Developmental changes in the human GH receptor and its signal transduction pathways

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Abstract

We previously reported the presence of functional human GH receptors (hGHRs) in the human fetal hepatocyte (FH) as early as the first trimester. Interestingly, fetal serum levels of hGH are in the acromegalic range, yet certain hGH-dependent factors are expressed at very low levels (IGF-I, IGF-binding protein-3), suggesting that fetal liver has limited responsiveness to hGH. To determine whether this is due to the fetal tissue levels of hGHR or factors in the hGH/hGHR axis that might influence hGHR function, we compared hGHR isoforms and downstream signaling proteins in FH versus human adult liver (HAL). Immunoprecipitation/immunoblotting (IB) analyses found similar precursor and mature hGHR forms while RT-PCR assays of truncated (T) hGHR_{1–279}, dominant negative for the full-length (FL) receptor, showed similar T/FL mRNA ratios in FH and HAL. IB demonstrated that Janus kinase (JAK) 2, signal transducers and activators of transcription (STAT(1, 3, 5A/B)),

and suppressors of cytokine signaling (SOCS(1, 2, 3, cytokine-inducible SH2-containing protein (CIS))) proteins were detectable in all FH and HAL tested (12 weeks of fetal age to 60 years); the levels were similar (STAT5B) or lower (JAK2/STAT1/STAT3/STAT5A: 38–53%, SOCS/CIS: 58–76%) in FH compared with HAL. Our studies to date demonstrate that, during hepatocyte development, hGHR levels are lower in the fetal cells but the hGHR isoforms, including the relative amount of truncated versus FL, remain unchanged. The JAK2/STAT/SOCS signaling molecules are present in the FH as early as the first trimester. However, they are generally at <50% level in postnatal liver. These data suggest that low expression of both hGHR and major hGHR signaling components may explain the limited responsiveness of the fetal cells to the high circulating levels of hGH.

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Introduction

The human growth hormone receptor (hGHR), a member of class I cytokine receptor superfamily, is a 620 amino acid peptide containing an extracellular hormone-binding region, a single transmembrane segment, and a cytoplasmic signaling domain. It is encoded by a single gene spanning more than 300 kb on chromosome 5p13.1–12 (Leung *et al.* 1987, Barton *et al.* 1989, Wei *et al.* 2006). Three hGHR isoforms have been reported to date: full-length (FL), exon 3 deleted (d3), and truncated (T). Transcripts for all three isoforms have been detected in human tissues from early in fetal life (Esposito *et al.* 1994, Zogopoulos *et al.* 1996b, Goodyer *et al.* 2001a).

d3 hGHR mRNAs are expressed in an individual-specific manner, likely due to a retrovirus-mediated recombination mechanism at the genomic level; they translate into a hGHR that binds specific hGH-related hormones with similar affinities as the FL receptor (Bass *et al.* 1991, Urbanek *et al.* 1992, Sobrier *et al.* 1993, Wickelgren *et al.* 1995, Zogopoulos *et al.* 1996b, Clackson *et al.* 1998, Pantel *et al.* 2000). Whether the d3 hGHR has a different functional ability relative to the

FL form remains controversial (Dos Santos *et al.* 2004, Audi *et al.* 2006, Binder *et al.* 2006, Blum *et al.* 2006, Carrascosa *et al.* 2006, Hujeirat *et al.* 2006, Jorge *et al.* 2006, Pilotta *et al.* 2006, Buzi *et al.* 2007, Kenth *et al.* 2007, Lettre *et al.* 2007, Mericq *et al.* 2007, Raz *et al.* 2008).

Two truncated hGHRs, 1–277 and 1–279, are produced at low levels in all human tissues examined, including fetal liver and placenta, with variable truncated to FL receptor (T/FL) ratios among the tissues (Dastot *et al.* 1996, Ballesteros *et al.* 2000, Fisker *et al.* 2001, 2004, Goodyer *et al.* 2001a). These truncated receptors are capable of binding hGH with high affinity, but they do not activate intracellular signal transduction pathways and, in fact, act as dominant-negative receptors (Ross *et al.* 1997, Ayling *et al.* 1999, Iida *et al.* 1999).

GH binds with high affinity to two molecules of hGHR, forming a stable heterotrimer complex (De Vos *et al.* 1992, Frank 2002, Gent *et al.* 2003, Waters *et al.* 2006, Brooks *et al.* 2008). Because the GHR is characterized by the absence of intrinsic catalytic activity within its intracellular domain, signal transduction through this receptor is mediated primarily by Janus kinase (JAK) 2, a cytoplasmic tyrosine kinase that

associates with Box 1 of the GHR cytoplasmic region (Argetsinger *et al.* 1993, Carter-Su *et al.* 1996). Rapid and transient activation of JAK2 results in phosphorylation of JAK2, GHR, and a number of intracellular signaling and adaptor proteins (Fig. 1; Argetsinger *et al.* 1993, Carter-Su *et al.* 1996, Herrington & Carter-Su 2001, Pivien-Pilipuk *et al.* 2002, Lanning & Carter-Su 2006, Brooks *et al.* 2008). The major GHR signaling factors in hepatocytes are the signal transducers and activators of transcription (STATs): STAT1, STAT3, STAT5A, and STAT5B associate with specific tyrosine phosphorylated sites on the activated GHR through their SH2 domains (Levy & Darnell 2002, Lanning & Carter-Su 2006, Rosenfeld *et al.* 2007). Once phosphorylated by JAK2, the STAT proteins form homo- or heterodimers that migrate to the nucleus and bind to specific sites on the promoters of GH-responsive genes (Herrington *et al.* 2000, Pivien-Pilipuk *et al.* 2002, Lanning & Carter-Su 2006, Brooks *et al.* 2008).

The family of cytokine-inducible proteins, termed suppressors of cytokine signaling (SOCS), is important in the negative regulation of GHR signaling in the hepatocyte (Krebs & Hilton 2001, Flores-Morales *et al.* 2006). SOCS1, SOCS2, SOCS3, and cytokine-inducible SH2-containing protein (CIS) expression are induced by GH in the rat liver with different kinetics (Adams *et al.* 1998, Ram & Waxman 1999, Tollet-Egnell *et al.* 1999). SOCS1 appears to inhibit JAK2 kinase activity directly, by binding to its kinase activation loop (Naka *et al.* 1997). SOCS2, SOCS3, and CIS, on the other hand, indirectly inhibit GHR signaling, by binding to the GHR and interfering with JAK2 activation and STAT5 binding (Hansen *et al.* 1999, Ram & Waxman 1999, Uyttendaele *et al.* 2007). Mice deficient in SOCS2 display gigantism (Metcalf *et al.* 2000), while those transgenic for CIS are growth retarded (Matsumoto *et al.* 1999), suggesting that SOCS2 and CIS may play especially important roles in the termination of GHR signaling. In addition, the SOCS proteins have been shown to be a new class of ubiquitin ligases that may regulate proteosomal degradation of the GHR and JAK2 (Flores-Morales *et al.* 2006, Lanning & Carter-Su 2006).

Pivotal roles for hGH and its receptor have been well established in the postnatal period (Veldhuis *et al.* 2005).

Figure 1 Schematic representation of the hGH/hGHR signaling molecules examined in the present study.

However, their functions during human gestation remain unclear. Only a subset of newborns with hGH deficiency or hGHR dysfunction have decreased birth length, suggesting that, while fetal hGH can affect fetal growth, the presence of other fetal growth factors (e.g., insulin, placental lactogen, prolactin) ensures relatively normal intrauterine growth (Gluckman *et al.* 1992, Wit & van Unen 1992, Woods *et al.* 1997, Jensen *et al.* 2005, Mehta *et al.* 2005, Osafo *et al.* 2005).

Interestingly, fetal plasma levels of immunoreactive hGH are significantly elevated relative to the normal adult, up to 150 ng/ml at mid-gestation (Kaplan *et al.* 1976). This is likely due to the greater sensitivity of the human fetal pituitary somatotrope to its hypothalamic stimulatory factor, GH-releasing hormone (GHRH), than its inhibitory factor, somatostatin, throughout *in utero* development (Delitala *et al.* 1978, Goodyer *et al.* 1993a,b). Similar hGH levels postnatally cause highly elevated serum levels of insulin-like growth factor-I (IGF-I) and IGF-binding protein-3 (IGFBP-3), resulting in gigantism in the prepubertal child and acromegaly in the adult (Kato *et al.* 2002). However, circulating levels of IGF-I and IGFBP-3 in the fetus are low, reaching significant levels only late in the third trimester (Butler & Le Roith 2001, Gohlke *et al.* 2004), suggesting a blunted responsiveness of fetal tissues to hGH during earlier stages in development, at either the hGH receptor or the signaling level.

hGHR mRNA and protein have been identified in a wide variety of human tissues from the first trimester of fetal life (Hill *et al.* 1992, Simard *et al.* 1996, Zogopoulos *et al.* 1996a,b). There is a decrease in lung, kidney, and intestinal levels of hGHR postnatally and a significant increase in liver when early to mid-gestation fetal versus adult tissues are compared (Goodyer *et al.* 2001a). We and others have previously demonstrated that the hGHR present in fetal hepatocytes (FH) specifically binds hGH, and that these cells have biological responses (proliferation, IGF production, glucose uptake) following exposure to hGH, although the effects are lower compared with adult hepatocytes (Strain *et al.* 1987, Hill *et al.* 1988, 1989, Goodyer *et al.* 2001a).

In the present study, we have tested the hypothesis that, in addition to lower tissue levels of hGHR, other factors, including the hGHR protein forms (FL versus truncated) and the JAK/STAT and SOCS/CIS families of downstream signal transduction molecules, may be responsible for the limited responsiveness of FHs to the high circulating levels of hGH.

Materials and Methods

Materials

A rabbit antibody raised against the intracellular domain of the hGHR (AL47) was a generous gift from Dr Stuart Frank (University of Alabama, Birmingham, AL, USA) (Zhang *et al.* 2001). Antibodies directed against hGH signaling proteins were obtained from the following

commercial sources: JAK2 from Imgenex (IMG-3007; San Diego, CA, USA); SOCS1 (ab3691-100) and SOCS2 (ab3692-100) from Abcam (Cambridge, MA, USA); SOCS3 (sc-9023) and CIS (sc-1529) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); and STAT1 (UBI 06-501), STAT3 (UBI 06-596), STAT5A (UBI 06-553), and STAT5B (UBI 06-554) from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Rabbit and mouse IgGs were obtained from Santa Cruz. A mouse monoclonal antibody for calnexin (C45520), the immunoblot loading control, was purchased from Transduction Laboratories (Lexington, KY, USA). The HRP-labeled secondary antibodies used were anti-rabbit (NEF812) and anti-mouse (NEF822) from Perkin-Elmer Life Sciences Inc. (Boston, MA, USA) and anti-goat (sc-2020) from Santa Cruz Biotechnology.

Tissue collections and cell lines

Human fetal tissues were obtained at the time of therapeutic abortion (10.75–19 weeks of fetal age (FA)); FA was determined by foot length (Munsick 1984). Postnatal liver specimens, perfused to remove all blood cells, were collected within 4–10 h after removal of organs for transplantation (15–60 years, donors were all males). Ethics approval for the study was obtained from local institutional research ethics boards, in accordance with the Canadian Council on Health Sciences Research Involving Human Subjects guidelines. Written informed consent was obtained in each case. Four primate cell lines were analyzed: CV1 (African green monkey kidney fibroblasts, ATCC), human embryonic kidney epithelial cells (HEK293 epithelial cells, ATCC), and two human postnatal hepatoma cell lines, HepG2 (ATCC) and Huh7 (kindly provided by Dr Ken K Ho, Garvan Institute of Medical Research, Sydney, New South Wales, Australia). CV1, HEK293, and HepG2 cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin G, and 1.6 mg/ml gentamicin sulfate. Huh7 cells were cultured in Earle's MEM (Invitrogen) supplemented with 10% FBS, 7.5 mM HEPES, 100 IU/ml penicillin G, and 1.6 mg/ml gentamicin sulfate. All cells were maintained at 37 °C with 5% CO₂ in a humid environment.

FH preparations

Fetal hepatic tissues were minced and dispersed by collagenase, followed by gravity separation, yielding >95% pure hepatocytes, as described previously (Zogopoulos *et al.* 1996a). The isolated hepatocytes were plated onto collagen-coated Petri dishes in William's E medium (Invitrogen) with 10% FBS, 100 IU/ml penicillin G, 1.6 mg/ml gentamicin sulfate, and 39.2 µg/ml dexamethasone. Two hours after plating, the hepatocytes were rinsed thoroughly (three to five times) with 1× PBS to remove all remaining traces of hematopoietic cells, replaced with fresh, complete William's

E medium with 39.2 µg/ml dexamethasone, and incubated overnight. After 24 h, hepatocytes were washed with 1× PBS and collected by scraping. The hematopoietic cells were pelleted, flash frozen, and stored at –80 °C until extracted for RNA.

Protein and RNA extractions

Homogenized tissue fragments or cell pellets were solubilized in ice-cold lysis buffer (50 mM Tris (pH 7.5), 0.1% Triton X-100, 150 mM NaCl, and 2 mM EDTA) containing protease and phosphatase inhibitors (complete cocktail tablets (Roche Diagnostics)) for 15 min on ice. To remove insoluble material, lysates were centrifuged at 13 000 *g* for 10 min at 4 °C. The supernatants were collected and protein concentrations measured with the Bradford kit (Bio-Rad Laboratories, Inc.), using BSA as a standard. Alternatively, tissue fragments and the cell pellets were extracted for RNA using the TRIzol method (Invitrogen).

RT-PCR assay for FL versus truncated hGHR

For each RT reaction, hepatic total RNA (5 µg) was reverse transcribed for 1 h at 42 °C in the presence of 200 U Superscript II (Invitrogen) and a specific exon 9 reverse primer (9AS) (5'-TAATCTTTGGAAGTGGAACT-3') designed to recognize both the FL and truncated (1–279) hGHR mRNAs (Goodyer *et al.* 2001a). RT products (3 µl) were amplified for 35 cycles with 1.25 U *Taq* DNA polymerase (Invitrogen), hGHR exon 7 sense (7S: 5'-ATAAGGAATATGAAGTGC GTGTGAG-3'), and hGHR exon 9 antisense primers. The cycles consisted of one cycle at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min, ending with a final elongation at 72 °C for 10 min. In each assay, an aliquot of H₂O was amplified in parallel as a negative PCR control. PCR products were separated on 10% polyacrylamide gels and stained with SybrGold (Molecular Probes, Eugene, OR, USA). Densitometric analyses were carried out using the Bio-Rad GelDoc system.

Immunoprecipitation and immunoblotting

Liver lysate protein (0.5–1 mg) were incubated by rotation in lysis buffer described above with anti-hGHR (αAL47, 1:250) overnight at 4 °C, followed by the addition of 50 µl protein G-agarose beads (Amersham Biosciences, Baie, D'urffé, QC, Canada) for 1 h at 4 °C. Antibody complexes were washed three times with lysis buffer followed by a single wash with 50 mM Tris (pH 8.0). hGHR immunoprecipitates or 50–100 µg whole cell extracts were boiled for 5 min in Laemmli loading buffer (50 mM Tris (pH 6.8), 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 100 mM DTT) and proteins were resolved by SDS-PAGE (8–15%) and transferred (Mini Trans-blot Cell; Bio-Rad Laboratories, Inc.) to polyvinylidene fluoride Immobilon-P transfer membranes (Millipore Corporation, Mississauga, ON, Canada).

The membranes were blocked overnight at 4 °C with 5% non-fat dry milk in PBST buffer, and then incubated with the appropriate primary antibody (hGHR AL47 (1:2000), JAK2 (1:700), STAT1 (1:1000), STAT3 (1:1000), STAT5A (1:1000), STAT5B (1:1000), SOCS1 (1:1000), SOCS2 (1:1000), SOCS3 (1:200), CIS (1:100), or calnexin (1:1000)) for 3 h at room temperature. After washing three times with PBST, the membranes were incubated with an anti-mouse (1:2000), anti-rabbit (1:1000), or anti-goat (1:1000) IgG conjugated to horseradish peroxidase for 1 h at room temperature. Protein bands were visualized by an enhanced chemiluminescence detection system (Perkin–Elmer Life Sciences Inc).

To probe the same membrane for another protein, the membranes were incubated for 30 min at 50 °C with stripping buffer (100 mM β -mercaptoethanol, 2% SDS, 62.5 mM Tris (pH 6.8)). Calnexin was used to normalize for protein loading; pilot western blots showed a linear increase in calnexin protein levels over 20–80 μ g of both fetal and postnatal hepatic protein (Supplementary Figure 1, see Supplementary data in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol198/issue1/>). Densitometric analyses were carried out using the Bio-Rad GelDoc system.

Statistical analyses

Data are presented as Mean $\pm$ S.E.M. Differences between two groups were analyzed using Student's *t*-test (two tailed). Differences among several groups were analyzed using ANOVA followed by Bonferroni's multiple group comparison test.

Results

Analysis of hGHR molecular forms in human FHs versus postnatal liver

We previously analyzed hGHR mRNAs from fetal and postnatal hepatocytes and found transcripts for FL, truncated (1–279), and exon 3 forms at both developmental stages (Zogopoulos *et al.* 1996b, Goodyer *et al.* 2001a). In the present study, FH (*n*=2, 13.8, and 15 weeks FA) and postnatal liver (*n*=2, 15, and 47 years) samples were analyzed for hGHR protein by immunoprecipitation (IP) followed by immunoblotting (IB), using the same antibody (AL47) (Fig. 2). Two major forms of the hGHR, precursor (~70 kDa) and mature (~120 kDa), were detected in all cases. Since the antibody used for the IP/IB analyses was directed against the intracellular domain of the hGHR, we were only able to identify the FL, as opposed to the truncated, forms (Zhang *et al.* 2001). These data demonstrate that the FL hGHR forms are similar at the two developmental stages.

Figure 2 Immunoblot (IB) analyses of immunoprecipitated (IP) hGHR in human adult liver (HAL) and fetal hepatocytes (FH) using the AL47 antibody raised against the intracellular domain of the GHR (Zhang *et al.* 2001). Molecular markers are indicated at the left side with arrowheads and arrows indicate the two major (~70 and ~120 kDa) IP/IB hGHR bands. IgG, rabbit IgG.

Truncated (1–279) hGHR mRNA in human FHs versus postnatal liver

To analyze whether increases in the major 1–279 dominant-negative truncated (T) form of the hGHR (Ross 1999) might play a role in the lower fetal biological responses to hGH, we determined the relative ratios of mRNAs for the FL and T forms. We used a specific RT-PCR assay that recognizes the FL and T hGHR mRNAs as distinct PCR products in each sample (Fig. 3A, C and E) (Goodyer *et al.* 2001a). Pilot studies with PCR cycle numbers ranging from 29 to 39 showed a parallel amplification of the two bands in both fetal and postnatal hepatic samples (Fig. 3B).

In a previous study, we determined T/FL hGHR mRNA ratios in intact fetal liver (0.18 ± 0.03 (Mean $\pm$ S.E.M.), *n*=8, 14–18 weeks) versus intact postnatal liver (0.09 ± 0.02 , *n*=3, 21–54 years) (Goodyer *et al.* 2001a). While the FL transcript always predominated, there was a trend for fetal liver to have a twofold higher ratio. However, fetal liver is the primary hematopoietic organ during this stage of development and blood cells have a number of cytokine receptors, including hGHR (Welniak *et al.* 2000). Therefore, for the present study, we dissociated the intact fetal liver into its two major cell components: hepatocytes and hematopoietic cells. This isolation permitted a more accurate comparison of fetal with postnatal liver, since the postnatal liver tissues used for these studies had been perfused for transplantation and, therefore, did not contain blood cells.

When intact postnatal liver, intact fetal liver, isolated FHs, and isolated fetal hematopoietic cells were compared, the highest T/FL hGHR mRNA ratios were detected in fetal liver (0.20 ± 0.02 , *n*=12, 12.7–19.5 weeks) and fetal hematopoietic cells (0.24 ± 0.04 , *n*=5, 12.7–19.5 weeks). FHs (0.07 ± 0.01 , *n*=7, 11.5–17.5 weeks) showed low ratios, similar to postnatal liver (0.09 ± 0.01 , *n*=3, 37–60 years) (Fig. 3C and D). Our findings of the same T/FL ratios in fetal and postnatal hepatocytes suggests that hGHR functional differences are not likely due to the dominant-negative form of the hGHR.

Figure 3 Analysis of full-length (FL) and truncated (T) (1–279) *hGHR* mRNA. (A) A specific primer in exon 9 (9AS) was designed to recognize both the FL and T forms of *hGHR* mRNA for RT–PCR assays. (B) Pilot RT–PCR assays showed that there were no significant differences in T versus FL *GHR* amplicon amplification over a wide range of PCR cycle number, in both fetal and postnatal hepatic samples ($n=3$ each). (C and D) Comparison of FL and T *hGHR* mRNAs in intact fetal liver ($n=12$, 12.7–19.5 weeks), postnatal liver ($n=3$, 37–60 years), fetal hepatocytes ($n=7$, 11.5–17.5 weeks), and fetal liver hematopoietic cells ($n=5$, 12.7–19.5 weeks) by RT–PCR. (C) Representative polyacrylamide gel of PCR products detects FL *hGHR* mRNA at 252 bp and T *hGHR* mRNA at 226 bp. (D) Comparative data for intact postnatal liver, intact fetal liver, fetal hepatocytes, and fetal hematopoietic cells, expressed as a ratio of T/FL transcripts (Mean $\pm$ S.E.M.). Statistical differences are calculated relative to fetal hepatocytes: *** $P<0.001$. (E and F) Comparison of FL and T *hGHR* mRNAs in various fetal tissues. (E) Representative polyacrylamide gel of PCR products. pBS-SK⁺/Hpa II marker is indicated at 242 bp. CNS, central nervous system. (F) T *hGHR* mRNA is detected in a wide variety of fetal tissues. Each tissue type was tested using three different samples and the final data are expressed as a ratio of T/FL transcripts (Mean $\pm$ S.E.M.). Statistical differences are calculated relative to intact fetal liver: * $P<0.05$, ** $P<0.01$. (G and H) Comparison of FL and T *hGHR* mRNAs in various primate cell lines. (G) Representative polyacrylamide gel of PCR products. pBS-SK⁺/Hpa II marker is indicated at 242 bp. (H) Graphic analysis of T/FL *hGHR* mRNA in three cell lines and human adult liver (HAL) (HepG2 band densities were too low to be accurately analyzed). Each sample was tested using three to five different RNA pools (Mean $\pm$ S.E.M.). Statistical differences are calculated relative to HAL: ** $P<0.01$, *** $P<0.001$.

Truncated (1–279) *hGHR* mRNA in fetal tissues

To place these T/FL ratios in a biological context, we also assessed the relative abundance of T and FL *hGHR* mRNAs in other fetal tissues. Like the fetal liver, all nine other tissues predominantly expressed the FL *hGHR* transcript (Fig. 3E and F). The dominant-negative T *hGHR* mRNA was also detected in all tissues, but T/FL *hGHR* mRNA ratios varied considerably, from a mean of 0.002 in skin to means of 0.53 and 0.65 in kidney cortex and medulla respectively (Fig. 3E and F).

Characterization of T/FL *hGHR* mRNA isoforms in human and primate cell lines

Four different human and primate cell lines (CV1, HEK293, HepG2, and Huh7) that are routinely used as cell models to examine *hGHR* binding and signaling pathways were also analyzed for the different *hGHR* isoforms. The Huh7 hepatoma and CV1 cell lines demonstrated relatively high T/FL ratios (Huh7: 1.48 ± 0.26 , CV1: 1.27 ± 0.19), while the HEK293 cell ratios were quite low (0.19 ± 0.05) (Fig. 3G and H).

Unfortunately, the HepG2 band densities were too low to be analyzed accurately.

Expression of JAK/STAT proteins in human fetal hepatocytes and intact liver versus postnatal liver

JAK2 and STAT1, STAT3, STAT5A, and STAT5B are major members of the hGH/hGHR intracellular signaling cascade (Lanning & Carter-Su 2006). To determine whether they are expressed in lower amounts in FHs, thereby decreasing responsiveness to hGH, we analyzed their levels in human intact fetal livers ($n=3$, 10·75–18 weeks), FHs ($n=3$, 12–18 weeks), and postnatal livers ($n=3$, 15–60 years). These five proteins were found in all of the samples tested demonstrating that they are expressed as early as the third month of fetal life (Fig. 4A and B). All but STAT5B were lower in FHs compared with postnatal liver; this difference was significant for STAT1 ($\downarrow 42\%$, $P<0\cdot05$) (Fig. 4A). Although a similar trend was seen for JAK2 ($\downarrow 38\%$), STAT3 ($\downarrow 38\%$), and STAT5A ($\downarrow 53\%$), they did not reach statistical significance, likely due to the small sample size. Unfortunately, hepatocyte preparations require substantial amounts of fetal liver tissue and these are in limited supply.

Interestingly, we observed quite different levels of the JAK/STATs when we analyzed intact human fetal versus

postnatal liver samples (Fig. 4B). While STAT1 and STAT3 were still lower than human adult liver (HAL) ($\downarrow 29\%$), JAK2, STAT5A, and STAT5B were markedly increased ($\uparrow 100\%$, $P<0\cdot06$; $\uparrow 680\%$, $P<0\cdot01$; and $\uparrow 130\%$, $P<0\cdot03$ respectively). These results, along with the T/FL hGHR data, indicate that the presence of hematopoietic cells in the intact fetal liver can markedly influence tissue biological expression patterns. In addition, the data suggest that the JAK2/STAT5A and STAT5B pathways may be very important for fetal hematopoietic cell function. Because of the limited number of hepatocyte preparations, we were, however, unable to carry out parallel studies in isolated hematopoietic cells.

Expression of SOCS proteins in human fetal hepatocytes and intact liver versus postnatal liver

SOCS proteins inhibit hGHR signaling in hGH target cells, including the hepatocytes (Flores-Morales *et al.* 2006). To test whether increased levels might be responsible for a lower response to hGH in fetal tissues, we compared the levels of SOCS/CIS proteins in human fetal livers ($n=3$, 10·75–18 weeks), FHs ($n=3$, 12–19 weeks), and postnatal livers ($n=3$, 15–60 years). The four SOCS proteins were detected in all of these samples (Fig. 5A and B). There were markedly lower levels of the inhibitory proteins in the FHs relative to the postnatal

Figure 4 Analysis of JAK/STAT proteins. Representative immunoblots of JAK/STAT and calnexin expression in (A) fetal hepatocytes and (B) fetal liver. Relative abundance of JAK2 (~120 kDa), STAT1 (~91 kDa), STAT3 (~92 kDa), STAT5A (~95 kDa), and STAT5B (~92 kDa) proteins in (A) fetal hepatocytes ($n=3$, 12–18 weeks) or (B) fetal liver ($n=3$, 10·75–18 weeks) (Mean $\pm$ s.e.m.). Data are normalized to calnexin (~90 kDa) and compared with postnatal liver (HAL: $n=3$, 15–60 years). Statistical differences are expressed relative to HAL: * $P<0\cdot05$, ** $P<0\cdot03$, *** $P<0\cdot01$.

Figure 5 Analysis of SOCS proteins. Representative immunoblots of SOCS/CIS and calnexin expression in (A) fetal hepatocytes and (B) fetal liver. Relative abundance of SOCS1 (~24 kDa), SOCS2 (~23 kDa), SOCS3 (~30 kDa), and CIS (~32 kDa) proteins in (A) fetal hepatocytes ($n=3$, 12–19 weeks) or (B) fetal liver ($n=3$, 10·75–18 weeks) (Mean $\pm$ S.E.M). Data are normalized to calnexin (~90 kDa) and compared with postnatal liver (HAL: $n=3$, 15–60 years). Statistical differences are expressed relative to HAL: ** $P<0\cdot04$.

livers: SOCS1 ($\downarrow 61\%$), SOCS2 ($\downarrow 76\%$), SOCS3 ($\downarrow 61\%$), and CIS ($\downarrow 58\%$), although these changes did not achieve statistical significance, again likely due to limited sample numbers (Fig. 5A). Thus, human FHs do contain the four SOCS family members from as early as 12 weeks of FA, but they are present at less than half the levels observed in postnatal livers.

When we analyzed the SOCS levels in intact human fetal livers, we observed that CIS was significantly decreased compared with the postnatal livers (CIS: $\downarrow 67\%$, $P<0\cdot04$), while there were consistent decreases in the other family members: SOCS1 ($\downarrow 31\%$), SOCS2 ($\downarrow 41\%$), and SOCS3 ($\downarrow 35\%$) (Fig. 5B). In contrast to the JAK/STAT results, these data are similar to the findings obtained for the FHs, suggesting that the SOCS/CIS family members are present at similar levels in both the fetal hematopoietic and hepatic cells.

Discussion

Although pivotal roles for GH, its receptor and their downstream signal transduction pathways have been well established in postnatal tissues, their functions during gestation remain unclear (Hill 1992, Symonds *et al.* 2001, Waters & Kaye 2002). Surprisingly, fetal serum levels of GH are tenfold higher than those in the postnatal period in both primates and subprimates (Gluckman *et al.* 1981). We have previously demonstrated that this may be because human fetal somatotropes have a much higher sensitivity to GHRH than

somatostatin (Goodyer *et al.* 1993a,b). In corroboration, clinical studies have shown that newborns have an exaggerated response to GHRH and a delayed and blunted response to somatostatin (Delitala *et al.* 1978, Shimano *et al.* 1985). Postnatally, these elevated hGH levels would cause high serum levels of IGF-I and IGFBP-3, resulting in gigantism in a prepubertal child and acromegaly in an adult (Kato *et al.* 2002). However, in the fetus, serum IGF-I and IGFBP-3 levels are very low or undetectable until late in the third trimester (Butler & Le Roith 2001, Gohlke *et al.* 2004). These data suggest that fetal tissues have a relatively low responsiveness to hGH.

In subprimates, the onset of significant *GHR* mRNA expression occurs late in gestation (Adams *et al.* 1990, Walker *et al.* 1992, Ymer & Herington 1992). In the human fetus, ubiquitous transcription of the *hGHR* gene has been documented from as early as the first trimester; postnatal changes in *hGHR* mRNA expression are tissue specific, with decreases in the lung, kidney, and intestine but a sixfold increase in liver after birth (Zogopoulos *et al.* 1996a,b, Goodyer *et al.* 2001b). Binding (Goodyer *et al.* 2001a), immunohistochemical (Hill 1992, Hill *et al.* 1992, Simard *et al.* 1996), and IB experiments (present study) have identified functional hGHR protein in multiple human tissues from as early as 9–11 weeks of fetal life. In the present study, we asked whether there are factors other than the levels of hGHR, which may be responsible for the decreased responsiveness to hGH in fetal tissues. We focused on

several hGHR isoforms and their downstream signaling pathway molecules, comparing their levels in FHs versus postnatal liver.

Based on its amino acid sequence, the nascent hGHR is predicted to be ~70 kDa in size while, when resolved on SDS-PAGE gels under reducing conditions, the mature hGHR migrates at 100–140 kDa (Hocquette *et al.* 1989, 1990, Alele *et al.* 1998, Zhang *et al.* 2001, Cowan *et al.* 2005). These differences are due primarily to the presence of five asparagine-linked glycosylation sites within the extracellular domain, a variable number of which are glycosylated in the mature hGHR (Harding *et al.* 1994). In addition, hGHRs can be modified by ubiquitination, a step that can target the receptor for internalization and degradation (Govers *et al.* 1997, Strous *et al.* 1997, van Kerkhof *et al.* 2002). cDNA sequencing (Zogopoulos *et al.* 1996a) and now IP and IB experiments indicate no major size differences between the fetal and postnatal hepatic hGHR: the mRNA sequences are identical and both the immature and mature forms of the fetal hGHR in hepatocytes have very similar SDS-PAGE migration patterns as the postnatal hGHR.

Ross *et al.* (1997) previously demonstrated that cells expressing a T/FL GHR cDNA ratio of 1:10 exhibited a 10–30% decrease in FL GHR activity, while a 1:1 T/FL ratio resulted in a 40–80% inhibition and a 10:1 ratio completely blocked activity of the FL GHR. When we examined the T/FL *hGHR* mRNA ratios in FHs and postnatal liver, we found that they were similar and quite low (~1:12), making it unlikely that the truncated form plays a role in the functional hGHR differences observed at these two developmental stages. However, we observed a relatively high T/FL *hGHR* mRNA ratio in hepatic hematopoietic cells (1:4), fetal kidney tissues (~1:2), and two primate cell lines (~1.4:1), suggesting that the dominant-negative isoforms of hGHR may play a functionally significant role in these cells and tissues, decreasing the ability of hGH to activate its intracellular signaling and, thus, its biological effectiveness. This is especially interesting in the case of the kidney, given the links between hGH and kidney function (Rabkin & Schaefer 2004, Reddy *et al.* 2007). It would be interesting, for example, to compare T/FL hGHR mRNA ratios in kidney tissue biopsies under normal versus diabetic conditions, to determine whether the T/FL ratio shifts from a high to low level with progression of the diabetic state, leading to increased responsiveness to hGH and diabetic nephropathy.

The only previous study of JAKs and STATs in human liver was undertaken by Hellgren *et al.* (1999): they determined by western blots, immunohistochemistry, and IP that JAK1, JAK2, JAK3, and TYK2 are all present in adult hepatocytes, and that the hGHR specifically associates with JAK1, JAK2, and TYK2. Our analysis of the hGHR downstream signaling factors demonstrates that JAK2 and STAT1, STAT3, STAT5A, and STAT5B are present in FHs from the 12th week of fetal life. With the exception of STAT5B, FHs contain ~40–50% lower levels of the JAK/STAT proteins compared with postnatal liver. Whether this actually creates a

functional difference in hGH signaling is impossible to know with our present data. To determine this, future experiments should examine the relative amounts of hGHR/JAK/STAT molecules that are phosphorylated or the relative responses of target genes following hGH treatment of fetal versus postnatal hepatocytes.

There have been two fetal versus postnatal liver analyses of JAK/STATs in the rat (Shoba *et al.* 1999, Phornphutkul *et al.* 2000). Contrary to our data (Goodyer *et al.* 2001a), Phornphutkul *et al.* (2000) found no difference in GH binding levels between late gestation (d17, d19) fetal and postnatal rat livers. *In vitro* or *in vivo* GH treatments caused a similar phosphorylation of GHR, JAK2, STAT1, and/or STAT5 in the fetal and postnatal hepatocytes. Finally, GH was able to enhance CAT expression of two GHRE reporter vectors transfected into FHs, leading Phornphutkul *et al.* (2000) to conclude that hepatic GH signaling mechanisms are functional in the late gestation rat. Similar to our data (Goodyer *et al.* 2001a), Shoba *et al.* (1999) found a marked increase in GHR mRNA and protein in fetal (d17) compared with postnatal (4 months) rat livers. Over the same time frame, surprisingly, JAK2, STAT1, and STAT3 protein levels dropped while STAT5 levels remained unchanged. These last findings are quite different from our present hepatocyte and intact human liver data and may represent major species differences.

One interesting note is that we observed significantly elevated levels of STAT5A and STAT5B expression in the human intact fetal liver when compared with the FHs, suggesting that this is due to the hematopoietic cell population. Several published reports support this conclusion. STAT5A-/STAT5B-deficient mice are severely anemic due to significant decreases in their hepatic erythroid precursor cell population (Socolovsky *et al.* 1999), demonstrating the importance of these two STATs for murine fetal liver hematopoiesis. The recent findings, by Rosenfeld *et al.*, of immune deficiencies in two patients with STAT5B mutations indicate an important role for STAT5B in the human immune system as well (Rosenfeld *et al.* 2005, Hwa *et al.* 2005).

The SOCS proteins are well recognized to act as inhibitors of GHR signaling (Flores-Morales *et al.* 2006). Although we had hypothesized that there might be increased levels in the FHs, our IB studies of FH lysates showed ~60–75% lower levels of all of the SOCS family members compared with postnatal liver. In the only other human study, Dey *et al.* (1998) examined a CLONTECH panel of fetal and adult human tissues: they also showed a lower level of SOCS2 mRNA levels in the fetal when compared with the postnatal liver. In the only animal study to date, Gentili *et al.* (2006) have reported that there is a significant increase in SOCS3 mRNA expression in the ovine fetal liver during late gestation (d125–d145), and that this expression is regulated by circulating levels of PRL but STAT5 does not appear to be involved.

One obvious explanation for the lower expression of the SOCS proteins in the human fetal liver is the lower amounts of hGHR, JAK2, and most of the STATs in the fetal cells. It

is well known that transcription of the SOCS genes is upregulated by hGH and other cytokines through the STATs: SOCS1 has STAT1, STAT3, and STAT6 binding sites while SOCS2, SOCS3, and CIS promoters have multiple STAT5 consensus response elements (Matsumoto *et al.* 1997, Adams *et al.* 1998, Davey *et al.* 1999, He *et al.* 2003, Hebenstreit *et al.* 2003, Gatto *et al.* 2004). However, an interesting caveat is that SOCS2 has dose-dependent effects on GHR signaling: at low concentrations it inhibits GH-induced STAT5-dependent gene transcription while restoration of GH signaling is observed at higher concentrations, demonstrating that SOCS2 functions by regulating cell sensitivity to GH (Favre *et al.* 1999, Greenhalgh *et al.* 2002). This interesting finding suggests that the relatively low levels of SOCS2 in the human FHs may, in fact, be acting to inhibit hGHR signaling and, thus, may contribute to the lower response to hGH.

Once induced, the SOCS3/CIS families are major inhibitors of GHR signaling (Adams *et al.* 1998, Hansen *et al.* 1999, Ram & Waxman 1999, Ronn *et al.* 2002, Flores-Morales *et al.* 2006), not only by blocking STAT phosphorylation but also by targeting the GHR for ubiquitination and subsequent degradation by the proteasome (Kamura *et al.* 1998, Zhang *et al.* 1999, Ram & Waxman 2000, Kile & Alexander 2001, Wormald & Hilton 2004, Flores-Morales *et al.* 2006). Whether the SOCS-ubiquitin-proteasome pathway is active in the FH remains unknown.

Several other aspects have yet to be investigated in any species. These include whether in the fetal liver: 1) the GH/GHR complex is working through alternative intracellular signaling pathways to regulate fetal-specific biological outcomes (e.g., the MAPK and PI3-kinase cascades, Ca^{2+} channels), 2) there are desensitization processes (e.g., more rapid dephosphorylation of the activated receptor, endocytosis, proteolysis) that influence GH responsiveness in general, and 3) gender has an effect on the levels of the signaling proteins, given that the fetal testes is producing significant amounts of testosterone at 10–20 weeks of fetal life (Tapanainen *et al.* 1981, Frank 2001, Herrington & Carter-Su 2001, Zhu *et al.* 2001, Choi *et al.* 2006).

In conclusion, our studies to date demonstrate that there are lower levels of hGHR in fetal versus postnatal hepatocytes but that the hGHR isoforms are unchanged, including the relative amounts of the truncated dominant-negative (hGHR_{1–279}) form. The JAK2/STAT/SOCS signaling molecules are present in the human hepatocyte as early as the first trimester. However, they are generally at <50% the level in postnatal perfused liver, suggesting that decreased hGHR signaling ability may help to explain the relatively low cell responsiveness to the very high fetal serum hGH levels.

Declaration of Interest

The authors have no conflict of interest that would prejudice the research described in this article. C G G is a member of

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