Partitioning of IGFBP-5 actions in myogenesis: IGF-independent anti-apoptotic function

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Accepted 1 December 2003
Journal of Cell Science 117, 1737-1746 Published by The Company of Biologists 2004
doi:10.1242/jcs.01028

Summary

Igfbp5 **is upregulated during the differentiation of several key cell lineages and in some tumours; the function of IGFBP-5 in these physiological and pathological situations is unknown. Since IGFBP-5 contains sequence motifs consistent with IGF-independent actions, the aim of these studies was to distinguish between IGF-dependent and -independent actions of IGFBP-5. Myc-tagged wild-type (termed wtIGFBP-5) and non-IGF binding mouse** ***Igfbp5*** **(termed mutIGFBP-5) cDNAs were generated and used to transfect C2 myoblasts, a cell line that undergoes differentiation to myotubes in an IGF- and IGFBP-5-regulated manner. WtIGFBP-5, but not mutIGFBP-5, inhibited myogenesis, as assessed by cell morphology, MHC immunocytochemistry and caveolin 3 expression. However, both wt- and mutIGFBP-5 increased cell survival and decreased apoptosis, as indicated by decreased caspase-3 activity and cell surface annexin V binding. Further examination of apoptotic pathways revealed that wt- and mutIGFBP-5 ameliorated the increase in caspase-9 but not the modest increase in caspase-8 during myogenesis, suggesting that IGFBP-5 increased cell survival via inhibition of intrinsic cell death pathways in an IGF-independent manner. The relationship between IGF-II and IGFBP-5 was examined further by cotransfecting C2 myoblasts with antisense** ***Igf2*** **(previously established to induce increased cell death) and** ***Igfbp5*****; both wt- and mutIGFBP-5 conferred equivalent protection against the decreased cell survival and increased apoptosis. In conclusion, we have partitioned IGFBP-5 action in myogenesis into IGF-dependent inhibition of differentiation and IGF-independent cell survival. Our findings suggest that, by regulation of cell survival, IGFBP-5 has an autonomous role in the regulation of cell fate in development and in tumourigenesis.**

Key words: Insulin-like growth factor binding protein-5 (IGFBP-5), Apoptosis, Cell survival, Myogenesis, Myoblast differentiation

Introduction

Recent studies have revealed that insulin-like growth factor binding protein-5 (*Igfbp5*) expression may be substantially upregulated in tumours. For example, *Igfbp5* accelerates progression of prostate cancer to androgen independence and induces increased proliferation in prostate cancer cells (Miyake et al., 2000a; Miyake et al., 2000b); increased levels of *Igfbp5* mRNA or protein have been consistently observed in thyroid carcinoma (Stolf et al., 2003), uterine leiomyomata (Tsibris et al., 2002) and breast cancers (Sheikh et al., 1993). Rhabdomyosarcoma (RMS) is a muscle-derived tumour and is one of the most common soft tissue sarcomas of childhood, accounting for 10-15% of childhood cancers (reviewed by Pappo, 1996). Two types of RMS have been characterised: embryonal and alveolar, of which the latter has a poor prognosis. Two independent cDNA microarray studies have highlighted *Igfbp5* as one of the most upregulated genes in myoblasts transfected with the PAX3-FKHR translocation that occurs in alveolar RMS (Khan et al., 1999; Astolfi et al., 2001).

Igfbp5 is expressed from early embryonic development (Green et al., 1994) and is substantially upregulated during the differentiation of specific key cell lineages (Thrailkill et al., 1995; Cheng et al., 1999), including myoblasts (James et al., 1993). Significantly, IGFBP-5 can determine head and neck induction in developing *Xenopus* (Pera et al., 2001). It is one of the most upregulated genes induced by the myogenic determination factor MyoD, which occurs in the absence of intermediate protein synthesis, implying direct transcriptional regulation (Bergstrom et al., 2002). Indeed, the *Igfbp5* promoter contains relevant response elements, including an E box (Kou et al., 1995). The upregulation of *Igfbp5* in neuroblastoma cells may be regulated directly at the transcriptional level by *Myb* genes (Tanno et al., 2002).

The function of IGFBP-5 in tumourigenesis and in development remains unidentified. IGFBP-5 may bind the IGFs with high affinity, inhibiting growth factor activity by preventing interaction with the IGF type 1 receptor; in vitro studies have further suggested that, in certain circumstances, IGFBP-5 may augment IGF activity by facilitating ligand-receptor interaction (Clemmons, 2001). Intriguingly however, an increasing number of IGF-independent actions of IGFBP-5 are being identified. IGFBP-5 stimulates proliferation of osteoblast and mesangial cells (Berfield., 2000) and intestinal smooth muscle cells (Kuemmerle and Zhou, 2002), as well as osteoblast activity in vivo (Richman et al., 1999; Miyakosi et al., 2001) via IGF-independent mechanisms which have not yet been characterised.

A cellular IGFBP-5 binding site has been reported (Andress, 1995) but to date, no receptor has been cloned.

IGFBP-5 is the most conserved of the IGFBPs (James et al., 1993). Structural and mutational studies have demonstrated that the primary IGF binding domain of IGFBP-5 resides within a hydrophobic patch located in the amino terminal domain (Kalus et al., 1998; Zestawski et al., 2001) with a secondary site of interaction in the C-terminal region, that is thought to stabilise binding (Shand et al., 2003). However, examination of the primary sequence of IGFBP-5 reveals the presence of additional putative functional motifs. The C-terminal domain contains a consensus nuclear localisation signal and nuclear import of IGFBP-5 has been demonstrated in vitro (Schedlich et al., 2000; Schedlich et al., 1998); moreover, IGFBP-5 interaction with the nuclear transcription factor FHL2 was recently demonstrated using osteoblasts (Amaar et al., 2002). This basic residue-rich region of the C-terminal domain is also responsible for IGFBP-5 interaction with extracellular matrix (Parker et al., 1996). IGFBP-5 may also be phosphorylated (Coverley and Baxter, 1997) but the significance of this has not been explored.

The aim of these studies is to investigate IGF-dependent and -independent actions of IGFBP-5 using a physiologically relevant model. The mouse C2 myoblast satellite cell line undergoes myogenesis in vitro, when cells irreversibly exit from the cell cycle and fuse to become multinucleated myotubes; myoblasts are subject to increased susceptibility to apoptosis until cell cycle exit has occurred when an apoptosis-resistant phenotype is acquired. Myoblasts secrete IGFBP-5 and IGF-II at the onset of myogenesis and both regulate the myogenic process (Florini et al., 1991; James et al., 1996). The effects of wild-type and non-IGF binding murine IGFBP-5 on myoblast differentiation, proliferation and survival were therefore examined using this model. We demonstrate partitioning of IGFBP-5 activity into IGF-dependent and -independent actions.

Materials and Methods

Cloning and mutagenesis

The coding region for murine *Igfbp5* [in pEMSVscribe α2/*Igfbp5* expression plasmid (James et al., 1996)] was PCR amplified using *PfuTurbo*® DNA polymerase (Stratagene) including insertion of a C-terminal *Myc* epitope (upstream primer: 5′ CCG GAA TTC CGG AGG TAA AGC CAG ACT CCG 3′; downstream primer: 5′ CCG GAA TTC CGG TCA CAG GTC CTC CTC GCT GAT CAG CTT CTG CTC CTC AAC GTT ACT GCT GTC GAA GGC GTG 3′). The *Igfbp5/Myc* cDNA (termed wtIGFBP-5) was cloned into an expression vector (termed pCAGGS) containing a CMV-enhancer and β-actin promoter (Niwa et al., 1991) and also into pBluescript®II SK(+) (pBS, Stratagene).

The IGF-binding site of murine IGFBP-5 was mutated (Lys^{64}, Pro^{69}, Leu^{70}, Leu^{73} and Leu^{74} to Asn, Gln, Gln, Gln and Gln, respectively), based on the structural studies of IGFBP-5/IGF-II interaction (Kalus et al., 1998) and the sequences derived for in vitro studies using human IGFBP-5 mutants (Imai et al., 2000), using PCR-based mutagenesis (sense primer: 5′ GGC AGG ACG AGG AGA ATC AGC AGC ACG CCC AGC AGC ACG GCC GCG GGG TTT GC 3′, its reverse complementary antisense copy and high fidelity *Pfu* DNA polymerase, Stratagene). Template DNA was digested using *Dpn*I (Promega), and the non-IGF-binding *Igfbp5* (termed mutIGFBP-5) was cloned into the pCAGGS vector.

Cell culture and transient transfections

All cell culture reagents were purchased from Invitrogen. C2 myoblasts (a gift from Dr C. E. H. Stewart, University of Bristol, UK), a line derived from murine muscle satellite cells, were seeded at 1×10^5 cells on 60 mm dishes coated with 2% gelatin. Cells were initially cultured in growth medium (GM, Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum and 10% foetal bovine serum). At 80% confluency (24 hours after transfection), myogenesis was induced by switching cells to differentiation medium (DM, DMEM supplemented with 2% horse serum). Media were refreshed every 24 hours. Myoblasts (in GM) were co-transfected with either pCAGGS (i.e. vector alone), wtIGFBP-5 or mutIGFBP-5 and pEGFP (Clontech Laboratories; to determine transfection efficiency) using Effectene™ transfection reagent (Qiagen) following the manufacturer's instructions. Transfection efficiency was consistently 30-35%. When interactions between IGF-II and IGFBP-5 were investigated, myoblasts were initially cotransfected with pEGFP and an *Igf2* antisense construct (Stewart and Rotwein, 1996a) or vector alone (pEMSV); cells were then selected for 3 passages over 10 days with G418 (800 μg/ml) to generate a population of bulk selected myoblasts; cotransfection with GFP and visualisation of the proportion of fluorescing cells indicated selection efficiency. Cells were subsequently transfected with the *Igfbp5* constructs in the presence of G418.

Cell morphology

C2 cells were washed 3 times with ice cold PBS, and fixed with 4% paraformaldehyde for 15 minutes at room temperature and analysed morphologically (Leica MZ6 microscope) following staining with Gill's Haematoxylin and Eosin (Langley et al., 2002).

Immunofluorescence

Myoblasts were washed with PBS and fixed in 4% paraformaldehyde for 10 minutes, incubated with 0.2% Triton-X-100 in PBS for 5 minutes and then with anti-MHC antibody at a dilution of 1:200 (MF20, University of Iowa) for 3 hours at room temperature. After washing in Triton-PBS, cells were incubated with Texas-Red-conjugated goat anti-mouse antibody (Jackson ImmunoResearch) for 45 minutes at room temperature. Nuclei were stained with DAPI (Vector Laboratories).

Cell survival analysis

Percentage cell survival was determined as previously described (Lawlor and Rotwein, 2000) by counting total and pEGFP fluorescent cells every 24 hours, using a haemocytometer.

Caspase activity

Caspase-3, -8 and -9 activities were determined by cleavage of the fluorigenic Ac-DEVD-AMC caspase-3 substrate, Z-IETC-AFC Granzyme B/caspase-8 substrate and Ac-LEHD-AFC caspase-9 substrate (Calbiochem). Cells (detached and adherent) were washed with ice cold PBS, harvested into caspase lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% Nonidet P-40) and incubated on ice for 20 minutes. Caspase activity was assayed using 50 μg protein from cell extracts and 50 μM (caspase-3) or 100 μM (caspase-8 or -9) substrate (Hermisson et al., 2000).

Annexin V staining flow cytometry

Adherent and detached cells were washed in PBS and binding buffer (10 mM Hepes pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). After centrifugation, cells were resuspended in binding buffer containing 10 μg/ml propidium iodide and 2 μg/ml Annexin V-FITC (both Sigma)

and incubated for 15 minutes in the dark at room temperature. Samples were analysed using CellQuest software on FACSCalibur (Beckon Dickinson).

BrdU incorporation assay

S phase occupancy as an indication of proliferation rate of C2 cells was measured using a BrdU Cell Proliferation Assay Kit (Oncogene Research Products) following the manufacturer's instructions; cells were incubated with BrdU for 24-hour periods.

Immuno- and ligand blotting

C2 cells on 60 mm plastic dishes were washed three times with PBS and harvested in 250 μl lysis buffer (20 mM Tris-HCl, pH 7.5; 137 mM NaCl; 1 mM EGTA pH 8.0; 1% Triton X-100; 10% glycerol; 1.5 mM $MgCl_2$; 10 mM NaF; 1 mM PMSF; 1 mM $NaVO_3$; 5 μg/ml aprotinin; 10 μg/ml leupeptin). Total protein concentrations were determined by the Bradford protein assay (BioRad). Cell lysates containing 20 μg protein (ligand blot) or 50 μg protein (immunoblot) were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) in the absence (ligand blot) or presence (immunoblot) of β-mercaptoethanol and transferred to Immobilon-P membranes (Millipore). For ligand blots, membranes were blocked in TBS containing 0.2% Tween 20 (TBST) with 2% BSA (RIA grade; Sigma). The membranes were incubated with 100 ng/ml biotinylated IGF-I or -II (GroPep) diluted in TBST, followed by HRP-conjugated streptavidin (Jackson Immunoresearch). Antibody-antigen complexes were visualised by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) following the manufacturer's instructions. For immunoblots, membranes were blocked in 0.2% I-block™ (Applied Biosystems) in TBS containing 0.1% Tween 20 for 1 hour at 37°C, and then probed with the appropriate primary (anti-caveolin 3, BD Biosciences; anti-Myc, Cell Signalling Technologies) and secondary antibodies (Jackson Laboratories). Antibody-antigen complexes were visualised by ECL.

Northern blot analysis

Total RNA was extracted from C2 cells using RNeasy® Mini Kit (Qiagen) following the manufacturer's instructions. 20 μg RNA per sample was separated on a 1.2% agarose/17% formaldehyde gel and transferred to Hybond N+ membrane (Amersham Pharmacia Biotech) in 20× SSC (1.5 M NaCl, 150 mM sodium citrate, pH 7.0) overnight. A cDNA probe for *Igf2* (Miguel Constancia, The Babraham Institute) was [α-^{32}P]γCTP labelled by random priming (HI-PRIME, Roche) and hybridisation was carried out at 65°C overnight.

Statistical analyses

Data were analysed by analysis of variance (for comparison between group means) or Student's *t*-test (to assess whether mean values were significantly different from 1.0) and are presented as means±s.e.m.

Results

Expression and IGF binding ability of wtIGFBP-5 and mutIGFBP-5

The expression of the transfected IGFBP-5 protein was determined in C2 myoblasts by presence of the C-terminal Myc epitope (Fig. 1A). Equivalent levels of IGFBP-5/Myc were observed for both wtIGFBP-5 and mutIGFBP-5 transfected cells at each time point, with maximum expression after 48 hours in DM; as expected, no Myc was detected in myoblasts transfected with vector alone.

The ability of wt- and mutIGFBP-5 to bind to IGFs was assessed by ligand blot analysis (Fig. 1B), as C2 myoblasts only synthesise IGFBP-5 (James et al., 1993). When IGF-I was the ligand, a single band was initially observed at approximately 32 kDa for the wtIGFBP-5 transfected cells, which co-migrated with the anti-Myc reactivity presented in Fig. 1A. Prolonged exposure revealed slightly lower M_r bands of similar intensity in both the control (i.e. vector only) and mutIGFBP-5-transfected cells, which did not have anti-Myc reactivity. Similar observations were made when IGF-II was the ligand (data not shown). It was concluded that the lower M_r band was endogenous IGFBP-5 and the higher M_r band (which would include the Myc tag) was transfected *Igfbp5*. Therefore, only the wtIGFBP-5, and not the mutIGFBP-5, had appreciable binding to either IGF-I or -II, confirming previous conclusions of Imai et al. (Imai et al., 2000) and Shand et al. (Shand et al.,2003) that this N-terminal mutant of IGFBP-5 has

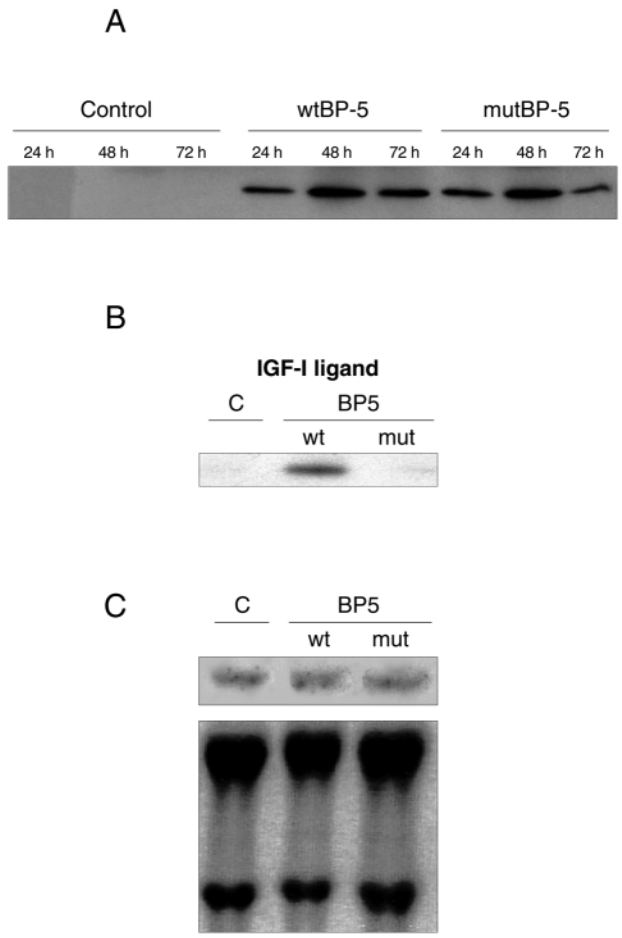

Fig. 1. Expression, IGF binding ability, and effects on *Igf2* mRNA levels of wtIGFBP-5 and mutIGFBP-5. (A) Western immunoblot to detect the Myc epitope in cell lysates from C2 myoblasts transiently transfected with control vector, wtIGFBP-5 (wtBP5) or mutIGFBP-5 (mutBP5) and transferred to DM for 24, 48 and 72 hours. (B) Ligand blot using biotinylated IGF-I to detect IGFBP-5 in cell lysates from C2 myoblasts transfected as in *A* and harvested after 48 hours in DM. (C) Northern blot to detect *Igf2* mRNA in myoblast lysates after transfection as in A and harvested after 72 hours; (upper panel) the major *Igf2* transcript at 3.9 kb and (lower panel) ethidium bromide staining of the 28S and 18S RNA bands as a loading control.

very much reduced IGF binding ability. A three- to fivefold increase in IGFBP-5 levels was quantified in lysates of cells transfected with *Igfbp5* constructs versus vector alone.

We investigated whether overexpression of *Igfbp5* might modulate endogenous IGF-II synthesis; northern blotting for *Igf2* mRNA revealed no change between vector alone, wtIGFBP-5 or mutIGFBP-5 transfected cells (Fig. 1C).

Non-IGF binding IGFBP-5 does not inhibit myogenesis

Transfection of C2 myoblasts with native sequence IGFBP-5 inhibits myogenesis (James et al., 1996). We therefore compared the ability of wtIGFBP-5 and mutIGFBP-5 to prevent myoblast differentiation. Morphological examination of C2 cells in DM using hours and E staining (Fig. 2A) revealed that even though no myotubes were observed in cells transfected with the IGF-binding wtIGFBP-5, myoblasts transfected with mutIGFBP-5 consistently differentiated into myotubes and resembled vector only transfected cells. Consistent with these observations, both vector alone and mutIGFBP-5 cells were undergoing differentiation, assessed by expression of the myogenic marker MHC, whereas cells transfected with wtIGFBP-5 exhibited greatly reduced MHC expression (Fig. 2B). Closer examination of the wtIGFBP-5 transfected cells, demonstrated that they aligned but fused more slowly than the vector alone or mutIGFBP-5-transfected cells. We therefore investigated expression of caveolin 3, which is essential for myoblast fusion (Galbiati et al., 1999). Equivalent protein levels of caveolin 3 were observed in vector alone and mutIGFBP-5 transfected cells but were significantly reduced in myoblasts transfected with wtIGFBP-5 (% vector alone transfected cells: wtIGFBP-5, 82.8±1.72, $P<0.01$; mutIGFBP-5 98.9±3.93, NS). Taken together, these data are therefore consistent with the hypothesis that IGFBP-5 inhibits myogenic fusion through the sequestration of IGFs.

IGFBP-5 is a survival factor in myogenesis

To assess the effects of IGFBP-5 on myoblast cell number, C2 cells were transiently co-transfected with pEGFP and control vector, wt- or mutIGFBP-5. Cell survival was assessed by counting the number of GFP-fluorescing cells during differentiation (Fig. 3A). Myoblast number was equivalent between the three groups at 0 hours (vector alone, 12.94±1.36; wtIGFBP-5, 11.94±0.84; mutIGFBP-5, 11.50±0.96 $\times 10^4$ cells/ml). Myoblasts transfected with vector alone exhibited an increase in cell number after 24 hours in DM, followed by a progressive decline at 48 and 72 hours. Cells overexpressing wt- and mutIGFBP-5 had significantly increased cell number at all time points after they were switched to DM, when compared with vector control cells ($P<0.001$).

Changes in cell number could be due to increased proliferation or to decreased cell death. Therefore myoblast proliferation was determined by BrdU incorporation. Consistent with previous observations (Coolican et al., 1997), proliferation rate increased over the initial 24 hours followed by a decline in all treatment groups (Fig. 3B). However, no differences in proliferation were observed between myoblasts transfected with either vector alone, wt- or mutIGFBP-5 at any time point. Therefore, the increased cell number induced by either wtIGFBP-5 or mutIGFBP-5 must be due to decreased cell death. These data suggest that IGFBP-5 may function as a survival factor during myogenesis, and that this activity is independent of IGF binding.

IGFBP-5 as an anti-apoptotic factor in myogenesis

In order to determine whether the increased survival seen in *Igfbp5*-overexpressing cells was due to decreased apoptosis, we assessed annexin V staining and caspase-3 activity. After 24 hours in DM, the proportion of annexin V-positive/

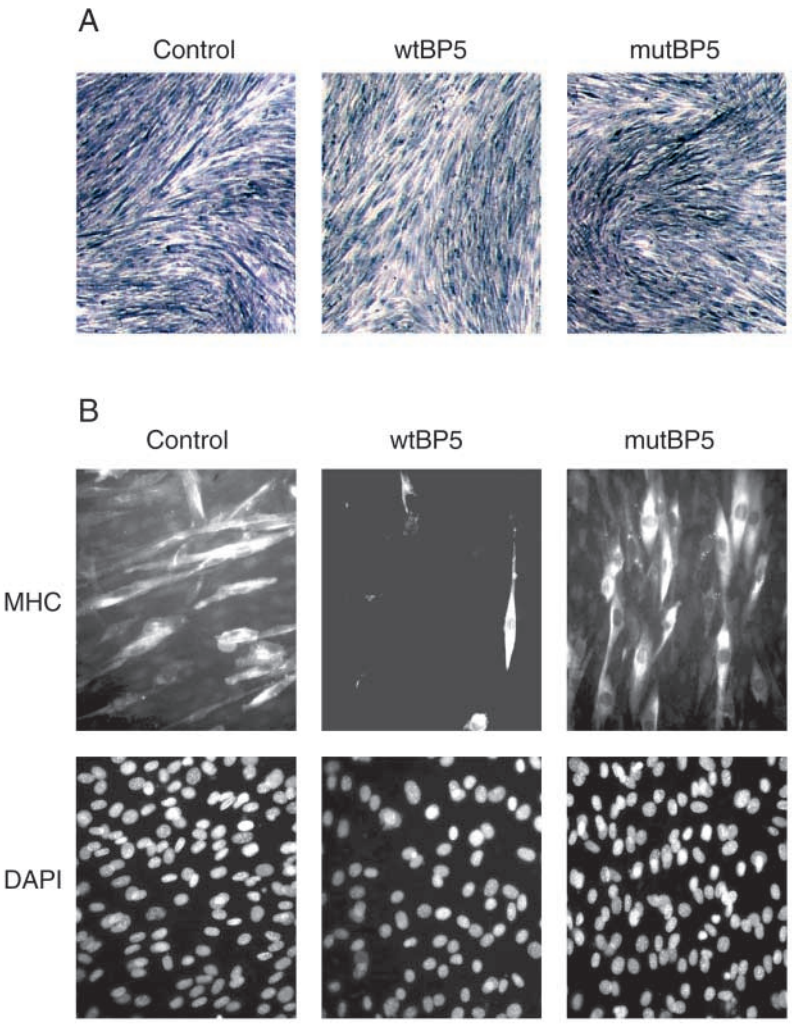

Fig. 2. WtIGFBP-5 but not mutIGFBP-5 inhibits myogenesis. (A) Haematoxylin and Eosin staining of C2 cells transfected with control vector, wtIGFBP-5 (wtBP5) or mutIGFBP-5 (mutBP5), 72 hours after transfer to DM (5× magnification). (B; upper panel) Fluorescent immunocytochemistry to show myosin heavy chain (MHC) protein in myoblasts transfected as in A; (lower panel) DAPI staining of nuclei (40× magnification).

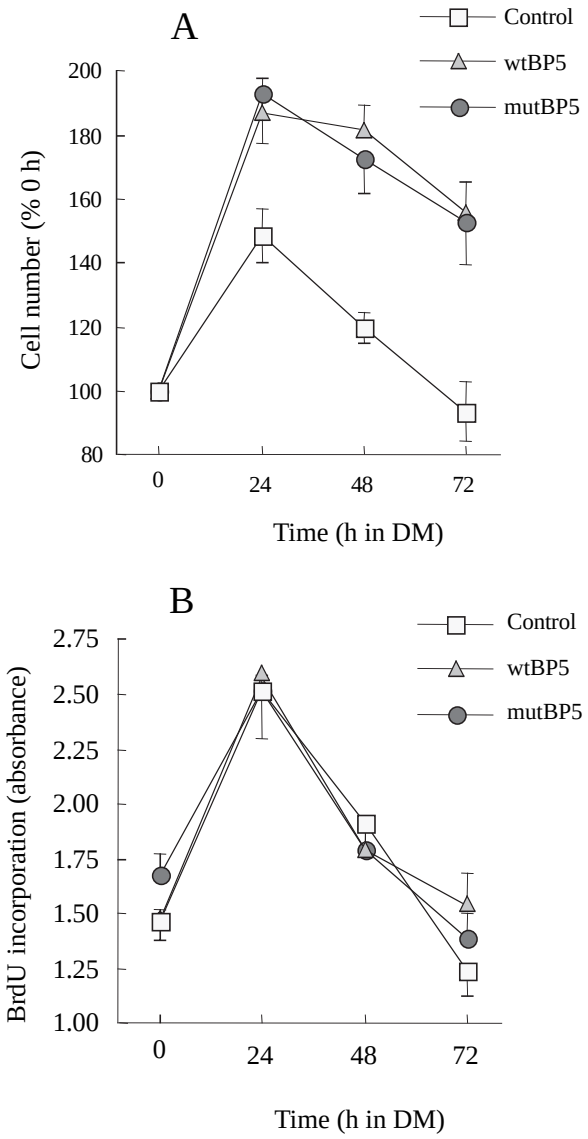

Fig. 3. WtIGFBP-5 and mutIGFBP-5 increase myoblast cell number but do not change proliferation rate. (A) Cell number, expressed as percentage of cells in GM compared with DM, in myoblasts transfected with control vector, wtIGFBP-5 (wtBP5) or mutIGFBP-5 (mutBP5); means±s.e.m.; n=3. (B) BrdU incorporation into myoblasts transfected as in A; mean±s.e.m., n=3.

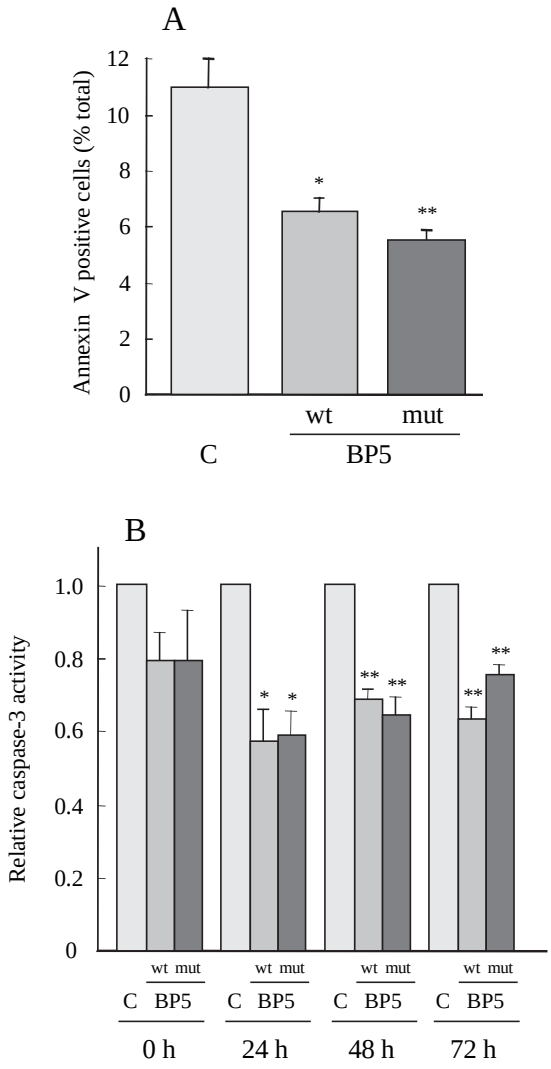

Fig. 4. WtIGFBP-5 and mutIGFBP-5 inhibit apoptosis in myoblasts. **(**A) Flow cytometric determination of the percentage of annexin V-positive, PI-negative myoblasts transfected with either control vector (C), wtIGFBP-5 (wtBP5) or mutIGFBP-5 (mutBP5) as an indication of apoptosis levels in C2 myoblasts after 24 hours in DM; mean±s.e.m., n=3; *P<0.05 compared with control vector; **P<0.01 compared with control vector. (B) Caspase-3 activity in myoblast lysates after transfection as in A, expressed as a fraction of control vector transfected cells at each time point after transfer to DM; means±s.e.m., n=3; significance that mean is less than 1.0: *P<0.05; **P<0.01.

propidium iodide-negative cells was significantly decreased in myoblasts transfected with either wtIGFBP-5 (P<0.05) or mutIGFBP-5 (P<0.01) compared with cells transfected with vector alone (Fig. 4A), indicating that IGFBP-5 was protecting the myoblasts from apoptosis rather than necrosis. Similar changes in annexin V staining were also observed at 48 hours, although more cells were both annexin V and PI positive, making it difficult to distinguish apoptotic death reliably. To confirm findings from annexin V staining, caspase-3 activation was also determined (Fig. 4B). In cells transfected with vector alone, accumulated caspase-3 activity increased during the first 48 hours of differentiation (fold increase above myoblasts in GM at: 24 hours, 9.17±2.74; 48 hours, 28.2±9.25; 72 hours, 42.1±11.9; n=3; P<0.05 for 24 hours versus GM and 48 versus 24 hours, NS for 72 versus 48 hours), confirming previous observations (Stewart and Rotwein, 1996a; Lawlor and Rotwein, 2000). Fig. 4B shows the change in caspase-3 activity in *Igfbp5*-transfected cells relative to vector alone-transfected cells. Intriguingly, wt- and mutIGFBP-5 induced a modest decrease in caspase-3 activity even in GM, which was exacerbated after transfer of myoblasts into low serum DM (P<0.05 at 24 hours; P<0.01 at 48 and 72 hours). Moreover, caspase-3 activity was equivalent for the IGF binding wtIGFBP-5 and the non-IGF binding mutIGFBP-5-transfected cells, further supporting the hypothesis that a cell survival role for IGFBP-5 is independent of direct IGF interaction. These

findings suggest that IGFBP-5 acts as an anti-apoptotic factor during myogenesis.

IGFBP-5 inhibits caspase-9 activity during myogenesis

Since caspase-3 activity represents the convergence of the major apoptotic pathways in cells, caspase-8 and -9 activities were also determined to indicate the extrinsic and intrinsic pathway activities respectively. After transfer to DM, caspase-8 activity increased only twofold (24 hours, 2.65±0.28; 48 hours, 1.60±0.25) whereas caspase-9 activity increased four- to sixfold (24 hours, 4.10±0.73; 48 hours, 6.13±2.11) in vector alone transfected cells. Caspase-8 activity was decreased, though only just significantly (P<0.05), in myoblasts transfected with both wt- and mutIGFBP-5 in GM, although neither form of IGFBP-5 protected cells from the subsequent increase in caspase-8 following transfer to DM (Fig. 5A). However, caspase-9 activity was reduced in both wt- and mutIGFBP-5-transfected cells in both GM and DM and therefore they partially prevented the rise in caspase-9 activity that would have occurred (Fig. 5B). Thus, IGFBP-5 appears to regulate caspase-9- rather than caspase-8-dependent cell death during differentiation.

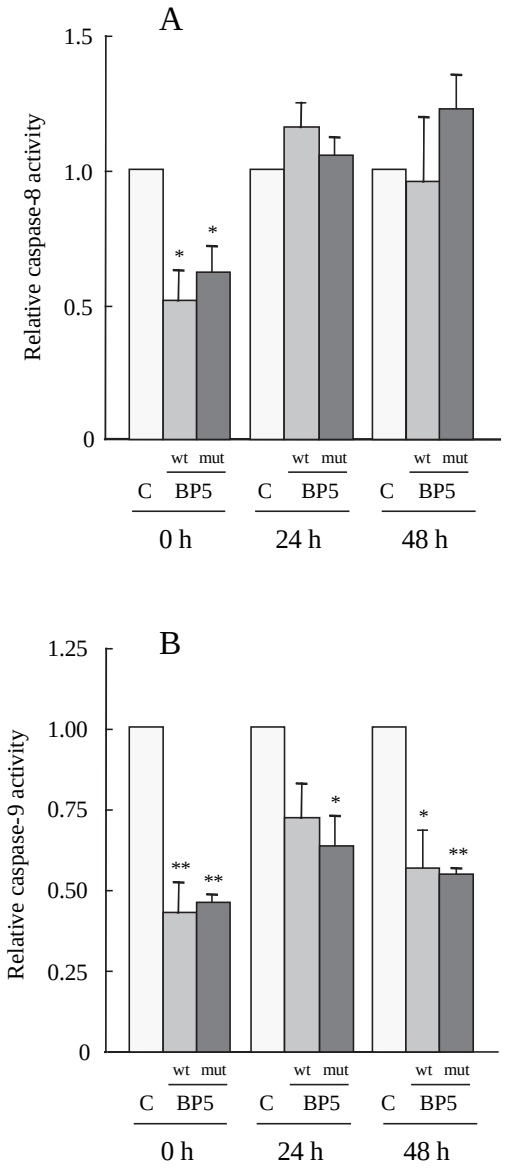

Fig. 5. Caspase-8 and caspase-9 activities in myoblasts during differentiation. Myoblasts were transfected with either control vector (C), wtIGFBP-5 (wtBP5) or mutIGFBP-5 (mutBP5) and (A) caspase-8, and (B) caspase-9 activities were determined in cell lysates during differentiation; means±s.e.m., n=3; data are expressed as a fraction of control values; significance that mean is less than 1.0: *P<0.05; **P<0.01.

IGFBP-5 counteracts decreased cell number induced by antisense *Igf2*

IGF-II is an important survival factor in myoblasts; C2 cells overexpressing antisense *Igf2* have, on growth factor withdrawal, significantly decreased survival (Stewart and Rotwein, 1996a). We therefore decided to use this model to further investigate the relationship between IGFs and IGFBP-5 during myogenesis. Populations of *Igf2* antisense transfected and pEMSV (control)-transfected cells were established (Stewart and Rotwein, 1996a), and enriched by use of antibiotic resistance to increase the percentage of transfected cells from 30-35% to 70-80%. During the first 24 hours following serum withdrawal, *Igf2* antisense expressing cells exhibited equivalent cell number compared with control pEMSV transfected cells (Fig. 6A, compare the V and antisense *Igf2* bars). However, significantly decreased myoblast number was observed at 48 and 72 hours (Fig. 6B). When cells were additionally transfected with IGFBP-5 control vector, cell number remained low but wtIGFBP-5 and mutIGFBP-5 transfection restored cell number almost to pEMSV values (Fig. 6B). Thus, IGFBP-5 prevented a reduction in cell number in *Igf2* antisense cells, irrespective of IGF binding, further supporting the hypothesis that IGFBP-5 acts as a potent survival factor during myogenesis.

IGFBP-5 protects C2 cells from the pro-apoptotic effects of *Igf2* antisense

The levels of apoptosis in *Igf2* antisense cells were assessed by both annexin V staining and caspase-3 activity. Fig. 7A shows apoptosis levels at 48 hours in DM, assessed by annexin V-positive/PI-negative staining in *Igf2* antisense cells additionally transfected with control vector, wt- or mutIGFBP-5. pEMSV populations exhibited significantly decreased apoptosis compared with *Igf2* antisense cells (P<0.01). Additional transfection of *Igf2* antisense cells with control vector induced a further increase in annexin V staining (P<0.01) which was prevented when either wt- or mutIGFBP-5 were the transfectant.

Fig. 7B shows caspase-3 activity in similar populations of pEMSV and *Igf2* antisense cells at 48 hours. As with annexin V staining, caspase-3 activity was induced several-fold in *Igf2* antisense cells (P<0.001). Unlike the results with annexin V but consistent with cell number analyses (described in Fig. 6), no further increase in caspase-3 activity occurred upon additional transfection with IGFBP-5 control vector alone. However, annexin V staining and caspase-3 activity represent temporarily distinct stages of the apoptotic cascade. When cells were transfected with either wt- or mutIGFBP-5, caspase-3

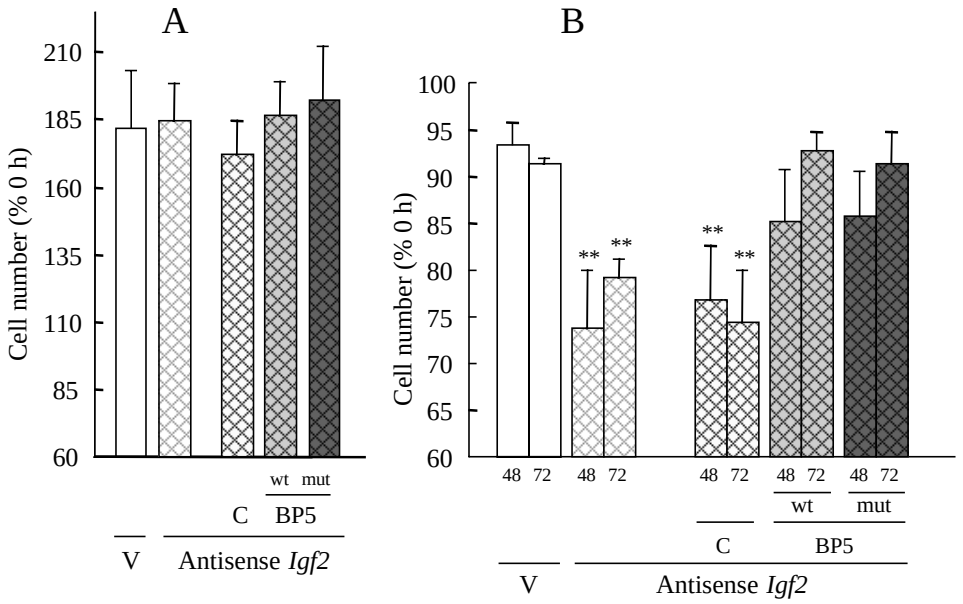

Fig. 6. WtIGFBP-5 and mutIGFBP-5 prevent the decrease in myoblast number induced by expression of antisense *Igf2*. Enriched myoblast populations expressing antisense *Igf2* or vector (V) were additionally transfected with wtIGFBP-5 (wtBP5) or mutIGFBP-5 (mutBP5) or their control vector (C). Cell number is expressed as a percentage of values at the time of transfer from GM to DM for myoblasts at (A) 24 hours and (B) 48 and 72 hours in DM; means±s.e.m., n=3; **P<0.01 compared with V.

activity was reduced by approximately 50% (P<0.05 for both) though not to pEMSV cell values. Thus presence of wt- or mutIGFBP-5 consistently reduced indicators of apoptosis when compared with control cells, and in a manner that is independent of IGF binding.

Discussion

The studies described here have revealed a novel, autonomous anti-apoptotic role for IGFBP-5 during myogenesis and have partitioned IGFBP-5 activities into IGF-dependent and -independent functions. Myogenesis was selected for investigation because myoblasts express and are regulated by both IGFBP-5 and the IGF ligands during differentiation; moreover, the fundamental processes of cell cycle exit, expression of lineage-specific genes and enhanced survival that occur in myogenesis make it an excellent model of cellular development. The significance of our findings is that successfully differentiated cells, which express increased levels of IGFBP-5, could have enhanced survival compared with non-differentiated counterparts. Thus *Igfbp5* could have a fundamental role in the determination of cell fate.

In terms of cell function, the dual actions of wtIGFBP-5 present a complex situation. The IGFs are established survival factors and their inhibition by IGFBPs such as IGFBP-5 could induce cell death, as well as reducing other IGF-stimulated actions. In contrast, the putative autonomous anti-apoptotic function of IGFBP-5 should promote cell survival. Consideration of recent findings on the actions of wtIGFBP-5, i.e. IGFBP-5 with the ability to bind IGFs, in vitro confirms this conflict. James et al. (James et al., 1996) reported that myoblasts transfected with sense *Igfbp5* cDNA had delayed differentiation, consistent with inhibition of IGF activity. However, Ewton et al. (Ewton et al., 1998) observed apparent potentiation of IGF-I stimulated differentiation in the presence of IGFBP-5. Our current studies using wt- and mutIGFBP-5 in myoblasts have identified IGFBP-5-mediated inhibition of differentiation as dependent on IGF binding (and presumably inhibition), confirming earlier conclusions derived from the use of non-IGFBP binding IGF analogs (James et al., 1996). Intriguingly, *Igfbp5* sense transfected myoblasts were reported to survive in culture for longer than those transfected with vector alone, although cell death was not formally quantified (James et al., 1996); stimulation of myoblast apoptosis by TNF-α suppressed IGFBP-5 secretion (Meadows et al., 2000). IGFBP-5 action in mammary epithelial cells is also inconsistent: IGFBP-5 has been reported to accelerate cell death (Butt et al., 2003) and inhibit the activity of established IGF signalling intermediates (Marshman et al., 2003) but to promote cell survival in the IGF-responsive MCF-7 breast cancer cell line (Perks et al., 2002). These conflicting findings could be caused by cell type-specific actions of IGFBP-5 or to method of administration but clearly it is important to utilise strategies to delineate IGF-dependent and -independent functions of IGFBP-5 for meaningful mechanistic analysis.

One approach is the use of cells that lack IGF signalling components. Since the IGF system is essential for normal cellular growth, differentiation and survival (Stewart and Rotwein, 1996b) and is often upregulated in tumourigenesis (Baserga, 1999), the spontaneous occurrence of cells lacking IGF signalling is unusual. However, the IGF-independent pro-apoptotic actions of IGFBP-3 were first identified using fibroblasts derived from IGF type 1 receptor null mice (Valentinis et al., 1995). A further model is the Hs578T breast cancer cell line that lacks functional IGF type 1 receptors and synthesises negligible amounts of IGF-I or -II; it is likely these cells must have acquired substantial compensatory mechanisms. Even with this consideration, IGFBP-5 promotes a modest increase in the survival of these cells if they have been exposed to a prior apoptotic stimulus, and in a manner that appears to be abolished when cell attachment is prevented (McCaig et al., 2002).

Mutagenesis has provided a powerful means of investigating IGFBP function (Clemmons, 2001). The stimulation of apoptosis by IGFBP-3 was unequivocally demonstrated to be independent of IGF interaction by generation of non-IGF

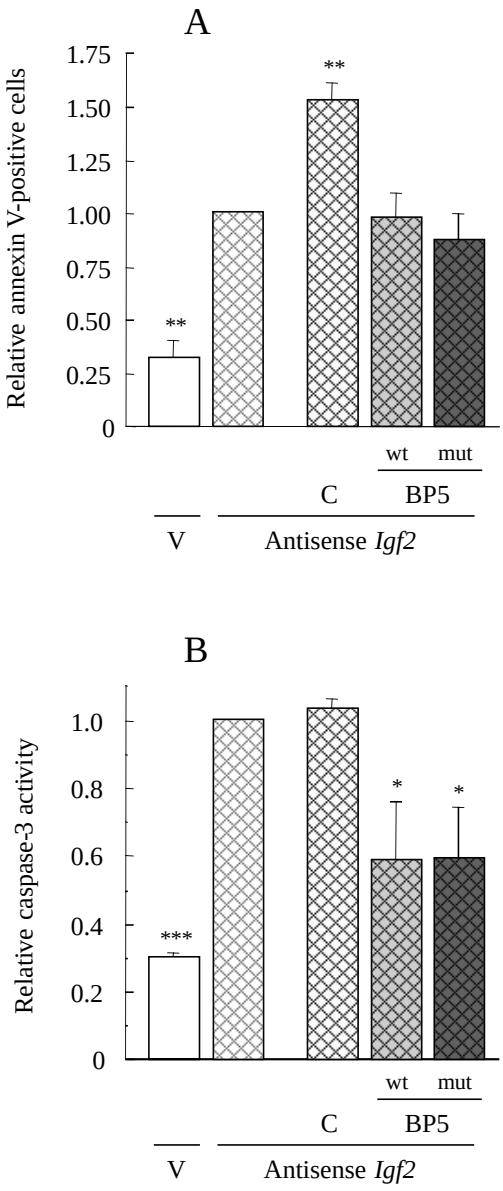

Fig. 7. WtIGFBP-5 and mutIGFBP-5 ameliorates the increased apoptosis induced by antisense *Igf2* expression in myoblasts. Enriched myoblast populations expressing antisense *Igf2* or vector (V) were additionally transfected with wtIGFBP-5 (wtBP5) or mutIGFBP-5 (mutBP5) or their control vector (C). (A) Flow cytometric determination of the percentage of annexin V-positive, PI-negative myoblasts after 48 hours in DM. (B) Caspase-3 activity in lysates derived from myoblasts transfected as in A. For A and B, data are expressed as a fraction of the values obtained for myoblasts transfected with antisense *Igf2* alone; means±s.e.m., n=3; significance that mean is less than 1.0: *P<0.05; **P<0.01. ***P<0.001.

binding mutants of IGFBP-3 (Hong et al., 2002). The non-IGF binding mutIGFBP-5 used in the current study has also clearly demonstrated autonomous anti-apoptotic actions of IGFBP-5. Our observations were extended further by investigation of the actions of the mutIGFBP-5 in myoblasts that exhibited increased cell death due to overexpression of antisense *Igf2*; even in this situation, nonIGF-binding IGFBP-5 was able to reverse the accelerated myoblast death. Perks et al., (Perks et al., 2002), using exogenous peptide, have suggested that, in IGF-responsive mammary cells, simultaneous and independent IGF signalling abolishes the cell survival action of IGFBP-5. Our findings strongly disagree with this hypothesis as it is established that C2 myoblasts express abundant endogenous IGF-II (our observations) (Florini et al., 1991); indeed preliminary studies in which mutIGFBP-5-transfected myoblasts were co-treated with exogenous IGF-I, suggested a further stimulation in survival and decrease in caspase-3 activity when compared with mutIGFBP-5 transfection or IGF-I alone (L.J.C. and J.M.P., unpublished observations).

The mechanism(s) by which IGFBPs exert IGF-independent actions have not been unequivocally determined. Cell surface binding sites have been identified in various cell types for both IGFBP-3 (e.g. Rajah et al., 1997) and IGFBP-5 (e.g. Liu et al., 2000) but no protein has been purified or sequenced to date. Yeast two-hybrid screens have identified nuclear binding partners for IGFBP-3 [RXR receptor (Liu et al., 2000)] and IGFBP-5 [FHL2 (Amaar et al., 2002)], which is supportive of their translocation to the nucleus. The molecular mass of IGFBP-3 indicates that it is secreted before translocation to the nucleus, consistent with the presence of a signal peptide sequence in IGFBPs and cell surface ‘receptors’; however internalisation of IGFBP-3 and -5 does not occur via known mechanisms (Schedlich et al., 1998). It is feasible therefore, that IGFBP-5 may have a role in the regulation of gene expression via interaction with transcription factors. It has also been suggested that multiple pathways exist for the growth inhibitory actions of IGFBP-3, because a mutant IGFBP-3 with reduced cell surface binding does not translocate to the nucleus and remains proapoptotic (Butt et al., 2002).

Two major forms of apoptotic cell death have been identified. The extrinsic pathway is, as the name suggests, initiated by extracellular insult, which triggers receptor (e.g. Fas)-mediated caspase-8 cleavage and activation which, in turn, catalyses cleavage of the downstream effector caspase-3 that acts on a series of protein targets, initiating the cellular changes leading to irreversible apoptotic cell death (Ashe and Berry, 2003). The intrinsic death pathway is activated by intracellular events, factors such as DNA damage, resulting in mitochondrial-mediated cell death (reviewed by van Loo et al., 2002). Pro-apoptotic members of the Bcl-2 family such as Bax undergo conformational change, oligomerization and translocation to the mitochondria, resulting in cytochrome c release that ultimately induces caspase-9 cleavage and activation; activated caspase-9 activates caspase-3 (Cain et al., 2002). Recent evidence also has identified a third endoplasmic reticulum stress-related apoptotic pathway in which caspase-12 mediates caspase-9 cleavage (Nakagawa et al., 2000).

During myogenesis, we observed an increase in the activity of the intrinsic pathway that was accompanied by a modest increase in the extrinsic pathway. IGFBP-5 ameliorated the increase in caspase-9 activity, suggesting that IGFBP-5 is acting on the Bcl-2-mediated mitochondrial death pathway or the caspase-12-mediated ER pathway. IGFBP-5 may therefore inhibit pro-apoptotic members of the Bcl-2 family such as Bax, or stimulate transcription of anti-apoptotic members such as Bcl-2 or Bcl-X_L; in this regard, wt- and mutIGFBP-5 reduced levels of activated Bax in myogenesis (L.J.C. and J.M.P., unpublished observations). Caspase-9 cleavage has frequently

been associated with the increased survival of many cancers, including colon (Wu et al., 2000), lung (Ding et al., 2002) and testicular cancers (Mueller et al., 2003). Using adenoviral delivery, Xie et al. (Xie et al., 2001) demonstrated that, in certain cell lines in vitro, and in vivo, expression of inducible caspase-9 caused significant apoptosis, leading to significant size reductions of xenograft tumours and greatly extended animal survival; consequently caspase-9 has been targeted for suicide gene therapy in prostate cancer. The upregulation of *Igfbp5* in specific tumours, including prostate, and its inhibition of caspase-9 activity is consistent with its potential contribution to tumour development. Interestingly, even though IGFBP-5 did not affect the increase in caspase-8 activity during myogenesis, it did reduce both caspase-8 and -9 activity before the induction of myoblast differentiation, i.e. when the cells were in high serum growth medium. The significance of this observation is not clear, as endogenous *Igfbp5* expression is not upregulated in myoblasts until after serum withdrawal, but it may indicate specific responses to IGFBP-5 in different physiological states.

In summary, we have partitioned IGFBP-5 action in myogenesis into an IGF-dependent inhibition of myoblast differentiation, and an IGF-independent, autonomous stimulation of cell survival. We speculate that the physiological increase in *Igfbp5* expression observed in the differentiation of many cell lineages and the pathological increase in *Igfbp5* expression observed in some tumours are related to the role of IGFBP-5 in cell survival, thus suggesting a key function for *Igfbp5* in cell fate.

We are very grateful to Dr Peter Rotwein, Oregon Health Sciences University for the gifts of *Igfbp5* sense- and *Igf2* antisense-containing plasmids. We thank the UK Biotechnology and Biological Sciences Research Council for Competitive Strategic, Responsive Mode (202/S15716) and Studentship (L.J.C.) funding for this work.

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