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Growth Inhibition in Giant Growth Hormone Transgenic Mice by Overexpression of Insulin-Like Growth Factor-Binding Protein-2

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ABSTRACT

To clarify the role of insulin-like growth factor (IGF)-binding protein-2 (IGFBP-2) in postnatal growth regulation, we crossed hemizygous CMV-IGFBP-2 transgenic mice with hemizygous PEPCK-bGH transgenic mice, which are characterized by serum GH levels in the range of 2 μg/ml. Four genetic groups were obtained: animals carrying both transgenes (GB), the GH (G) or the IGFBP-2 transgene (B), and nontransgenic controls (C). Male offspring were analyzed for serum levels of IGF-I, for serum and tissue levels of IGFBP-2, and for body and organ growth. Serum IGF-I levels were 2- to 3-fold increased ($P < 0.001$) in the GH-overexpressing groups, with no difference between G and GB mice. Serum IGFBP-2 levels were 4- to 9-fold ($P < 0.001$) increased both in B and GB *vs.* C and G mice. Western immunoblot analysis did not reveal differences in tissue IGFBP-2 levels between B and GB mice. IGFBP-2 levels were highest in pancreas, followed by skeletal muscle, heart, kidney, brain, skin, and spleen. No elevation of IGFBP-2 was found in the liver. Body weight gain of G and GB mice was significantly increased *vs.* C and B mice, resulting in almost 2-fold increased body weights at the age of 15 weeks. However, there was a significant reduction in body weight of GB *vs.* G mice (17%; $P < 0.001$) and of B *vs.* C mice (13%; $P < 0.05$). This was primarily caused by a marked reduction of carcass weight (GB *vs.* G, 27%; B *vs.* C, 21%; $P < 0.001$). Measurements of nose-rump-length, organ (brain, heart, spleen, liver, pancreas, kidney), and tissue weights (skin, carcass, abdominal fat) in 5- and 15-week-old mice revealed several indications that the growth-inhibiting effect of IGFBP-2 overexpression was more marked in high-GH/IGF-I mice: 1) At 5 weeks of age, GB mice displayed a significant reduction of all growth parameters except for the weight of abdominal fat, when compared with G mice, whereas only brain weight was significantly reduced in B *vs.* C mice. 2) In 15-week-old animals, a significant reduction in all growth parameters, except for spleen and abdominal fat weights, was seen in GB *vs.* G mice, whereas only nose-rump-length and the weights of carcass and brain were significantly reduced in B *vs.* C mice. Our study demonstrates, for the first time, the potential of IGFBP-2 to inhibit GH-stimulated growth in giant transgenic mice, providing further evidence for an inhibitory effect of this IGFBP *in vivo*. (*Endocrinology* **142:** 1889–1898, 2001)

INSULIN-LIKE GROWTH FACTOR (IGF)-BINDING protein (IGFBP)-2 is particularly prominent in late fetal and neonatal serum, but it is also found at significant levels in adult serum. Serum and tissue levels of IGFBP-2 are subject to complex physiological regulation (for review, see Ref. 1). We have recently shown that IGFBP-2 messenger RNA (mRNA) levels are increased by exogenous IGFs already during preimplantation embryonic development (2). Elevated IGFBP-2 levels were found in the milk of transgenic rabbits overexpressing IGF-I in the mammary gland (3) and in the serum of transgenic mice overexpressing IGF-II under the control of the PEPCK promoter (4), demonstrating up-regulation of IGFBP-2 expression by IGFs. Increased hepatic IGFBP-2 mRNA and serum IGFBP-2 levels were observed in mice that are small because of long-term selection for low body weight (5). In addition, IGFBP-2 has been shown to be up-regulated in a variety of pathological conditions, such as interstitial lung disease, liver cirrhosis, or chronic renal failure (for review, see Ref. 6). However, until recently, it was unclear whether elevation of IGFBP-2 has intrinsic effects or merely represents an epiphenomenon without direct consequences for growth and metabolism.

Effects of IGFBP-2 overexpression have been studied in a variety of *in vitro* models, with a wide spectrum of consequences, from growth inhibition (7–10) to growth stimulation (Refs. 11 and 12; for review, see Ref. 6). Overexpression of IGFBP-2 in transfected 293 human embryonic kidney cells reduced cell proliferation. The same effect was seen for IGF-responsive colon carcinoma cell lines cultured in conditioned media containing high levels of IGFBP-2. In both systems, the proliferation-inhibiting effect of IGFBP-2 could be overcome by addition of exogenous IGFs, Long R^3 IGF-I being markedly more effective than IGF-I (8). On the other hand, long-term overexpression of IGFBP-2 in transfected mouse adrenocortical tumor cells (Y-1) was associated with increased cell proliferation and tumorigenic potential. These effects were independent of exogenous IGFs (12).

To understand the function of IGFBP-2 *in vivo*, the IGFBP-2 gene has been inactivated in mice by gene targeting. However, only minor phenotypic changes, *i.e.* increased liver weights and

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reduced spleen weights, in adult males were observed. In this model, the serum activities of IGFBP-1, IGFBP-3, and IGFBP-4 were increased, eventually compensating for the lack of IGFBP-2 (13, 14).

To investigate the consequences of elevated IGFBP-2 *in vivo*, we generated transgenic mice overexpressing IGFBP-2 under the control of the CMV-promoter. These mice displayed reduced postweaning body weight gain, which was mostly caused by a reduction in carcass weight, identifying IGFBP-2, for the first time, as a growth inhibitor *in vivo* (15). Because the phenotype of IGFBP-2 transgenic mice was, in many aspects, opposite to that of IGF-I-overexpressing transgenic mice, we concluded that IGFBP-2 might inhibit IGF-I effects.

To further clarify this point, we studied consequences of IGFBP-2 overexpression under conditions of increased levels of GH and IGF-I. Transgenic mice overexpressing homologous or heterologous GH provide unique models for studying long-term effects of elevated GH and IGF-I. These models have been extensively studied, with respect to body (16, 17), skeletal (18–20), and organ growth (16, 21–24). In addition to markedly stimulated growth, long-term GH overproduction in transgenic mice causes a spectrum of pathological lesions, which develop primarily in kidney and liver (for review, see Refs. 25–27) and result in a dose-dependent shortened life-span (28).

To study effects of elevated IGFBP-2, in the context of GH excess, we crossed hemizygous CMV-IGFBP-2 transgenic mice with hemizygous PEPCK-bGH transgenic mice, generating four groups of offspring harboring either the CMV-IGFBP-2 (B) or the PEPCK-bGH transgene (G), both transgenes (GB), and nontransgenic controls (C). Analysis of a large panel of growth parameters revealed that elevated IGFBP-2 can indeed partially block GH-stimulated growth processes. Interestingly, the growth-inhibiting activity of IGFBP-2 was even more pronounced in high-GH/IGF-I mice than in mice with normal GH and IGF-I levels.

Materials and Methods

Generation of transgenic mice and animal husbandry

IGFBP-2 transgenic mice were generated by microinjection of an expression vector containing the murine IGFBP-2 complementary DNA (cDNA) (kindly donated by Dr. S. Drop, Rotterdam, The Netherlands) and the CMV-promoter for transcriptional control of the transgene as described before (28). IGFBP-2 transgenic mice were identified by real-time PCR using the SYBR Green technology (PE Applied Biosystems, Weiterstadt, Germany). PCR conditions were according to the manufacturer's instructions. Primers used were as follows: mIGFBP-2 sense: 5′ GCG CGG GTA CCT GTG AAA 3′; mIGFBP-2 antisense: 5′ TCC CTC AGA GTG GTC GTC ATC 3′. Genomic DNA used for the PCR analysis was isolated from tail tips of 3-week-old offspring using the Puregene genomic DNA purification system (Biozym, Hess. Oldendorf, Germany).

PEPCK-bGH transgenic mice, originally generated on a C57BL/6 × SJL genetic background (29), were kindly provided by Dr. T. E. Wagner, Edison Biotechnology Center, Ohio University, Athens, OH. Hemizygous PEPCK-bGH transgenic mice used in this study were derived from the 12th generation of sequential crossing with NMRI outbred mice (Charles River Laboratories, Inc.-Wiga, Sulzfeld, Germany). PEPCK-bGH transgenic mice were identified by PCR using 5′ CGG ACC GTG TCT ATG AGA AGC 3′ sense and 5′ GGA AAG GAC AGT GGG AGT GG 3′ antisense oligonucleotides (30).

Female hemizygous CMV-IGFBP-2 transgenic mice were mated with male hemizygous PEPCK-bGH transgenic mice to generate four different genetic groups of offspring, of which only male mice were investigated in the present study: animals harboring both transgenes (GB), the GH (G) or the IGFBP-2 (B) transgene, and nontransgenic controls (C).

All mice were maintained under standard (nonbarrier) conditions and had free access to a standard diet (V1534; Ssniff, Soest, Germany) and tap water.

Measurement of serum bGH, IGFBPs, and IGF-I

Blood samples were obtained from 15-week-old mice by puncture of the retroorbital sinus in ether anesthesia. Serum was separated by centrifugation, and IGFBP-2 and IGF-I concentrations were quantified by specific RIAs as described previously (31–33). For all assays, dilution curves of mouse serum samples were linear and paralleled those of human standards. Serum GH was quantified by an enzyme-linked immunosorbent assay specific for bGH (34). Serum samples were analyzed for IGF-binding activity by Western ligand blot (WLB) analysis as previously described (15). Data were analyzed by ANOVA as described below.

IGFBP-2 levels in different tissues

IGFBP-2 levels in different tissues were determined by Western immunoblot analysis as described previously (15). Briefly, tissue samples were homogenized in extraction buffer [10 mM Na_2HPO_4, pH 7.0; 0.2% (wt/vol) SDS; 10% (vol/vol) glycerin] using a cell homogenizer (ART, Mühlheim, Germany). Thirty micrograms of protein were boiled (5 min) and separated under reducing conditions on a 5% stacking/12% separating SDS-polyacrylamide gel using the Mini Protean II system (Bio-Rad Laboratories, Inc., Munich, Germany). Separated proteins were transferred to a nitrocellulose membrane (Millipore Corp., Eschborn, Germany). The blots were blocked with 1% fish gelatin. IGFBP-2 was identified using a specific antiserum raised in rabbit (15). Bound antibodies were detected with peroxidase-coupled antibodies against rabbit IgG (Dianova Germany, Hamburg, Germany) using an ECL detection kit (Amersham Pharmacia Biotech, Freiburg, Germany). Three mice per genetic group were analyzed at 5 and 15 weeks of age.

Analysis of body and organ growth

Body weight of all mice was recorded, in weekly intervals, to the nearest 0.1 g. To estimate average growth of the individual groups, data were transformed to a weighing age of n × 7 days, by linear interpolation, as described previously (17). At the age of 15 weeks, mice were ether-anesthetized and killed by bleeding from the retroorbital sinus. In addition, mice from each genetic group were analyzed at the age of 5 weeks. Nose-rump-length (NRL) was measured as the distance between nose and base of the tail, as described before (17). The weight of mesentery and fat tissue surrounding the genital organs and kidneys, which is correlated with total body fat content, was determined as the amount of abdominal fat tissue. For the analysis, organs were removed, blotted dry, and weighed to the nearest milligram. Carcasses were weighed, after removal of the organs, without skin, head, and tail. Data for body growth and organ/tissue weights were analyzed by ANOVA, taking the effect of group into account. Means were compared by using LSD *post hoc* tests [SPSS, Inc. (Chicago, IL) program package]. To compare the effects of IGFBP-2 transgene expression, under conditions of normal *vs.* increased GH/IGF-I levels, relative differences between B and C mice, as well as GB and G mice, were calculated for all parameters. Therefore, values recorded for B and GB mice were divided by the corresponding means calculated for C and G mice, respectively. The relative effects (percent difference in body weight, organ/tissue weights, or NRL) of IGFBP-2 overexpression in a high (GB *vs.* G) and a normal (B *vs.* C) GH/IGF-I background were compared using Student's *t* test.

Histology

Livers from 6 G, 6 GB, 3 B, and 6 C mice were selected for histological analysis. Immediately after determination of liver weight, the organ was fixed by immersion in 10% neutral buffered formalin for 48 h at room temperature. Approximately 3-mm-thick slices of the left, the median, and the right lobes were routinely processed and were embedded in paraffin wax. Histological sections were cut at 4 μm and stained with hematoxylin and eosin according to standard methods. Histological analyses were carried out on coded slides to avoid knowledge of the nature of the experimental group.

Results

Circulating levels of GH, IGF-I, and IGFBPs

Mean serum concentrations of GH were in the range of 2 μg/ml in the groups carrying the PEPCK-bGH transgene and were not affected by IGFBP-2 overexpression in the double transgenic mice. The endogenous GH in C and B mice was not detected by the enzyme-linked immunosorbent assay used in our study (Fig. 1A).

Serum IGFBP-2 levels were 4- to 9-fold ($P < 0.001$) increased both in B and GB mice, compared with C and G mice (Fig. 1B). The activities of other serum IGFBPs were determined by WLB analysis, which showed an increased signal for proteins of 39/43 kDa (IGFBP-3) and of 24 kDa (nonglycosilated IGFBP-4) in the PEPCK-bGH transgenic groups. The increase in immunoreactive IGFBP-2 in the B and GB groups was confirmed by WLB, demonstrating functional activity of the transgene-encoded IGFBP-2 (Fig. 2).

Serum IGF-I levels were 2- to 3-fold increased ($P < 0.001$) in the GH-overexpressing groups, with no difference between G and GB mice (Fig. 1C).

IGFBP-2 levels in different tissues

Western immunoblot analysis did not reveal obvious differences in tissue IGFBP-2 levels between 15-week-old B and GB mice. IGFBP-2 levels were highest in pancreas, followed by skeletal muscle and heart, kidney, brain, skin, and spleen. No elevation of IGFBP-2 was found in the liver (Fig. 3). Analysis of tissue samples from 5-week-old mice led to similar results (data not shown).

Body weight gain and body dimensions

As expected, body weight gain was markedly stimulated by expression of the PEPCK-bGH transgene. This effect became significant at the age of 5 weeks and resulted in an almost 2-fold increased body weight at the age of 15 weeks (Fig. 4A). Body weight of B *vs.* C mice was reduced (13%, $P < 0.05$), confirming our first report on the consequences of IGFBP-2 overexpression in transgenic mice (15). However, the growth-inhibiting effect of IGFBP-2 was also evident on the background of GH overexpression and increased levels of IGF-I. Body weight of GB *vs.* G mice was reduced by 17% ($P < 0.001$).

In accordance with the findings for body weight, the nose-rump-length (NRL) of the G mice was significantly increased *vs.* C and B mice, at both the ages of 5 and 15 weeks (Fig. 4B). IGFBP-2 overexpression in GB mice abolished the effect of the PEPCK-bGH transgene on NRL in 5-week-old mice and reduced it significantly ($P < 0.001$) in 15-week-old animals. In addition, there was a significant ($P < 0.05$) reduction in NRL in 15-week-old B *vs.* C mice. The NRL-body weight$^{1/3}$-ratio was not different between the groups at 5 weeks of age. Although the relative NRL was significantly ($P < 0.05$) increased in 15-week-old G mice, IGFBP-2 overexpression in GB mice completely reduced normalized body size in the condition of GH excess (Fig. 4C), strongly supporting an enhanced IGFBP-2 effect in the context of high levels of GH and IGF-I.

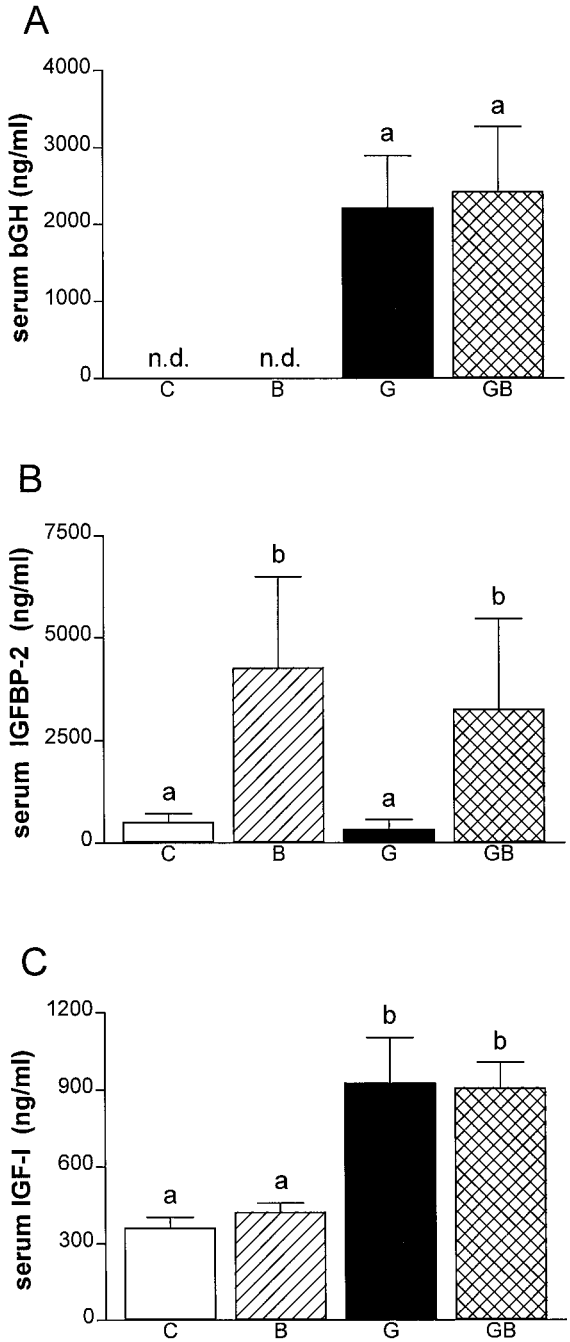

FIG. 1. Serum concentrations of bGH (panel A), IGFBP-2 (panel B), and IGF-I (panel C) in mice carrying the PEPCK-bGH transgene (G; n = 12), the CMV-IGFBP-2 transgene (B; n = 7), both transgenes (GB; n = 10), and nontransgenic controls (C; n = 14). The figure shows means and SDs. Means marked by *different superscripts* (a, b) are significantly (at least $P < 0.05$) different; n.d., not detectable.

Organ and tissue weights

These parameters were investigated in 5-week-old mice because this was the age when the first significant differences in body weight were observed. The absolute weights of spleen (127%), liver (70%), skin (41%), kidneys (35%), heart (33%), and pancreas (23%) were already significantly (at least

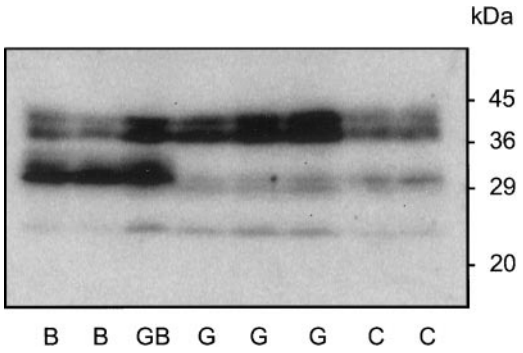

FIG. 2. WLB analysis of serum samples from mice carrying the PEPCK-bGH transgene (G), the CMV-IGFBP-2 transgene (B), both transgenes (GB), and nontransgenic controls (C).

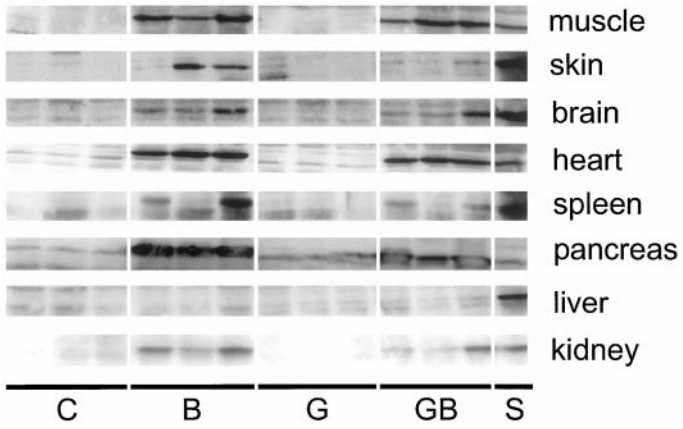

FIG. 3. Western immunoblot analysis of IGFBP-2 levels in different tissues from mice carrying the PEPCK-bGH transgene (G), the CMV-IGFBP-2 transgene (B), both transgenes (GB), and nontransgenic controls (C). For comparison of the blots, 30 μg protein extract of skeletal muscle from a B mouse was loaded on each gel (lane S).

$P < 0.05$) increased in G *vs.* C mice (Table 1). The carcass weight of G mice was, as a tendency, increased (13%, $P = 0.158$), compared with C mice.

Overexpression of IGFBP-2 in GB mice markedly reduced the effect of elevated GH on the weights of all organs and tissues, most of which were not different from those measured in controls (C). The reduction was most prominent for the weights of spleen, pancreas, and kidneys (Fig. 5A). Pancreas weights of GB mice were even lower than those of C mice. Liver weights of GB mice were also significantly reduced *vs.* G mice ($P < 0.001$) but were still higher ($P < 0.05$) than in C and B mice. Interestingly, a reduction ($P < 0.05$) in brain weight was seen in B and GB mice *vs.* C and G mice. These data show that elevated IGFBP-2 potently reduces effects of GH overexpression at the age of 5 weeks.

Relative to body weight, the weights of skin, abdominal fat, and heart were not different between the genetic groups (Table 2). Whereas the relative weights of carcass and brain were significantly ($P < 0.05$) reduced in G and GB *vs.* C and B mice, GH overexpression resulted in an overproportionate increase of spleen and liver weights. These effects were similar to those observed in 15-week-old mice (see below). However, in contrast to the older age class, relative pancreas weights were not yet increased in 5-week-old GH-overexpressing mice but were reduced ($P < 0.05$) in GB mice *vs.* all other groups. The relative kidney weight was increased ($P < 0.01$) in B mice *vs.* all other groups.

At the age of 15 weeks, all organ and tissue weights were significantly increased in G *vs.* C and B mice ($P < 0.05$ for abdominal fat; $P < 0.001$ for all other parameters; Table 3). However, coexpression of the CMV-IGFBP-2 transgene in GB mice reduced the effect of GH excess on the weights of carcass (27%, $P < 0.001$), kidneys (21%, $P < 0.001$), pancreas (17%, $P < 0.05$), liver (15%, $P < 0.001$), brain (12%, $P < 0.001$), heart (12%, $P < 0.05$), spleen (11%, $P = 0.07$), and skin (9%, $P < 0.05$; Fig. 5B). In contrast, a significant reduction in B *vs.* C mice was seen only for the weights of carcass (21%, $P < 0.001$) and brain (15%, $P < 0.001$).

As a proportion of body weight, the weights of heart and kidneys were not different among the genetic groups (Table 4). However, the GH transgenic groups (G, GB) displayed an overproportionate increase in the weights of liver ($P < 0.001$), skin ($P < 0.001$), spleen ($P < 0.001$ *vs.* C; $P < 0.05$ *vs.* B), and pancreas ($P < 0.01$ *vs.* C; $P < 0.05$ *vs.* B). In contrast, the relative weights of brain ($P < 0.001$) and carcass ($P < 0.01$) were significantly reduced in G and GB *vs.* C and B mice. Effects of the CMV-IGFBP-2 transgene were also seen on relative organ weights. The relative weight of the carcass was decreased ($P < 0.001$), whereas the relative weight of the skin was increased ($P < 0.05$) in GB *vs.* G mice. Furthermore, B mice displayed reduced relative carcass weights ($P < 0.001$) but increased relative spleen and liver weights ($P < 0.05$), compared with C mice. As a proportion of body weight, the abdominal fat weight was, as a tendency, reduced in G *vs.* C mice and in GB *vs.* B mice. A significant ($P < 0.05$) difference was found between B and G mice.

To provide further evidence for an enhanced growth inhibitory effect of IGFBP-2 in giant GH transgenic mice, we compared the relative effects (percent difference in body weight, organ/tissue weights, or NRL) of IGFBP-2 overexpression in a high (GB *vs.* G) and a normal (B *vs.* C) GH/IGF-I background, using Student's *t* tests.

In 5-week-old mice, the reduction in spleen, pancreas, kidney, and skin weights, as well as NRL of GB *vs.* G mice, was significantly ($P < 0.05$) greater than the effect observed in B *vs.* C mice. A tendency ($P < 0.1$) of an enhanced inhibitory effect of IGFBP-2 overexpression in giant GH transgenic mice was seen for body weight and the weights of heart, abdominal fat, liver, and brain (Fig. 5A).

In 15-week-old mice, the relative reduction in liver weights of GB *vs.* G mice was significantly ($P < 0.05$) greater than in B *vs.* C mice, and a tendency ($P < 0.1$) of an augmented IGFBP-2 effect in high-GH/IGF-I mice was seen for kidney weight and NRL. Opposite effects of IGFBP-2 overexpression were seen for spleen weight, which was, as a tendency, reduced in GB *vs.* G mice but increased in B *vs.* C mice (Fig. 5B).

Histology of the liver

Because overexpression of IGFBP-2 markedly reduced growth effects of GH excess, we studied pathological alterations of the liver that are a well known consequence of GH excess in transgenic mice. Histologically, liver lesions were consistently

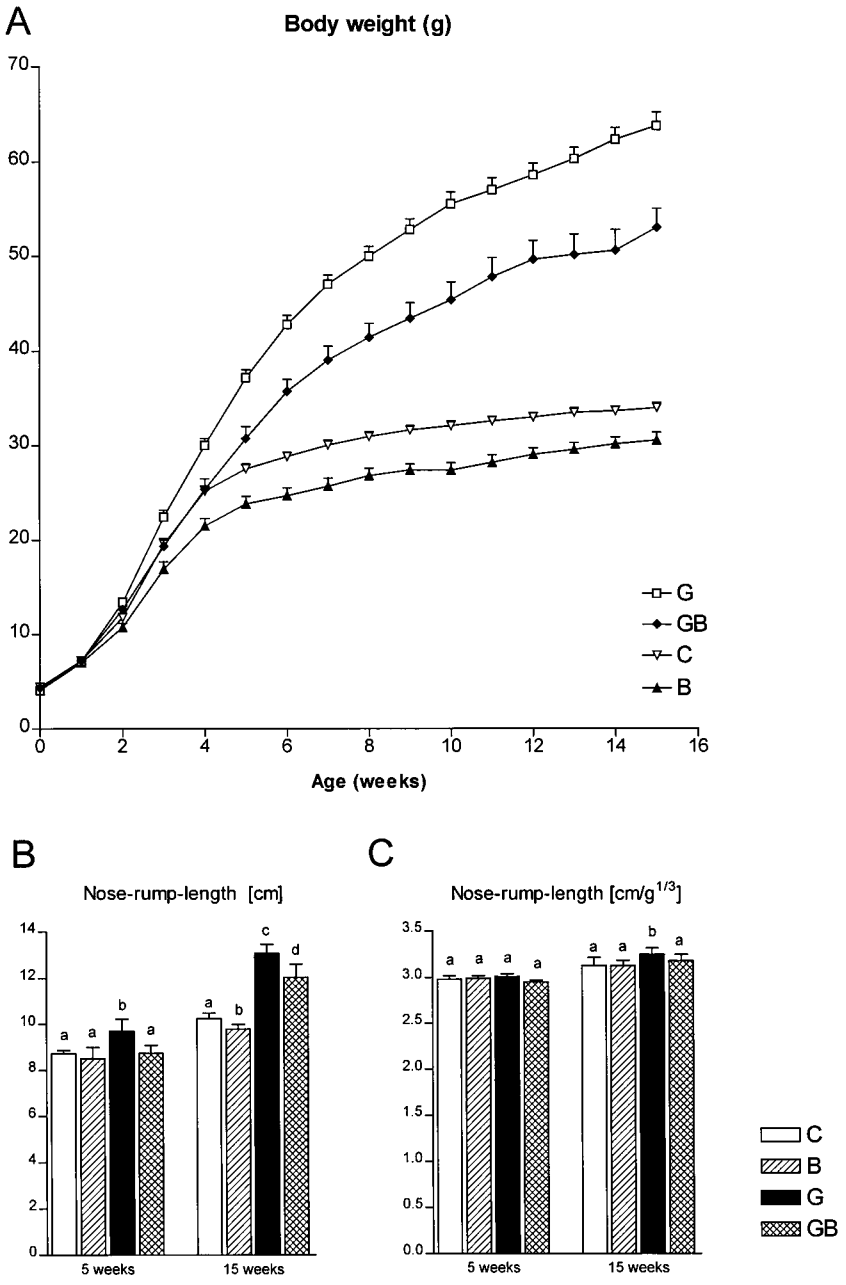

FIG. 4. Body weight gain (panel A), NRL (panel B), and relative NRL (rNRL; panel C) of mice carrying the PEPCK-bGH transgene (G; n = 13), the CMV-IGFBP-2 transgene (B; n = 7), both transgenes (GB; n = 10), and non-transgenic controls (C; n = 15). To calculate rNRL, NRL was divided by the cube root of body weight, to keep the same dimension. The figures show means and SDs. In panels B and C, means marked by *different superscripts* (a, b, c, d) are significantly (at least $P < 0.05$) different within age class.

found in both 15-week-old G and GB mice, and the pattern of histological liver alterations was similar in these groups (Fig. 6). The most striking feature was the presence of abnormally large hepatocytes with karyomegaly. The megalocytic change was restricted to hepatocytes and was commonly more pronounced in the centrilobular areas. Significant enlargement and pleomorphism of almost all the hepatocytes and their nuclei were observed in two out of six G mice and in one out of six GB mice. Hypertrophic liver cell nuclei frequently demonstrated pseudo-inclusions resulting from enfolding of the nuclear membrane by cytoplasmic material. Further changes consistently observed in livers from G and GB mice, respectively, included single liver cell necroses and mild degrees of oval cell proliferation. Livers from B and C mice (Fig. 6) were indistinguishable by light microscopy and demonstrated slight variation in the size of liver cell nuclei and low numbers of lymphoid cells in scattered portal tracts.

Discussion

According to previous findings *in vitro* (7–10) and *in vivo* (3, 5, 15), IGFBP-2 is likely to be an inhibitor of IGF actions. The present study was performed to clarify: 1) whether IGFBP-2 has an inhibitory effect on growth also in the context of GH excess and increased levels of IGF-I; and 2) whether this effect is general or tissue-/organ-specific.

Serum GH levels of both G and GB mice were in the range of 2 μg/ml, which is consistent with our previous reports on PEPCK-bGH transgenic mice (28, 35) and indicates that phe-

TABLE 1. Absolute organ and tissue weights of 5-week-old mice carrying the PEPCK-bGH transgene (G), the CMV-IGFBP-2 transgene (B), both transgenes (GB), and nontransgenic controls (C)

Parameter	C (n = 9) Mean (SD)	B (n = 7) Mean (SD)	G (n = 3) Mean (SD)	GB (n = 5) Mean (SD)
Carcass (g)	10.26^{ab}	8.76^{a} (1.84)	11.29^{b} (1.87)	8.53^{a} (0.88)
Skin (g)	4.38^{a} (0.28)	4.27^{a} (0.69)	6.16^{b} (1.37)	4.75^{a} (0.46)
Liver (g)	1.45^{a} (0.10)	1.45^{a} (0.32)	2.47^{b} (0.58)	1.99^{c} (0.12)
Abdominal fat (mg)	457^{a} (135)	521^{a} (148)	635^{a} (255)	510^{a} (97)
Brain (mg)	418^{a} (21)	380^{b} (23)	438^{a} (33)	370^{b} (29)
Pancreas (mg)	256^{a} (37)	223^{ab} (40)	323^{c} (80)	205^{b} (11)
Kidney[d] (mg)	172^{a} (15)	175^{a} (55)	227^{b} (57)	159^{a} (14)
Heart (mg)	118^{a} (8)	112^{a} (21)	163^{b} (33)	128^{a} (10)
Spleen (mg)	113^{ab} (11)	100^{a} (15)	249^{c} (111)	156^{b} (34)

Significant differences ($P < 0.05$) between the groups are indicated by *different superscripts* (a, b, c); [d] mean of the weights of left and right kidney per animal.

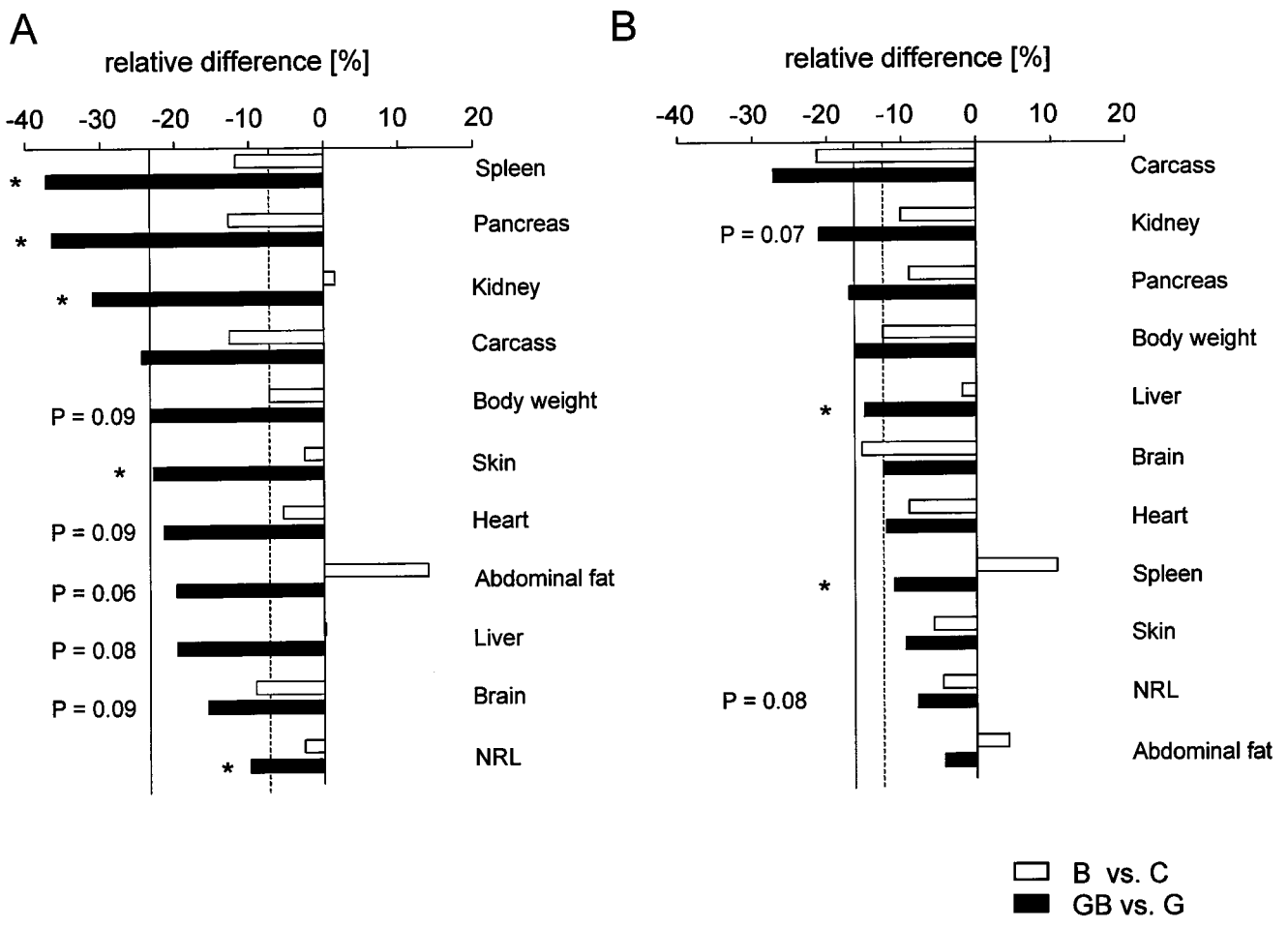

FIG. 5. Reduction (%) of body weight, body dimensions, and organ/tissue weights by IGFBP-2 overexpression in the context of normal GH levels (B *vs.* C; *white bars*) and in the context of GH excess (GB *vs.* G; *black bars*) in 5-week-old (panel A) and 15-week-old mice (panel B). The levels of body weight reduction are indicated by a *dotted line* (B *vs.* C) and a *solid line* (GB *vs.* G), respectively. The relative effects (percent difference in body weight, organ/tissue weights or NRL) of IGFBP-2 overexpression in a high (GB *vs.* G) and a normal (B *vs.* C) GH/IGF-I background were compared using Student's *t* tests. Significant differences ($P < 0.05$) are indicated by an *asterisk*.

notypic differences between G and GB mice are not attributable to altered PEPCK-bGH transgene expression by coexpression of the CMV-IGFBP-2 transgene in GB mice. Conversely, GH overexpression did not affect the expression of the CMV-IGFBP-2 transgene, as shown by similar tissue levels of IGFBP-2 in B and GB mice (Fig. 3). Serum IGFBP-2 levels of B and GB mice, investigated in the present experiment, were higher than those observed in our previous study (15). This is most likely attributable to the different genetic background produced by our crossing experiment.

GH overproduction caused a marked increase in all organ weights except for the brain. In 15-week-old mice, the increase was proportionate to body weight for kidney and heart but overproportionate for skin, pancreas, liver, and spleen. An overproportionate growth of liver and spleen, but not of skin and pancreas, was seen in 5-week-old G mice,

TABLE 2. Relative organ and tissue weights of 5-week-old mice carrying the PEPCK-bGH transgene (G), the CMV-IGFBP-2 transgene (B), both transgenes (GB), and nontransgenic controls (C)

Parameter	C (n = 9) Mean (SD)	B (n = 7) Mean (SD)	G (n = 3) Mean (SD)	GB (n = 5) Mean (SD)
Carcass (g)	39.85^{a} (1.50)	37.46^{b} (1.27)	33.40^{c} (0.58)	32.91^{c} (1.15)
Skin (%)	17.43^{a} (1.12)	18.40^{a} (1.04)	18.12^{a} (1.03)	18.35^{a} (0.71)
Liver (%)	5.76^{a} (0.31)	6.22^{a} (0.58)	7.26^{b} (0.49)	7.69^{b} (0.50)
Abdominal fat (%)	1.82^{a} (0.57)	2.22^{a} (0.36)	1.83^{a} (0.42)	1.96^{a} (0.27)
Brain (%)	1.66^{a} (0.08)	1.67^{a} (0.23)	1.31^{b} (0.11)	1.43^{b} (0.14)
Pancreas (%)	1.01^{a} (0.12)	0.96^{a} (0.06)	0.95^{a} (0.08)	0.80^{b} (0.07)
Kidney[d] (%)	0.68^{a} (0.05)	0.74^{b} (0.11)	0.66^{ac} (0.07)	0.62^{c} (0.04)
Heart (%)	0.47^{a} (0.03)	0.48^{a} (0.02)	0.48^{a} (0.02)	0.50^{a} (0.03)
Spleen (%)	0.45^{a} (0.03)	0.43^{a} (0.06)	0.72^{b} (0.19)	0.60^{b} (0.09)

Significant differences ($P < 0.05$) between the groups are indicated by *different superscripts* (a, b, c); [d] mean of the weights of left and right kidney per animal.

TABLE 3. Absolute organ and tissue weights of 15-week-old mice carrying the PEPCK-bGH transgene (G), the CMV-IGFBP-2 transgene (B), both transgenes (GB), and nontransgenic controls (C)

Parameter	C (n = 15) Mean (SD)	B (n = 7) Mean (SD)	G (n = 13) Mean (SD)	GB (n = 10) Mean (SD)
Carcass (g)	14.75^{a} (1.29)	11.67^{b} (0.82)	22.82^{c} (1.30)	16.62^{d} (2.00)
Skin (g)	5.88^{a} (0.82)	5.54^{a} (0.70)	15.43^{b} (1.71)	13.97^{c} (2.39)
Liver (g)	1.51^{a} (0.20)	1.48^{a} (0.18)	4.73^{b} (0.45)	4.02^{c} (0.55)
Abdominal fat (g)	1.31^{a} (0.84)	1.38^{ac} (0.53)	2.00^{b} (0.59)	1.92^{bc} (0.37)
Brain (mg)	446^{a} (26)	377^{b} (44)	491^{c} (28)	430^{a} (19)
Pancreas (mg)	246^{a} (34)	224^{a} (40)	579^{b} (121)	480^{c} (138)
Kidney[e] (mg)	237^{a} (24)	213^{a} (20)	448^{b} (38)	355^{c} (54)
Heart (mg)	165^{a} (20)	150^{a} (13)	311^{b} (42)	274^{c} (45)
Spleen (mg)	85^{a} (23)	95^{a} (17)	244^{b} (49)	217^{b} (33)

Significant differences ($P < 0.05$) between the groups are indicated by *different superscripts* (a, b, c, d); [e] mean of the weights of left and right kidney per animal.

TABLE 4. Relative organ and tissue weights of 15-week-old mice carrying the PEPCK-bGH transgene (G), the CMV-IGFBP-2 transgene (B), both transgenes (GB), and nontransgenic controls (C)

Parameter	C (n = 15) Mean (SD)	B (n = 7) Mean (SD)	G (n = 13) Mean (SD)	GB (n = 10) Mean (SD)
Carcass (%)	41.96^{a} (2.14)	38.00^{b} (1.67)	35.15^{c} (2.15)	30.55^{d} (2.22)
Skin (%)	16.67^{a} (1.56)	17.96^{a} (0.84)	23.68^{b} (1.55)	25.53^{c} (2.07)
Liver (%)	4.29^{a} (0.46)	4.81^{b} (0.27)	7.27^{c} (0.51)	7.38^{c} (0.63)
Abdominal fat (%)	3.60^{ab} (1.91)	4.37^{b} (1.37)	3.04^{a} (0.72)	3.51^{ab} (0.53)
Brain (%)	1.27^{a} (0.09)	1.24^{a} (0.21)	0.76^{b} (0.05)	0.79^{b} (0.06)
Pancreas (%)	0.70^{a} (0.10)	0.73^{a} (0.13)	0.88^{b} (0.15)	0.87^{b} (0.18)
Kidney[e] (%)	0.68^{a} (0.07)	0.69^{a} (0.05)	0.69^{a} (0.05)	0.65^{a} (0.05)
Heart (%)	0.47^{a} (0.05)	0.49^{a} (0.03)	0.48^{a} (0.05)	0.50^{a} (0.05)
Spleen (%)	0.24^{a} (0.07)	0.31^{b} (0.06)	0.37^{c} (0.05)	0.40^{c} (0.06)

Significant differences ($P < 0.05$) between the groups are indicated by *different superscripts* (a, b, c, d); [e] mean of the weights of left and right kidney per animal.

demonstrating organ- and time-specific sensitivity of different organs to GH excess. The weights of carcass and especially of brain were less increased than total body weight both in 5- and 15-week-old G mice.

The effects seen for elevated IGFBP-2 in the absence of GH overexpression largely confirmed our previous study. However, presumably because of the higher IGFBP-2 expression levels, the weight of the brain and the NRL were significantly reduced in B *vs.* C mice, in addition to the previously described significant reduction in carcass weight (15).

Interestingly, the growth-inhibiting effect of IGFBP-2 overexpression was even more marked in high-GH/IGF-I mice, as indicated by several observations: 1) At 5 weeks of age, GB mice displayed a significant reduction of all growth parameters except for the weight of abdominal fat, compared with G mice; whereas only brain weight was significantly reduced in B *vs.* C mice (Table 1); 2) In 15-week-old animals, a significant reduction in all growth parameters, except for spleen and abdominal fat weights, was seen in GB *vs.* G mice; whereas only NRL and the weights of carcass and brain were significantly reduced in B *vs.* C mice (Table 3); 3) IGFBP-2 overexpression in GB mice completely reduced normalized body size (NRL-body weight$^{1/3}$-ratio) in the condition of GH excess (Fig. 4C); and 4) A direct comparison of the relative effects (percent difference in body weight, organ/tissue weights or NRL) of IGFBP-2 overexpression in a high (GB *vs.* G) and a normal (B *vs.* C) GH/IGF-I background showed statistically significant differences in both 5- and 15-week-old mice (Fig. 5, A and B).

Theoretically, there are three major routes to explain the enhanced inhibitory activity of IGFBP-2 in giant GH transgenic mice: 1) IGFBP-2 blocks IGF-I-mediated effects of GH

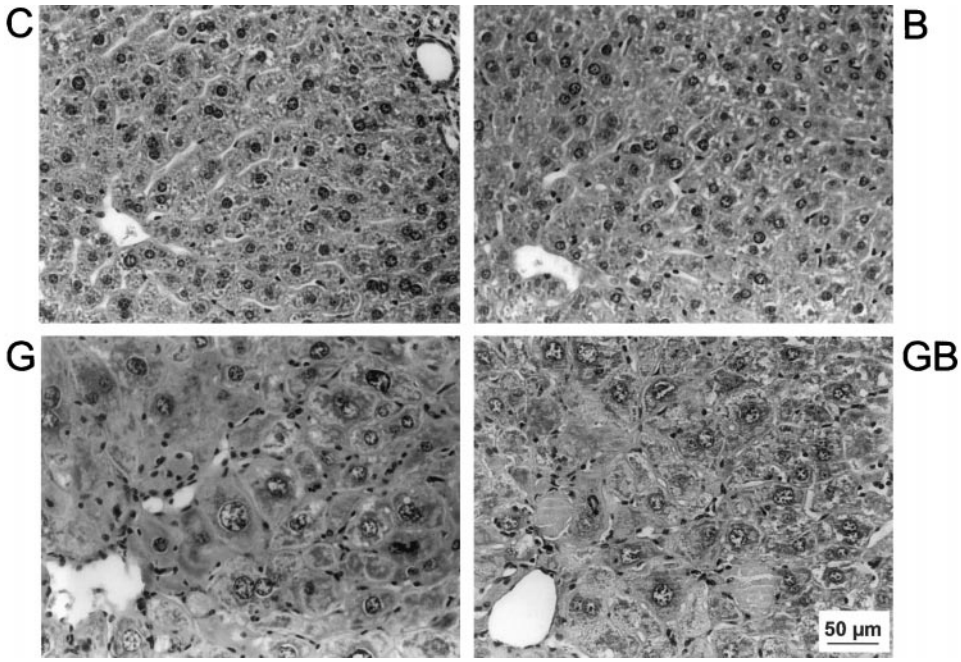

FIG. 6. Representative histological sections of liver tissue from mice carrying the PEPCK-bGH transgene (G), the CMV-IGFBP-2 transgene (B), both transgenes (GB), and nontransgenic controls (C).

excess; 2) IGFBP-2 impairs direct effects of GH overexpression; or 3) IGFBP-2 inhibits growth in a GH/IGF-I independent manner, but this effect is via yet-unknown mechanisms augmented in high-GH/IGF-I mice.

Several reasons argue for an IGF-I-dependent mechanism of growth inhibition by IGFBP-2 overexpression in giant GH transgenic mice. The essential role of IGF-I, to stimulate growth *in vivo*, has been shown by elegant studies in transgenic and knockout mouse models. Whereas overexpression of IGF-I in GH-deficient transgenic mice restored normal somatic growth (36), treatment with GH was inefficient in stimulating growth of IGF-I-deficient mice (37). Furthermore, the growth of organs that were particularly affected by IGFBP-2 overexpression has previously been shown to be regulated by IGF-I. For instance, the weights of the spleen, pancreas, and kidneys were most markedly reduced in 5-week-old GB *vs.* G mice. In IGF-I-overexpressing transgenic mice, spleen and pancreas were among the organs showing the greatest increase in weight (38). Furthermore, a specific increase in weights of spleen and kidney was observed in GH-deficient rats treated with IGF-I (39–41). In contrast, markedly reduced serum IGF-I levels in mice lacking hepatic *Igf1* gene expression were associated with significantly reduced kidney weights, in spite of elevated GH levels (42).

The weight of the carcass, which consists mainly of skeletal muscle tissue, was most markedly reduced by IGFBP-2 overproduction in 15-week-old mice, both in the absence and presence of the PEPCK-bGH transgene. In hypophysectomized rats, skeletal muscle was the tissue that showed the greatest increase (9.7-fold) in IGF-I mRNA levels after GH treatment (43). Furthermore, transgenic mice overexpressing IGF-I displayed an increase in body weight by 1.3-fold, which was primarily explained by increased muscle and/or connective tissue growth (38). In contrast, lack of IGF-I expression in *Igf1* knockout mice results in generalized muscle hypoplasia (44) and dystrophy that is most prominent in the diaphragm (45), causing a high incidence of perinatal death attributable to respiratory failure. These findings underline the importance of IGF-I for muscle development and growth.

A role of IGF-I for the growth of the heart has been demonstrated by cardiac-specific overexpression, in transgenic mice, of a human IGF-I cDNA under the control of the α-myosin heavy chain promoter. These mice developed cardiomegaly because of an increased number of cells in the heart (46). Also, for brain, IGF-I seems to be an important growth factor. Increased brain weights were found in IGF-I-overexpressing transgenic mice (38, 47), even in the near absence of GH (36). In contrast, reduced brain growth was a common finding in several transgenic mouse models overexpressing IGFBP-1 (Refs. 48–54; for review, see Ref. 55), which seems to inhibit IGF-dependent cellular growth and differentiation (1, 56).

However, there are also indications that IGFBP-2 might inhibit direct growth effects of GH excess or act as a growth inhibitor independent of GH or IGF-I. A number of studies suggested that liver growth is directly stimulated by GH and does not depend on IGF-I. First, transgenic mice overexpressing a human IGF-I cDNA under the control of the mouse metallothionein I promoter were characterized by 3-fold-increased IGF-I peptide levels in the liver but did not show increased liver growth (38). Second, expression of the same transgene in mice lacking somatotrophic cells completely normalized linear growth and weights of most organs but not the weight of the liver, which was still lower than in pituitary-intact controls (36). Third, liver-specific inactivation of the *Igf1* gene in mice eliminated IGF-I production in the liver and decreased circulating IGF-I levels by 75%; however (possibly as a consequence of elevated GH levels), the weight of the liver was increased in these animals (42, 57). Our study demonstrated a significant reduction of liver weight in GB *vs.* G mice, at both the ages of 5 and 15 weeks, suggesting that IGFBP-2 overexpression inhibits liver

growth by partially blocking the GH stimulus or directly via a yet-unknown mechanism. The latter is supported by the fact that male mice lacking IGFBP-2 expression displayed increased liver weights (14). However, relative liver weights of G and GB mice were not different, both being disproportionately increased, compared with C and B mice. Therefore, the reduction of absolute liver weight in GB *vs.* G mice might be a general size effect, *i.e.* the expected organ size change, given a certain overall weight change and a particular coefficient of growth allometry (for review, see Ref. 21). Importantly, characteristic pathological lesions observed in GH transgenic mice (16, 25, 26, 58) were also seen in GH/IGFBP-2 double transgenic mice. The influence of IGFBP-2 on long-term effects of GH on liver growth and pathology, including tumorigenesis (59, 60), deserves further investigation.

The effects of elevated IGFBP-2, to reduce body and organ growth, parallel those observed in transgenic mice overexpressing IGFBP-1 in several aspects. In phosphoglycerate kinase promoter-rat IGFBP-1 transgenic mice, the birth weight was significantly reduced, to approximately 83–92% of the weight of wild-type animals (49). The growth retardation persisted in the postnatal period, particularly after weaning. Except for brain weight (which was disproportionately reduced) and spleen weight (which was heavier), the absolute weights of individual organs were reduced in transgenic mice, but similar to those of control mice when considered as a function of body weight. The specific increase in spleen weight, in this transgenic model, has been discussed as a consequence of IGF-independent effects of elevated IGFBP-1. Overexpression of human IGFBP-1 under the control of the α1 antitrypsin promoter in transgenic mice resulted in growth retardation within the first weeks of postnatal life. Body weight of adult mice was negatively correlated with plasma IGFBP-1 concentration (50). In contrast, no major effects on total body weight were observed in MT-hIGFBP-1 transgenic mice (61). These different findings may be attributable to differences in level and tissue specificity of transgene expression in the various models (for review, see Ref. 55). Other alterations, such as altered glucose homeostasis and impaired female fertility, which were seen in part of the IGFBP-1-overexpressing transgenic mouse models (49, 50, 62), were not obvious in CMV-IGFBP-2 transgenic mice (15), although detailed analyses of metabolic and reproductive functions in this model remain to be done.

In summary, our study demonstrates that IGFBP-2 is an inhibitor of normal and GH-stimulated growth of mice, and that growth inhibition by IGFBP-2 overexpression is more effective in high-GH/IGF-I mice. This may be attributable to inhibition of indirect (IGF-I-mediated) or direct effects of GH excess by elevated IGFBP-2 or may represent a direct effect of IGFBP-2 that might be potentiated in high-GH/IGF-I mice. The crossing experiment used for this study provides a unique model system to understand tissue-specific effects and interactions between members of the GH/IGF system and to identify, by expression profiling, other factors involved in growth regulation of specific organs.

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