Effect of insulin-like growth factor binding protein-1 on integrin signalling and the induction of apoptosis in human breast cancer cells

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ABSTRACT

Interaction of epithelial cells with the extracellular matrix is mediated through integrin receptors, which transmit signals regulating cell growth, differentiation and death. Occupation of these receptors, via Arg-Gly-Asp (RGD) recognition sequences, leads to activation of focal adhesion kinase (FAK).

We treated human breast cancer cell lines with RGD-containing peptides, which can disrupt integrin attachment, and investigated alterations in FAK phosphorylation, cell detachment and death. Cells grown in vitro were treated with insulin-like growth factor-binding protein-1 (IGFBP-1) and a small, synthetic RGD-containing peptide (Gly-Arg-Gly-Asp-Thr-Pro) and its negative control peptide RGE (Arg-Gly-Glu-Ser) for either 30 min followed by immunoprecipitation of cell lysates with anti-phosphotyrosine and Western immunoblotting with anti-FAK or for 24 h followed by cell counting, immunocytochemistry and flow cytometry.

Both IGFBP-1 (0–800 ng/ml) and the synthetic RGD-containing peptide (1–100 µg/ml) caused significant dephosphorylation of FAK. Furthermore, after 24 h both peptides caused detachment from the matrix and the induction of apoptosis.

We conclude from these data that IGFBP-1 can interact with integrin receptors to induce FAK dephosphorylation and subsequently influence attachment and cell death.

INTRODUCTION

The normal growth of cells generally requires attachment to a substrate and the presence of growth factors (Ingber 1990). Cells which become transformed or malignant are characterised by the ability to undergo anchorage-independent growth (Freedman & Shin 1974, Tucker et al. 1981). Cell adhesion to the extracellular matrix (ECM) in vivo is mediated by integrin receptors. These bind to matrix proteins outside the cell and associate with cytoskeletal proteins within the cell. ECMs are composed of several macromolecules including fibronectin, laminin, collagens and proteoglycans (Ruoslathi & Pierschbacher 1987). A number of these adhesive proteins, for example fibronectin (Pierschbacher & Ruoslathi 1984), contain the three amino acid sequence Arg-Gly-Asp (RGD), which is specifically recognised by corresponding integrin receptors (Ruoslathi & Pierschbacher 1987).

The binding of integrins to the ECM initiates assembly of actin microfilaments and the accumulation of numerous different proteins, including signalling molecules, integrins and components of the cytoskeleton, into structures called focal adhesions (Burrige et al. 1988). A number of these proteins, including focal adhesion kinase (FAK), paxillin (Burridge et al. 1992) and tensin (Bockholt & Burridge 1993), are tyrosine phosphorylated upon integrin ligation, suggesting a role for these proteins in integrin signalling. Specifically, integrin-dependent signals have been shown to modulate the control of growth (Giancotti & Ruoslathi 1990) and cell survival (Frisch & Francis 1994).

Maintenance of integrin linkages is essential for cell adhesion. It has been demonstrated previously that disruption of these attachments, via addition of antibodies or peptides, can induce cells to detach from the substratum (Knudson et al. 1981,
Hayman et al. 1985) with a resultant induction of programmed cell death.

Insulin-like growth factor binding protein-1 (IGFBP-1) belongs to a family of six closely related proteins (IGFBP-1 to -6) that are known to modulate the actions of insulin-like growth factors (IGFs) (Shimasaki et al. 1991, Oh et al. 1996). These proteins bind IGF-I and -II with high affinity, thereby modulating their actions on target cells (Clemmons 1992). However, evidence now suggests that these binding proteins may be able to exert IGF-independent effects. Specifically, IGFBP-1, which contains an RGD integrin recognition sequence (Brewer et al. 1988, Hynes 1992), can stimulate cell migration in CHO cells. This action of IGFBP-1 was demonstrated to be specifically via binding to the α5β1 integrin receptor and is independent of the IGFs (Jones et al. 1993). IGFBP-1 is expressed in a strictly tissue-specific manner, being normally restricted to the liver and a small number of other tissues, including decidualised endometrium (Julkunen et al. 1988), the ovary (Suikkari et al. 1989) and regenerating liver (Mohn et al. 1991), all of which exhibit high rates of tissue remodelling.

However, it has also been demonstrated that IGFBP-1 is expressed in the breast both in vitro (Clemmons et al. 1990) and in vivo (Pekonen et al. 1992). IGFBP-1 mRNA expression increased in human breast tumours in comparison to their adjacent normal tissue. The upregulation of IGFBP-1 was associated with the malignant transformation of breast tissue (Pekonen et al. 1992).

Therefore, we chose to study the effects of RGD-containing peptides, including IGFBP-1, on FAK phosphorylation, cell attachment and death in our human breast cancer cell lines, since IGFBP-1 may have a significant pathological role in this tissue. Furthermore, one of our lines, Hs578T, is an ideal model in which to study IGF-independent effects of this binding protein, since it is non-responsive to IGFs and lacks a functional IGF receptor. In fact, we have shown previously in this cell line that IGFBP-3 could predispose cells to programmed cell death in an IGF-independent manner (Gill et al. 1997). We also studied three additional breast cancer cell lines, MCF-7, T47D and ZR-75–1, which are IGF-responsive.

The results of this study provide us with evidence to suggest that IGFBP-1 is capable of eliciting integrin-mediated IGF-independent cellular effects.

**MATERIALS AND METHODS**

Human IGFBP-1 peptide was a gift from Dr. J. Cox, Synergen Inc. (Boulder, CO, USA). The RGD-containing synthetic peptide (Gly-Arg-Gly-Asp-Thr-Pro) together with its negative control peptide, RGE (Arg-Gly-Glu-Ser) were bought from Sigma Chemical Co., Poole, Dorset, UK. All other reagents were purchased from Sigma Chemical Co. or Merck Sharp and Dohme Ltd (Hoddesdon, Herts, UK) unless otherwise stated.

**Cell culture**

The human breast cancer cell lines T47D, Hs578T, MCF-7 and ZR-75–1 were purchased from ECACC (Porton Down, Wilts, UK) and grown in a humidified 5% CO2 atmosphere at 37 °C. They were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with glutamax-1, Minimum Essential Medium (MEM), Eagle’s medium and RPMI 1640 medium respectively supplemented with 10% foetal calf serum (Advanced Protein Products Ltd, Brierley Hill, West Midlands, UK), penicillin (5000 IU/ml), streptomycin (5 mg/ml) and L-glutamine (2 mM).

**Immunoprecipitation and Western immunoblotting**

Cells (1 × 10^6) were grown to 80% confluence in 90 mm dishes (Nunclon, Rochester, NY, USA) and then washed twice with PBS. The growth medium was replaced with serum-free Dulbecco’s MEM and Ham’s nutrient mix F-12 buffered with Hepes and supplemented with sodium bicarbonate (0-12%), BSA (0.2 mg/ml) and transferrin (0.01 mg/ml) (SFM) for 24 h. Cells were treated, incubated at 37 °C for 30 min and then lysed on ice for 10 min (1 ml; 10 mM Tris–HCl, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 µM sodium orthovanadate, 1% Triton, 1 mM phenylmethylsulphonyl fluoride; pH 7.6). Lysates were then centrifuged at 14 000 g for 15 min at 4 °C. The supernatant fractions were incubated at 4 °C with anti-phosphotyrosine antibody (4 µg; Upstate Biotechnology, Lake Placid, NY, USA) for 2 h, goat anti-mouse IgG (5 µl; Calbiochem, Beeston, Nottingham, UK) for 1 h and then 25 µl protein-A–Sepharose beads (Calbiochem) for 1 h. The samples were washed three times with lysis buffer (500 µl), centrifuged at 14 000 g for 3 min and the supernatant removed. Laemml loading buffer (2 ×) was added and proteins were separated by 8% SDS-PAGE and then transferred onto a nylon membrane. Non-specific binding sites were blocked by washing the membranes in 5% milk and then they were probed with anti-FAK (1 µg/ml; Upstate Biotechnology) overnight. Following the removal of excess unbound antibody, an anti-mouse

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antibody conjugated to peroxidase (1:2000) was added for 1 h. Binding of the peroxidase was visualised by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham International, Amersham, Bucks, UK). Optical density measurements were determined using a scanning densitometer (Bio-Rad, Hemel Hemstead, Herts, UK) and analysed using Molecular Analyst software (Biorad).

**Cell counting**

Aliquots of cells (50 µl) were loaded onto a haemocytometer and the total cell number was determined.

**Flow cytometry**

This technique was used to assess the amount of apoptosis in a given sample. Apoptotic cells have a lower DNA stainability than normal cells and appear as a pre-G1 peak on a DNA cell cycle histogram. Cells (1–2 × 10⁶) were washed in PBS and fixed for 30 min by the addition of 70% ethanol (1 ml). Cells were pelleted (1600g; 5 min) and washed three times with PBS. 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; NP-40, 0·3%; pH 8·3) vortexed and incubated at 4°C for 30 min. All cells were then measured on a FACS CALIBUR flow cytometer (Becton Dickinson, Cowley, Oxford, UK) with an argon laser at 488 nm for excitation and analysed using Cell Quest (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 and can be used as a crude measure of cell viability. Cells were seeded at 5 × 10⁴/ml (150 µl growth medium) in 96-well plates and allowed to grow for 24 h. Growth medium was replaced with SFM (100 µl) 24 h prior to the assay. 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) and the tetrazolium crystals were dissolved by mixing on a plate shaker for 20 min at room temperature. The samples were measured on a Bio-Rad 450 plate reader at a test wavelength of 595 nm and a reference wavelength of 650 nm. Results, which are expressed as a percentage optical density of SFM controls, represent the mean ± S.E.M. of five wells from one experiment which is representative of experiments repeated at least three times.

**RESULTS**

**Measurement of FAK phosphorylation in cells treated with RGD-containing peptides over 30 min**

The RGD-containing peptides caused significant dose-dependent decreases in the phosphorylation of FAK in the T47D cells (Fig. 1A and C). It is important to note that no cell detachment was
evident after 30 min treatment and no significant differences were found between the total protein concentrations of the individual plates. The synthetic RGD-containing peptide (Fig. 1A and C) reduced FAK phosphorylation significantly by 45.7% ($P<0.01$) and 66.5% ($P<0.001$) at 25 and 100 µg/ml respectively relative to control values. This reduction in FAK phosphorylation was also evident in the T47Ds after treatment with IGFBP-1 (Fig. 1A and C) at 400 ng/ml (19.1%), becoming significant at 800 ng/ml (39.4%; $P<0.001$). We also showed that IGFBP-1 and the synthetic RGD-containing peptide can similarly decrease FAK phosphorylation in another breast cancer cell line, Hs578T (Fig. 1B). The RGD-containing peptide reduced FAK phosphorylation by 31.5 and 62% at 25 and 100 µg/ml respectively. Similarly, IGFBP-1 was also able to induce the dephosphorylation of FAK in this cell line by 33.2 and 35% at 400 and 800 ng/ml respectively. The control synthetic peptide (RGE) had no significant effects on FAK phosphorylation in either cell line (Fig. 1D).

**Measurement of detached cells treated with RGD-containing peptides for 24 h**

Data demonstrated that treatment with both the RGD-containing synthetic peptide and IGFBP-1 induced cells to detach from the plate over 24 h in T47D cultures (Fig. 2A and B). The RGD-containing synthetic peptide (Fig. 2A; 100 µg/ml) caused a significant ($P<0.01$) increase (35.3%) in the

**FIGURE 1.** Effects of a synthetic RGD-containing peptide (0–100 µg/ml) and IGFBP-1 (0–800 ng/ml) on FAK phosphorylation in T47D and Hs578T cells after treatment for 30 min. Cells were plated in 90 mm Petri dishes, grown to 80% confluence and switched to SFM for 24 h prior to treatment. FAK phosphorylation was assessed by immunoprecipitation and Western immunoblotting as outlined in Materials and Methods. (A and B) Show representative Western immunoblots of T47D and Hs578T cells respectively; a=untreated cells, b=RGD (25 µg/ml), c=RGD (100 µg/ml), d=IGFBP-1 (400 ng/ml) and e=IGFBP-1 (800 ng/ml). (C) Optical density measurements of FAK phosphorylation from Western immunoblots following immunoprecipitation in T47D cells. Results represent the mean ± s.e.m. of experiments performed at least three times, where ***$P<0.001$ and **$P<0.01$. (D) Optical density measurements of FAK phosphorylation from Western immunoblots following immunoprecipitation in T47D and Hs578T cells after treatment with the control RGD peptide (RGE) in comparison to untreated control (CT) cells for 30 min. Results represent the mean ± s.e.m. of experiments performed at least three times.
number of detached cells in comparison to untreated cells. Similarly, IGFBP-1 (Fig. 2B; 400 and 800 ng/ml) also caused significant ($P$<0.05) increases in detached cells at both doses tested (14.5 and 13.8% respectively) in comparison to control cells. Similarly, in the Hs578T cell line the RGD-containing peptide caused a 2.5 and 10% increase in the number of detached cells in comparison to untreated cells at 25 and 100 µg/ml respectively. Likewise, IGFBP-1 (400 and 800 ng/ml) also caused increases in detached cells in this cell line at both doses tested (9 and 6.7% respectively). The control synthetic peptide (RGE) had no significant effects on cell detachment in either cell line (Fig. 2C).

**Flow cytometric analysis of cells treated with RGD-containing synthetic peptides for 24 h**

Results showed that treatment with both the RGD-containing synthetic peptide and IGFBP-1 induced apoptosis after treatment for 24 h in T47D cells (Fig. 3A and B). Pre-G1 values significantly increased (6.7%; $P$<0.01) over control values after treatment with 100 µg/ml synthetic RGD-containing peptide (Fig. 3A). Similarly, IGFBP-1 (Fig. 3B; 400 and 800 ng/ml) also caused significant increases (2.8 and 3.2%; $P$<0.05) in the extent of apoptosis relative to untreated samples. Morphological analysis also indicated classical features associated with apoptosis in the T47D cells after IGFBP-1 treatment (Fig. 3C and D). Cell shrinkage, cytoplasmic condensation and loss of nuclear membrane integrity were evident.

The effects of the RGD-containing peptide and IGFBP-1 in the Hs578T cell line were also comparable to data in the T47D cells. The RGD-containing peptide (25 and 100 µg/ml) caused increases in the rate of apoptosis relative to control values (3.2 and 17.9% respectively) as did IGFBP-1 (400 and 800 ng/ml) at 1.2 and 4.6% respectively.

We also demonstrated, using the MTT assay, which is a crude measure of cell viability, that the synthetic RGD-containing peptide had differential effects on cell survival depending on cell type, with the Hs578T cells being the most sensitive (Fig. 4).

**Localisation of $\alpha_5$ and $\beta_1$ integrin subunits by immunocytochemistry**

We demonstrated using immunocytochemistry that the cell lines T47D and Hs578T stained positively for $\alpha_5$ and $\beta_1$ integrin subunits. These are the components of the $\alpha_5\beta_1$ fibronectin receptor, which
has been demonstrated conclusively to be specifically bound by IGFBP-1 (Jones et al. 1993). The antibody to the β1 subunit identified the membrane-associated region and the anti-α5 recognised the cytoplasmic domain of this integrin in both cell lines (Fig. 5A–C).

**DISCUSSION**

It was originally believed that the integrins were a family of receptors which simply allowed cells to adhere to the ECM. However, it is becoming increasingly evident that they can transmit signals regulating cell growth, differentiation and death.

In this study we have shown that treatment of human breast cancer cells, T47D and Hs578T, with a synthetic RGD-containing peptide or with IGFBP-1 resulted in FAK dephosphorylation after 30 min and subsequent cell detachment and death by apoptosis after 24 h. FAK is thought to play a key role in integrin-mediated signal transduction pathways (Zachary & Rozengurt 1992). FAK phosphorylation is increased by integrin-mediated cell adhesion, for example when platelets (Lipfert et al. 1992) or benign and malignant cells attach to fibronectin (Guan & Shalloway 1992). Clustering of β1 and β3 integrins also induces the phosphorylation of FAK (Guan et al. 1991, Kornberg et al. 1991). In addition, extracellular agents, including platelet-derived growth factor (Rankin & Rozengurt 1994), bombesin or endothelin (Zachary et al. 1992), have also been shown to induce FAK phosphorylation in Swiss 3T3 cells.

The complete deletion of FAK causes a marked reduction in cell motility (Illic et al. 1995). Conversely, the overexpression of FAK is associated with increased cell motility as demonstrated by motile melanoma cells (Akasaka et al. 1995).

**FIGURE 3.** Measurement of apoptosis in T47D cells treated with a synthetic RGD-containing peptide (A) and IGFBP-1 (B) for 24 h. Cells were grown in six-well plates, allowed to settle and switched to SFM for 24 h prior to treatment. Cells were then incubated with either (A) a synthetic RGD-containing peptide (0–100 µg/ml) or (B) IGFBP-1 (0–800 ng/ml) for a further 24 h. Cells were analysed for apoptosis by flow cytometry as outlined in Materials and Methods. Results represent the mean ± s.e.m. of three wells from experiments repeated at least three times. (C and D) Represent photomicrographs of control cells and those treated with 800 ng/ml IGFBP-1 respectively. Cells were cyto spun and stained with Wright’s stain in an automated stainer (Lillie 1977). Photomicrographs were taken under oil immersion at a magnification of × 100.
Furthermore, the invasive and metastatic phenotype in solid tumours has also been correlated with increased FAK expression (Owens et al. 1995).

In this paper we have demonstrated that a synthetic RGD-containing peptide and IGFBP-1 caused FAK to be dephosphorylated. Previous data have shown that generally FAK requires to be phosphorylated for subsequent effects to be elicited. However, one paper did report the dephosphorylation of FAK by insulin in HIRc cells (Pillay et al. 1995) although its functional significance at that time was unknown. In our model the dephosphorylation of FAK after 30 min was followed by subsequent cell detachment and deletion by apoptosis. As with other acute signalling events, the link with subsequent changes in cell function remains to be proven.

Apoptosis is a highly regulated mode of cell death which is characterised by a number of morphological and biochemical features including cell blebbing of the plasma membrane, cell shrinkage, chromatin condensation and DNA fragmentation into membrane-bound vesicles (Wyllie 1980). This process allows harmful, redundant or abnormal cells to be removed without harm to the organism. It allows a balance between cell proliferation and death ensuring homeostasis in a multicellular organism. However, dysregulation of apoptosis can lead to the emergence of many diseases including AIDS and neurodegenerative disorders where apoptosis is upregulated, and cancer where it is inhibited (Thompson 1995).

Our results show that IGFBP-1 has the ability to induce cell detachment in both the Hs578T and T47D cell lines and the degree of detachment was much greater in the T47Ds in comparison to the Hs578T cells. This could reflect that each cell line possesses a different complement of integrin receptors, varying in both type and number. This is further highlighted by the differential dose-dependent effects of the synthetic RGD-containing peptide in the different breast cancer cell lines.
We have demonstrated previously that Hs578T cells have no functional type I IGF receptors and cannot be rescued by IGF-I from ceramide-induced apoptosis (Gill et al. 1997). These data provide evidence that the induction of apoptosis 24 h after IGFBP-1 treatment described in this cell line is therefore independent of IGF-I. The rates of cell detachment and subsequent apoptosis in this cell line are also fairly comparable. In contrast, T47D cells showed high rates of cell detachment which were followed by comparably lower rates of apoptosis. These data suggest that a large proportion of the T47D cells which have been detached from the plate by both RGD and IGFBP-1 are still alive due to IGF-mediated cell survival. Therefore, the amount of apoptosis we have found in the T47D cell line, induced by RGD and IGFBP-1, is likely to be underestimated.

Finally, the higher rates of cell death induced by RGD in the Hs578T in comparison to the T47D cells were corroborated by the MTT assay, which is a crude measure of cell survival. These data confirmed that the Hs578T cells were the most sensitive to RGD-induced apoptosis. In addition, they illustrated that all the different cell lines responded differently to RGD, which was probably dependent on their respective complement of integrin receptors.

IGF-independent effects of IGFBP-1 could have particularly important physiological implications in the body, where it is expressed. The IGF system is thought to mediate steroid hormone actions in both the ovary and endometrium. The biological effects of the IGFs within the endometrium are modulated by specific binding proteins which can inhibit or enhance effects at the cellular level (Rutanen et al. 1994). It has been shown that IGFBP-1 is expressed in the endometrium only during the secretory phase of the menstrual cycle (Zhou et al. 1994), which is associated with upregulation of apoptosis. Interestingly, in endometrial cancer the expression of IGFBP-1 is suppressed with no cyclic variation (Rutanen et al. 1994). We have demonstrated that IGFBP-1 can induce apoptosis independently of IGFs. Therefore, in the normal cycle during the secretory phase IGFBP-1 could be contributing to the increased rate of apoptosis required for tissue remodelling. The downregulation of IGFBP-1 which occurs in endometrial cancer could cause an imbalance between cell growth and death.

IGF-independent effects of IGFBP-1 could also play a role in the process of cytotrophoblast invasion. Trophoblasts upregulate $\alpha_5\beta_1$ receptors as they invade decidual ECM matrix (Damsky et al. 1992). IGFBP-1 is localised in stromal cells and the ECM of the maternal decidua (Rutanen et al. 1984). Therefore, IGFBP-1 is present at the maternal–foetal interface, which may suggest that it could bind to the $\alpha_5\beta_1$ receptor on the trophoblast and regulate its invasion into the maternal decidua.

We have demonstrated previously that Hs578T cells have no functional type I IGF receptors and cannot be rescued by IGF-I from ceramide-induced apoptosis (Gill et al. 1997). These data provide evidence that the induction of apoptosis 24 h after IGFBP-1 treatment described in this cell line is therefore independent of IGF-I. The rates of cell detachment and subsequent apoptosis in this cell line are also fairly comparable. In contrast, T47D cells showed high rates of cell detachment which were followed by comparably lower rates of apoptosis. These data suggest that a large proportion of the T47D cells which have been detached from the plate by both RGD and IGFBP-1 are still alive due to IGF-mediated cell survival. Therefore, the amount of apoptosis we have found in the T47D cell line, induced by RGD and IGFBP-1, is likely to be underestimated.
In summary, we have demonstrated that IGFBP-1 and a synthetic RGD-containing peptide can induce FAK dephosphorylation after 30 min with subsequent cell detachment and death by apoptosis. Furthermore, these data suggest that IGFBP-1 has the ability to evoke integrin-mediated IGF-independent effects in vivo.

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