Altered expression of IGFs and IGF-binding proteins during intrauterine growth restriction in guinea pigs

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Abstract

The IGF system is one of the most important endocrine and paracrine growth factor systems that regulate fetal and placental growth. We hypothesized that intrauterine growth restriction (IUGR) in guinea pigs is mediated by the altered expression of IGFs and/or IGF binding protein (BP) mRNAs in tissues and is related to growth of specific tissues. IUGR was induced by unilateral uterine artery ligation on day 30 of gestation, and fetal plasma, amniotic fluid and tissue samples were collected at 55–57 days (term about 68 days) from paired IUGR and control fetuses ($n=6$). Western ligand blotting and immunoblotting were used to compare IGFBP levels in plasma and amniotic fluid. Total RNA was extracted from placenta and fetal tissues, and the relative abundance of IGF-II and IGFBP-1–6 mRNA was determined by Northern blotting, using species-specific probes where available. IUGR fetuses had decreased ($P<0.01$, by Student's *t*-test) placental weight

and body weight with an increase in the brain:liver weight ratio. The principal IGFBPs in fetal plasma migrated at 40–35, 30 and 25 kDa and were identified as IGFBP-3, -2 and -4 respectively. IUGR was associated with elevated plasma IGFBP-2 and IGFBP-4 and reduced IGFBP-3 levels. IGFBPs were detected at low levels in amniotic fluid of control fetuses but at higher levels in IUGR fetuses. In IUGR placentae, there was a small increase in IGFBP-4 mRNA ($P<0.05$). IGFBP-2 mRNA increased ($P<0.001$) in liver of IUGR fetuses. IGF-II and IGFBP mRNA expression did not change in fetal muscle. The results are consistent with reduced IGF action, directly or through inhibition by IGFBPs, particularly by circulating and tissue IGFBP-2, as a potential causal factor in decreased growth of the placenta and certain fetal tissues.

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Introduction

Insulin-like growth factors I and II (IGF-I and -II) are polypeptides with structural homology to proinsulin and are important in regulating fetal and placental growth (DeChiara *et al.* 1990, Liu *et al.* 1993). They promote proliferation, differentiation, migration and aggregation, and inhibit apoptosis; all processes involved in development. The bioavailability and biologic actions of IGFs are regulated by a family of six high-affinity binding proteins (BP) (IGFBP-1–6) (Clemmons 1997). In general, IGFBPs inhibit the actions of IGFs, by competing with the IGF receptors for the peptide. Slowing of fetal growth, as in intrauterine growth restriction (IUGR), probably involves decreased expression of IGFs and increased expression of IGFBPs.

Guinea pigs provide a valuable model for studying the developmental regulation of the IGF system. As in

humans, IGF-II persists in the serum of adult guinea pigs, and IGF-II mRNA is expressed in adult tissues (Levinovitz *et al.* 1992). A further important similarity between guinea pig and human is the presence of IGFBP-3 in fetal plasma. This is in contrast to rats and mice, which lack postnatal expression of IGF-II (Soares *et al.* 1986, Rotwein & Hall 1990) and prenatal expression of IGFBP-3 (D'Ercole & Underwood 1980, Lee *et al.* 1999). There is 100% homology in the amino acid sequences of human and guinea pig IGF-I and -II and 96% and 94% homology in the nucleotide sequences of the respective mRNAs (Bell *et al.* 1990, Levinovitz *et al.* 1992). The cellular localization of IGF and IGFBP mRNAs in the placenta has been described in greater detail for the guinea pig than for any other nonprimate species (Han *et al.* 1999, Han & Carter 2000).

Fetal growth restriction can be induced in guinea pigs by uterine artery ligation (Lafeber *et al.* 1984). Fetal body

weight is reduced by about 40%, but with brain sparing and an increase in brain:liver weight ratio. This model has been well characterized in terms of fetal endocrine and cardiovascular responses (Carter 1993). A previous report showed that circulating IGF-I levels are decreased and IGF-II levels are increased (Jones *et al.* 1987). Since IGF-II is a more important IGF during fetal development, the role of circulating IGFs in mediating the growth phenotype in this model is unclear. We therefore hypothesized that, in guinea pigs, IUGR is mediated by the altered expression of IGFBP mRNAs in tissues, and that this would be reflected in the protein levels in fetal plasma and related to changes in growth of specific tissues.

Materials and Methods

Uterine artery ligation

Unilateral uterine artery ligation was performed on day 30 of gestation in guinea pigs that had been given atropine (0.05 mg s.c.) and anesthetized with ketamine (4 mg/kg i.m.) and xylazine (50 mg/kg i.m.), as previously described (Detmer & Carter 1992).

Tissue collection

At 55–57 days of gestation, the dam was again anesthetized with ketamine and xylazine, and hysterotomy was performed. A fetus was taken from each horn of the uterus (ligated and control) in six guinea pigs. Unilateral pregnancies ($n=4$) and experiments in which ligation resulted in fetal demise ($n=1$) or failed to cause growth restriction ($n=1$) were not included. As in our previous studies (Detmer & Carter 1992, Carter 1993), we arbitrarily defined a growth-restricted fetus as one with a brain:liver weight ratio of >0.9 .

We took samples of amniotic fluid and collected fetal and maternal blood by cardiac puncture. Samples were collected on ice and the plasma and amniotic fluid stored at -20°C . The dam was then killed by an overdose of pentobarbital sodium. The placenta (less the subplacenta), fetus and fetal organs were weighed. The placenta and fetal liver and muscle were divided into two parts. One part was snap frozen and stored at -80°C for total RNA; one part was immersion fixed in 4% paraformaldehyde in 70 mM phosphate buffer at 4°C for 48 h for *in situ* hybridization.

Western ligand blot analysis

The profile and relative levels of IGFBPs in fetal guinea pig plasma and amniotic fluid were determined by Western ligand blot analysis, as previously described (Delhanty & Han 1993, Winger *et al.* 1997). Samples of plasma

(10–20 μl) or amniotic fluid (100 μl) were added to 20 μl 6 $\times$ Laemmli buffer (160 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol and 0.02% bromophenol blue), and sterile water was added to a total volume of 120 μl . The samples were boiled for 5 min, quenched on ice for 2 min and then loaded onto a 6–12% gradient non-reducing SDS-polyacrylamide gel and electrophoresed at constant voltage (50 V) overnight. The proteins were then transferred to a 0.2 μm nitrocellulose membrane (Schleicher and Schuell, Keene, NH, USA) by electroblotting in Tris-glycine buffer (25 mM Tris, 1.192 mM glycine and 20% (v/v) methanol) at 4°C at constant voltage (50 V) overnight. After transfer, the blots were washed in Tris-NaCl buffer (150 mM NaCl and 10 mM Tris-HCl (pH 7.4)) and blocked with Tris-NaCl buffer containing 1% (w/v) fatty acid-free bovine serum albumin (BSA) and 0.1% Tween-20. The blots were hybridized with ^{125}I -labeled IGF-II for 20 h at 4°C in 10–15 ml Tris-NaCl buffer, 1% (w/v) BSA and 0.1% Tween-20. The blots were then washed in Tris-NaCl buffer (pH 7.4), with and without 0.1% Tween-20, air dried and exposed to radiographic film (BMR; Eastman Kodak, Rochester, NY, USA) at -70°C for 3–7 days. They were analyzed by densitometry (PhosphorImager SI; Molecular Dynamics, Sunnyvale, CA, USA).

To identify IGFBPs present in plasma, parallel Western blots were prepared for ligand blotting and immunoblotting. Membranes for immunoblotting were blocked overnight at 4°C in TTBS buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.4) containing 0.05% Tween-20) and 4% (w/v) fatty acid-free BSA. The blot was then washed three times with TTBS and incubated for 20 h in TTBS with 1% (w/v) BSA and rabbit antiserum against bovine IGFBP-2 (no. 06–317; Upstate Biotechnology, Lake Placid, NY, USA) (1:2000), which cross-reacted with the guinea pig protein. After incubation with primary antiserum, the membranes were washed three times with TTBS and then incubated for 2 h with antirabbit immunoglobulin (Ig) G biotin conjugates (Sigma, Oakville, Ontario, Canada) (1:1000) diluted in TTBS with 1% (w/v) BSA. Membranes were washed three times in TTBS, in ExtraAvidin (Sigma) for 1 h, and three times in TTBS. Bands were visualized by a 3,3'-diaminobenzidine tetrahydrochloride (Sigma)/3% hydrogen peroxide reaction. The reaction was then quenched in 50 mM Tris-HCl (pH 7.5), and the membranes were air dried.

Immunoblotting of amniotic fluid revealed two bands that were immunopositive for IGFBP-2. Therefore, we performed additional experiments to determine whether a phosphorylated or glycosylated form was present. First, Western immunoblot was performed with amniotic fluid samples containing 30 or 60 μg total protein. The antibody used to detect phosphorylated proteins was antiphosphoserine clone PSR-45 (mouse ascites fluid; Sigma) (1:5000). On the same blot, guinea pig IGFBP-2 was detected as before with rabbit antiserum against bovine IGFBP-2.

Table 1 Primers used to clone partial cDNA sequences of guinea pig IGF-II, IGFBP-2, IGFBP-3, IGFBP-5 and IGFBP-6

Gene	Expected cDNA length (bases)	Primer end	Primer length (bases)	Primer sequence	Restriction enzyme
IGF-II	329 bases	5'	21	5' GCCCAGGCCCCAGGAAGATAC 3' (cut in plasmid)	Nco I
		3'	20	5' CGGAAGCACGGCCAAAGACG 3' (cut in plasmid)	Pst I
IGFBP-2	167 bases	5'	30	5' GGAAGAATTCACGTGTCACAAGCATGGCCTG 3'	Eco R I
		3'	28	5' GGAAGGATCCTCCTGCTGCTCATTGTAG 3'	BamH I
IGFBP-3	209 bases	5'	29	5' GGAAGAATTCAGAACTTCTCCTCCGAGTC 3'	EcoR I
		3'	31	5' GGAAGGATCCTATCCACACACCAGCAGAAGC 3'	BamH I
IGFBP-5	195 bases	5'	30	5' GGAAGAATTCGCTCAAGCCAGCCGCGCAT 3'	EcoR I
		3'	30	5' GGAAGGATCCGTCGAAGCCGTGGCACTGAA 3'	BamH I
IGFBP-6	344 bases	5'	31	5' GGAAGAATTCGACGAGGCGCCTTTGCGGGC 3'	EcoR I
		3'	31	5' GGAAGGATCCGGAGGAGCGGCACTGCCGCT 3'	BamH I

Secondly, amniotic fluid, containing 30 µg total protein, was treated with the N-glycosylation F deglycosylation kit (Roche Canada, Laval, Quebec, Canada). A further sample of the amniotic fluid was not treated with the enzyme but contained the remaining reagents of the kit. The protocol was performed according to the manufacturer's instructions. Western immunoblot was performed as before with the antbovine IGFBP-2 antibody (1:10 000). To determine whether the aliquots of amniotic fluid loaded on the gels contained similar amounts of protein, duplicate determinations of total protein concentration were made by the bicinchoninic acid assay (BCA Protein Assay; Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

RNA extraction

Total RNA was extracted from the tissues of control and IUGR fetal guinea pigs with a guanidinium thiocyanate-phenol-chloroform extraction protocol (Chomczynski & Sacchi 1987). Final RNA pellets were dissolved in diethylpyrocarbonate-treated water, and the yield of RNA was quantified by measuring optical density at 260 nm.

Reverse transcription (RT)

RNA was reverse transcribed by oligo(dT) priming and reverse transcriptase. An amount of 1 µg total adrenal RNA extracted from fetal tissues was incubated with 0.5 µg oligo(dT) primer (Gibco BRL, Burlington, Ontario, Canada) for 10 min at 70 °C in 12 µl. Samples were then cooled on ice, and the following components added: 1 µl deoxyribonucleotides (each 10 mM), 4 µl RT-buffer (supplied with the reverse transcriptase kit), 1.5 µl MgCl₂ (25 mM), 2 µl dithiothreitol (100 mM), 0.5 µl RNase inhibitor (20 IU/µl) and 1 µl reverse transcriptase (200 U/µl; SuperScript II, Gibco BRL). Samples were incubated for 1 h at 42 °C and the reaction stopped by

heating to 70 °C for 15 min. After cooling for 10 min at 4 °C, 1 µl RNase (2 U/µl, RNase H, Gibco BRL) was added and the solution was then heated at 37 °C for 20 min. The DNA was stored at -20 °C until used.

PCR

Known cDNA sequences encoding IGF-I (Bell *et al.* 1990) and IGF-II (Levinovitz *et al.* 1992) were used to design DNA oligomers for amplification by PCR. As the guinea pig IGFBP sequences were unknown, primer pairs for IGFBP-1-6 were designed from regions of identity between the known cDNA sequences for human, rat, mouse, sheep and cattle. To facilitate cloning, the oligonucleotides were synthesized with adapters containing the restriction sites shown in Table 1. Primer pairs were synthesized at the Core Molecular Biology Facility, London Regional Cancer Centre, University of Western Ontario.

For PCR, the cDNA was supplied with 1 µl of each primer (100 pmol/µl), 1 µl deoxyribonucleotides (10 mM), 5 µl 10 × PCR buffer, 2.5 µl MgCl₂ (50 mM) and water to a final volume of 50 µl. The tubes were incubated for 5 min at 94 °C to denature the template, followed by the addition of 0.5 µl Taq polymerase (5 units/µl; Gibco BRL). cDNA was then amplified for 25-35 cycles in a DNA thermal cycler (Masterflex, Eppendorf Scientific Instruments, Westbury, NY, USA), with each cycle including denaturation (30 s at 94 °C), annealing (1 min at 60-65 °C) and extension (1 min at 72 °C). A final extension period of 7 min at 72 °C was allowed.

PCR products were mixed with 6 × gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol) and were electrophoresed with 1 × Tris acetate EDTA buffer on 2% agarose gels at 100 V against a 1 kb DNA ladder (Gibco BRL). Appropriately sized bands were excised from the gel and purified with a gel extraction kit (Qiagen, Mississauga, Ontario, Canada).

Cloning of cDNA

PCR products were ligated into pGEM-T vector (Promega) for heat shock uptake into competent *Escherichia coli* (DH5 α , Gibco BRL) by standard methods (Maniatis *et al.* 1989). Insert sequences were verified by sequencing in both directions, with SP6 and T7 polymerases, at the London regional genome-sequencing core facility. The cDNAs were then isolated by restriction digestion and purified with a gel extraction kit (Qiagen).

Partial cDNA sequences of IGF-II, IGFBP-2, IGFBP-3, IGFBP-5 and IGFBP-6 were cloned successfully. The IGF-II insert was identical to the published sequence, and the IGFBP probes showed 89–96% nucleotide sequence identity with the known cDNAs of mouse and human (data not shown).

Preparation of cRNA probes

Probes were labeled by random priming with [32 P]deoxy-CTP, using an oligolabeling kit (Pharmacia, Baie d'Urfe, Quebec, Canada). Human probes were used for IGF-I, IGFBP-1 and IGFBP-4; we have previously demonstrated their utility in *in situ* hybridization experiments on guinea pig tissues (Han *et al.* 1999).

Northern blot hybridization

RNA samples (20 μ g) and a 0.24–9.5 kb RNA ladder (Gibco BRL) were electrophoresed on a 1% denaturing agarose gel in 1 $\times$ Northern buffer (0.2 M 3-(N-morpholino)propane sulfonic acid, 5 mM Na $_2$ HPO $_4$ and 0.5 mM EDTA, pH 7.0). The RNAs were blotted by capillary transfer onto a Zetaprobe membrane (Bio-Rad Laboratories, Richmond, CA, USA), cross-linked to the membrane and then baked at 80 °C for 1 h.

The blots were prehybridized in Zetaprobe hybridization buffer (containing 7% SDS, 50% formamide, 0.1 mg/ml denatured salmon sperm DNA and 5 $\times$ SSPE (0.75 M NaCl, 44 mM Na $_2$ HPO $_4$ ·2H $_2$ O and 5 mM EDTA)) at 42 °C. After prehybridization, the buffer was replaced with a new Zetaprobe buffer containing the 32 P-labeled probe (10 6 c.p.m.). After hybridization overnight at 42 °C, the blots were washed twice for 15 min each in 1 $\times$ SSC and 0.1% SDS at 42 °C, once for 30 min with 0.1 $\times$ SSC and 0.1% SDS solution at 42 °C and twice for 30 min each with 0.1 $\times$ SSC and 0.1% SDS at 60 °C. The blots were air dried and exposed to radiographic film (BMR or XAR, Eastman Kodak) for 90 min to 10 days at –70 °C with intensifying screens.

Before hybridizing with a new probe, the blot was stripped by washing twice for 30 min with 0.01 $\times$ SSC and 0.5% SDS at 80–100 °C. The blots were hybridized finally with a 32 P-labeled 18S rRNA cDNA probe to check for uniformity of RNA loading among lanes.

In situ hybridization

In situ hybridization was performed on fetal liver and placenta as described previously (Han *et al.* 1999). Briefly, 5 μ m thick sections were deparaffinized and rehydrated. After prehybridization with hybridization buffer, they were hybridized with 35 S-labeled complementary RNA (cRNA) probes overnight at 55 °C and washed at the maximum stringency of 0.1 $\times$ SSC (standard saline citrate) at 55–65 °C for 30 min. The 35 S-labeled antisense and sense cRNA probes were generated from the partial cDNA sequences of guinea pig IGFBP-2 and IGFBP-3 (this paper).

After hybridization, sections were dehydrated, coated with photoemulsion (NTB-3 nuclear track emulsion (Eastman Kodak)) and exposed at 4 °C for 4–16 weeks, depending on the strength of the autoradiographic signal on exposure to radiographic film (Biomax MR; Eastman Kodak). The photoemulsion was developed with a D-19 developer (Eastman Kodak), fixed, stained with Mayer's hematoxylin and eosin, and mounted with Permount (Fisher Scientific, Nepean, Ontario, Canada). The specificity of *in situ* hybridization was demonstrated by the absence of specific hybridization signal when adjacent tissue sections were subjected to an identical *in situ* hybridization procedure with radiolabeled sense cRNA probes.

Data analysis

Relative levels of IGF and IGFBP mRNAs on autoradiograms of Northern blots and levels of IGFBPs in plasma and amniotic fluid, as analyzed by Western ligand blotting, were quantified by laser densitometry. To correct for minor differences in loading of total RNA on Northern blots, a ratio of the relative density of each specific band to the relative density of the 18S rRNA band was calculated. Comparisons between fetuses from uterine horns with uterine artery ligation and control fetuses from the contralateral horns were made by Student's *t*-test for paired samples. Results were considered significant at $P < 0.05$.

Results

Fetal morphometry

Unilateral uterine artery ligation resulted in a smaller fetus with a smaller placenta (Table 2). Although all organs weighed less than in the control fetuses, liver weight was reduced more than body weight, while brain and adrenal glands were reduced proportionately less than body weight (Table 3).

IGFBP proteins in plasma and amniotic fluid

The principal IGFBPs in fetal guinea pig plasma migrated at 40–35, 30 and 25 kDa, corresponding to IGFBP-3, -2

Table 2 Weights of fetus, placenta and fetal organs after unilateral uterine artery ligation in the guinea pig

	Non ligated horn	Ligated horn	Change from non ligated (%)
Fetal weight (g)	54.2 ± 6.3	34.5 ± 5.8***	-36.5 ± 7.3
Placental weight (g)	3.82 ± 0.65	2.45 ± 0.29**	-34.4 ± 13.6
Liver weight (g)	2.85 ± 0.39	1.58 ± 0.29***	-44.6 ± 8.5
Kidney weight (g)	0.49 ± 0.14	0.30 ± 0.09***	-38.6 ± 6.8
Adrenal weight (g)	0.022 ± 0.005	0.016 ± 0.004**	-25.7 ± 12.3
Heart weight (g)	0.30 ± 0.04	0.21 ± 0.04**	-30.7 ± 14.7
Lung weight (g)	1.12 ± 0.13	0.70 ± 0.14***	-37.4 ± 8.5
Brain weight	2.13 ± 0.19	1.95 ± 0.14**	-8.5 ± 3.6
Brain:liver weight ratio	0.75 ± 0.07	1.26 ± 0.20***	+67.3 ± 20.7

Mean ± S.D. for paired data (n=6).

P<0.01, *P<0.001 for differences between means of paired values.

and -4 respectively. IGFBP-2 was identified by immunoblotting with antiserum against bovine IGFBP-2 (Fig. 1B). By molecular size, the 40–35 kDa doublet was identified as the glycosylated and nonglycosylated forms of IGFBP-3,

Table 3 Ratio of fetal organ weights to body weight (%) after unilateral uterine artery ligation in the guinea pig

	Non ligated horn	Ligated horn
Liver	5.27 ± 0.42	4.59 ± 0.42*
Kidneys	0.90 ± 0.22	0.87 ± 0.21
Adrenals	0.040 ± 0.009	0.047 ± 0.008*
Heart	0.56 ± 0.02	0.60 ± 0.08
Lungs	2.07 ± 0.13	2.03 ± 0.12
Brain	3.94 ± 0.31	5.77 ± 1.01**

Means ± S.D. for paired data (n=6).

*P<0.05, **P<0.001 for differences between means of paired values.

Figure 1 Parallel ligand and immunoblots of plasma from a control fetus (lane C) and a fetus affected by uterine artery ligation (lane L). (A) Ligand blot probed with ^{125}I -labeled IGF-II. (B) Immunoblot with antiserum against bovine IGFBP-2. Numbers show molecular size markers. The principal binding proteins migrated at 40–35, 30 and 25 kDa. The major 30 kDa band is shown by immunoblotting to be IGFBP-2. By molecular size, the 40–35 kDa bands were identified as the IGFBP-3 doublet and the 25 kDa band as IGFBP-4.

and the 25 kDa band as IGFBP-4. Antisera against human or rodent IGFBP-3 and -4 did not specifically identify these binding proteins due to lack of cross-reactivity. After uterine artery ligation, IGFBP-2 and IGFBP-4 were elevated ($P<0.05$) and IGFBP-3 was depressed ($P<0.05$; Fig. 2A and C).

IGFBPs were found only at low levels in amniotic fluid of control fetuses, but amniotic fluid contained a higher molecular mass protein species not seen in plasma that was immunoreactive to IGFBP-2 (denoted IGFBP-2 L). In additional immunoblots of amniotic fluid, a phosphoserine antibody showed no band the size of IGFBP-2. The IGFBP-2 antibody detected two bands as before. There was no difference between amniotic fluid treated with glycosidase F enzyme and samples not treated with the enzyme. Thus, IGFBP-2 L appears not to contain phosphorylated serine residues, nor is it glycosylated by N-linkage. IGFBP-2 L and -3 were present at higher concentrations in amniotic fluid of fetuses from the horn with uterine artery ligation ($P<0.05$), with a similar trend for IGFBP-2 (Fig. 2B and D). The total protein concentrations were similar in amniotic fluid from control fetuses and those from the horn with uterine artery ligation (2.15 ± 0.36 vs 2.11 ± 0.50 g/l (mean ± S.D.)), indicating that the increase in IGFBP levels in the amniotic fluid of uterine artery ligated animals was not due to oligohydramnios.

IGF and IGFBP mRNA expression in normal fetal tissues and placenta

The relative expression of IGF and IGFBP mRNAs is shown in Fig. 3. IGF-I and -II mRNA was identified by Northern blot in all tissues examined. There were three IGF-I transcripts (about 4.1, 2.7 and 2.3 kb). IGF-II was present as two major (about 5.5 and 3.1 kb) and six minor transcripts (not shown) with relatively greater expression in liver than in placenta or muscle. IGFBP-1 mRNA (about 2.6 kb) was expressed at very low levels in fetal

Figure 2 (A and B) Ligand blots of guinea pig fetal plasma (A) and amniotic fluid (B) at 55–57 days of gestation probed with ^{125}I -labeled IGF-II. The first six lanes in each blot are from control fetuses and the second six lanes are from fetuses affected by uterine artery ligation. Numbers show molecular size markers. Amniotic fluid contained a protein not seen in plasma that was immunoreactive to IGFBP-2 (denoted IGFBP-2 L). (C) Densitometric analysis for fetal plasma expressed as percentage change relative to control plasma. Levels of IGFBP-2 and -4 increased after uterine artery ligation, whereas IGFBP-3 decreased. (D) Densitometric analysis of amniotic fluid expressed as percentage change relative to control amniotic fluid. IGFBPs were present at low concentration in control conditions, but IGFBP-2 L and -3 rose after uterine artery ligation, with a similar trend for IGFBP-2. * $P < 0.05$ for difference between means of paired values.

liver (not shown) and could not be detected in placenta or muscle. IGFBP-2 mRNA was present as one major transcript (about 3.3 kb), the relative level of expression being liver>placenta>muscle. The major IGFBP-3 mRNA transcript (about 3.7 kb) had a similar pattern of relative expression. Expression of the major IGFBP-4 mRNA transcript (about 4.1 kb) was similar in the three tissues. IGFBP-5 mRNA was found in placenta and muscle as three transcripts (about 7.0, 3.2 and 2.1 kb), with the 7.0 kb transcript being most prominent. In liver, the most prominent transcript was 2.1 kb; the 7.0 kb transcript could not be detected. Two transcripts of

IGFBP-6 mRNA were identified (about 3.4 and 2.9 kb), the relative level of expression of the smaller transcript being muscle>placenta>liver. The larger transcript was seen mainly in liver.

In sections of fetal liver, expression of IGFBP-2 mRNA was limited to the hepatocytes (Fig. 4); mRNA was not expressed in hematopoietic tissues. The signal for IGFBP-3 mRNA was restricted to a subset of cells, probably Kupffer cells (data not shown), as described previously in rodents (Zimmermann *et al.* 2000). Expression of IGFBP-2 and -3 mRNA in placenta was as previously described (Han *et al.* 1999).

Figure 3 Northern blot analysis of total RNA from guinea pig placenta, fetal liver and fetal muscle at 55–57 days of gestation. Similar amounts of total RNA (20 µg) were loaded in each lane, as confirmed by the signal for 18S rRNA. Relatively higher levels of IGF-II, IGFBP-2 and IGFBP-3 were seen in fetal liver. The principal IGFBP-5 transcript in fetal liver differed from that in placenta and fetal muscle, with the 7.0 kb transcript being predominant in placenta and muscle while the 2.1 kb transcript was predominant in fetal liver. IGFBP-6 was more strongly expressed in fetal muscle than in either placenta or fetal liver.

IGF and IGFBP mRNA expression in fetal tissues and placenta from the ligated horn

In fetal liver, expression of IGFBP-2 mRNA increased 1.7-fold after uterine artery ligation ($P < 0.01$; Fig. 5A and B). No significant change occurred in hepatic expression of IGF-I and -II mRNA or IGFBP-3 and -5 mRNA. There was no significant change in IGF or IGFBP mRNA expression in fetal skeletal muscle.

In placenta, a small increase in expression of IGFBP-4 mRNA (14%; $P < 0.01$) was observed after uterine artery ligation (Fig. 5C and D). There was a trend toward a 62% decrease in IGFBP-5 mRNA expression that reached borderline significance ($P = 0.06$; Fig. 5C and E). No change occurred in placental expression of IGF-I and II mRNA or IGFBP-1, -2 or -5 mRNA.

Figure 4 Localization of IGFBP-2 mRNA in fetal liver by *in situ* hybridization. (A and B) In both control fetuses and fetuses affected by uterine artery ligation, the IGFBP-2 mRNA is restricted to the hepatocytes (inset); message is not expressed by hematopoietic tissue. (C) No specific signal was seen when liver sections were incubated with a sense IGFBP-2 cRNA probe. Magnification $\times 20$. Inset in (B) is magnified $\times 40$.

Figure 5 Northern blot analysis of total RNA from guinea pig fetal liver (A) and placenta (C) at 55–57 days of gestation. The first six lanes in each blot are from control fetuses and the second six lanes from fetuses affected by uterine artery ligation. In addition, RNA from chorionic villi (CV) and basal plate decidua (BP) of human placenta were run as routine controls. Histograms show the ratio of the specific mRNA to 18S rRNA (mean $\pm$ S.D.) for the two groups of fetuses. In fetal liver (B), there was a significant increase in IGFBP-2 mRNA ($P<0.01$). In placenta (D), there was a small but significant increase in IGFBP-4 mRNA after uterine artery ligation ($P<0.01$), and a trend toward decreased IGFBP-5 expression ($P=0.06$).

Discussion

As previously shown (Lafeber *et al.* 1984, Carter & Detmer 1990, Detmer *et al.* 1991), uterine artery ligation in guinea pigs led to a decrease in placental size and asymmetric restriction of fetal growth. This is a useful model to study

IUGR caused by a reduction in oxygen and substrate supply to the fetus (Carter 1993). Previous studies have demonstrated that IGF-I and -II are required to achieve normal fetal and placental growth. In mice carrying a null mutation for either *Igf1* or *Igf2*, fetal size is reduced by 40% (DeChiara *et al.* 1990, Liu *et al.* 1993). Our results are

consistent with a role for the IGF system in slowing fetal growth after uterine artery ligation. They suggest that tissue-specific alterations in the expression of IGFBPs may be important.

Proteins in plasma and amniotic fluid

The most abundant IGFBP in adult guinea pig plasma is IGFBP-3 (Sohlström *et al.* 1998b), and this was present in fetal plasma as a 40–35 kDa doublet corresponding to the glycosylated and nonglycosylated forms. However, the principal IGFBP in fetal guinea pig plasma was identified by molecular mass and immunoblotting as IGFBP-2 (Fig. 1). IGFBP-4 was present as a 25 kDa band. We did not detect the additional band that has been described for adult guinea pigs and is thought to include IGFBP-1 protein (Sohlström *et al.* 1998b).

After uterine artery ligation, we observed a strong increase in the concentration of IGFBP-2, the principal BP in fetal plasma. This mimics the rise in IGFBP-1 and IGFBP-2 levels seen in human IUGR (Chard 1994, Langford *et al.* 1994) or after mechanical restriction of uterine blood flow in sheep (McLellan *et al.* 1992). In the rat, uterine artery ligation is accompanied by an increase in IGFBP-1, while IGFBP-2 remains constant (Price *et al.* 1992a, Unterman *et al.* 1993). There was a decrease in the concentration of IGFBP-3 in fetal plasma after uterine artery ligation. Similarly, there is a positive correlation of birth weight and placental weight to IGFBP-3 levels in cord blood (Ong *et al.* 2000). It should be noted that IGFBP-3 is absent in rat plasma (Lee *et al.* 1999). Elevated levels of IGFBP-2 and reduced IGFBP-3 levels have also been seen in maternal blood after food restriction in guinea pigs (Sohlström *et al.* 1998b, Roberts *et al.* 2001, 2002). IGFBP-4 levels in fetal plasma were elevated after uterine artery ligation, as is the case after food restriction in adult, nonpregnant guinea pigs (Sohlström *et al.* 1998a).

The amniotic fluid of control fetuses contained only small amounts of IGFBPs, but higher concentrations were seen after uterine artery ligation. There is, again, a similarity to human IUGR, where elevated IGFBP-1 levels are seen in amniotic fluid (Hakala-Ala-Pietila *et al.* 1993). The increase in IGFBP-2 concentration was greater in amniotic fluid than plasma and included a band of larger molecular size that did not appear in plasma. Moreover, IGFBP-3 decreased in plasma but increased in amniotic fluid. Total protein concentration was similar in amniotic fluid from control fetuses and those from the horn with uterine artery ligation. Thus, we do not think the increased concentrations can be explained by similar amounts of IGFBP being distributed in a smaller volume of amniotic fluid due to oligohydramnios. It is possible that renal handling of IGFBPs is altered in growth-restricted fetuses.

IGF and IGFBP mRNA expression in fetal tissues and placenta

IGF-II and IGFBP-2, -3, -5 and -6 mRNA expression in fetal tissues and placenta was examined by cDNA probes, which were generated by RT-PCR of guinea pig total RNA, using oligonucleotide primers based on the known human sequences. Human rather than rodent sequences were used because previous hybridization studies suggested that the guinea pig mRNAs may be more similar to those of humans than rodents (Han *et al.* 1999). There was a satisfactory degree of homology between the nucleotide sequences of the probes and published cDNAs for human as well as mouse. Human probes were used for IGF-I, IGFBP-1 and IGFBP-4; we have previously demonstrated their utility in *in situ* hybridization experiments on guinea pig tissues (Han *et al.* 1999).

Altered levels of IGFs and IGFBPs in maternal and fetal plasma are probably of limited importance for placental growth and function, which is more likely to be regulated by the IGF system through paracrine and autocrine mechanisms (Han & Carter 2000). There was a small increase in placental IGFBP-4 mRNA expression after uterine artery ligation. In addition, we saw a strong tendency for decreased expression of IGFBP-5 mRNA (Fig. 5C and D). We have shown by *in situ* hybridization that IGFBP-5 mRNA is expressed in the nonvascularized (marginal and interlobular) syncytium of the placenta and IGF-II mRNA in the labyrinth (Han *et al.* 1999). Earlier, we suggested that interaction between IGF-II and IGFBP-5 mRNA is involved in the vascularization of the placenta by fetal vessels (Han *et al.* 1999). Thus, the decreased expression of IGFBP-5 mRNA indicated by the present study, probably in the marginal and interlobular syncytium, could contribute to the restriction of placental growth.

Growth restriction in the fetus was asymmetric, with a proportionately greater reduction in liver weight and sparing of the brain (Table 3). We observed a strong increase in IGFBP-2 mRNA in fetal liver. This observation is consistent with a role for IGFBP-2 in regulating fetal growth after uterine artery ligation in guinea pigs. Muscle mass also is disproportionately reduced after uterine artery ligation. IGFBP-6 was highly expressed in fetal muscle and has the potential to affect muscle growth by inhibiting proliferation and differentiation (Bach *et al.* 1994, Ewton & Florini 1995). However, we saw no change in IGF or IGFBP mRNA expression in skeletal muscle.

Origin of circulating IGFBPs

A consistent finding in human fetal growth restriction is a rise in the IGFBP-1 levels of fetal blood (Chard 1994). Later it was shown that the increase in IGFBP-1 is restricted to the phosphorylated isoform, which strongly inhibits IGF action, whereas levels of the nonphosphorylated isoform of IGFBP-1 are similar in small and average

for gestational age infants (Iwashita *et al.* 1998). Circulating IGFBP-1 levels also rise in response to fetal hypoxia, and the *Igfbp-1* gene has a consensus sequence for the hypoxia-response element that binds hypoxia-inducible factor (Tazuke *et al.* 1998). The question arises of whether the IGFBP in fetal blood is placental or fetal in origin. This has not been resolved by experiments with overexpression of hIGFBP-1 in transgenic mice. In one hIGFBP-1 transgenic line, expression of the transgene in the mother led to growth restriction that was independent of the fetal genotype, suggesting it was due to placental insufficiency (Crossey *et al.* 2002). On the other hand, when the hIGFBP-1 transgene was controlled by the α -fetoprotein promoter, restricting expression to fetal liver, gut and brain, fetal weight was reduced by 20% from E14.5 (Watson *et al.* 2002, Carter *et al.* 2004).

In the guinea pig fetus, IGFBP-1 mRNA was expressed at very low levels in fetal liver and did not change after uterine artery ligation. IGFBP-1 protein could not be detected in fetal plasma or amniotic fluid by ligand blot. Some anomalies in the endocrine system of guinea pigs are attributed to divergences in the amino-acid sequences of regulatory hormones, including pancreatic insulin (Keightley & Fuller 1996). A reduced role for IGFBP-1 in guinea pigs would fit this pattern, since IGFBP-1 normally is regulated by insulin (O'Brien *et al.* 2001). In guinea pigs, IGFBP-2 seems to play a more prominent role than IGFBP-1, and this BP was elevated in fetal plasma after uterine artery ligation. Increased plasma levels of IGFBP-2 have been reported in growth-restricted human fetuses (Langford *et al.* 1994). In growth-restricted rat fetuses, there is increased abundance of both IGFBP-1 and IGFBP-2 mRNA in liver, but only IGFBP-1 rises in fetal plasma (Price *et al.* 1992a,b). The reason for the increased prominence of IGFBP-2 in guinea pig fetuses subjected to stress such as hypoxia and undernutrition is unknown, but may be due to differences in the regulatory regions of the IGFBP-1 and IGFBP-2 genes. Thus, further elucidation of the guinea pig genes for IGFBP-1 and IGFBP-2 is needed to explain the phylogenetic differences among species. IGFBP-2 in guinea pig fetal plasma is probably of hepatic origin, since the relative level of expression of IGFBP-2 mRNA is greater in liver than in other tissues examined (Fig. 3) and increases further after uterine artery ligation (Fig. 5A and B).

The evidence from our studies suggests that the increase in circulating IGFBPs in growth-restricted fetuses is hepatic, and not placental, in origin (Han & Carter 2000). For example, the plasma level of IGFBP-1 rises and hepatic IGFBP-1 mRNA expression increases in the sheep fetus after maternal fasting (Osborn *et al.* 1992) or mechanical restriction of uterine blood flow (McLellan *et al.* 1992), yet the sheep has a nondeciduate placenta. Elevated IGFBP-1 in maternal blood is more likely to be of decidual origin (Martina *et al.* 1997). In addition, the proportional increase in IGFBP-2 in the circulation of

growth-restricted fetuses without changes in IGFs indicates that there is a causal relationship between the increase in IGFBP-2 and fetal growth. That IGFBP-2 is a predominant IGFBP, in contrast to IGFBP-1 in other species, may be of phylogenetic significance.

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