 2.

with C2 alone (Fig. 4D) is dramatically reduced with a much higher proportion of live attached cells still remaining.

Ligand and Western Immunoblotting

Figure 5A demonstrates that the apoptotic dose of C2 (Fig. 5A, lane c) caused an approximately 3.4-fold increase in endogenously produced IGFBP-5 (29-kDa) protein but a 2.2-fold decrease of IGFBP-4 (24-kD) protein relative to control cells (Fig. 5A, lane b). Using flow cytometry, we have shown that exogenously added IGFBP-2 (like exogenously added IGFBP-1 and -6) had no effect on C2-induced apoptosis and on the ligand blot IGFBP-2 (Fig. 5A, lane d) and IGFBP-2 and C2 (Fig. 5A, lane e) gave identical binding protein profiles as the control and C2, respectively. The exogenously added IGFBP-2 is also detected on the ligand blot at 35 kDa. As we saw previously [Gill et al., 1997], the added IGFBP-3 is masked on the ligand blot at 30 kDa. The profile for exogenously added IGFBP-3 (Fig. 5A, lane f) and C2 and IGFBP-3 (Fig. 5A, lane g) is again the same as control and C2 profiles, respectively. Exogenously added IGFBP-4 (Fig. 5A, lane h) has the same profile as control cells (Fig. 5A, lane a), except for the obvious increase in IGFBP-4 (endogenous and exogenous). The addition of C2 and IGFBP-4 produces IGFBP-5 levels equivalent to C2 alone (Fig. 5A, lane c), but the C2 has not been able to reduce IGFBP-4 concentrations, as it can on its own. Finally, exogenously added IGFBP-5 alone increases concentrations of IGFBP-5 on the gel (exogenous and endogenous), but there is also an approximately 1.8-fold increase in IGFBP-4 concentrations relative to the control profile. On co-incubation of C2 with IGFBP-5 (Fig. 5A, lane k), the C2 can still reduce levels of IGFBP-4, but only to control levels (Fig. 5A, lane b), and not down to the concentrations it can achieve on its own (Fig. 5A, lane c), and there is still a rise in IGFBP-5.

The ligand blots of conditioned media were subsequently immunoblotted for IGFBP-2, -3, and -4 to investigate any proteolytic modification of these binding proteins. We confirmed that IGFBP-3 was proteolysed (Fig. 5C), as seen previously [Gill et al., 1997]. In addition, we demonstrated that IGFBP-4 and -2 remained intact, regardless of treatment (Fig. 5B,D).

DISCUSSION

It is now becoming increasingly clear that the IGFBPs are more than simply modulators of IGF activity, tightly controlling their supply. We have shown previously that IGFBP-3 has the ability to markedly accentuate ceramide-induced apoptosis in Hs578T cells. These cells lack a functional type 1 IGF receptor, therefore IGFBP-3 is interacting with C2-induced programmed cell death in an IGF-independent manner [Gill et al., 1997]. IGFBP-3 therefore has the capacity to influence both the availability of the IGFs to act on the cells but also has the ability to directly affect apoptotic signalling.

We have now further demonstrated that this effect of IGFBP-3 is specific for this binding protein and that other binding proteins also have differential effects on ceramide induced apoptosis in Hs578T human breast cancer cells. In addition to ceramide, we have also used a synthetic RGD-containing peptide as an alternative physiological initiator of apoptosis. It acts by disrupting integrin attachment, causing cells to detach from the extracellular matrix with the subsequent induction of programmed cell death.

In contrast to IGFBP-3 [Gill et al., 1997], we found that IGFBPs -1, -2, and -6 did not have the ability to interact with C2-induced apoptosis, but that IGFBP-4 and -5 each were able to significantly inhibit this apoptotic signalling pathway. In additon, although IGFBP-3 had no effect on RGD-induced apoptosis, IGFBP-5 was also able to significantly inhibit this apoptotic pathway.

All the binding proteins share some structural and functional similarities, but other particular characteristics are shared by only a few [Clemmons, 1992]. They undergo differential phosphorylation or glycosylation, which may affect their actions on cell function. Specifically, both IGFBP-1 and -2 possess an RGD sequence (arginine-glycine-aspartic acid). This sequence

Fig. 2. Effects of IGFBP-3 and -5 on apoptosis induced by a synthetic RGD-containing peptide. Graphs represent the percentage of cells in the pre-G1 peak of each treated sample. **A:** Untreated cells, 100 ng/ml IGFBP-3 alone, C2 (10 μM) alone, C2 + IGFBP-3, synthetic RGD-containing peptide (100 μg/ml) alone, and IGFBP-3 + RGD, where C2 > CT; $P < 0.05$, and C2 + IGFBP-3 > C2; $P < 0.05$.**B:** Untreated cells, 100 ng/ml IGFBP-5 alone, RGD (100 μg/ml) alone and IGFBP-5+ RGD, where a : b, $P > 0.05$, c + d > a; $P < 0.001$; and d < c; $P < 0.05$. Graphs show the mean of data from at least three experiments which were each performed in triplicate.

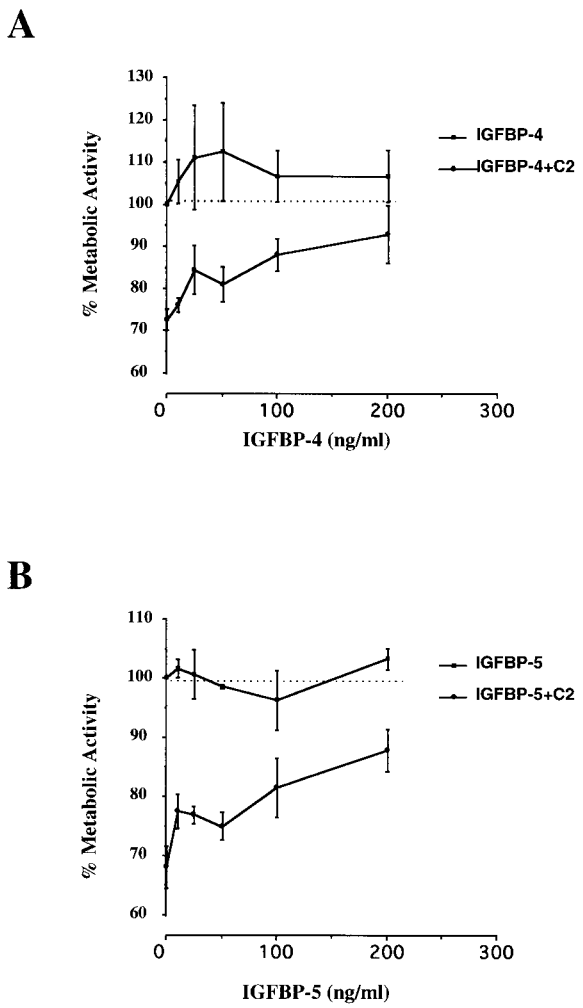


Fig. 3. Addition of IGFBP-4 or -5 (100 ng/ml) to the Hs578T cells inhibits C2-induced apoptosis. Cells were seeded in 96-well plates, and all were switched to SFM before a preincubation with IGFBP-4 or -5 alone (0–200 ng/ml) for 24 h. After this preincubation, all plates were incubated with either IGFBP-4 or -5 alone or co-incubated with IGFBP-4 or -5 and the ceramide-analogue -C2 for a further 24 h. MTT activity was assayed as described under Experimental Procedures. The results represent the mean of three different experiments. Each experiment is composed of five replicates.

enables IGFBP-1 to associate with the cell surface via binding to the fibronectin receptor [Jones et al., 1993]. The effects of IGFBP-1 seen on cell migration [Jones et al., 1993] and FAK dephosphorylation and subsequent apoptosis [Perks et al., 1999] were all achieved at concentrations which were 8- to 10-fold in excess of those used in this study. These higher doses were required specifically to study the effects of IGFBP-1, which were mediated via its RGD sequence. IGFBP-3 possesses a nuclear localization sequence [Jaques et al., 1997]. In addition, all binding proteins contain heparin binding sites. These are of highest affinity in IGFBF-3 and -5, which permit attachment to the cell surface [Fowlkes et al., 1997].

Previously we determined that IGFBP-3 could accentuate ceramide-induced apoptosis in Hs578T cells [Gill et al., 1997]. Whereas IGFBP-3 and -5 are the most structurally similar proteins, they had opposite effects on ceramide-induced apoptosis in this model. However, specific putative receptors for both IGFBP-3 and IGFBP-5 have been described [Oh et al., 1993; Andress et al., 1998], which may explain differential effects despite their structural similarity.

We also showed previously that added IGFBP-3 was proteolytically modified by the cells, indicating that the enhanced ceramide-induced apoptosis could have been effected by the intact IGFBP-3 or a fragment thereof, particularly since a fragment of IGFBP-3 has been reported to inhibit the growth of chick embryo fibroblasts [Lalou et al., 1996]. We further demonstrated in this article that IGFBP-2 and -4 remain intact.

The C2-induced apoptosis appeared to be accompanied by relative changes in endogenously produced levels of IGFBP-4 and -5, IGBP-5 was increased concomitant with a decrease in IGFBP-4. In vivo there are numerous examples of tissue remodelling in which dramatic upregulation occurs in the levels of these binding proteins, such as in atretic human ovarian follicles [El Roiey et al., 1994], and in the involuting mammary gland [Tonner et al., 1997]. These previous studies indicated that increases in either IGFBP-4 or -5 were associated with apoptosis, whereas we have provided the first evidence of direct anti-apoptotic actions of each of these binding proteins on ceramide-induced apoptosis. Furthermore, we also showed C2-induced apoptosis was associated with an increase in IGFBP-5 but a decrease in IGFBP-4, indicating that a balance in levels of these proteins may also be an important aspect in the initiation of apoptosis.

Although IGFBP-4 and -5 are relatively dissimilar in strucure, they had identical effects on programmed cell death. We first speculated that perhaps these added binding proteins were affecting the proteolysis of endogenous IGFBP-3. However, we then established that IGFBP-5 could rescue the cells from both RGD and C2-induced apoptosis, whereas IGFBP-3 can only accentuate C2-induced pro-

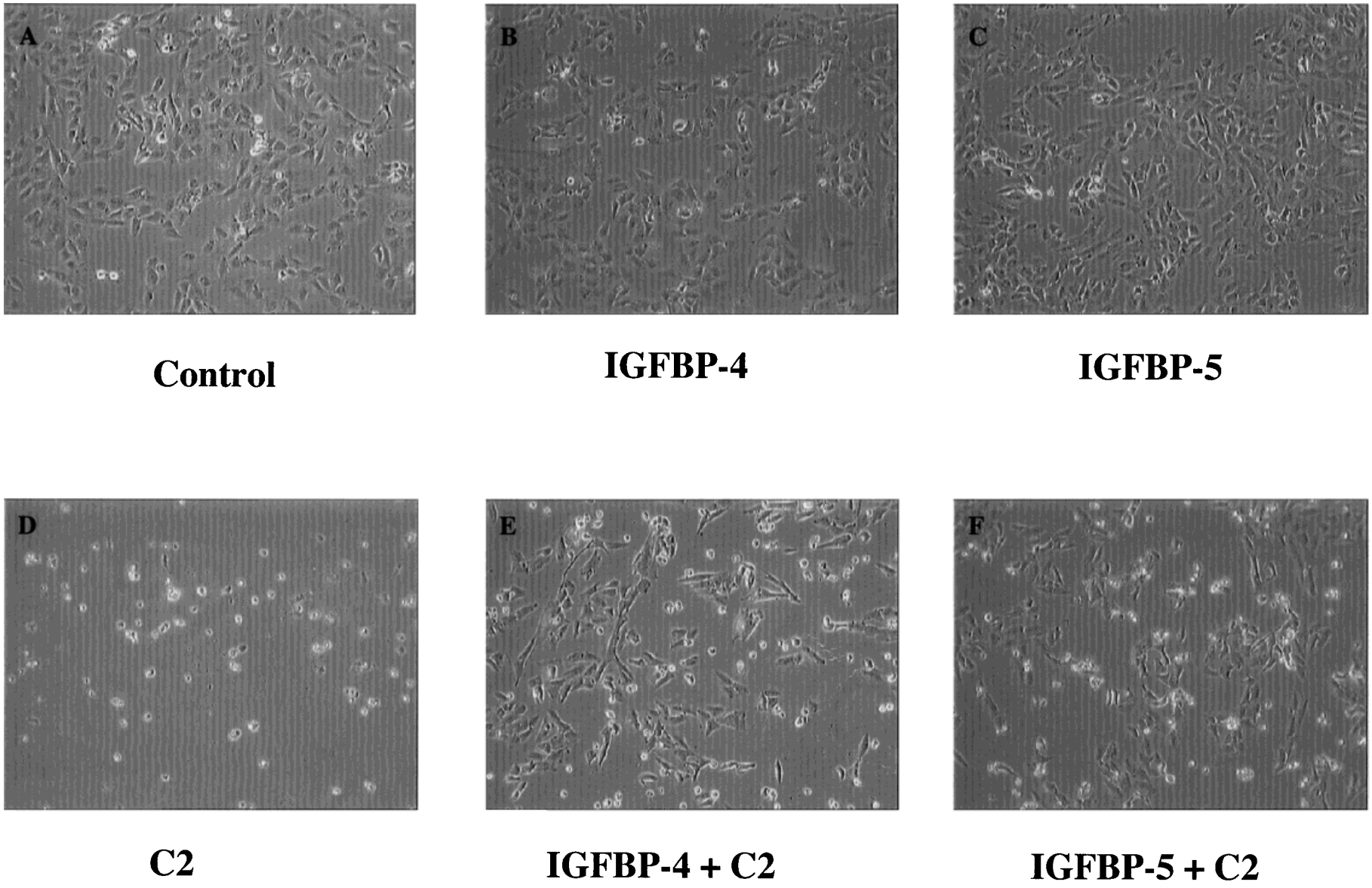


Fig. 4. Morphological evidence to demonstrate that C2-induced apoptosis in Hs578T cells is inhibited by the addition of IGFBP-4 or -5 (100 ng/ml). **A:** Untreated cells. **B:** IGFBP-4 (100 ng/ml) alone. **C:** IGFBP-5 (100 ng/ml) alone. **D:** Ceramide analogue (C2; 5 μm) alone. **E:** IGFBP-4 + C2. **F:** IGFBP-5 + C2.

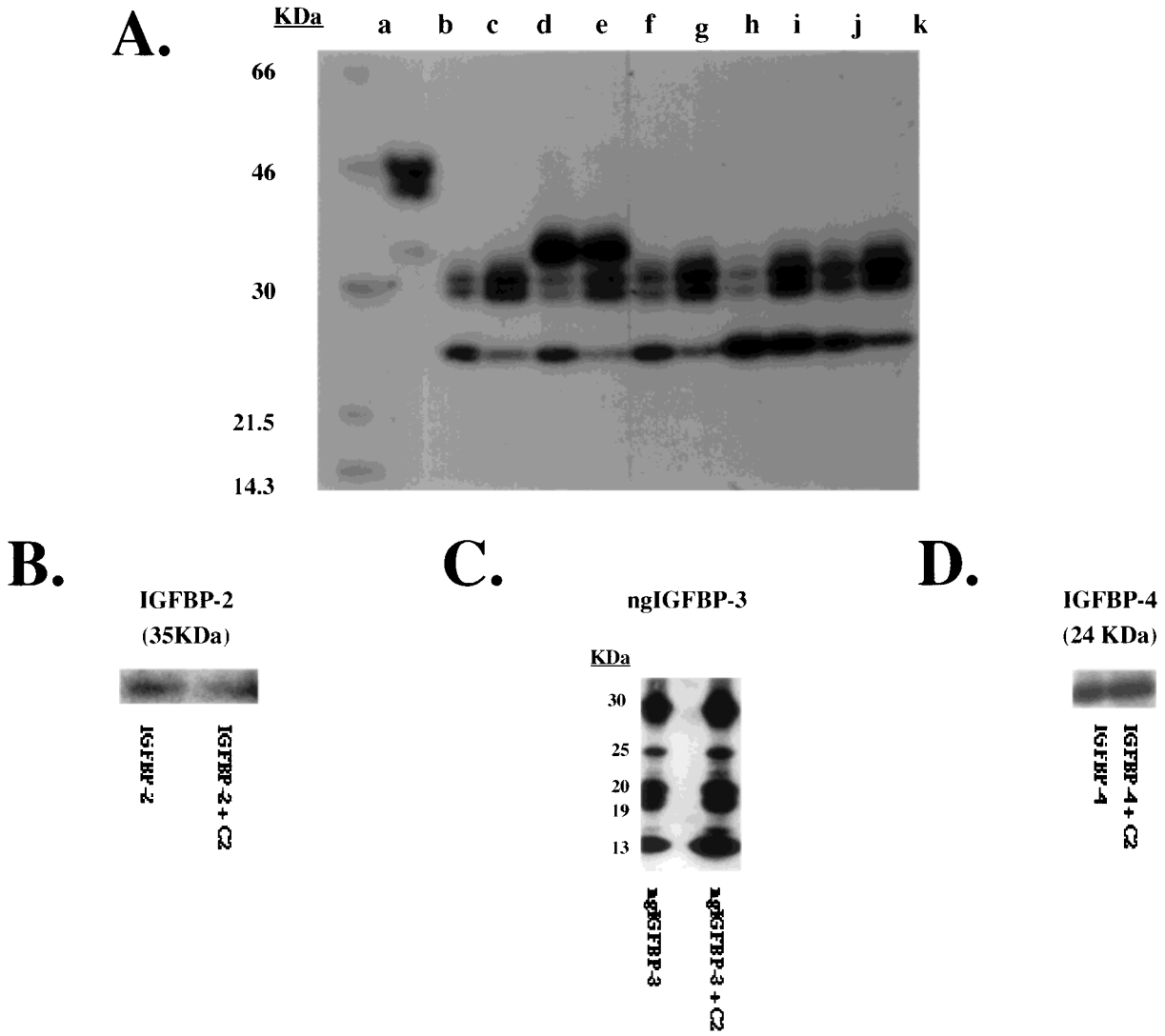


Fig. 5. Representative ligand blot and Western immunoblots of concentrated conditioned media from Hs578T cells treated with IGFBP alone (100 ng/ml) or with or without an apoptotic dose of C2. **A:lane a**, normal human serum; **lane b**, CT; **lane c**, C2; **lane d**, IGFBP-2 alone; **lane e**, IGFBP-2 + C2; **lane f**, IGFBP-3 alone; **lane g**, IGFBP-3 + C2; **lane h**, IGFBP-4 alone; **lane i**, IGFBP-4 + C2; **lane j**, IGFBP-5 alone; **lane k**, IGFBP-5 + C2. Molecular weights are indicated on the right-hand side of the blot (66–14.3 Kda). **B–D:** Representative immunoblots for IGFBP-2, -3, and -4, respectively, after treatment with binding protein alone, with or without an apoptotic dose of ceramide.

grammed cell death but not that initiated by RGD. The mechanism of action therefore does not appear to be dependent on the proteolysis of endogenous IGFBP-3, but it suggests that there are alternative mechanisms of action for these binding proteins. We speculate that IGFBP-3 could be acting specifically upstream of IGFBP-5 in the apoptotic signalling pathway.

In summary, we have established that the binding proteins have differential effects on apoptotic signalling, which are independent of IGF-I. Specifically, IGFBP-3 can accentuate whereas IGFBP-4 and -5 can protect against apoptosis. These data illustrate that the binding proteins play an active and direct role in influencing cell survival independently of the IGFs. These findings demonstrate that the IGFBPs can play a crucial role in maintaining tissue homeostasis, both indirectly, by sequestering IGFs and protecting against inappropriate cell proliferation, and directly, by having the ability to modulate the degree of cell death.

ACKNOWLEDGMENT

We are grateful to The Wellcome Trust for their generous support in enabling us to purchase a flow cytometer.

REFERENCES

Andress DL. 1998. Insulin-like growth factor-binding protein-5 (IGFBP-5) stimulates phosphorylation of the IGFBP-5 receptor. Am J Physiol Endocrinol Metab 274: E744–E750.

Andress DL, Birnbaum RS. 1992. Human osteoblast-derived insulin-like growth factor (IGF) binding protein-5 stimulates osteoblast mitogenesis and potentiates IGF action. J Biol Chem 267:22467–22472.

Baxter RC. 1993. IGF binding protein-3 and the acid-labile subunit: formation of the ternary complex in vitro and in vivo. Adv Exp Med Biol 343:237–244.

Blum WF, Jenne EW, Reppin F, Kietzmann K, Ranke MB, Bierich JR. 1989. Insulin-like growth factor I (IGF-I)-binding protein complex is a better mitogen than free IGF-I. Endocrinology 125:766–772.

Clemmons DR. 1992. IGF binding proteins: regulation of cellular actions. Growth Regul 2:80–87.

Clemmons DR, Camacho-Hubner C, Coronado E, Osborne CK. 1990. Insulin-like growth factor binding protein secretion by breast carcinoma cell lines: correlation with estrogen receptor status. Endocrinology 127:2679–2686.

El Roiey A, Chen X, Roberts VJ, Shimasaki S, Ling N, LeRoith D, Roberts CT Jr, Yen SSC. 1994. Expression of the genes encoding the insulin-like growth factors (IGF-I and II), the IGF and insulin receptors, and IGF-binding proteins 1–6 and the localization of the gene products in normal and polycystic ovary syndrome ovaries. J Clin Endocrinol Metab 78:1488–1496.

Fowler CA, Perks CM, Holly JMP, Farndon JR. 1998. The role of IGFBP-3 in modulating paclitaxel-induced apoptosis in human breast cancer cells. Br J Surg 85:1569.

Fowlkes JL, Thrailkill KM, George-Nascimento C, Rosenberg CK, Serra DM. 1997. Heparin-binding, highly basic regions within the thyroglobulin type-1 repeat of insulin-like growth factor (IGF)-binding proteins (IGFBPs) -3, -5, and -6 inhibit IGFBP-4 degradation. Endocrinology 138: 2280–2285.

Freedman VH, Shin S. 1974. Cellular tumorigenicity in nude mice: correlation with cell growth in semisolid medium. Cell 3:353–359.

Frisch SM, Francis H. 1994. Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol 124: 619–626.

Giancotti FG, Ruoslahti E. 1990. Elevated levels of $\alpha_5\beta_1$, fibronectin receptor suppress the transformed phenotype of CHO cells. Cell 60:849–859.

Gill ZP, Perks C M, Newcomb PV, Holly JMP. 1998. IGFBP-3 can differentially accentuate apoptotic pathways in Hs578T human breast cancer cells in a non-IGF-dependent manner. J Endocrinol 156:OC25.

Gill ZP, Perks CM, Newcomb PV, Holly JMP. 1997. Insulin-like growth factor binding protein (IGFBP-3) predisposes breast cancer cells to programmed cell death in a non-IGF dependent manner. J Biol Chem 272:25602–25607.

Gucev ZS, Oh Y, Kelley KM, Rosenfeld RG. 1996. Insulin-like growth factor binding protein 3 mediates retinoic acid- and transforming growth factor beta2-induced growth inhibition in human breast cancer cells. Cancer Res 56:1545–1550.

Hayman EG, Pierschbacher MD, Ruoslahti E. 1985. Detachment of cells from culture substrate by soluble fibronectin peptides. J Cell Biol 100:1948–1954.

Hollowood AD, Lai T, Perks CM, Newcomb PV, Holly JMP, Alderson D. 1998. Insulin-like growth factor binding protein-3 (IGFBP-3) predisposes oesophageal cancer cells to apoptosis in a p53-dependent manner. In: BACR RSM meeting on carcinogenesis and chemoprevention, London. Abstract 1.5.

Huynh H, Yang XF, Pollack M. 1996. Estradiol and antiestrogens regulate a growth inhibitory insulin-like growth factor binding protein 3 autocrine loop in human breast cancer cells. J Biol Chem 271:1016–1021.

Jaques G, Noll K, Wegmann B, Witten S, Kogan E, Radulescu RT, Havemann K. 1997. Nuclear localization of insulin-like growth factor binding protein 3 in a lung cancer cell line. Endocrinology 138:1767–1770.

Ingber DE. 1990. Fibronectin controls capillary endothelial cell growth based on its ability to modulate cell shape. Proc Natl Acad Sci USA 87:3579–3583.

Jones JI, Clemmons DR. 1995. Insulin-like growth factors and their binding proteins: biological actions. Endocrinol Rev 16:3–34.

Jones JI, Gockerman A, Busby WH, Clemmons DR. 1993. IGFBP-1 stimulates cell migration and binds to the $\alpha_5\beta_1$ integrin by means of its Arg-Gly-Asp sequence. Proc Natl Acad Sci USA 90:10553–10557.

Knudson KA, Rao PE, Damsky CH, Buck CA. 1981. Membrane glycoproteins involved in cell-substratum interactions. Proc Natl Acad Sci USA 78:6071–6075.

Lalou C, Lassarre C, Binoux M. 1996. A proteolytic fragment of insulin-like growth factor (IGF) binding protein-3 that fails to bind the IGFs inhibits the mitogenic effects of IGF-I and insulin. Endocrinology 137:3206–3212.

Noll K, Wegmann B, Havemann K, Jaques G. 1996. Insulin-like growth factors stimulate the release of insulin-like growth factor-binding protein-3 (IGFBP-3) and degradation of IGFBP-4 in nonsmall cell lung cancer cell lines. J Clin Endocrinol Metab 81:2653–2662.

Obeid LM, Linardic CM, Karolak LA, Hannun YA. 1993. Programmed cell death induced by ceramide. Science 259:1769–1771.

Oh Y, Möller, HL, Lamson G, Rosenfeld RG. 1993. Insulin-like growth factor (IGF)-independent action of IGF-binding protein-3 in Hs578T human breast cancer cells. Cell surface binding and growth inhibition. J Biol Chem 268:14964–14971.

Oh Y, Möller HL, Ng L, Rosenfeld RG. 1995. Insulin-like growth factor binding protein 3 mediates retinoic acid- and transforming growth factor beta 2-induced growth inhibition in human breast cancer cells. J Biol Chem 270:13589–13592.

Oh Y, Nagalla SR, Yamanaka Y, Kim H-S, Wilson E, Rosenfeld RG. 1996. Synthesis and characterisation of insulin-like growth factor-binding protein (IGFBP)-7. J Biol Chem 271:30322–30325.

Perks CM, Newcomb PV, Norman MR, Holly JMP. 1999. Effect of insulin-like growth factor binding protein-1 on integrin signalling and the induction of apoptosis in human breast cancer cells. J Mol Endocrinol 22:141–150.

Rajah R, Valentinis B, Cohen P. 1997. Insulin-like growth factor (IGF)-binding protein-3 induces apoptosis and mediates the effects of transforming growth factor-beta1 on programmed cell death through a p53- and IGF-independent mechanism. J Biol Chem 272:12181–12188.

Rechler MM. 1993. Insulin-like growth factor binding proteins. Vitam Horm 47:1–114.

Ritvos O, Ranta T, Jalkanen J, Suikkari AM, Voutilainen R, Bohn H, Rutanen EM. 1988. Insulin-like growth factor (IGF) binding protein from human decidua inhibits the binding and biological action of IGF-I in cultured choriocarcinoma cells. Endocrinology 122:2150–2157.

Ruoslahti E, Pierschbacher MD. 1987. New perspectives in cell adhesion. Science 238:491–497.

Sheikh MS, Shao Z-M, Clemmons DR, LeRoith D, Roberts CT Jr, Fontana JA. 1992. Identification of the insulin-like growth factor binding proteins 5 and 6 (IGFBP-5 and 6) in human breast cancer cells. Biochem Biophys Res Commun 183:1003–1010.

Shimasaki S, Shimonaka M, Zhang HP, Ling N. 1991. Identification of five different insulin-like growth factor binding proteins (IGFBPs) from adult rat serum and molecular cloning of a novel IGFBP-5 in rat and human. J Biol Chem 266:10646–10653.

Thompson CB. 1995. Apoptosis in the pathogenesis and treatment of disease. Science 267:1456–1462.

Tonner E, Barber MC, Travers MT, Logan A, Flint DJ. 1997. Hormonal control of insulin-like growth factor-binding protein-5 production in the involuting mammary gland of the rat. Endocrinology 138:5101–5107.

Tucker RW, Butterfield CE, Folkman J. 1981. Interaction of serum and cell spreading affects growth of neoplastic and nonneoplastic cells. J Supramol Struct 15:29–40.

Valentinis B, Reiss K, Baserga R. 1998. Insulin-like growth factor-I-mediated survival from anoikis: a role of cell aggregation and focal adhesion kinase. J Cell Physiol 176:648–57.

Wyllie AH. 1980. Cell death: the significance of apoptosis. Int Rev Cytol 60:251–306.