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Signalling Pathways Involved in the Direct Effects of IGFBP-5 on Breast Epithelial Cell Attachment and Survival

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Abstract We have demonstrated previously that IGFBP-5 can confer survival against apoptosis induced by ceramide, C2, or a small synthetic arginine–glycine-aspartic acid (RGD)-containing peptide in a direct manner. The endogenous ceramide-induced pathway is normally counter-balanced by survival signals mediated by sphingosine kinase (SK) and protein kinase C (PKC). In order to investigate whether these pathways are involved in the IGFBP-5 survival effect, we have used inhibitors of SK (N, N-di-methyl sphingosine, DMS) and PKC (chelerythrine chloride, CC). The effect of pre-incubating Hs578T breast cancer cells with IGFBP-5 on cell adhesion or on subsequent cell death induced by C2 or RGD was investigated with and without the presence of DMS or CC. Cell death was determined by trypan blue cell counts and apoptosis confirmed by morphological assessment and flow cytometry. Cell attachment was determined by a cell adhesion assay. The presence of IGFBP-5 significantly inhibited cell death induced by C2 or RGD, compared to the triggers of apoptosis alone ($P < 0.01$ in both cases). In the presence of either IGFBP-5, CC or DMS, there was no significant effect on cell death compared to the control. IGFBP-5 in the presence of either inhibitor resulted in a significant increase in cell death; IGFBP-5 also lost its ability to confer survival on C2 and RGD-induced apoptosis and in contrast significantly increased cell death. In the cell adhesion assay, IGFBP-5 significantly increased cell attachment over basal levels. In the presence of either inhibitor the IGFBP-5 effect on cell adhesion was reversed and cell attachment was reduced to below basal levels. These data suggest that IGFBP-5 promotes the attachment and survival of Hs578T cells by modulating the balance between ceramide and opposing survival signals. J. Cell. Biochem. 84: 784–794, 2002.

Key words: IGFBP-5; breast epithelial cells; apoptosis; attachment; sphingosine kinase; protein kinase C

The IGF system comprises the ligands IGF-I and IGF-II, their receptors and six high affinity-binding proteins (IGFBP). The IGFs are potent stimulators of cell growth in many cell types and their binding proteins serve to modulate these actions [Jones and Clemmons, 1995]. IGFBP-3 is the major binding protein found in adult human serum. Together with the acid labile subunit (ALS), IGFBP-3 can prolong the half-life of the circulating IGFs and inhibit or enhance their binding to IGF receptors on the cell surface. As well as IGF-dependent actions, it is becoming increasingly evident that the IGF binding proteins also exert intrinsic actions that can affect aspects of cell growth and death. Specifically IGFBP-3 was shown to inhibit the growth of Hs578T human breast cancer cells [Oh et al., 1993], while in prostate cancer cells IGFBP-3 induced apoptosis [Rajah et al., 1997]. We have demonstrated previously that IGFBP-3, in Hs578T cells, could accentuate apoptosis induced by either the ceramide analogue, C2, [Gill et al., 1997] or the mitochondrial respiratory chain inhibitor, Antimycin A [Perks et al., 2000].

IGFBP-5 is structurally similar to IGFBP-3 [Jones and Clemmons, 1995]. They have each been shown to possess a nuclear localisation sequence [Schedlich et al., 1998], as well as binding sites for the ALS [Twigg et al., 2000] and heparin. By sequestration of IGF, IGFBP-5 has

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been associated with increased apoptosis in the involuting breast [Flint et al., 2000; Tonner et al., 2000] and prostate [Thomas et al., 1998]. There is now evidence to suggest that IGFBP-5 may have opposing intrinsic effects on cell survival. It has been found in high concentrations in bone and it was reported that IGFBP-5 could stimulate the proliferation of osteoblast cultures [Miyakoshi et al., 2001]. In addition we have also demonstrated previously, in Hs578T cells, that IGFBP-5 can confer survival by reducing apoptosis induced by either C2 or the synthetic arginine–glycine-aspartic acid (RGD)-containing peptide [Perks et al., 1999]. It has also been further demonstrated that these direct effects of IGFBP-5 can induce cell migration of rat mesangial cells in an RGD-independent manner [Abrass et al., 1997; Berfield et al., 2000]. Furthermore, we have demonstrated that IGFBP-5 can promote attachment of Hs578T human breast cancer cells on a general extracellular matrix (ECM) gel [McCaig et al., 2001].

A balance between cell proliferation and death must be achieved to maintain tissue homeostasis. Activation of growth factor receptors and integrins can activate protein kinase C (PKC). Protein kinase C is a serine/threonine kinase of which there are eight known isoforms that fall into three classes; classic, novel and atypical. Protein kinase C can activate sphingosine kinase (SK) which converts ceramide into sphingosine-1-phosphate (SPP). Sphingosine-1-phosphate can then overcome apoptosis by promoting phosphorylation of Bcl-2 or inducing gene transcription [Spiegel et al., 1996; Mathias et al., 1998].

It is still unclear how IGFBP-5 exerts its intrinsic actions on cell survival and adhesion. There have been reports of a putative IGFBP-5 receptor [Andress, 1998], which to date is still uncharacterised. Furthermore, signalling by IGFBP-5 directly has not yet been explored.

It has been recently reported that blocking sphingosine kinase and protein kinase C with their respective inhibitors (N, N-di-methyl sphingosine, DMS and chelerythrine chloride, CC) prevented vitamin D from exerting its potent survival effect on HL-60 cells [Kleuser et al., 1998]. Hence having established previously that IGFBP-5 also acted as a potent survival factor against C2 and RGD-induced apoptosis in Hs578T human breast cancer cells, we employed the same inhibitors to establish if they could also negate the survival effects of IGFBP-5 in this cell line.

MATERIALS AND METHODS

Materials

Recombinant human IGFBP-5 was purchased from Austral Biologicals, CA. The ceramide analogue (C2) was obtained from Calbiochem (Nottingham, UK). The protein kinase C inhibitor, chelerythrine chloride (CC), the sphingosine kinase inhibitor, N-N-dimethyl sphingosine (DMS), a synthetic RGD-containing peptide (Gly-Arg-Gly-Asp-Thr-Pro) and extracellular matrix (ECM) gel were obtained from Sigma Chemical Co. (Poole, UK). Tissue culture plastics were purchased from Greiner Labortechnik Ltd. (Stonehouse, UK).

Cell Culture

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

Dosing Protocol

Cells were seeded in 6-well plates and grown in GM for 24 h before switching to SFM for a further 24 h prior to dosing. Cells were pre-incubated with IGFBP-5 (100 ng/ml) for a further 24 h followed by a co-incubation of IGFBP-5 and an apoptotic dose of ceramide or RGD for a further 24 h. The optimal dose of IGFBP-5 was determined previously [Perks et al., 1999]. Experiments were repeated following the same protocol with and without the addition of DMS (0.5 μM) or CC (0.5 μM) alongside the IGFBP-5. The apoptotic dose of ceramide used was between 10–15 μM in order to achieve 40–60% cell death. The final dose used in each experiment was dependent on the confluency and passage number of the cells. The apoptotic dose of RGD used for all these experiment was 75 μg/ml.

Trypan Blue Dye Exclusion

Aliquots of cells were loaded onto a haemocytometer (1:1) with trypan blue dye. Viable cells exclude the dye. Both live and dead cells were counted, from which the percentage of dead cells was calculated.

Flow Cytometry

This technique was used to determine the amount of apoptosis in any given sample. The fragmented DNA of an apoptotic cell has less capacity to stain than normal cell and appears as a pre-G1 peak on a DNA cell cycle histogram. Cells ($1-2 \times 10^6$) were washed in phosphate buffered saline (PBS) and fixed in 70% ethanol for a minimum of 30 min prior to analysis. The fixed cells were pelleted (6,500*g*, 5 min) and washed with PBS. The cells were resuspended in 500 μl of reaction buffer (propidium iodide, 0.05 mg/ml; sodium citrate, 0.1%; RNase A, 0.02 mg/ml; NP-40, 0.3% pH 8.3). This was incubated for 30 min at 4°C prior to measurement using a FACS Calibur Flow Cytometer (Becton Dickinson, Plymouth, UK) with an argon laser at 488 nm for excitation. Analysis was by Cell Quest software package (Becton Dickinson, Plymouth, UK)

Adhesion Assay

Cell adhesion assays were undertaken following a protocol modified from that described previously [Yeh et al., 1998]. Hs578T cells were grown to confluency in T75 flasks in GM and switched to SFM 24 h prior to dosing. Twenty-four well plates were coated in 500 μl of ECM solution (30 μg/ml; additive free DMEM) for 1 h at 37°C. Wells were then washed with PBS before non-specific binding was blocked with 500 μl of PBS containing 0.1% BSA for at least 2 h at 37°C. Meanwhile cells were trypsinised and collected using SFM. Pellets were resuspended in 1 ml of SFM and 50 μl of the cell solution was counted to determine cell number. Cells were further diluted, using SFM to 0.3×10^6 cells/1.5 ml to which IGFBP-5 (100 ng/ml) with or without DMS (0.5 μM) or CC (0.5 μM) were added as described in results. The cells were placed on a shaker and incubated for 1 h at room temperature. Wells were washed twice with PBS before control and pretreated cells were applied at 0.1×10^6 cells/well and incubated at 37°C for 30 min. Unattached cells were collected and wells washed with PBS. Cell pellets were collected and resuspended in 100 μl PBS. Adherent cells were trypsinised and collected. Cell pellets were again resuspended in 100 μl PBS. Fifty microlitres of each cell suspension was counted following trypan blue cell staining from which the percentage of cells attached was determined.

Morphological Assessment

To assess changes in levels of apoptosis, cells were viewed under phase contrast with a 10 × objective. The image was captured using a JVC TK 1281 colour video camera coupled to time lapse video recorder using Adobe Premiere 4.1

Statistical Analysis

Data were analysed using Microsoft Excel 2000 software package. Significant differences were determined using ANOVA. Statistically significant differences were considered to be present at $P < 0.05$.

RESULTS

Confirmation of IGFBP-5's Ability to Confer Survival on C2 and RGD-Induced Apoptosis

Figure 1A demonstrates that IGFBP-5 alone had no effect on basal levels of cell death, while the ceramide analogue, C2, significantly ($P < 0.01$) increased cell death from 15.5% to 45.7%. The addition of IGFBP-5 to C2, significantly ($P < 0.01$) inhibited the levels of cell death compared to C2 alone, as previously described [Perks et al., 1999]. Apoptosis was confirmed using flow cytometry (Fig. 1B). IGFBP-5 had no effect on apoptosis compared to the control, whereas C2 significantly ($P < 0.05$) increased apoptosis over basal levels. The co-incubation of C2 and IGFBP-5 significantly ($P < 0.05$) decreased the levels of apoptosis compared to C2 alone.

Figure 1C demonstrates that a synthetic RGD-containing peptide, at 75 μg/ml, significantly ($P < 0.001$) increased basal cell death from 20.0% to 47.4%. IGFBP-5 alone had no effect but when co-incubated with RGD, significantly ($P < 0.01$) negated the amount of cell death compared to RGD alone. Similarly the flow data in Figure 1D shows that IGFBP-5 had no effect on basal levels of apoptosis, while RGD significantly ($P < 0.01$) increased cells in the pre-G1 peak compared to the control. The

co-incubation of IGFBP-5 with RGD significantly ($P < 0.001$) reduced the levels of apoptosis compared to RGD alone.

The photomicrographs in Figure 1E shows that IGFBP-5, panel II, has no effect on the number of cells attached or on morphological appearance compared to the untreated cells in panel I. The apoptotic trigger, C2 (panel III), dramatically decreased the number of cells attached compared to control cells. Dead cells round up and float off into the surrounding media. Panel IV shows the addition of IGFBP-5 to C2 dramatically increased the number of cells attached compared to C2 alone. Incubation of the cells with RGD decreased the number of cells attached and made them more spiked in appearance (panel V). Co-incubation of IGFBP-5 and RGD, panel VI, increased the number of cells attached compared to RGD alone.

Effects of the Sphingosine Kinase Inhibitor, DMS, on the Ability of IGFBP-5 to Confer Survival on C2 and RGD-Induced Apoptosis

Figure 2A shows that the ceramide analogue, C2, significantly ($P < 0.001$) increased cell death to 45% compared to basal levels of 15.1%. The addition of IGFBP-5 to C2 significantly ($P < 0.05$) reduced the amount of cell death to 36.8% compared to C2 alone. The sphingosine kinase inhibitor, DMS, had no effect on cell death alone and no effect on C2-induced cell death. IGFBP-5, in the presence of DMS, significantly ($P < 0.01$) increased cell death from 16.8% to 25.2% compared to IGFBP-5 alone. In the presence of DMS, IGFBP-5 could no longer reduce C2-induced cell death. Apoptosis was again confirmed by flow cytometry analysis (data not shown).

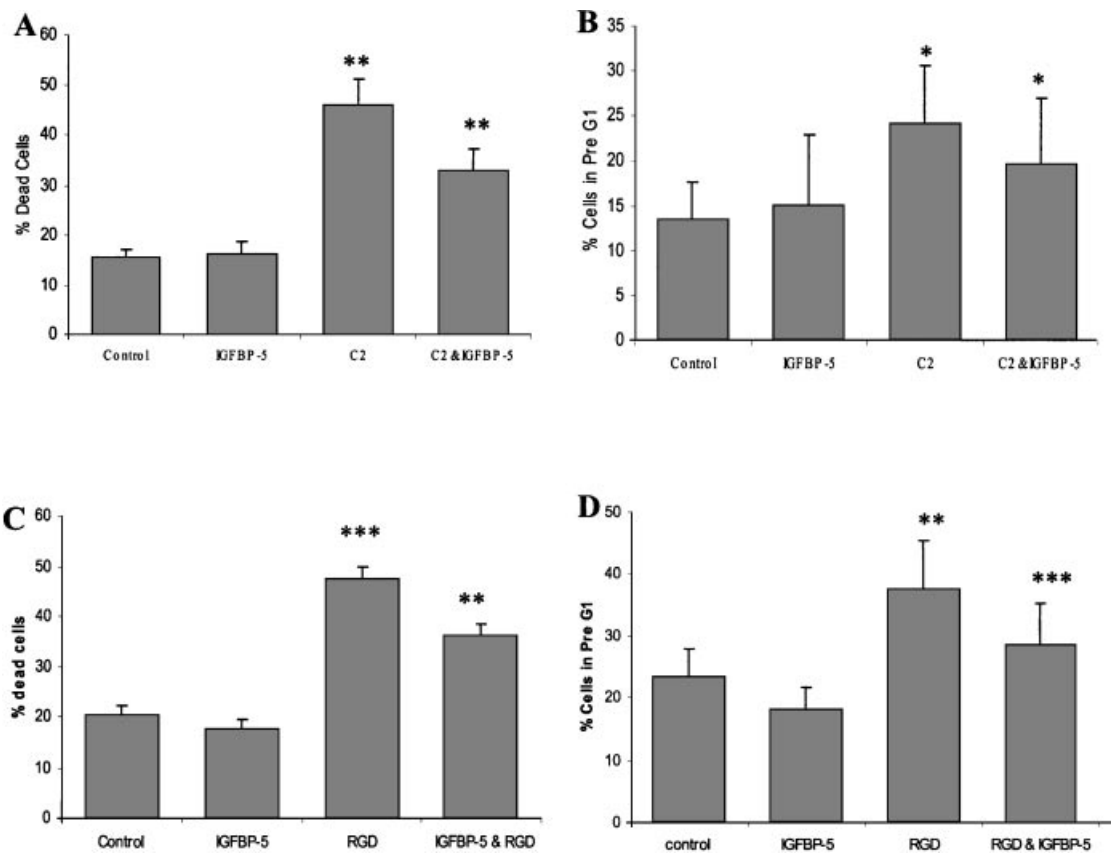

Fig. 1. Confirmation that IGFBP-5 can confer survival on C2 and RGD-induced apoptosis. Graphs represent the percentage of dead cells (**A**,**C**) and the percentage of cells in the pre-G1 peak (**B**,**D**). Cells were pre-treated with IGFBP-5 (100 ng/ml) for 24 h prior to a co-incubation with either ceramide (A + B) or RGD (75 μg/ml) (C + D). Where *C2 > CT and IGFBP-5 + C2 < C2, $P < 0.05$, **C2 > CT; RGD > CT; IGFBP-5 + C2 < C2 and IGFBP-5 + RGD < RGD, $P < 0.01$, ***RGD > CT and IGFBP-5 + RGD < RGD, $P < 0.001$. Experiments were performed in triplicate at least three times. **E**: Morphological evidence demonstrates that IGFBP-5 inhibits C2 and RGD-induced apoptosis.

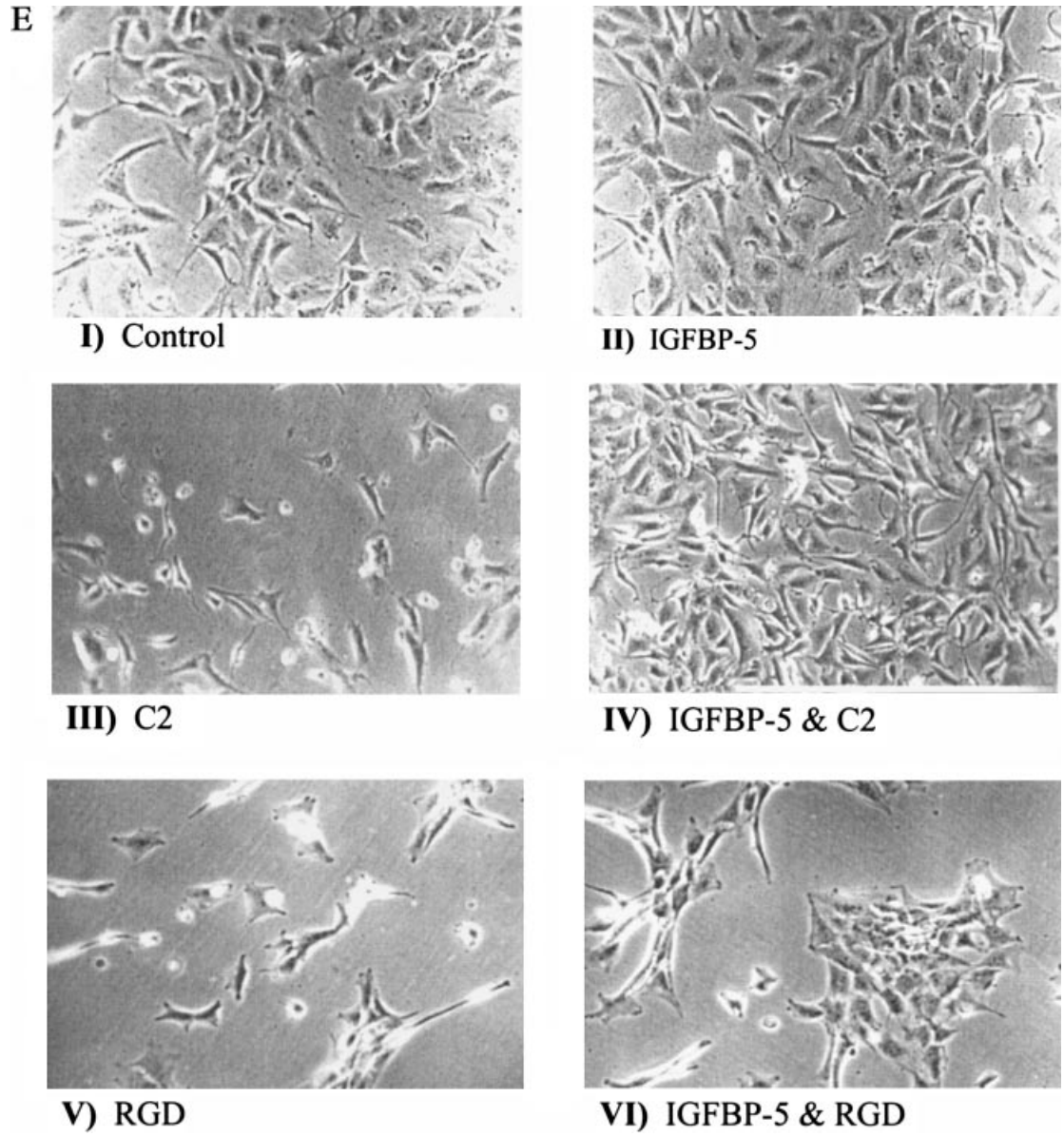

Fig. 1. *(Continued)*

The trypan blue cell counts in Figure 2B shows that RGD significantly ($P < 0.001$) increased cell death to 48.8% compared to basal levels of 20.9%, while the addition of IGFBP-5 to RGD significantly ($P < 0.01$) inhibited cell death compared to RGD alone. DMS had no effect on cell death alone, although in its presence, RGD-induced apoptosis was significantly ($P < 0.01$) inhibited compared to RGD alone. The co-incubation of IGFBP-5 with DMS significantly ($P < 0.01$) increased cell death compared to IGFBP-5 alone, while the addition of IGFBP-5 to DMS and RGD was unable to have any effect on cell death compared to RGD and DMS together. Apoptosis was confirmed by flow cytometry analysis (data not shown).

Figure 2C show representative photographs. IGFBP-5 (panel II) and DMS (panel III) each alone had no effect on cell number or on morphology compared to control cells (panel I). The ceramide analogue (panel V) greatly reduced the number of cells attached compared to the control, while the addition of IGFBP-5 to C2 (panel VI) increased the number of cells attached compared C2 alone. The presence of DMS had no effect on cells incubated with C2 (panel VII) compared to C2 treated cells. Furthermore, DMS incubated with IGFBP-5

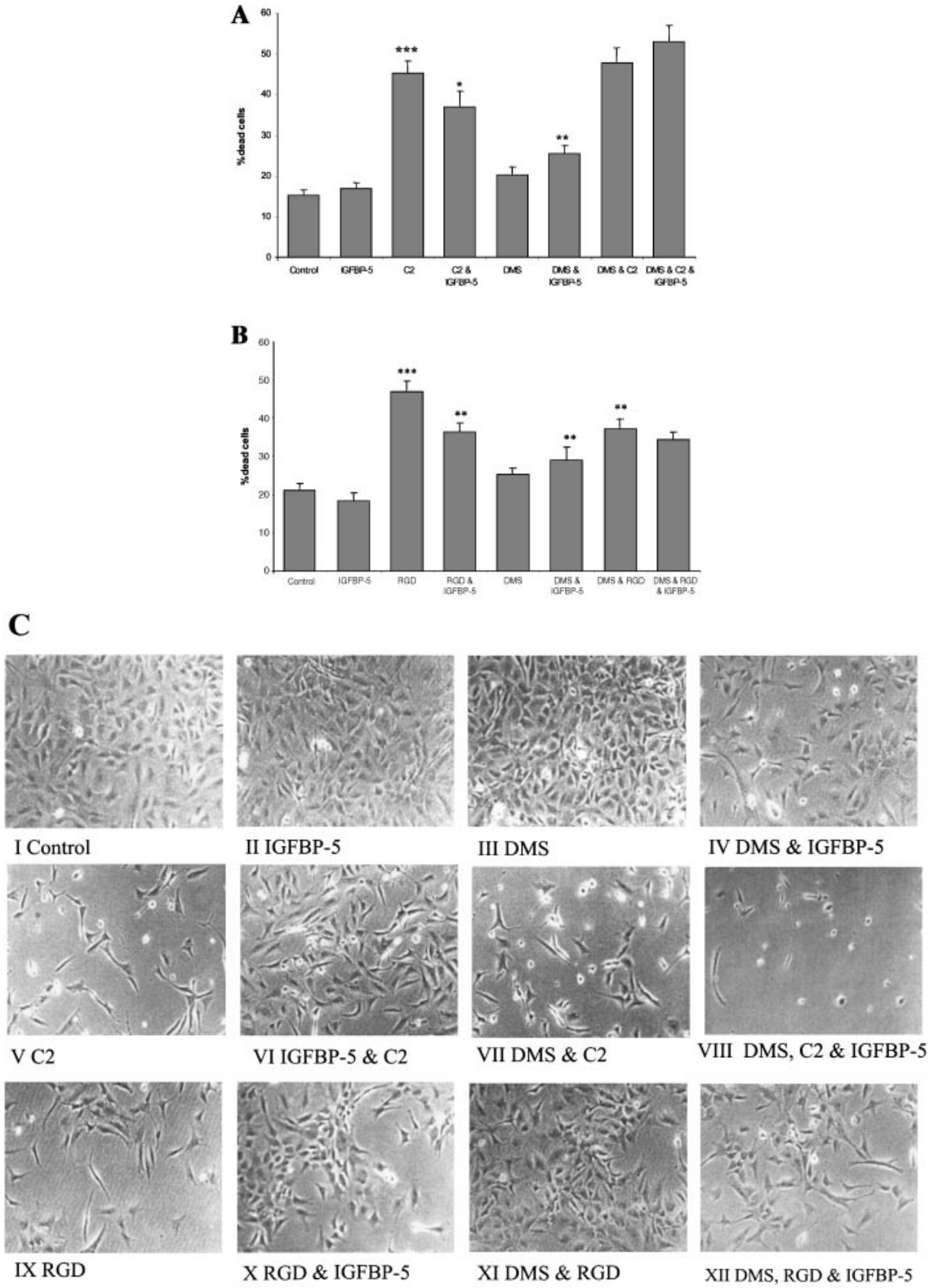

Fig. 2. The effects of the sphingosine kinase inhibitor, DMS, on IGFBP-5's ability to confer survival on C2- and RGD-induced apoptosis. Graphs represent percentage of dead cells. Cells were pre-treated with IGFBP-5 (100 ng/ml) with or without DMS (0.5μM) for 24 h followed by a co-incubation with either (**A**) ceramide or (**B**) RGD (75 μg/ml). Where *C2 + IGFBP-5 < C2, $P < 0.05$, **DMS + IGFBP-5 > IGFBP-5; RGD + IGFBP-5 < RGD and DMS + RGD < RGD, $P < 0.01$, ***RGD > CT and C2 > CT, $P < 0.001$. **C**: Morphological evidence to demonstrate the effects of DMS on the direct effects of IGFBP-5 on C2 and RGD-induced apoptosis. Experiments were performed in triplicate at least three times.

(panel IV) decreased the number of cell attached compared to IGFBP-5 alone. The addition of IGFBP-5 to DMS and C2 (panel VIII) was unable to increase the number of cells attached compared to C2 and DMS together. RGD (panel IX) greatly decreased the amount of cells attached compared to the control, while the co-incubation of IGFBP-5 with RGD (panel X) increased the number of cells attached compared to RGD alone. DMS and RGD (panel XI) together increased the number of cells attached compared to RGD alone. The addition of IGFBP-5 to DMS and RGD did not further increase the degree of cell attachment seen with DMS and RGD together (panel XII).

Effects of the Protein Kinase C Inhibitor, CC, on IGFBP-5's Ability to Confer Survival on C2 and RGD-Induced Apoptosis

The trypan blue cell counts in Figure 3A show that C2 significantly ($P < 0.001$) increased cell death to 61.1% compared to basal levels of 23.7%, while a co-incubation with IGFBP-5 significantly ($P < 0.01$) reduced this to 49.5%. The protein kinase C inhibitor, CC, had no effect on cell death alone but on co-incubation with IGFBP-5 significantly ($P < 0.001$) increased cell death to 45.7% compared to IGFBP-5 alone, 26.4%. The addition of CC to C2 significantly ($P < 0.01$) inhibited cell death compared to C2 alone. The co-incubation of IGFBP-5 with CC and C2 was unable to inhibit cell death further compared to C2 and CC. Apoptosis was confirmed by flow cytometry analysis (data not shown).

The trypan blue cell counts in Figure 3B show that RGD significantly ($P < 0.001$) increased cell death to 49.7 % compared to control levels of 20.9%. The co-incubation of RGD with IGFBP-5 significantly ($P < 0.01$) inhibited cell death to 38.2% compared to RGD alone. The PKC inhibitor, CC, had no effect on RGD-induced apoptosis, although co-incubation of IGFBP-5 with CC significantly ($P < 0.01$) increased cell death compared to IGFBP-5 alone. Furthermore, IGFBP-5 was unable to confer survival on RGD-induced apoptosis in the presence of CC; the levels of cell death remained the same as that of RGD alone. Apoptosis was confirmed by flow cytometry analysis (data not shown).

The photomicrographs (Fig. 3C) show that IGFBP-5 and CC (panel II and panel III, respectively) each had no effect on cell number or morphology compared to the control cells in panel I, but the co-incubation of both (panel IV) decreased the number of cells attached compared to IGFBP-5 alone. Cells treated with C2 (panel V) greatly reduced the number of cells attached compared to the control, while co-incubation with IGFBP-5 (panel VI), greatly increased the number of cells attached compared to C2 alone. The addition of CC to C2 (panel VII) increased the number of cells attached compared to C2 alone. The addition of CC to IGFBP-5 and C2 could not increase cell attachment further than IGFBP-5 and C2 (panel VIII), but cell attachment and number was similar to that seen in IGFBP-5 and CC

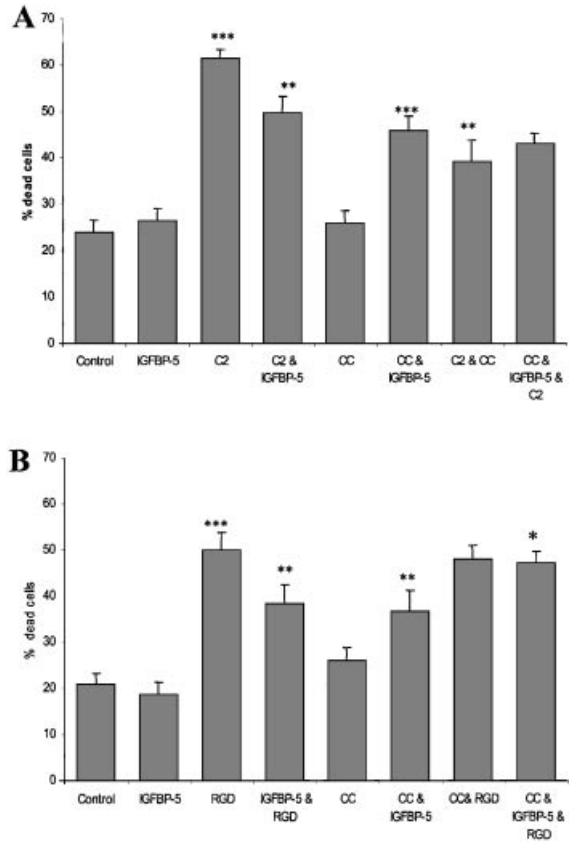

Fig. 3. The effects of the protein kinase C inhibitor, CC, on IGFBP-5's ability to confer survival on C2 and RGD-induced apoptosis. Graphs represent percentage of dead cells. Cells were treated with IGFBP-5 (100 ng/ml) with or without CC (0.5 μM) for 24 h followed by a co-incubation with either (**A**) ceramide or (**B**) RGD (75 μg/ml). Where *RGD + IGFBP-5 + CC > IGFBP-5 + RGD, $P < 0.05$, **C2 + IGFBP-5 < C2; RGD + IGFBP-5 < RGD; CC + IGFBP-5 > IGFBP-5 and C2 + CC < C2, $P < 0.01$, ***IGFBP-5 + CC > IGFBP-5; RGD > GT, and C2 > CT, $P < 0.001$. **C**: Morphological evidence to demonstrate the effects of CC on the direct effects of IGFBP-5 on C2 and RGD-induced apoptosis. Experiments were performed in triplicate at least three times.

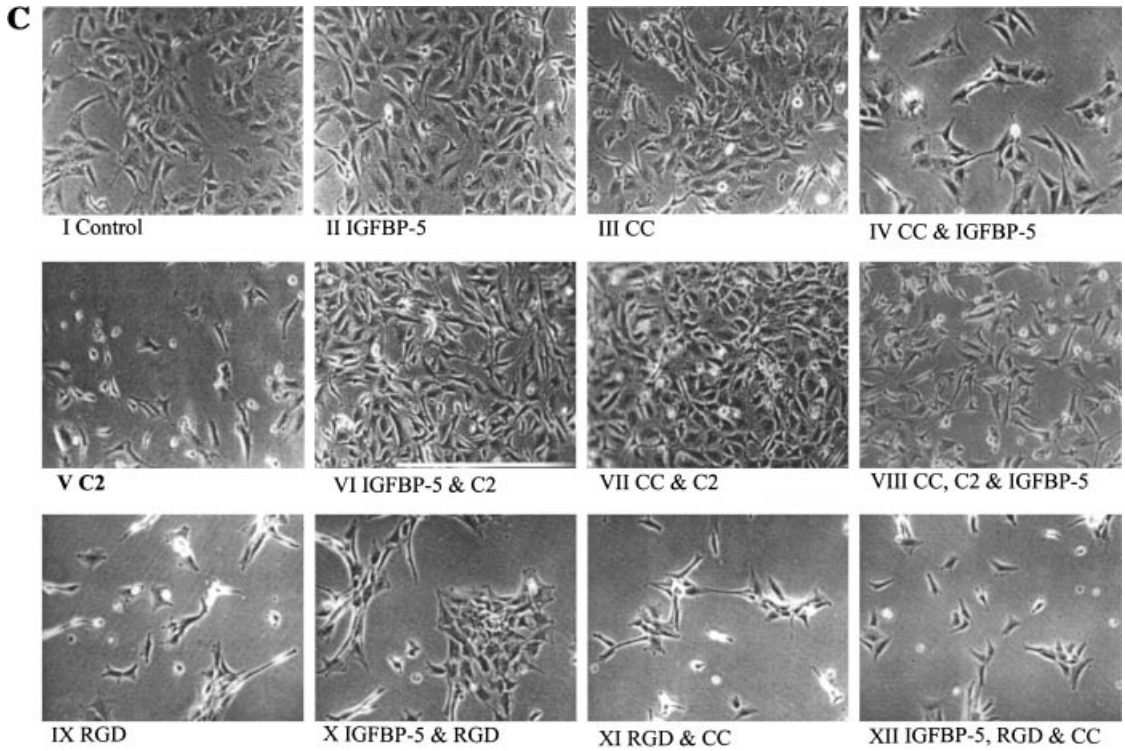

Fig. 3. *(Continued)*

together (panel IV). RGD (panel IX) greatly decreased cells attached and the addition of IGFBP-5 to RGD (panel X) increased the number of cells attached compared to RGD alone. The PKC inhibitor, CC, did not have any effect on cell number when it was co-incubated with RGD (panel XI). Co-incubation of IGFBP-5 with CC and RGD (panel XII) did not increase the number of cells attached in comparison to RGD alone as seen in panel IX.

Sphingosine Kinase and Protein Kinase C Inhibitors Can Each Negate the Actions of IGFBP-5 on Cell Adhesion

Figure 4A shows that a non-apoptotic dose of RGD (10 μg/ml) significantly ($P < 0.001$) decreased cell attachment to 44.2% compared to control levels of 58.8%. Incubation with IGFBP-5 significantly ($P < 0.05$) increased cell adhesion to 65.0% over basal levels, while cells incubated with DMS alone had no effect on cell adhesion compared to the control. The co-incubation of IGFBP-5 and DMS not only negated the effect seen with IGFBP-5 alone but significantly ($P < 0.001$) also inhibited cell adhesion to 45.7%.

In Figure 4B, the non-apoptotic dose of RGD again significantly ($P < 0.001$) decreased cell adhesion (35% compared to basal levels of 53.0%), while IGFBP-5 significantly ($P < 0.05$) promoted cell adhesion to 58.9% compared to control. CC had no effect on cell adhesion while the co-incubation of IGFBP-5 and CC not only negated the effect of IGFBP-5 alone but significantly also ($P < 0.001$) also inhibited cell adhesion to 30.6%.

DISCUSSION

The IGFBPs are becoming increasingly recognised for their direct effects on cell growth and survival. While IGFBP-5 was originally thought to be an apoptotic factor in the involuting mammary and prostate due to its ability to sequester IGFs and remove their survival effects [Flint et al., 2000], recent evidence however suggests that IGFBP-5 can also act intrinsically as a potent survival factor. Reports have also shown that IGFBP-5 can induce cell proliferation in osteoblast cells [Andress and Birnbaum, 1992; Miyakoshi et al., 2001]. We described previously that the IGFBPs could have differential intrinsic actions on apoptosis induced by the physiological triggers, C2 and RGD in the Hs578T breast cancer cell line. While IGFBP-1, -2 and -6

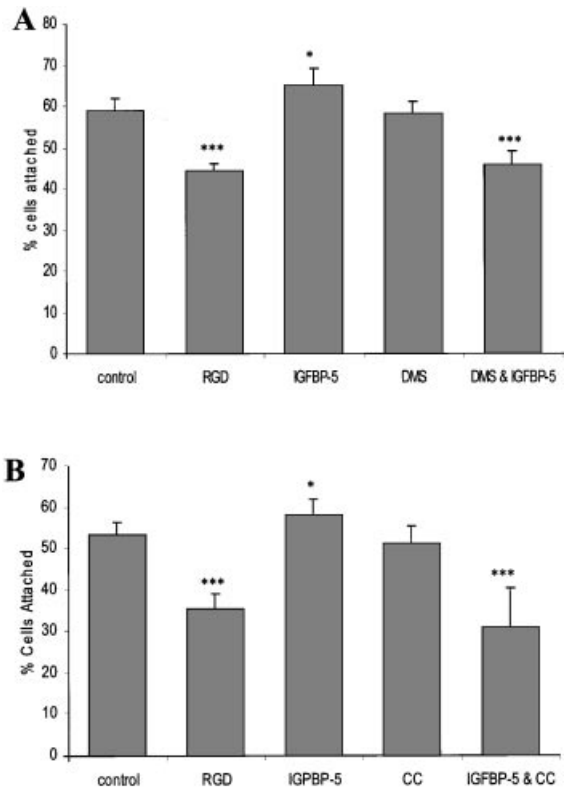

Fig. 4. The effects of DMS and CC on the direct actions of IGFBP-5 on cell adhesion. The graphs represent percentage of cells attached. Cells were treated with RGD (10 μg/ml), IGFBP-5 (100 ng/ml), or either (**A**) DMS (0.5 μM) +/− IGFBP-5 or (**B**) CC (0.5μM) +/− IGFBP-5, where *IGFBP-5 > CT, ***RGD < CT, DMS + IGFBP-5 < IGFBP-5 and IGFBP-5 + CC < IGFBP-5, $P < 0.001$. Experiments were performed in triplicate at least three times.

had no effect on cell death, IGFBP-4 and -5 (as confirmed in this study) conferred survival on both triggers of cell death. Furthermore IGFBP-3 could accentuate C2-induced apoptosis but had no effect on RGD-induced cell death [Gill et al., 1997; Perks et al., 1999].

Ceramide plays a pivotal role in controlling cell apoptosis. An increase in endogenous levels of ceramide pushes the cell towards apoptosis, but the elevation in ceramide levels can be overcome by its conversation to sphingosine-1-phosphate (SPP) through the actions of sphingosine kinase (SK). As SPP levels are increased, it counter-balances the actions of ceramide on apoptosis, allowing the cells to proliferate and survive [as reviewed by Spiegel et al., 1996; Mathias et al., 1998]. RGD can induce apoptosis or anoikis by disruption of the integrin receptors that anchor the cell to the surrounding ECM. Activation of integrins by the ECM leads to the recruitment of molecules such as focal adhesion kinase (FAK) and paxillin to large signalling complexes, where they are activated by phosphorylation on specific residues to mediate survival signals and inhibit apoptosis [Clark and Brugge, 1995; Cary and Guan, 1999].

A recent study described that vitamin D_3, reduced apoptosis induced by sphingomyelinase in HL-60 cells [Kleuser et al., 1998]. Furthermore, they found that vitamin D_3 increased sphingosine kinase activity, accompanied by an increase in SPP levels, suggesting that its survival effect may be mediated by conversation of ceramide to SPP. When vitamin D_3 was incubated with a sphingosine kinase inhibitor, DMS, SK activity and SPP levels were severely inhibited and the anti-apoptotic properties of vitamin D_3 were abolished. We have demonstrated that in the presence of DMS, the survival effects of IGFBP-5 were lost and IGFBP-5 then promoted death. This suggests that IGFBP-5 may modulate the balance between ceramide and SPP within the cell. If IGFBP-5 enhances the production of endogenous ceramides and their conversion to SPP, this could contribute to its ability to reduce apoptosis. When SK is inhibited, the conversion of SPP is blocked, ceramides would then accumulate and IGFBP-5 would promote cell death.

Sphingosine kinase has also been reported to be activated by protein kinase C. Activation of growth factor and integrin receptors can promote the generation of diacylglycerol (DAG) and an increase in intracellular calcium levels, both of which are required to activate PKC. Protein kinase C activity can promote the expression of genes that are involved in cell survival as well as activation of other survival pathways [Spiegel et al., 1996]. When vitamin D_3 pre-treated HL-60 cells were co-incubated with the general PKC inhibitor, CC, SK activity and SPP levels were greatly decreased and again the anti-apoptotic effect of vitamin D_3 was abolished [Kleuser et al., 1998].

We found that similar to the inhibition of SK, when PKC was inhibited, IGFBP-5 survival effects were lost and indeed IGFBP-5 promoted cell death. These data suggest that with PKC inhibited, IGFBP-5 may not be able to increase SPP levels through SK and so survival actions are lost and the cell undergoes apoptosis more readily. It has been reported that the atypical PKCs can be activated by ceramide and further transduce the actions of ceramide within the

cell. This could explain the inhibition of C2 observed when co-incubated with the general PKC inhibitor, CC [Jarvis et al., 1996]. Further experiments will be required to determine the point at which IGFBP-5 can modulate this pathway and whether PKC is acting upstream or downstream of the balance between ceramide and SPP.

To provide further evidence for an effect of IGFBP-5 through SK and PKC, we also examined the effects of these inhibitors on cell adhesion. Cell adhesion to the ECM is mediated through integrin receptors. There have been reports that IGFBPs can interact with integrin receptors. IGFBP-1 and IGFBP-2 have been shown to induce cell migration in different cell lines through their RGD sequence [Jones et al., 1993; Schutt et al., 2000]. We have shown that IGFBP-3 in an RGD-independent manner decreased cell adhesion to an ECM gel as well as decreasing FAK phosphorylation [Perks and Holly, 1999]. There have been other reports that IGFBP-5, again independently of RGD, induces cell migration in rat mesangial cells [Abrass et al., 1997; Berfield et al., 2000]. We have also shown that IGFBP-5 increased Hs578T cell attachment over basal levels to an ECM gel [McCaig et al., 2001]. Sphingosine-1-phosphate has been implicated in cell spreading and attachment [Wang et al., 1999], while it has also been demonstrated that the PKCα isoform can influence the trafficking of β1 integrin subunit to the cell surface [Ng et al., 1999]. In the adhesion assay co-incubation of IGFBP-5 with either of the inhibitors (DMS and CC) not only inhibited the ability of IGFBP-5 to promote cell adhesion but cell attachment was also greatly decreased. This again suggests that IGFBP-5 can modulate balance of signals within the cells involving PKC and SK.

In this study, we have investigated the intrinsic actions of IGFBP-5 in Hs578T cells. It is well established that the binding proteins modulate the actions of IGF-I by either inhibiting or enhancing its activity [Jones and Clemmons, 1995]. Likewise in vivo the direct actions of IGFBP-5 will be modulated in the presence of IGFs. We have shown previously that in the presence of IGF-I, IGFBP-3 was no longer able to accentuate ceramide-induced apoptosis. It was suggested that IGF-I prevented IGFBP-3 from binding to the cell surface [Maile et al., 1999].

In summary, we have shown that the ability of IGFBP-5 to confer survival on C2 and RGD-induced apoptosis was inhibited by the PKC and SK inhibitors CC and DMS. Furthermore the promotion of cell attachment by IGFBP-5 was greatly reduced in the presence of each inhibitor. These data suggest that the direct actions of IGFBP-5 on cell survival and attachment may be mediated through PKC, which in turn could activate SK to influence the balance between ceramide and SPP.

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