Effect of recombinant porcine IGF-binding protein-3 on proliferation of embryonic porcine myogenic cell cultures in the presence and absence of IGF-I

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Abstract

IGF-binding protein (IGFBP)-3 is produced by cultured porcine embryonic myogenic cell (PEMC) cultures and is secreted into the medium. Levels of secreted IGFBP-3 and IGFBP-3 mRNA are significantly reduced during differentiation and increase after differentiation is complete, suggesting that IGFBP-3 may play some role in myogenesis and/or in changes in myogenic cell proliferation that accompany differentiation. IGFBP-3 reportedly may either suppress or stimulate proliferation of cultured cells depending on cell type. Additionally, IGFBP-3 has been shown to affect proliferation via both IGF-dependent and IGF-independent mechanisms in some cell types but not all. Currently, the effect, if any, of IGFBP-3 on myogenic cell proliferation is not known. Consequently, the goal of this study was to assess the IGF-I-dependent and IGF-I-independent actions of recombinant porcine IGFBP-3 on proliferation of cultured porcine myogenic cells. To facilitate these investigations, we have expressed porcine

IGFBP-3 in the baculovirus system, purified and characterized the expressed recombinant porcine IGFBP-3 (rpIGFBP-3), and produced and characterized an anti-porcine IGFBP-3 antibody that neutralizes the biological activity of porcine IGFBP-3. rpIGFBP-3 suppressed IGF-I-stimulated proliferation of PEMCs in a concentration-dependent manner with equimolar concentrations of IGF-I and rpIGFBP-3, resulting in complete suppression of IGF-I-stimulated proliferation. rpIGFBP-3 also suppressed Long-R3-IGF-I-stimulated proliferation of PEMC, indicating that rpIGFBP-3 possesses IGF-independent activity in this cell system. These data have established that IGFBP-3 has the potential to affect proliferation of PEMCs during critical periods of muscle development that may impact ultimate muscle mass achievable postnatally.

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Introduction

Insulin-like growth factors (IGF)-I and -II have significant effects on both differentiation and proliferation of myogenic cells (Florini *et al.* 1996, Lawlor & Rotwein 2000, Tureckova *et al.* 2001). The actions of IGFs are regulated by members of the IGF-binding protein (IGFBP) family consisting of six proteins that bind IGF-I and -II with high affinity (Clemmons 1998, Hwa *et al.* 1999, Baxter 2000). Binding of IGF to IGFBPs has been shown to increase the stability of the IGFs and to affect their biological activity (Clemmons 1998, Hwa *et al.* 1999, Baxter 2000). Additionally, IGFBP-1, -3 and -5 reportedly exert IGF-independent actions, possibly by binding to cell surface receptors (Clemmons 1998, Hwa *et al.* 1999, Baxter 2000).

IGFBP-3 is produced by a number of different cell types including porcine embryonic myogenic cells (PEMCs) and

porcine muscle satellite cells (Hembree *et al.* 1996, Johnson *et al.* 1999, Dahlfors & Arnqvist 2000, Granata *et al.* 2000, Jennische & Hall 2000, Wabitsch *et al.* 2000, Drivdahl *et al.* 2001, Kveiborg *et al.* 2001, Yi *et al.* 2001). Although IGFBP-3 has generally been shown to suppress proliferation of cultured cells, it may also stimulate proliferation depending upon the cell type and the assay conditions (Baxter 2000, Baxter *et al.* 2000). IGFBP-3 binds to both IGF-I and -II with a high affinity thus making IGFs unavailable to bind to the type I IGF receptor that is responsible for many of their biological effects. IGFBP-3 also suppresses proliferation of cells that lack the type I IGF receptor suggesting that IGFBP-3 has effects on cell proliferation that are independent of IGF (Valentinis *et al.* 1995). Numerous studies using IGF analogs that bind to the type I IGF receptor but only weakly to IGFBP-3 have supported this initial hypothesis. Further support for the IGF-independent actions of

IGFBP-3 is provided by the recent reports that IGFBP-3 stimulates phosphorylation of Smad proteins via the transforming growth factor- β (TGF β) receptor pathway in T47D breast cancer cells (Fanayan *et al.* 2000, 2002). IGFBP-3 also reportedly binds to the type V TGF β receptor and to several other less well-characterized cell surface proteins (Leal *et al.* 1999).

Cultured PEMCs produce IGFBP-3, and levels of IGFBP-3 mRNA and protein decrease significantly during differentiation and then increase after differentiation is complete (Hembree *et al.* 1996, Johnson *et al.* 1999, Yang *et al.* 1999). These changes in IGFBP-3 mRNA concentration during differentiation suggest that IGFBP-3 may play a role in myogenesis and/or alterations in proliferation of cultured PEMCs during differentiation. The goal of this study was to assess the IGF-I-dependent and IGF-I-independent actions of recombinant porcine IGFBP-3 (rpIGFBP-3) on proliferation of cultured porcine myogenic cells. In order to facilitate these investigations, we have sequenced the full open reading frame of porcine IGFBP-3 cDNA, expressed this protein in the baculovirus system, purified and characterized the expressed rpIGFBP-3, and produced and characterized an anti-porcine IGFBP-3 antibody that neutralizes the biological activity of porcine IGFBP-3.

Materials and Methods

cDNA production

The nucleotide sequence of porcine IGFBP-3 (including more than 100 nt of 5'-UTR sequence that was previously unreported) was obtained by using 5'-RACE PCR using porcine liver RNA (Accession no. AF085482). cDNA containing the full open reading frame of IGFBP-3 was amplified from porcine liver total RNA and cloned into a pT-Adv vector (Clontech, Palo Alto, CA, USA). IGFBP-3 was sequenced in both directions by fluorescent automated DNA sequencing (ABI Prizm Model 377; Applied Biosystems, Foster City, CA, USA). In order to reduce secondary structure, GC melt (Clontech) was included in the amplification mixture and 10% DMSO was included in the sequencing mixture.

Protein production

Recombinant IGFBP-3 was produced in a baculovirus expression system by ATG Laboratories Inc. (Eden Prairie, MN, USA). IGFBP-3 cDNA was PCR cloned into the pGEM-T Easy plasmid and then subcloned into the baculovirus transfer vector (pBacHIS3) containing the sequence necessary to add six histidines to the N-terminus (IGFBP-3-6 $\times$ HIS). Finally, IGFBP-3-6 $\times$ HIS was subcloned into a baculovirus expression vector (pACGP67B) containing a secretion signal. This construct produced

rpIGFBP-3 with an N-terminal baculovirus secretion signal and a six-histidine tag on the N-terminus. The recombinant protein was produced in *Trichoplusia ni* (*T. ni*) 5B1-4 cells (High Five Cells, Invitrogen, Carlsbad, CA, USA) cells grown in serum-free conditions. Conditioned media were collected 48 h post-infection and stored at -80°C .

Protein purification

Nickel-NTA affinity chromatography resin (Qiagen, Avalencia, CA, USA) was equilibrated in 10 mM imidazole, 50 mM NaPO₄, 600 mM NaCl, pH 7.0, and utilized to bind His-tagged rpIGFBP-3 as described in the Results section. An IGF-I affinity column was made by coupling 2 mg recombinant IGF-I (GroPep, Adelaide, SA, Australia) to Affi-Gel 10 (BioRad Laboratories, Hercules, CA, USA) in 50 mM HEPES, pH 6.5. The column was run in 50 mM NaPO₄, 600 mM NaCl, pH 7.0. An affinity column containing bound soluble proteins from uninfected *T. ni* cells was produced by coupling soluble proteins to a CnBr-activated Sepharose 4B column which was run in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂ HPO₄ $\cdot$ 7H₂O, 1.8 mM KH₂PO₄).

Antibody production

Polyclonal antibodies to affinity-purified IGFBP-3 were produced in rabbits by Covance Research Products (Denver, PA, USA) using their standard protocols. The IgG was purified from serum by protein A chromatography.

Electrophoresis

Samples were run on 10% polyacrylamide SDS gels according to the method of Laemmli (1970). Gels were stained with either GelCode Blue Stain (Pierce Chemical Company, Rockford, IL, USA) or with the GelCode Color Silver Stain Kit (Pierce Chemical Company).

Western blotting

Proteins were electrophoresed on a 10% polyacrylamide SDS gel and then transferred from the gel to a nitrocellulose membrane in 15 mM glycine, 5% methanol for 1 h at 100 V. After the membrane was rinsed in Tris-buffered saline (TBS) (10 mM Tris-HCl, 150 mM NaCl, pH 7.4), it was blocked for 1.5 h in TBS containing 3% bovine serum albumin (BSA). The membrane was incubated in blocking buffer containing anti-rpIGFBP-3 (1:5000) for 1 h followed by several washes in TBS containing 0.1% Tween 20. The membrane was then incubated for 1 h with phosphatase-labeled goat anti-rabbit IgG (H+L) (KPL, Gaithersburg, MD, USA)

(1:2500) in blocking buffer and washed several times with TBS containing 0.1% Tween 20. Bands were detected using Lumi-Phos (Pierce Chemical Company).

Ligand blotting

Proteins were run on non-reducing SDS-PAGE and then transferred from the gel to a nitrocellulose membrane. Binding proteins were detected with ^{125}I -IGF-I as described previously (Hembree *et al.* 1996, Yang *et al.* 1999).

Isolation and culture of PEMCs

PEMCs for culture were isolated from 50- to 55-day-old porcine fetuses and stored in liquid nitrogen as described in detail previously (Pampusch *et al.* 1990, Hembree *et al.* 1991, 1996). To establish cultures from frozen stocks, rapidly thawed cell suspensions were diluted with the appropriate amount of Dulbecco's modified Eagle's medium (DMEM) containing 7% (v/v) swine serum (SS) (Hembree *et al.* 1991) and 3% (v/v) chicken embryo extract (CEE) (Hembree *et al.* 1991) and plated in dishes coated with reduced growth factor basement membrane matrigel (BD, Bedford, MA, USA) diluted 1:100 (v/v) in DMEM. All cultures were maintained at 37 °C, 5% CO₂, 95% air in a water-saturated environment. After a 24-h attachment period, cultures were fed with 10 ml DMEM containing 7% SS and 3% CEE.

^3H -Thymidine incorporation

^3H -Thymidine incorporation was measured as previously described (Yang *et al.* 1999). PEMC cultures were established in 2 cm² wells as described previously. After 48 h in culture, serum-containing medium was removed and replaced with a serum-free medium (DMEM containing 250 µg IGFBP-3-free fetuin and 100 µg BSA-linoleic acid/ml medium) containing IGF-I, Long-R3-IGF-I, rpIGFBP-3, and/or anti-rpIGFBP-3 antibody as indicated in specific Figure legends. IGFBP-3-free fetuin was prepared according to procedures described previously (Yang *et al.* 1999). After 6 h, this washout medium was removed and 1 ml fresh, serum-free medium identical to the media used in the washout was added to each culture. At 72 h in culture, ^3H -thymidine was added to culture media (1 µCi/ml final concentration) and allowed to incubate at 37 °C for 3 h. Cells were rinsed with cold serum-free DMEM, and fixed with 1 ml cold 5% trichloroacetic acid (TCA) overnight at 4 °C. Unincorporated ^3H -thymidine was removed by aspirating the cold 5% TCA and rinsing wells with additional cold 5% TCA. ^3H -Thymidine incorporation into cellular DNA was measured by dissolving cell material in 0.5 M NaOH and counting it in a scintillation counter. All data points are the average of values obtained from triplicate cultures.

Figure 1 Silver-stained, 10% polyacrylamide SDS gel of *T. ni* cell medium and rpIGFBP-3-containing fractions obtained at various steps in the purification of rpIGFBP-3. A: *T. ni* cell medium; B: dialyzed medium; C: rpIGFBP-3 fraction eluting from the nickel-NTA affinity column; D: rpIGFBP-3 fraction eluting from the IGF-I affinity column; S: molecular weight standards.

Results

Expression and purification of rpIGFBP-3

Medium conditioned for 48 h by *T. ni* cells infected with baculovirus containing the rpIGFBP-3 construct contained rpIGFBP-3 that could be detected using ^{125}I -IGF-I ligand blotting. This medium was dialyzed for 24 h against 10 mM imidazole, 50 mM NaPO₄, 600 mM NaCl, pH 7.0, and then for an additional 24 h against the same buffer at pH 8.0. The dialyzed, conditioned medium was mixed with nickel-NTA affinity chromatography resin for 1.5 h and then poured into a column. The column was washed with three column volumes of 15 mM imidazole, 50 mM NaPO₄, 600 mM NaCl, pH 8.0, and the rpIGFBP-3 was then eluted with 50 mM imidazole, 50 mM NaPO₄, 600 mM NaCl, pH 8.0. A silver-stained SDS polyacrylamide gel showed that, although rpIGFBP-3 was greatly enriched in the column eluate as compared with the culture media, significant contaminants remained (Fig. 1). In order to further purify the nickel column eluate, its pH was lowered to 7.4 by addition of 1 M HCl and it was loaded onto an IGF-I affinity column. The column was washed with five column volumes of column buffer (50 mM NaPO₄, 600 mM NaCl, pH 7.0), two column volumes of column buffer without NaCl, and 0.5 column volumes of 1.0% (v/v) acetic acid prior to elution of IGFBP-3 with 3 column volumes of 1.0% (v/v) acetic acid. Silver-stained SDS polyacrylamide gels showed that the rpIGFBP-3 fraction eluted from the IGF-I affinity column in 1.0% (v/v) acetic acid contained proteins migrating at 41 and 39 kDa (Fig. 1). These proteins were identified as IGFBP-3 based on the ability of both the 41 and 39 kDa bands to bind ^{125}I -IGF-I in the ligand blot assay (Fig. 2). Scanning densitometry showed that more

Figure 2 ^{125}I -IGF-I Western ligand blot of purified rpIGFBP-3 and of porcine serum. Serum IGFBP-3 (B) bands at apparent molecular weights of 39 and 43 kDa. rpIGFBP-3 (A) bands at apparent molecular weights of 39 and 41 kDa.

than 98% of the protein in this fraction was contained in the two rpIGFBP-3 bands. Two minor contaminating proteins banding at approximately 14 kDa were also present in the purified rpIGFBP-3 fraction. Results obtained during production and characterization of anti-rpIGFBP-3 showed that these proteins most likely originated from the *T. ni* cells (see sections on antibody

production and characterization). Approximately 5 mg purified rpIGFBP-3 could be isolated from 1 liter cell culture medium.

Effect of rpIGFBP-3 on IGF-I-stimulated proliferation of PEMC cultures

Figure 3 demonstrates that when IGF-I and rpIGFBP-3 were added simultaneously to PEMC cultures, rpIGFBP-3 suppressed IGF-I (1.33 nM)-stimulated proliferation in a concentration-dependent manner. When IGF-I and rpIGFBP-3 were added to the cultures at equimolar concentrations, rpIGFBP-3 completely suppressed the mitogenic activity of IGF-I (data not shown).

Effect of rpIGFBP-3 on Long-R3-IGF-I-stimulated proliferation of PEMC cultures

In order to assess the IGF-I-independent activity of rpIGFBP-3 in PEMC cultures, we examined its ability to inhibit Long-R3-IGF-I-stimulated proliferation. Long-R3-IGF-I is an IGF-I analog that has very low affinity for the IGFBPs but retains its ability to bind to the type I IGF receptor and thereby stimulate proliferation. Consequently, suppression of Long-R3-IGF-I-stimulated proliferation by IGFBP-3 is believed to result from IGF-I-independent association of IGFBP-3 with cell surface receptors rather than binding and inactivation of IGF-I. Figure 4a shows that rpIGFBP-3 suppressed

Figure 3 Effect of rpIGFBP-3 on IGF-I-stimulated proliferation of PEMCs. The bar labeled B shows ^3H -thymidine incorporation in cultures treated for 24 h with basal medium containing no IGF-I. All other bars represent ^3H -thymidine incorporation into cultures treated for 24 h with 10 ng IGF-I/ml (1.33 nM) and the indicated amount of rpIGFBP-3 prior to measuring ^3H -thymidine incorporation as described in the Materials and Methods section. *Values are different from 0 value ($P < 0.05$).

Figure 4 (a) Effect of rpIGFBP-3 on Long-R3-IGF-I-stimulated proliferation of PEMCs. The bar labeled B shows ³H-thymidine incorporation in cultures treated for 24 h with basal medium containing no Long-R3-IGF-I. The bar labeled Anti-BP3 shows ³H-thymidine incorporation in cultures treated for 24 h with basal medium containing 25 ng Long-R3-IGF-I/ml (3.33 nM), 7.14 nM rpIGFBP-3 and 80 mg anti-rpIGFBP-3 IgG/ml prior to measuring ³H-thymidine incorporation. All other bars represent ³H-thymidine incorporation into cultures treated for 24 h with 25 ng Long-R3-IGF-I/ml (3.33 nM) and the indicated amount of rpIGFBP-3 prior to measuring ³H-thymidine incorporation. *Values are different from 0 value ($P < 0.05$). (b) Effect of rpIGFBP-3 on PEMC proliferation in the presence of a large excess of Long-R3-IGF-I. Basal Media: cultures were treated for 24 h with basal medium prior to measuring ³H-thymidine incorporation; LR3: cultures were treated for 24 h with basal medium containing 15.2 nM Long-R3-IGF-I prior to measuring ³H-thymidine incorporation; LR3/rpIGFBP-3: cultures were treated for 24 h with basal medium containing 15.2 nM Long-R3-IGF-I and 3.5 nM rpIGFBP-3 prior to measuring ³H-thymidine incorporation. *Values are different from LR3 ($P < 0.05$).

Long-R3-IGF-I-stimulated proliferation in PEMC cultures in a concentration-dependent manner. Since Long-R3-IGF-I still has some affinity for IGFBP-3 and the molar concentrations required to suppress Long-R3-IGF-I-stimulated proliferation are high, it is possible that the suppression of proliferation observed in Fig. 4a results from binding and inactivation of Long-R3-IGF-I by rpIGFBP-3. To eliminate this possibility, we have examined the ability of rpIGFBP-3 to suppress proliferation of PEMC in the presence of a large molar excess of

rpIGFBP-3. Figure 4b shows that 3.5 nM rpIGFBP-3 suppressed proliferation in PEMC cultures containing 15.2 nM Long-R3-IGF-I. These data established that rpIGFBP-3 has IGF-I-independent effects on proliferation of cultured PEMC.

Characterization of anti-IGFBP-3 antibody

Purified rpIGFBP-3 was used to elicit antibody production in rabbits and anti-IGFBP-3 IgG was isolated as

Figure 5 Western immunoblot showing the interaction of anti-rpIGFBP-3 against rpIGFBP-3. The lane labeled Anti-BP-3 was incubated with anti-rpIGFBP-3 IgG and then with phosphatase-labeled goat anti-rabbit IgG (H+L) as described in the Materials and Methods. The lane labeled Affinity-Purified Anti-BP-3 IgG was incubated with anti-IGFBP-3 IgG that had been preabsorbed by passing through an affinity column containing bound soluble proteins from uninfected *T. ni* cells.

described in the Materials and Methods. Western immunoblots show that anti-IGFBP-3 IgG recognizes rpIGFBP-3 as well as proteins banding at approximately 14 kDa (Fig. 5). In order to remove the antibodies recognizing these unidentified proteins, the anti-rpIGFBP-3 IgG was passed through an affinity column containing bound soluble protein from *T. ni* cells that had not been infected with the virus containing the rpIGFBP-3 construct. This column bound antibodies specific for insect proteins while allowing the anti-IGFBP-3 IgG to pass through. Figure 5 (right-hand lane) shows that after passage through this column, the anti-rpIGFBP-3 IgG recognized only IGFBP-3.

Anti-IGFBP-3 recognized IGFBP-3 in PEMC conditioned medium (Fig. 6). Other bands recognized in the conditioned medium were recognized by the second antibody (Fig. 6) and were present in the fetuin and

Figure 6 Western immunoblot showing the interaction of affinity-purified anti-rpIGFBP-3 with IGFBP-3 in cell culture medium conditioned for 24 h by PEMC cultures. Medium was concentrated approximately 10-fold, mixed with the appropriate amount of tracking dye, electrophoresed on a 10% polyacrylamide SDS gel, and the proteins were electrophoretically transferred to a nitrocellulose membrane. The membrane was probed with affinity-purified anti-rpIGFBP-3 IgG followed by a phosphatase-labeled goat anti-rabbit IgG (H+L) as described in the Materials and Methods. Lane A: PEMC culture medium incubated with second antibody only. Lane B: PEMC culture medium incubated with affinity-purified anti-rpIGFBP-3 followed by second antibody. The anti-rpIGFBP-3 recognizes only the 43 and 39 kDa IGFBP-3 proteins (lane B). Other bands indicated by arrowheads are also present in the second antibody control (lane A) and are the result of interactions of the second antibody with proteins in the BSA and fetuin present in the serum-free medium used to grow the PEMCs (data not shown).

BSA–linoleic acid that are components of the serum-free medium in which the PEMCs were cultured (data not shown).

Neutralization of IGF-I-dependent and IGF-I-independent IGFBP-3 suppression of PEMC proliferation by anti-rpIGFBP-3 IgG

Anti-rpIGFBP-3 was able to suppress the ability of either rp-IGFBP-3 (Fig. 7) or commercially available recombinant human IGFBP-3 (data not shown) to inhibit IGF-I-dependent proliferation in PEMC cultures. Furthermore, these data showed that high concentrations of the antibody neither suppress nor stimulate proliferation of PEMC cultures, indicating that the anti-rpIGFBP-3 had no non-specific mitogenic effects on the PEMC cultures. Additionally, 80 µg non-immune IgG had no effect on proliferation of PEMC cultures, further establishing that the effects of the anti-rpIGFBP-3 IgG were specific. Figure 4a shows that anti-rpIGFBP-3 IgG also inhibited

Figure 7 Effect of affinity-purified anti-IGFBP-3 IgG on the ability of rpIGFBP-3 to suppress IGF-I-stimulated proliferation of PEMC cultures. IGF-I: ³H-thymidine incorporation stimulated by treatment with 10 ng IGF-I/ml (1:33 nM); IGF/BP3: ³H-thymidine incorporation in the presence of 10 ng IGF-I and 53 ng rpIGFBP-3/ml (1:33 nM); 5, 10, 20, 40 and 80: ³H-thymidine incorporation in the presence of 10 ng IGF-I and 53 ng rpIGFBP-3/ml plus the indicated quantity of affinity-purified anti-rpIGFBP-3 IgG (mg/ml); IgG: ³H-thymidine incorporation in the presence of 10 ng IGF-I and 53 ng rpIGFBP-3/ml plus 80 mg non-immune IgG/ml. *Values are different for IGF/BP3 ($P < 0.05$).

the ability of rpIGFBP-3 to suppress LR3-IGF-I-stimulated proliferation of PEMCs. This result established that the interaction of rpIGFBP-3 with the antibody inhibits the IGF-I-independent actions of rpIGFBP-3 on PEMC.

Discussion

We have utilized a baculovirus expression system to produce mg quantities of rpIGFBP-3, purified the rpIGFBP-3 to greater than 98% homogeneity using nickel and IGF-I affinity chromatography and demonstrated its ability to bind IGF-I. In addition, we have shown that IGFBP-3 suppresses proliferation of cultured myogenic cells via both IGF-I-dependent and IGF-I-independent mechanisms.

Finally, purified rpIGFBP-3 has been used to elicit production of an anti-rpIGFBP-3 antibody that is able to neutralize both IGF-dependent and IGF-independent actions of IGFBP-3.

It is well established that IGFBP-3 exists as a glycoprotein; however, glycosylation does not appear to be required for biological activity (Conover 1992, Firth & Baxter 1999). Native porcine IGFBP-3 exists as two glycosylation variants migrating at apparent molecular weights of 39 and 43 kDa on non-reducing SDS polyacrylamide gels (Yang *et al.* 1999). There are three potential glycosylation sites in the IGFBP-3 molecule and

the 39 kDa glycosylation variant is glycosylated at two of these sites while the 43 kDa variant is glycosylated at all three sites (Firth & Baxter 1999). Non-glycosylated IGFBP-3 migrates as a single band with a molecular weight of approximately 29 kDa (Firth & Baxter 1999). Since the rpIGFBP-3 in this study was produced and secreted from a eukaryotic cell system, the protein should be glycosylated. Comparison of the apparent molecular weights of rpIGFBP-3 (39 and 41 kDa), glycosylated native IGFBP-3 (39 and 43 kDa) and non-glycosylated IGFBP-3 (29 kDa) on non-reducing SDS gels strongly indicates that rpIGFBP-3 is glycosylated. It is likely that the production of a 41 kDa glycoform in the baculovirus system reflects differences in glycosylation by insect cells versus mammalian cells.

Several studies have shown that if IGF-I and IGFBP-3 are mixed together in equimolar quantities prior to addition to cultured cells, IGFBP-3 binds IGF-I and effectively makes it unavailable to the type I IGF-I receptor (Jones & Clemmons 1995). This results in total suppression of IGF-I-stimulated proliferation. This suppression is referred to as IGF dependent because it requires that IGFBP-3 binds IGF-I. Our data showed that rpIGFBP-3 suppresses IGF-I-stimulated proliferation of PEMC cultures in a concentration-dependent manner and that approximately 1 nM rpIGFBP-3 completely abolishes the proliferation-stimulating activity of 1 nM IGF-I. These data have established that rpIGFBP-3 has IGF-dependent activity in the PEMC system that is

similar to that reported for native IGFBP-3 in other culture systems.

In order to assess the IGF-I-independent activity of rpIGFBP-3 in PEMC cultures, we examined its ability to inhibit Long-R3-IGF-I-stimulated proliferation in these cultures. Long-R3-IGF-I is an IGF-I analog that has very low affinity for the IGFBPs but retains its ability to bind to the type I IGF receptor and thereby stimulate proliferation (Kubler *et al.* 2002). Consequently, suppression of Long-R3-IGF-I-stimulated proliferation by IGFBP-3 is believed to result from IGF-I-independent association of IGFBP-3 with cell surface receptors rather than binding and inactivation of IGF-I. rpIGFBP-3 suppresses Long-R3-IGF-I-stimulated proliferation of PEMC cultures in a concentration-dependent manner, strongly suggesting that rpIGFBP-3 possesses IGF-independent activity in this culture system. These results also suggested that PEMCs possess receptors for IGFBP-3; however, the identity of these receptors is currently unknown.

The polyclonal antibody specific for rpIGFBP-3 is able to recognize native IGFBP-3 and Western ligand blotting shows that it is specific for only IGFBP-3. Additionally, the anti-rpIGFBP-3 antibody neutralizes the activity of both rpIGFBP-3 and commercially available recombinant human IGFBP-3. This antibody will be a useful reagent in studying the effects of endogenous IGFBP-3 on proliferation and differentiation of PEMC cultures.

In summary, we have shown that recombinantly produced porcine IGFBP-3 affects proliferation of cultured PEMCs via both IGF-I-dependent and IGF-I-independent mechanisms. These data have established that IGFBP-3 produced by myogenic cells has the potential to affect proliferation of embryonic myogenic cells during critical periods of muscle development that may impact the ultimate muscle mass achievable postnatally.

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