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, in mass, 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...
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 probed 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-1 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 MgSO4, 1.6-mM Na2HPO4, 0.4-mM KH2PO4 and 25-mM NaHCO3) 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 NaHPO4 and 10-mM d-glucose) containing 2.7% (w/v) CaCl2 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 Na2HPO4 and 1.4-mM KH2PO4) 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 liver-specific IGF-I knock-out (Yakar et al., 2005). 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-1 and ALS in hepatocytes (Brameld et al., 1995, 1999; Jin et al., 2004), has been reported in this species.

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% CO2 (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

<table>
<thead>
<tr>
<th>Item</th>
<th>Direction</th>
<th>5' to 3' sequence</th>
<th>Location in cDNA</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I</td>
<td>Forward</td>
<td>TCCTCTTCGACATCTCTTCTTA</td>
<td>#25-44</td>
<td>Tavakkol et al., 1988</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ACTTGTGTATTTCATGTTGGGG</td>
<td>#537-556</td>
<td></td>
</tr>
<tr>
<td>IGFBP-2</td>
<td>Forward</td>
<td>TAGAAGAGATGCACACTCGGG</td>
<td>#548-567</td>
<td>Badinga et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TAGAAGAGATGCACACTCGGG</td>
<td>#957-976</td>
<td></td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>Forward</td>
<td>AAACCTCACTCTGTCCACAC</td>
<td>#385-404</td>
<td>Schimasaki et al., 1990</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGTTAGAAGCCCTTCTGTGC</td>
<td>#616-635</td>
<td></td>
</tr>
<tr>
<td>ALS</td>
<td>Forward</td>
<td>GAGGGAATGGCCCTGAGAAAA</td>
<td>#32-51</td>
<td>Lee et al., 2001;</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGGAGAAGTGTGGCCTGTCCA</td>
<td>#278-298</td>
<td>Jeong et al., 2002</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward</td>
<td>GACATCAAGGAGAAGCTCTG</td>
<td>#267-286</td>
<td>Foss et al., 1998</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATCTTGATGTTCCATGGTGCT</td>
<td>#667-686</td>
<td></td>
</tr>
</tbody>
</table>

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 Na125I (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 antiseras (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 [125I]IGF-II (200,000 cpm/ml; 0.1 ml/cm² 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 autoradiographic 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), was also 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 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 1×10⁵ cells (Table 2).

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

| 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  |

Table 2.
Table 3. Relative abundance of IGFBPs secreted into the culture medium monitored by Western ligand blotting and scanning densitometry

<table>
<thead>
<tr>
<th>Duration of culture</th>
<th>Molecular mass of IGFBP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>43 kDa</td>
</tr>
<tr>
<td>One day</td>
<td>8.51</td>
</tr>
<tr>
<td>Two days</td>
<td>4.14</td>
</tr>
</tbody>
</table>

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.

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 -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.

REFERENCES


Brameld, J. M., R. S. Gilmour and P. J. Buttery. 1999. Glucose and


a,b,c,d,e,f,g,h,i,j,k,l,m,n,o,p,q,r,s,t,u,v,w,x,y,z,A,B,C,D,E,F,G,H,I,J,K,L,M,N,O,P,Q,R,S,T,U,V,W,X,Y,Z,a,b,c,d,e,f,g,h,i,j,k,l,m,n,o,p,q,r,s,t,u,v,w,x,y,z