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Expression and Secretion of the Insulin-like Growth Factor System Components by Pig Liver Cells*

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ABSTRACT : The aim of the present study was to delineate the expression and secretion of insulin-like growth factor (IGF) system components by pig liver cells. Hepatocytes were prepared from 3-wk-old weanling piglets following a two-step collagenase perfusion procedure, after which the cells were incubated for 24 or 48 h at a density of 2×10^5 cells per 35-mm dish in 2-ml Williams' medium E. The cells were found to express the genes encoding IGF-I, IGF-binding proteins (IGFBPs)-2 and -3 and acid-labile subunit (ALS) by reverse transcription-polymerase chain reaction (RT-PCR) following the culture. However, IGF-I was localized to hepatocytes by immunohistochemical analysis, whereas IGFBP-3 was localized to endothelial cells, but not to hepatocytes. This indicated that the IGFBP-3 gene expression detected by RT-PCR was likely to have been contributed by unidentified non-parenchymal cells that had not been removed during the hepatocyte preparation. The conditioned culture medium (CCM) of the cells contained immunoreactive IGF-I and IGF-II, with the latter being seven-fold more abundant than the former. The CCM also contained 43-, 40-, 34-, 31-kDa doublet and 26-kDa IGFBPs as examined by Western ligand blotting. The 40-, 34- and 31-kDa doublet IGFBPs were approximately three-fold as abundant as the 43- and 26-kDa IGFBPs. Moreover, the 43- and 40-kDa doublet and the 34-kDa IGFBPs were immunoprecipitable with IGFBP-3 and IGFBP-2 antibodies, respectively. Overall, these results are similar to those known in the rat, which suggests that the IGF system components are likely to be expressed and secreted in pig liver in a manner similar to that in rat liver. (**Key Words :** IGF, IGFBP, Gene Expression, Hepatocyte, Pig)

INTRODUCTION

The insulin-like growth factor (IGF) system consists of IGF-I and IGF-II ligands, two types of IGF receptors, six species of IGF-binding proteins (IGFBPs) and acid-labile subunit (ALS) which is a 'secondary' IGFBP that possesses no intrinsic IGF-binding activity (Jones and Clemmons, 1995; Hwa et al., 1998; LeRoith et al., 2001). IGF-I is a 7.5-kDa endocrine- as well as autocrine/paracrine-acting peptide that mediates most of the growth-promoting action of growth hormone (GH) in postnatal animals. IGF-II, which is structurally similar to IGF-I, possesses an activity similar to that of the latter *in vitro*, but its *in-vivo* function in postnatal animals is not clear. IGFBPs are structurally related peptides which *en masse* are IGF carriers and reservoirs as well as modulators of IGF actions (Rechler and Clemmons, 1998). The majority of plasma IGFs are bound to 40- to 45-kDa IGFBP-3 and 85-kDa ALS to form 150-kDa ternary complexes, whereas a minor portion of the IGFs form binary complexes with 25- to 34-kDa IGFBPs other than IGFBP-3.

The liver has received special attention for the past several decades as a major source of circulating IGF since Salmon and Daughaday (1957) published the somatomedin hypothesis which states that the growth-stimulating action of GH on skeletal tissue is elicited indirectly by "sulfation

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factor" now known as IGF secreted upon stimulus of GH mostly likely from the liver. The somatomedin hypothesis, as well as the postulated hepatic origin of plasma IGF, has been at least partially proven through numerous studies including the hepatic perfusion (Schwander et al., 1983; Scott et al., 1985) and liver-specific IGF-I knock-out (Yakar et al., 1999; LeRoith et al., 2001) models in rodents. It is now known, however, that not only endocrine but also autocrine/paracrine IGF (D'Ercole et al., 1984) is a significant mediator of the action of GH (LeRoith et al., 2001; Yakar et al., 2005).

Plasma IGFBPs also are believed to be secreted mostly from the liver in the rodent (Baxter, 1986), although a solid proof for this has not been published to date. In this regard, it has been well established that besides IGF-I, IGFBPs-1, -2 and -4 and ALS are secreted by hepatocytes in rat liver, whereas IGFBP-3, the major plasma IGF carrier in adult rats, is expressed only in non-parenchymal tissue mostly consisting of endothelial cells and Kupffer cells (Takenaka et al., 1991; Chin et al., 1994; Scharf et al., 2001). However, such information is available only to a limited extent in the pig, although expression of both IGFs, IGFBPs-2 and -3 in the whole liver (Lee et al., 1993; Jeong et al., 2002), as well as the expression of IGF-I and ALS in hepatocytes (Brameld et al., 1995, 1999; Jin et al., 2004), has been reported in this species.

It is suggestible from the studies in rodents that plasma IGFs and IGFBPs be expressed and secreted from the liver in large animal species (larger mammals) in a manner similar to that in the former. Strictly speaking, however, results obtained from rodents are not necessarily applicable to larger mammals, because substantial differences exist between these two groups in the expression of the IGF system components. For instance, hepatic IGF-II gene expression and plasma concentration of this peptide decline to virtually undetectable levels postnatally in rodents, whereas in larger mammals, including humans, pigs and cows, plasma IGF-II concentration increases to a level higher than that of IGF-I during postnatal development (Lee, 2000). Little is known, however, as to how IGF system components are expressed and secreted from the liver in larger mammals. The present study was therefore initiated with an aim of delineating the expression and secretion of the major IGF system components by hepatic cells in the pig as an alternative to the rat.

MATERIALS AND METHODS

Preparation of hepatocytes

The animal handling procedures of the present study conformed to the guidelines of the Care and Use of Animals released by the Ministry of Agriculture and Forestry, Korea, and were also approved by the Animal Experimentation Ethics Committee of Korea University. Hepatocytes were prepared by the two-step collagenase perfusion method (Seglen, 1975) as described by Chung et al. (2002). Three-wk-old weanling piglets weighing approximately 7 kg were anesthetized by intramuscular injection of 1.5-ml rompun (23.32 mg xylazine-HCl/ml; Bayer Korea, Seoul) and 0.5 ml of 5% (w/v) ketamine-50 (Yuhan Inc., Seoul) following intraperitoneal injection of 5,000 IU of heparin (Jungooe Pharmaceuticals, Seoul). The peritoneal cavity was exposed by incising the belly along the median line, after which a 1.6-mm silicone tube was inserted into the portal vein at a site approximately 5 cm below the liver. Half a liter of buffer I (pH 7.4; 2-mM EDTA, 5-mM KCl, 0.8-mM $MgSO_4$, 1.6-mM Na_2HPO_4, 0.4-mM KH_2PO_4 and 25-mM $NaHCO_3$) was perfused through the liver at a flow rate of 200 ml/min. The liver was dissected following an excision of the suprahepatic inferior vena cava and infrahepatic inferior vena cava. After perfusing the dissected liver with buffer I for 10 min, the liver was further perfused by recirculating 300 ml of buffer II (Leffert solution (Leffert et al., 1979); 10-mM Hepes, 3-mM KCl, 130-mM NaCl, 1-mM $NaHPO_4$ and 10-mM *d*-glucose) containing 2.7% (w/v) $CaCl_2$ and 0.1% (w/v) type IV collagenase (Gibco-Invitrogen, Carlsbad, CA, USA) in a beaker under a hood. Both buffers I and II were maintained at 37°C and oxygenated through a silicone tube during the entire perfusion which was continued until the hepatic parenchymal tissue exhibited yellowish color and fissures. The collapsed hepatic tissue was washed with phosphate-buffered saline (PBS (pH 7.3); 137-mM NaCl, 2.7-mM KCl, 4.3-mM Na_2HPO_4 and 1.4-mM KH_2PO_4) and sequentially filtered through 250- and 150-μm sieves. Washed cells enriched with hepatocytes were centrifuged for 10 min at 50×*g* at 4°C followed by suspension of the pelleted cells with Williams' medium E (Sigma Chemical Co., Saint Louis, MO, USA) and this centrifugation and suspension procedure was repeated three times.

Primary cell culture

The liver cells were cultured under serum-free conditions on type I collagen-coated dishes. In brief, 2×10^5 cells per 35-mm dish were cultured in 2-ml Williams' medium E containing 0.02% BSA, 0.22% sodium bicarbonate, 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco-Invitrogen) at 37°C for 24 or 48 h in atmosphere/5% CO_2 (all the reagents from Sigma unless indicated otherwise). At the end of the culture, conditioned culture medium (CCM) was collected and concentrated using the ultraconcentrator Centricon-3 (Amicon Corp., Beverly, MA, USA).

Reverse transcription-polymerase chain reaction

Total cellular RNA was extracted with 1-ml Trizol

Table 1. Nucleotide sequences of the PCR primer pairs

Item	Direction	5' to 3' sequence	Location in cDNA	References
IGF-I	Forward	TCCTCTTCGCATCTCTTCTA	#25-44	Tavakkol et al., 1988
	Reverse	ACTTGTGTATTTCATTGGGG	#537-556	
IGFBP-2	Forward	TAGAAGAGATGACACTCGGG	#548-567	Badinga et al., 1999
	Reverse	TAGAAGAGATGACACTCGGG	#957-976	
IGFBP-3	Forward	AAACTCCACTCTGTCCACAC	#385-404	Schimasaki et al., 1990
	Reverse	TTGTAGAAGCCCTTCTTGTC	#616-635	
ALS	Forward	GAGGGATGGCCCTGAGGAAA	#32-51	Lee et al., 2001;
	Reverse	AGGAGAAGTTGTTGCTGTCCA	#278-298	Jeong et al., 2002
β-actin	Forward	GACATCAAGGAGAAGCTCTG	#267-286	Foss et al., 1998
	Reverse	ATCTTGATGTTCATGGTGCT	#667-686	

(Gibco-Invitrogen) per 35-mm dish following 48-h culture of the cells and removal of CCM. First-strand cDNA was synthesized by reverse transcription (RT) using the Cell to cDNA II kit (Ambion Co., Austin, TX, USA) according to manufacturer's instructions. PCR was performed using the RT product as template in the presence of 10 pmol of each of the primer pair in each selected IGF system component and β-actin (Table 1), 0.2-mM each dNTP and 1.25 U of *Taq* polymerase (Takara, Otsu, Japan) in a 25-μl total volume of reaction buffer (10-mM Tris-HCl (pH 8.5), 50-mM KCl and 1.5-mM $MgCl_2$). The thermal condition of PCR was 94°C for 5 min→ (94°C/1 min → 55°C/1 min → 72°C/1 min)×35 cycles → 72°C/10 min → 4°C.

Immunohistochemistry

Hepatic tissue preparation and immunohistochemistry were performed as described by Cheon et al. (2002). Briefly, after dissection of the liver from a weanling piglet under anesthesia, the hepatic tissue was fixed in 4% formaldehyde in 0.1 M phosphate buffer and frozen in embedding medium in liquid nitrogen. The embedded tissue was cryosectioned to an approximately 12-μm thickness, mounted onto gelatin-coated slides and stored at -70°C until used for immunohistochemistry. After thawing, the tissue specimen mounted on the slide was incubated with 1:200 rabbit antiserum to IGF-I (#AFP4892898, distributed by National Hormone and Peptide Program, NIDDK, USA), IGF-II (Gropep, Adelaide, Australia), pIGFBP-3 (Lee and Chung, 2000), or hIGFBP-2 (Upstate Biotechnology, Inc. (UBI), Lake Placid, NY, USA) with 5% normal goat serum (NGS) overnight at 4°C; negative control was incubated with 5% NGS only. The specimen was next incubated at room temperature sequentially with secondary antibodies (biotinylated goat immunoglobulin (Ig)G against rabbit IgG) for 1 h, with the avidin-biotin complex for 1 h and with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 10 min on a rocking shaker. Finally, the specimen was stained for 2 to 5 min after adding hydrogen peroxide to the DAB solution to a final concentration of 0.01% and washed with PBS.

IGF-I and IGF-II RIAs for CCM

IGF-I and IGF-II (Gropep) were iodinated by the chloramines-T method as previously described (Lee and Henricks, 1990). Half a microgram each of IGF-I and IGF-II was iodinated using 0.3-μCi $Na^{125}I$ (Amersham-Pharmacia Biotech, St. Louis, MO, USA) and 30-μg chloramines-T for 45 sec to a specific activity of approximately 250 μCi/μg. The iodination reaction was quenched with 75-μg sodium metabisulfite, followed by removal of free iodine on a Sephadex G-50 column (1.5×20 cm).

Four hundred microliters of a 50-fold concentrated pool of 24- and 48-h CCM of the liver cells were fractionated on a Sephadex G-50 column (1.5×20 cm) that had been pre-equilibrated with 1 M acetic acid, as described by Lee and Henricks (1990). Fractions between 50% and 80% of bed volume containing free IGFs separated from IGFBPs were pooled, dried by Speed-vac in six aliquots and stored at 4°C until used. The dried aliquot was dissolved in 0.1 M acetic acid prior to double-antibody IGF-I and IGF-II RIAs using commercial antisera (Gropep) as previously described (Lee et al., 1991; Lee and Chung, 2000; Kim et al., 2005).

Western ligand blotting (WLB) of IGFBPs

Relative abundance of IGFBPs with different molecular masses contained in the CCM was determined by WLB (Hossenlopp et al., 1986) as previously described (Lee et al., 1991; Lee and Rechler, 1995; Yun et al., 2001). Twenty microliters of the 10-fold concentrated CCM were electrophoresed on each lane of the SDS-12.5% polyacrylamide gel, and separated proteins were transferred onto nitrocellulose membranes. After blocking with 1% BSA, the membrane was incubated with [^{125}I]IGF-II (200,000 cpm/ml; 0.1 ml/cm^2 membrane) overnight at 4°C, followed by extensive washing and autoradiography. The intensity of each IGFBP band was scanned using a densitometer (Lee et al., 1991) and normalized to the average of three most intense IGFBP bands of a reference serum which had been loaded on two lanes at the time of SDS-polyacrylamide gel electrophoresis (PAGE) in each WLB.

To identify the species of secreted IGFBPs, 0.25 ml of the 10-fold concentrated CCM was incubated with 3-μl each of rabbit polyclonal antisera to hIGFBP-2 to -5 (UBI), pIGFBP-3 (Lee and Chung, 2000), or normal rabbit serum (negative control) overnight at 4°C. The antigen-antibody complex was precipitated by centrifugation following incubation of the reaction mixture with 0.2-ml 10% protein A-coated *Staphylococcus aureus* suspension at 4°C on a rotating mixer as previously described (Lee and Rechler, 1995). The pellet was extensively washed by repeated suspension with Tris buffer and re-centrifugation and finally suspended in SDS-PAGE sample buffer, followed by SDS-PAGE and WLB as described above.

Statistical analysis

The authoradiographic intensity of the IGFBP band in WLB was analyzed by the General Linear Model procedure of SAS (SAS Inst., Cary, NC, USA). The model included the IGFBP band, duration of culture and an interaction of these.

RESULTS

Liver cells in culture

The cells of the hepatocyte preparation exhibited the typical polygonal morphology of primary hepatocytes (Figure 1). Moreover, non-parenchymal cells were unidentifiable in the present hepatocyte preparation by the morphological examination. The morphology and number of the liver cells did not change up to 48 h in culture to any significant extent, but by 72 h, the cell number diminished to varying extents (data not shown). Accordingly, the liver cells were cultured for 24 or 48 h in subsequent experiments.

Expression of the IGF system components in liver cells

Reverse transcription-polymerase chain reaction (RT-PCR) was performed to examine whether genes of selected IGF system components are expressed in pig liver cells. Genes encoding IGF-I, IGFBP-2 and ALS, as expected, were confirmed to be expressed in the liver cells (Figure 2). Surprisingly, however, the IGFBP-3 gene, which is known to be expressed only in non-parenchymal cells in rat liver (Takenaka et al., 1991; Chin et al., 1994; Scharf et al., 2001), also was found to be expressed in these cells. Moreover, the RT-PCR result was reproducible with different liver cell preparations derived from additional piglets, which implies that the present hepatocyte preparation was adulterated with a minor portion of unidentified non-parenchymal cells, or that the IGFBP-3 gene is actually expressed in porcine hepatocytes.

Immunohistochemistry was performed to determine in what cell types the IGF system components are expressed. The immunohistochemical signal of IGF-I was expectedly

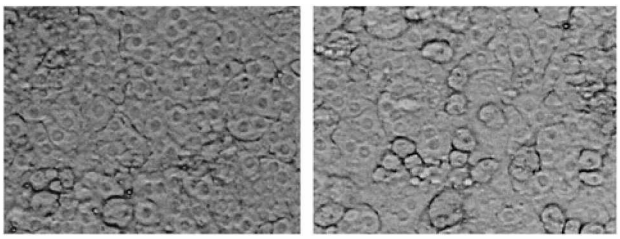

Figure 1. Pig liver cells following the serum-free culture. Cells of the porcine hepatocyte preparation derived from a weanling piglet were cultured for 24 h (left) or 48 h (right) in serum-free Williams' medium E.

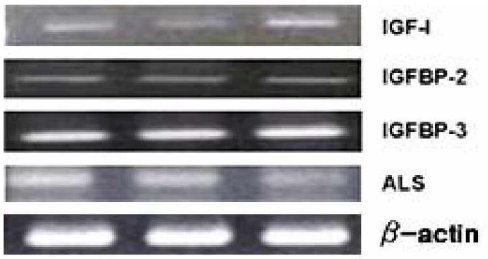

Figure 2. Reverse transcription-polymerase chain reaction (RT-PCR). Each indicated RT-PCR was performed three times using three RNA preparations isolated from the pig liver cells following the 48-h culture in three 35-mm dishes. The PCR products were electrophoresed on agarose gels and stained with ethidium bromide. For clarity, only PCR products of expected sizes are shown in this figure. The five DNA bands in each vertical array of the lanes have originated from a same RNA preparation.

localized to hepatocytes (Figure 3, lower left panel). In contrast, the IGFBP-3 signal was localized to endothelial cells (Figure 3, lower right panel), but not to hepatocytes, implicating that the IGFBP-3 gene expression detected by RT-PCR (Figure 2) must have been contributed by non-parenchymal cells presumably adulterated in the hepatocyte preparation. Further immunohistochemistry using polyclonal antisera against hIGF-II and hIGFBP-2 was unfruitful, because neither antiserum exhibited any discernable immunoreactivity towards the corresponding porcine peptide in the tissue specimens.

Secretion of IGFs and IGFBPs by liver cells in culture

The amounts of IGF-I and IGF-II that had been secreted from the hepatocyte-enriched liver cells during the primary culture were determined by corresponding RIAs following concentration of a pool of conditioned cultured media (CCM) and removal of IGFBPs by acid gel filtration on a Sephadex G-50 column. The IGF-I and IGF-II contents were approximately 0.33 and 2.37 ng, respectively, per milliliter native CCM or 1×10^5 cells (Table 2).

The species and relative abundance of IGFBPs contained in CCM were resolved by WLB using [^{125}I]IGF-II. Both 1-d and 2-d CCM contained 43-, 40-, 34-, 31-kDa

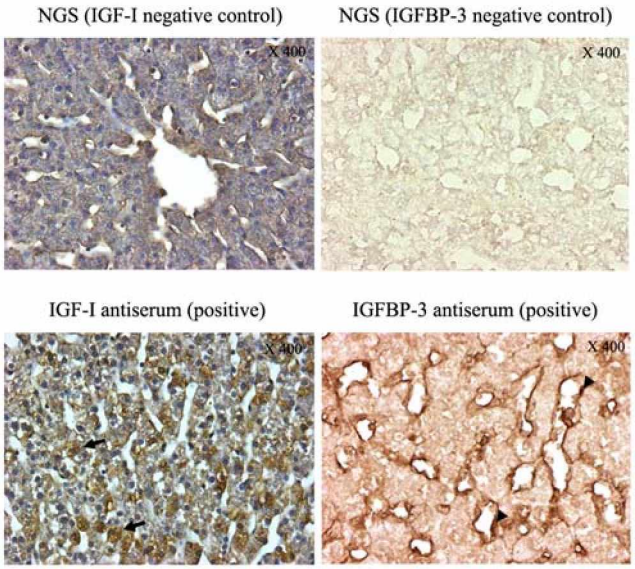

Figure 3. Immunohistochemical localization of IGF-I and IGFBP-3 peptides in pig liver. Hepatic tissue specimen of a weanling piglet was incubated with 1:200 indicated antiserum (lower panels) in 5% normal goat serum (NGS) or with 5% NGS alone as negative controls (upper panels), sequentially followed by further incubation with biotinylated secondary antibodies, avidin-biotin complex and 3,3'-diaminobenzidine tetrahydrochloride for the development of the chemical signal. Note the positive IGF-I (arrows) and IGFBP-3 (arrowheads) signals in hepatocytes and endothelial cells in lower left and right panels, respectively.

doublet and 26-kDa IGFBPs, all of which, except the 'small' 31-kDa IGFBP, were also present in sera of the weanling piglets (Figure 4, left three panels). When the abundance of these IGFBPs in CCM was quantitated by autoradiographic densitometry of the IGFBP bands, the overall abundance of secreted IGFBPs did not differ

Table 2. Secretion of IGF-I and IGF-II from cultured pig liver cells

	IGF-I	IGF-II
Concentration in native CCM (pg/ml)	332	2,374

Hepatocyte-enriched pig liver cells prepared from weanling piglets were cultured at a density of 2×10^5 cells per 35-mm dish in 2-ml Williams' medium E for 24 or 48 h. Conditioned cultured media (CCM) were pooled and concentrated 50 folds using an ultraconcentrator prior to removal of IGF-binding proteins by acid gel filtration and the IGF RIAs.

between the 1-d and 2-d CCM (Table 3). The 40-, 34- and 'big' 31-kDa IGFBPs were approximately three-fold more abundant ($p<0.01$) than the 43- and 26-kDa IGFBPs. Moreover, the small 31-kDa IGFBP, which also was more abundant than the 43- and 26-kDa IGFBPs ($p<0.05$), was as abundant as the 40- and big 31-kDa IGFBPs but less abundant than the 34-kDa IGFBP ($p<0.05$). It was thus evident that the 40-, 34- and 31-kDa IGFBPs were almost equally abundant in CCM, whereas in serum, the 43- and 40-kDa IGFBPs were the most abundant followed by the 34-kDa one. In other words, the 43- and 40-kDa doublet IGFBPs were minimally secreted by the liver cells compared with their relative abundance in serum, whereas the reverse was true for the 31-kDa doublets IGFBPs. The 43- and 40-kDa IGFBPs could be immunoprecipitated using a pIGFBP-3 antiserum, but not by a hIGFBP-3 antiserum (Figure 4). The 34-kDa IGFBP was also immunoprecipitated with hIGFBP-2 antibodies, but none of the other IGFBPs was precipitated with hIGFBP-4 or -5 antibodies.

DISCUSSION

The present hepatocyte preparation contained mostly hepatocytes as judged from the morphology of the cells. However, the liver cell preparation was likely to contain a

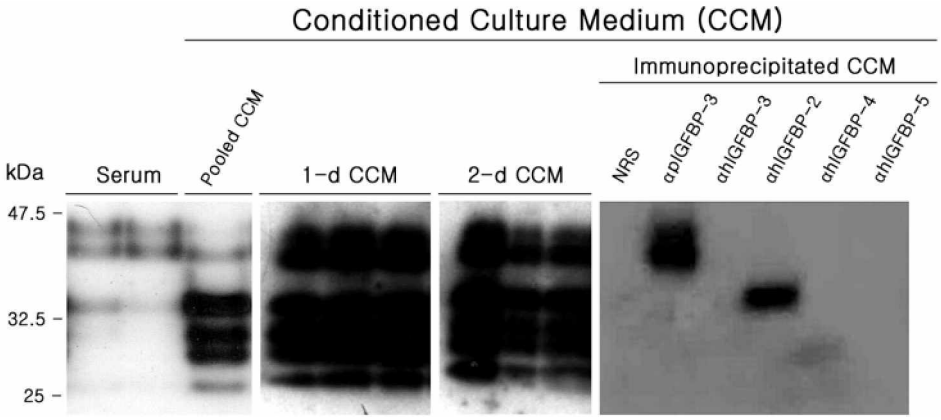

Figure 4. Western ligand blotting (WLB) of conditioned culture medium (CCM) of liver cells. Left three panels, 1-μl serum from each of two weanling piglets, 25 μl of 10-fold concentrated pooled CCM (leftmost panel), or 25-μl 10-fold-concentrated CCM from each of three culture dishes (middle two panels) was electrophoresed on each lane, after which separated proteins were transferred onto nitrocellulose membranes followed by WLB using [^{125}I]IGF-II and autoradiography. Rightmost panel, 10-fold concentrated CCM (0.25 ml) was immunmoprecipitated using the indicated antiserum or normal rabbit serum (NRS) as negative control prior to electrophoresis for WLB.

Table 3. Relative abundance of IGFBPs secreted into the culture medium monitored by Western ligand blotting and scanning densitometry

Duration of culture	Molecular mass of IGFBP					
	43 kDa	40 kDa	34 kDa	31 kDa (big)	31 kDa (small)	26 kDa
One day	8.51	18.31	20.41	22.88	16.91	6.17
Two days	4.14	15.96	21.13	14.68	10.65	5.29
	---------- Pooled SE = 2.75 ----------					

The intensities of IGFBP bands in Figure 4 were quantitated by scanning densitometry according to an arbitrary unit. The effect of the species of the IGFBP was significant ($p<0.01$). Effects of the duration of culture and an interaction of this with the IGFBP species were not significant.

small portion of non-parenchymal cells as well, which was suggested from the detected expression and secretion of IGFBP-3 from the liver cells in culture in spite of the immunohistochemical detection of this peptide only in endothelial cells. One may ask how a significant amount of IGFBP-3 comparable to that of any other IGFBP could be secreted from minimally adulterated non-parenchymal cells that are known to be less than one-fourth as abundant as hepatocytes in neonatal pig liver (Caperna et al., 1985). In this regard, the ability of the former to secrete IGFBP-3 appears to be much greater than that of the latter to secrete other IGFBPs, because in our study, liver perfusates of adult rats, like sera, contain greatest amounts of IGFBP-3 of all the secreted IGFBPs (Lee et al., 1996). However, the relative amount of IGFBP-3 in the present conditioned culture medium (CCM) was much greater than the trace amount of secreted IGFBP-3 in rat hepatocyte culture reported by Schmid et al. (1989). It thus remains to be known why the hepatocyte preparation was apparently adulterated with non-parenchymal cells more significantly in the present study than in the previous one.

The immunohistochemical localizations of IGF-I and IGFBP-3 to the hepatocytes and endothelial cells, respectively, in the present study are consistent with previous reports in the rat (Chin et al., 1994; Scharf et al., 1996). It remains to be studied, however, if Kupffer cells, which are known to express IGFBP-3 in rat liver, express IGFBP-3 in pig liver and also why this major plasma IGFBP is expressed only in non-parenchymal cells in the pig and other species whereas IGFs, ALS and other IGFBPs are expressed in hepatocytes.

The present study did not include an investigation of hormonal and nutritional regulations of the expression of the IGF system components. In this regard, it has been reported that IGF-I gene expression in isolated porcine hepatocytes is greatly induced by glucose, thyroid hormone, glucocorticoid and GH (Brameld et al., 1999) in addition to insulin which is known to increase the viability of pig and rat hepatocytes in culture (Brameld et al., 1995) and also to suppress the expression of IGFBPs-1 and -2 genes in rat liver (Rechler et al., 1991). It thus seems likely that the amounts of secreted IGFBPs did not increase after 24 h in culture in the present study apparently due to inadequate hormonal and/or nutritional supports for the cells. Nevertheless, substantial amounts of both IGFs and all the IGFBPs with different molecular masses found in serum were present in the CCM as monitored by RIAs and WLB. Moreover, the IGF-I content in the CCM was approximately one-seventh that of IGF-II, which was close to an approximately 1:4 ratio of serum concentrations of these peptides in 3-wk-old piglets (Lee et al., 1991) used in the present study.

The immunoprecipitability of the 43- and 40-kDa doublet and 34-kDa IGFBPs contained in the CCM with IGFBP-3 and IGFBP-2 antibodies, respectively, was consistent with previous results obtained with porcine serum (Lee et al., 1991). It is also almost certain that the 26-kDa IGFBP is the truncated form of IGFBP-4 which is found in many biological fluids including serum and CCM of various cell types (Shimonaka et al., 1989; Pampusch et al., 2005). However, the identities of the 31-kDa doublets IGFBPs, which apparently correspond to the 28-kDa doublets found in CCM of porcine embryonic myogenic cells (Pampusch et al., 2005), need to be confirmed, although the latter doublets were identified as IGFBP-5 and a mixture of IGFBPs-4 and -5, respectively, by immunoblotting by Pampusch et al. (2005).

In summary, IGF-I and IGFBP-3 have been identified to be expressed in hepatocytes and endothelial cells, respectively, in the pig in the present study. Moreover, detection of hepatic expression of IGFBP-2 and ALS genes and secretion of all the different-size plasma IGFBPs in the present conditioned cell culture medium (CCM) suggests that the IGF system components are likely to be expressed and secreted in pig liver in a manner similar to that in rat liver. It remains to be known, however, whether the IGFBPs other than IGFBP-3 detected in the present CCM are secreted from hepatocytes in pig liver.

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