Production of recombinant porcine IGF-binding protein-5 and its effect on proliferation of porcine embryonic myoblast cultures in the presence and absence of IGF-I and Long-R3-IGF-I

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Abstract

IGF-binding protein-5 (IGFBP-5) is produced by porcine embryonic myogenic cell (PEMC) cultures and is secreted into the medium. IGFBP-5 may play some role in myogenesis and/or in changes in myogenic cell proliferation that accompany differentiation. IGFBP-5 reportedly may either suppress or stimulate proliferation or differentiation of cultured cells depending on cell type and culture conditions. Additionally, IGFBP-5 has been shown to possess both IGF-dependent and IGF-independent actions in some cell types. The goal of this study was to produce recombinant porcine IGFBP-5 (rpIGFBP-5) and assess its IGF-I-dependent and IGF-I-independent actions on the proliferation of PEMCs. To accomplish this, we have expressed porcine IGFBP-5 in the baculovirus system, purified and characterized the expressed rpIGFBP-5 and produced an anti-porcine IGFBP-5 antibody that neutralizes the biological activity of porcine IGFBP-5. rpIGFBP-5, purified to 98% homogeneity using nickel affinity chromatography and IGF-I affinity chromatography, suppressed IGF-I-stimulated proliferation of PEMCs in a concentration-dependent manner ($P < 0.05$). rpIGFBP-5 also suppressed Long-R3-IGF-I-stimulated proliferation of PEMCs

($P < 0.05$), even in the presence of significant molar excess of Long-R3-IGF-I compared with rpIGFBP-5, demonstrating the IGF-independent activity that rpIGFBP-5 possesses in PEMCs, since Long-R3-IGF-I is an IGF analog that has very low affinity for the IGFBPs but retains its ability to bind to the type I IGF receptor and thereby can stimulate proliferation. The anti-rpIGFBP-5 IgY produced against rpIGFBP-5 specifically recognized native porcine IGFBP-5 in PEMC media that also contained porcine IGFBP-2, -3, and -4. This antibody is capable of neutralizing the effects of both rpIGFBP-5 and endogenously produced porcine IGFBP-5 on PEMCs as well as detecting IGFBP-5 in Western blots. The production of rpIGFBP-5 and a neutralizing antibody to porcine IGFBP-5 provides a powerful tool to investigate the role of IGFBP-5 in porcine myogenic cell proliferation and differentiation. The data provided here demonstrated that IGFBP-5 has the potential to affect proliferation of PEMCs during critical periods of *in vitro* muscle cell development and therefore may impact the capacity for ultimate postnatal muscle mass development *in vivo*.

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Introduction

Insulin-like growth factors (IGF)-I and -II are potent regulators of both differentiation and proliferation of myogenic cells (Florini *et al.* 1996, Lawlor & Rotwein 2000, Tureckova *et al.* 2001). The availability and 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). In addition to their IGF-dependent actions, IGFBP-1, -3, and -5 have been shown

to exert IGF-independent actions by binding to cell surfaces (Clemmons 1998, Hwa *et al.* 1999, Baxter 2000) and IGFBP-3 and -5 possess nuclear localization sequences which suggest that they can travel to the nucleus (Schedlich *et al.* 2000).

IGFBP-5 has been shown to both stimulate and suppress cell survival, proliferation, and differentiation (Andress & Birnbaum 1992, James *et al.* 1996, Ewton *et al.* 1998, Baxter 2000, Meadows *et al.* 2000, Yin *et al.* 2004). Additionally, IGF-independent actions of IGFBP-5 have been demonstrated in various cell types (Andress & Birnbaum 1992, Schneider *et al.* 2002). IGFBP-5 reportedly binds to an uncharacterized 420 kDa membrane protein which causes serine phosphorylation when bound

to IGFBP-5 (Andress 1998). Furthermore, evidence exists indicating that there are mechanisms for nuclear transport of IGFBP-5 and that IGFBP-5 can function as a transcription regulator (Schedlich *et al.* 1998, Xu *et al.* 2004). The intracellular transport and nuclear localization of IGFBP-5 as well as putative IGFBP-5 receptors may be responsible for the IGF-independent actions of IGFBP-5 on cells.

IGFBP-5 is produced by myogenic cell lines, 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). Several studies have shown that IGFBP-5 expression in muscle cell lines is altered during myogenesis and muscle development (James *et al.* 1996, Gerrard *et al.* 1999, Cobb *et al.* 2004), suggesting that IGFBP-5 may play an important role in critical periods of muscle development that may impact postnatal muscle mass. In contrast, IGFBP-5 mRNA levels remain constant in differentiating PEMC cultures (Johnson *et al.* 2003), raising the possibility that regulation of IGFBP-5 expression and/or function may be different in PEMC cultures than it is in immortalized murine myogenic cell lines. Consequently, the goal of this study was to express porcine IGFBP-5 cDNA (White *et al.* 1996) in the baculovirus system, purify and characterize the expressed recombinant porcine IGFBP-5 (rpIGFBP-5), and assess the IGF-I-dependent and IGF-I-independent actions of rpIGFBP-5 on proliferation of cultured porcine myogenic cells. In order to facilitate these investigations, we also have produced and characterized an anti-porcine IGFBP-5 antibody that neutralizes the biological activity of both endogenous and exogenous porcine IGFBP-5.

Materials and Methods

cDNA production

The nucleotide sequence of porcine IGFBP-5 was obtained by our laboratory previously (White *et al.* 1996) (GenBank accession number U41340 from a neonatal porcine skeletal muscle Lambda ZapII cDNA Library prepared by Stratagene Cloning Systems (La Jolla, CA, USA)) and the cDNA was cloned into Bluescript SK phagemids. IGFBP-5 was sequenced in both directions using the dideoxy Sanger termination DNA sequencing method with a ³⁵S-labeled dATP (Sanger *et al.* 1977).

Protein production

Recombinant IGFBP-5 was produced in a baculovirus expression system by ATG Laboratories Inc. (Eden Prairie, MN, USA). IGFBP-5 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-5-6 XHIS). Finally, IGFBP-5-6 XHIS was subcloned into a baculovirus expression vector (pACGP67B) containing a secretion signal. This construct produced rpIGFBP-5 with an N-terminal baculovirus secretion signal and a six-histidine tag on the N terminus. The recombinant protein was produced in T. ni cells grown in serum-free conditions. Conditioned medium (CM) was collected 48-h post-infection and stored at -80 °C.

Protein purification

Nickel-NTA affinity chromatography resin (Qiagen, Valencia, CA, USA) was equilibrated in 10 mM imidazole, 50 mM NaPO₄, and 600 mM NaCl, pH 7.0, and utilized to bind His-tagged rpIGFBP-5. 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 and 600 mM NaCl, pH 7.0.

Antibody production

Polyclonal antibodies to affinity purified IGFBP-5 were produced in chickens by Covance Research Products (Denver, PA, USA) using their standard protocols. The IgY was purified from egg yolk by Eggstact IgY Purification System (Promega Corp., Madison, WI, USA).

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 Tris, 120 mM glycine, and 5% methanol overnight at 50 V. After the membrane was rinsed in TBS (10 mM Tris-HCl and 150 mM NaCl, pH 7.4), it was blocked for 1.5 h in blocking buffer (TBS containing 0.1% Tween 20 plus 3% bovine serum albumin (BSA)). The membrane was incubated in blocking buffer containing anti-rpIGFBP-5 (1:250) overnight followed by six washes in TBS containing 0.1% Tween 20. The membrane was then incubated for 1 h with peroxidase-labeled rabbit anti-chicken IgY (Promega Corp.) (1:5000) in blocking buffer and washed six times with TBS

containing 0.1% Tween 20. Bands were detected using Super Signal West Pico Chemiluminescent substrate (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 (Hembree *et al.* 1996).

Isolation and culture of PEMCs

PEMCs for culture were isolated from 50- to 55-day 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 Eagles' medium (DMEM) containing 7% (v/v) swine serum (SS) and 3% (v/v) chicken embryo extract (CEE) 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 in 5% CO₂ and 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-free fetuin and 100 µg BSA-linoleic acid/ml medium) containing IGF-I, Long-R3-IGF-I, rpIGFBP-5, and/or anti-rpIGFBP-5 antibody as indicated in the specific Figure legends. Long-R3-IGF-I is an IGF-I analog that has a 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-5 is believed to result from IGF-I-independent actions. IGFBP-free fetuin was prepared according to procedures described previously (Yang *et al.* 1999). After 6 h, the washout medium was removed and 1 ml fresh, serum-free medium identical to the media used in the washout was added to each culture and allowed to incubate for an additional 18 h. After this 24-h treatment, ^3H -thymidine was added to the 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 and are representative of duplicate or triplicate assays.

Statistical analysis

All data were analyzed using the mixed procedure of SAS (SAS 2001). All data points are the average of values obtained from triplicate cultures and are representative of duplicate or triplicate assays. When significant interactions were detected ($P < 0.05$), least squares means were separated using Fischer's LSD test ($P < 0.05$).

Results

Expression and purification of rpIGFBP-5

Medium conditioned for 48 h by T. ni cells infected with baculovirus containing the rpIGFBP-5 construct contained rpIGFBP-5 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, and 600 mM NaCl, pH 7.0, and then for an additional 24 h against the same buffer at pH 8.0. The dialyzed, CM was mixed with nickel-NTA affinity chromatography resin for 1.5 h and then poured into a column. The column was washed with 3 column volumes of 15 mM imidazole, 50 mM NaPO₄, and 600 mM NaCl, pH 8.0, and the rpIGFBP-5 was then eluted with 50 mM imidazole, 50 mM NaPO₄, and 600 mM NaCl, pH 8.0. A silver-stained SDS polyacrylamide gel showed that although rpIGFBP-5 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 the addition of 1 M HCl and it was loaded onto an IGF-I affinity column. The column was washed with 5 column volumes of column buffer (50 mM NaPO₄ and 600 mM NaCl, pH 7.0), 2 column volumes of column buffer without NaCl, and 0.5 column volumes of 1.0% (v/v) acetic acid prior to elution of IGFBP-5 with 3 column volumes of 1.0% (v/v) acetic acid. Silver-stained SDS polyacrylamide gels showed that the rpIGFBP-5 fraction eluted from the IGF-I affinity column in 1.0% (v/v) acetic acid contained a single protein band migrating at 37 kDa in a reducing SDS-PAGE gel (Fig. 1). Scanning densitometry showed that more than 98% of the protein in this fraction was in the rpIGFBP-5 band. This protein ran as a doublet on silver-stained non-reducing SDS-PAGE and was identified as IGFBP-5 based on its ability to bind ^{125}I -IGF-I in the ligand blot assay (Fig. 2) which was performed on non-reducing SDS-PAGE. The two rpIGFBP-5 bands observed using non-reducing SDS-PAGE ran at

Figure 1 Reduced, silver-stained, 10% polyacrylamide gel of T. ni cell medium and rpIGFBP-5-containing fractions obtained at various steps in the purification of rpIGFBP-5. M, T. ni cell medium; Ni, rpIGFBP-5 fraction eluting from the nickel-NTA affinity column; IGF, rpIGFBP-5 fraction eluting from the IGF-I affinity column. The rpIGFBP-5 band migrates at approximately 37 kDa.

apparent molecular sizes of approximately 34 and 28 kDa. Both rpIGFBP-5 bands observed in non-reducing SDS-PAGE ligand blots were capable of binding ^{125}I -IGF-I and are likely conformation and/or glycosylation variants of rpIGFBP-5 that run at similar molecular weights under reducing conditions. Approximately 5 mg purified rpIGFBP-5 could be isolated from 1 liter of cell culture medium.

Effect of rpIGFBP-5 on IGF-I-stimulated proliferation of PEMC cultures

Figure 3 demonstrates that when IGF-I and rpIGFBP-5 were added simultaneously to PEMC cultures, rpIGFBP-5 suppressed IGF-I (1.3 nM)-stimulated proliferation in a concentration-dependent manner. Most of the IGF-stimulated proliferation was suppressed when 1–2.7 nM rpIGFBP-5 was added to 1.3 nM IGF-I.

Effect of rpIGFBP-5 on Long-R3-IGF-I-stimulated proliferation of PEMC cultures

In order to assess the IGF-I-independent activity of rpIGFBP-5 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. Conse-

Figure 2 Non-reduced, silver-stained, 10% polyacrylamide gel (S) and ^{125}I -IGF-I Western ligand blot (LB) of purified rpIGFBP-5. Non-reduced rpIGFBP-5 appears as a doublet with apparent molecular weights of approximately 34 and 28 kDa.

quently, suppression of Long-R3-IGF-I-stimulated proliferation by IGFBP-5 is believed to result from IGF-I-independent actions of IGFBP-5 with the cell rather than binding and inactivation of IGF-I. Figure 4a shows that rpIGFBP-5 suppressed Long-R3-IGF-I-stimulated proliferation in PEMC cultures in a concentration-dependent manner. Significant suppression of Long-R3-IGF-I-stimulated proliferation was observed when rpIGFBP-5 was added in the same equimolar quantities as Long-R3-IGF-I. However, complete suppression was not observed until an eightfold molar excess of rpIGFBP-5 was added. In order to ensure that the suppression of Long-R3-IGF-I-stimulated proliferation in PEMCs by rpIGFBP-5 was not due to weak interaction of rpIGFBP-5 with Long-R3-IGF-I, we incubated PEMCs in a molar excess of Long-R3-IGF-I (20 nM) compared with rpIGFBP-5 (13.5 nM). These data indicated that rpIGFBP-5 was able to suppress Long-R3-IGF-I-stimulated proliferation even

Figure 3 Effect of rpIGFBP-5 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 1.33 nM IGF-I and the indicated concentration of rpIGFBP-5 prior to measuring ³H-thymidine incorporation as described in Materials and Methods. Values with different letters are different from each other ($P < 0.05$).

in the presence of a large excess of Long-R3-IGF-I. This result indicated that rpIGFBP-5 was not suppressing Long-R3-IGF-I-stimulated proliferation by weakly interacting with and sequestering Long-R3-IGF-I away from the cells preventing it from binding to the type I IGF receptor (Fig. 4b). Rather, rpIGFBP-5 was instead functioning through an IGF-independent mechanism. In order to further confirm that rpIGFBP-5 does not bind and inactivate Long-R3-IGF-I, data from our laboratory have demonstrated that rpIGFBP-5 affects IGF-I-stimulated proliferation and differentiation of L6 myoblasts but *does not* affect Long-R3-IGF-I-stimulated proliferation or differentiation (data not shown). These data confirm previous reports by Ewton *et al.* (1998). Furthermore, others have reported that IGFBP-5 does not inhibit Long-R3-IGF-I-stimulated phosphorylation of insulin receptor substrate (IRS), establishing that the IGF-I receptor is still activated by Long-R3-IGF-I when IGFBP-5 is present (Ricort & Binoux 2002). Furthermore, McCusker & Novakofski (2004) have demonstrated that ¹²⁵I-Long-R3-IGF-I does not bind to IGFBP-5 and that IGFBP-5 does not affect the binding of ¹²⁵I-Long-R3-IGF-I to the type I IGF receptor. Taken together, we feel that our data and a large body of supporting literature have established the functionality of rpIGFBP-5 and that rpIGFBP-5 does not bind to Long-R3-IGF-I making it unavailable to the type I IGF receptor.

Characterization of anti-IGFBP-5 antibody

Because the amino acid sequence homology between porcine IGFBP-5 and other mammalian IGFBP-5 is

so highly conserved, we chose chickens as the species to produce antibodies against rpIGFBP-5. Purified rpIGFBP-5 was used to elicit antibody production in chickens and anti-IGFBP-5 IgY was isolated as described in Materials and Methods. Western immunoblots showed that anti-IGFBP-5 IgY recognizes rpIGFBP-5 (Fig. 5). Western immunoblots of PEMC CM demonstrated that anti-IGFBP-5 can specifically recognize the endogenously produced 28 kDa porcine IGFBP-5 protein (Fig. 6). CM was obtained from PEMCs that had been incubated in the basal media described in Materials and Methods containing Long-R3-IGF-I and transforming growth factor- β which has been shown to enhance IGFBP-5 production in porcine muscle cells (Yi *et al.* 2001, Johnson *et al.* 2003). As demonstrated by ¹²⁵I-IGF-I ligand blots in Fig. 6, this CM also contained IGFBP-2, -3, and -4 in addition to porcine IGFBP-5, yet anti-rpIGFBP-5 IgY only recognized the endogenous porcine IGFBP-5 confirming the specificity of the antibody. Anti-IGFBP-5 also recognized a protein banding at approximately 17 kDa which does not bind ¹²⁵I-IGF-I and is most likely an IGFBP-5 fragment. ¹²⁵I-IGF-I ligand blots of PEM CM also showed a light, 30 kDa IGFBP-5 band (Fig. 6) that does not show up on the anti-IGFBP-5 Western blot of PEM CM. This is likely because the quantity of IGFBP-5 in this band is beyond the detection threshold of the Western blot.

Neutralization of IGFBP-5 suppression of PEMC proliferation by anti-rpIGFBP-5 IgY

Anti-rpIGFBP-5 was able to suppress the ability of rp-IGFBP-5 to inhibit IGF-I-dependent proliferation in

Figure 4 (a) Effect of rpIGFBP-5 on Long-R3-IGF-I (LR3-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. All other bars represent ³H-thymidine incorporation into cultures treated for 24 h with 3.33 nM Long-R3-IGF-I and the indicated concentration of rpIGFBP-5 prior to measuring ³H-thymidine incorporation as described in Materials and Methods. Since Long-R3-IGF-I has very little affinity for IGFbps, the effects of rpIGFBP-5 on Long-R3-IGF-I-stimulated proliferation of PEMCs is likely to be an IGF-independent effect of rpIGFBP-5. Values with different letters are different from each other ($P < 0.05$). (b) Effect of rpIGFBP-5 on PEMC proliferation in the presence of a large excess (5 nM) of Long-R3-IGF-I. B, 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 20 nM Long-R3-IGF-I prior to measuring ³H-thymidine incorporation; LR3/rpIGFBP-5, cultures were treated for 24 h with basal medium containing 20 nM Long-R3-IGF-I and 13.5 nM rpIGFBP-3 prior to measuring ³H-thymidine incorporation. Values with different letters are different from each other ($P < 0.05$).

Figure 5 Anti-IGFBP-5 Western blots (W) of non-reduced rpIGFBP-5 and a control lane (C) that was incubated with non-specific IgY before the secondary antibody.

PEMC cultures (Fig. 7) as well as IGF-independent proliferation of PEMCs (data not shown). Non-immune IgY up to 1200 µg/ml had no effect on proliferation of PEMC cultures, thus establishing that the effects of the anti-rpIGFBP-5 IgY are specific. Furthermore, anti-rpIGFBP-5 IgY was able to neutralize the ability of endogenous porcine IGFBP-5 in PEM CM to inhibit proliferation in PEMC cultures (Fig. 8). These data demonstrated that both endogenous porcine IGFBP-5 and exogenously added rpIGFBP-5 have the same biological actions in PEMCs and that immunoneutralization of either exogenous rpIGFBP-5 or endogenous porcine IGFBP-5 increases the proliferation of PEMCs.

Discussion

We have utilized a baculovirus expression system to produce mg quantities of rpIGFBP-5, purified the

Figure 6 Anti-IGFBP-5 Western blots (W) and ^{125}I -IGF-I Western ligand blot (LB) of PEM CM. Control lane (C) is a negative control lane of PEM CM that was incubated with non-specific IgY before the secondary antibody. Non-reduced native porcine IGFBP-5 in the PEM CM appears as a light band at approximately 28 kDa and a fragment that appears to be less than 17 kDa. The very light 30 kDa IGFBP-5 band indicated in the ligand blot is not observed in the Western blot likely because the quantity of this molecular weight IGFBP-5 band is beyond the detection limits of anti-IGFBP-5 Western blot assay.

rpIGFBP-5 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-5 suppresses proliferation of cultured myogenic cells via both IGF-I-dependent and IGF-I-independent mechanisms.

Finally, purified rpIGFBP-5 has been used to elicit production of an anti-rpIGFBP-5 antibody that is able to neutralize the actions of both rpIGFBP-5 and endogenously produced porcine IGFBP-5.

IGFBP-5 expression remains constant during the differentiation of PEMC cultures (Johnson *et al.* 2003); however, several studies have shown that IGFBP-5 expression in muscle cell lines is altered during myogenesis and muscle development (James *et al.* 1996, Gerrard *et al.* 1999, Cobb *et al.* 2004). This observation raises the possibility that the regulation of IGFBP-5 expression and/or function may be different in PEMC cultures than it is in immortalized murine myogenic cell lines.

Many studies have shown that IGFBP-5 can have either stimulatory and/or inhibitory actions on cell proliferation that may be tissue specific or dependent on culture conditions (Schneider *et al.* 2002). Several studies have

Figure 7 Effect of anti-IGFBP-5 IgY on the ability of rpIGFBP-5 to suppress IGF-I-stimulated proliferation of PEMC cultures. IGF-I, ³H-thymidine incorporation stimulated by treatment with 1.33 nM IGF-I; IGF+BP5, ³H-thymidine incorporation in the presence of 1.33 nM IGF-I and 2.7 nM rpIGFBP-5; 600, 1200, and 1800, ³H-thymidine incorporation in the presence of 1.33 nM IGF-I and 2.7 nM rpIGFBP-5 plus the indicated quantity of anti-rpIGFBP-3 IgY (μg/ml); ns-IgY (non-specific IgY, 1200 μg/ml); ³H-thymidine incorporation in the presence of 1.33 nM IGF-I and 2.7 nM rpIGFBP-5 plus 1200 μg non-immune IgY/ml. Values with different letters are different from each other ($P < 0.05$).

shown that IGFBP-5 binds IGF-I and effectively makes it unavailable to the type I IGF-I receptor (Jones & Clemmons 1995, Xu *et al.* 2004), resulting in suppression of IGF-I-stimulated proliferation. This suppression is referred to as IGF dependent because it requires that IGFBP-5 bind IGF-I. Our data have shown that rpIGFBP-5 suppresses IGF-I-stimulated proliferation of PEMC cultures in a concentration-dependent manner. Significant suppression of IGF-I-stimulated proliferation

Figure 8 Immunoneutralization of endogenous porcine IGFBP-5 results in increased proliferation rate in PEMC cultures. C, ³H-thymidine incorporation of PEMCs in CM containing endogenous porcine IGFBP-5 (Fig. 6); Anti-BP-5, ³H-thymidine incorporation in the presence of 1200 μg/ml anti-IGFBP-5 IgY; IgY, ³H-thymidine incorporation of PEMCs in CM in the presence of 1200 μg/ml non-specific IgY. Values with different letters are different from each other ($P < 0.05$).

was observed at equimolar concentrations of IGF-I and rpIGFBP-5 and at a twofold excess of rpIGFBP-5 to IGF-I. ³H-thymidine incorporation was not different from basal media. These data have established that rpIGFBP-5 has IGF-dependent activity in the PEMC system.

It has also been demonstrated in many tissue and cell types that IGFBP-5 possesses IGF-independent actions (Schneider *et al.* 2002). In order to assess the IGF-I-independent activity of rpIGFBP-5 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 (Schneider *et al.* 2001). Consequently, suppression of Long-R3-IGF-I-stimulated proliferation by IGFBP-5 is believed to result from IGF-I-independent actions of IGFBP-5 on the cell rather than binding and modulation of IGF-I. Further confirmation of this comes from Ewton *et al.* (1998) and data from our laboratory demonstrating that rpIGFBP-5 affects IGF-I-stimulated proliferation and differentiation of L6 myoblasts but *does not* affect Long-R3-IGF-I-stimulated proliferation or differentiation. If rpIGFBP-5 could bind Long-R3-IGF-I, it should also be able to affect Long-R3-IGF-I-stimulated proliferation and differentiation of L6 myoblasts, since IGF-I and Long-R3-IGF-I both function through the type 1 IGF receptor. Furthermore, others have reported that IGFBP-5 does not inhibit Long-R3-IGF-I-stimulated phosphorylation of IRS, establishing that

the IGF-I receptor is still activated by Long-R3-IGF-I when IGFBP-5 is present (Ricort & Binoux 2002). Moreover, McCusker & Novakofski (2004) have demonstrated that ^{125}I -Long-R3-IGF-I does not bind to IGFBP-5 and that IGFBP-5 does not affect the binding of ^{125}I -Long-R3-IGF-I to the type 1 IGF receptor. Taken together, we feel that our data and a large body of supporting literature have established the IGF-independent functionality of rpIGFBP-5. Since rpIGFBP-5 suppresses Long-R3-IGF-I-stimulated proliferation of PEMC cultures in a concentration-dependent manner, even when excess Long-R3-IGF-I is present, our data and the supporting literature strongly suggest that rpIGFBP-5 possesses IGF-independent activity in this culture system. These data also suggest that PEMCs possess receptors for IGFBP-5; however, the identity of these receptors is currently unknown.

The polyclonal antibody specific for rpIGFBP-5 was able to neutralize the activity of exogenous rpIGFBP-5 and recognized the 28 kDa native IGFBP-5 band endogenously produced by PEMCs. In addition, the anti-rpIGFBP-5 IgY was able to neutralize the ability of endogenous porcine IGFBP-5 in PEMC CM to inhibit proliferation in PEMC cultures. These data demonstrated that both endogenous porcine IGFBP-5 and exogenously added rpIGFBP-5 have the same biological actions in PEMCs and that immunoneutralization of either of these increases the proliferation of PEMCs.

PEMC CM also contains a light, 30 kDa IGFBP-5 band that did not show up on the anti-IGFBP-5 Western blot of PEMC CM. This is likely because the quantity of IGFBP-5 in this band is beyond the detection threshold of the Western blot. Although this antibody has a low titer requiring the use of larger concentrations, there is no affect of similar quantities of non-specific IgY. Therefore this antibody can be a useful reagent in studying the effects of endogenous IGFBP-5 on proliferation and differentiation of PEMC cultures.

In summary, we have purified rpIGFBP-5 and it appears to have significant effects on porcine myogenic cells similar to those reported for murine myogenic cell lines. Recombinantly produced porcine IGFBP-5 affects proliferation of cultured PEMCs via both IGF-I-dependent and IGF-I-independent mechanisms. IGF-independent effects of IGFBP-5 have not been previously reported for skeletal muscle myogenic cells. These data have established that IGFBP-5 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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