Overexpression of Insulin-Like Growth Factor-Binding Protein-4 (IGFBP-4) in Smooth Muscle Cells of Transgenic Mice through a Smooth Muscle α-Actin-IGFBP-4 Fusion Gene Induces Smooth Muscle Hypoplasia

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ABSTRACT

Insulin-like growth factor I (IGF-I) has been postulated to function as a smooth muscle cell (SMC) mitogen and to play a role in the pathogenesis of bladder hypertrophy, estrogen-induced uterine growth, and restenosis after arterial angioplasty. IGF-binding protein-4 (IGFBP-4) inhibits IGF-I action in vitro and is the most abundant IGFBP in the rodent arterial wall. To explore the function of this binding protein in vivo, transgenic mouse lines were developed harboring fusion genes consisting of a rat IGFBP-4 complementary DNA cloned downstream of either a -724 bp fragment of the mouse smooth muscle α-actin 5'-flanking region (SMP2-BP-4) or -1074 bp, 63 bp of 5'-untranslated region, and 2.5 kb of intron 1 of smooth muscle α-actin (SMP8-BP-4). SMP2-BP-4 mice expressed low levels of the exogenous IGFBP-4 messenger RNA (mRNA), which was not specifically targeted to SMC-rich tissue environments, and were therefore not analyzed further. Six SMP8-BP-4 transgenic lines derived from separate founders were characterized. Mating of hemizygous SMP8-BP-4 mice with controls produced about 50% transgenic offspring, with equal sex distribution. Expression of IGFBP-4 mRNA in nontransgenic littermates was maximal in liver and kidney. By contrast, transgenic IGFBP-4 mRNA expression, distinguished because of a smaller transcript size, was confined to SMC-containing tissues, with the following hierarchy: bladder > aorta > stomach > uterus. There was no transgene expression in skeletal muscle, brain, or cardiac myocytes. The abundance of IGFBP-4 measured by Western ligand blotting or by immunoblotting, was 8- to 10-fold higher in aorta and bladder of SMP8-BP-4 mice than in their nontransgenic littermates, with no change in plasma IGFBP-4 levels. Transgenic mice exhibited a significant reduction in wet weight of SMC-rich tissues, including bladder, intestine, aorta, uterus, and stomach, with no change in total body or carcass weight. In situ hybridization showed that transgene expression was targeted exclusively to the muscular layers of the arteries, veins, bladder, ureter, stomach, intestine, and uterus. Overexpression of IGFBP-4 was associated with SMC hypoplasia, a reciprocal phenotype to that of transgenic mice overexpressing IGF-I under control of the same promoter (SMP8-IGF-I). Double transgenic mice derived from mating SMP8-BP-4 with SMP8-IGF-I animals showed a modest decrease in wet weight at selected SMC tissues. Although we cannot exclude that the effects of IGFBP-4 may be IGF independent, these data suggest that IGFBP-4 is a functional antagonist of IGF-I action on SMC in vivo. (Endocrinology 139: 2605–2614, 1998)
icate that some IGFBPs may have direct, receptor-mediated effects themselves, independent of IGFs (reviewed in Ref. 2, 3).

IGFBP-4 was originally isolated and cloned from a human osteosarcoma cell line (4). It exists in biological fluids as a 28-kDa glycosylated and/or a 24-kDa nonglycosylated form. It consistently inhibits IGF-mediated cell proliferation of all cell types tested in vitro. IGFBP-4 does not bind to the cell membrane and is found associated with connective tissue, although the precise nature of this interaction is not known. We and others (5–7) have observed that adult arterial SMC release IGFBP-4 into serum-free conditioned medium. IGFBP-4 is subject to proteolysis by a cation-dependent serine protease that cleaves it only in the presence of IGF-I (7–9). IGFBP-4 is also expressed in the rat artery wall in vivo, where it is found primarily in the extracellular compartment. It is important to note that individual IGFBPs may have opposite activities on IGF action in vitro according to whether the IGFBP is in solution or bound to the cell membrane or matrix (2). Thus, a more physiological appraisal of the action of a particular IGFBP requires that it be expressed in the appropriate tissue setting in vivo, as it will presumably interact with its putative partners and be targeted to its natural compartment. In this paper, we report the characterization of transgenic mice overexpressing IGFBP-4 selectively in smooth muscle through a mouse smooth muscle α-actin (SM-α-actin) promoter. To our knowledge, together with a companion paper in which we generated mice overexpressing IGF-I under control of the same promoter (10), these reports represent the first examples of overexpression of functional proteins selectively in SMC, capable of modifying their properties in vivo. Indeed, the SMP8 smooth muscle α-actin promoter directs high levels of expression that are entirely confined to SMC of all tissue beds. Furthermore, SMP8-BP-4 transgenic mice demonstrate that overexpression of this binding protein is associated with smooth muscle hypoplasia and support the concept that IGFBP-4 inhibits IGF action, presumably by preventing IGF access to its cellular receptor.

Materials and Methods

Construction of SMP2-BP-4 and SMP8-BP-4 fusion genes

The SMP2-BP-4 chimeric gene was constructed by fusing a 0.77-kilobase (kb) fragment of the mouse SM-α-actin to a rat IGFBP-4 (rIGFBP-4) complementary DNA (cDNA) followed by the simian virus 40 (SV40) small T intron and early polyadenylation signal fragment (Fig. 1). SMP-2 contains −724 bp of the proximal 5′-flanking region plus 43 bp 5′-untranslated (5′-UT) region (exon 1). The rIGFBP-4 cDNA contains the entire coding sequence as well as 184 bp 5′-UT and 318 bp 3′-UT. Plasmid pSMP2 (11) was digested with BamHI and NotI to delete a 0.67-kb chloramphenicol acetyltransferase fragment; the rat IGFBP-4 cDNA fragment was released by EcoRI from pRBP-4–503 (a gift from Dr. Shimazaki) and inserted into pSMP2 after filling in both vector and insert with DNA polymerase I.

The SMP8-BP-4 chimeric gene was constructed by fusing a 3.6-kb fragment of the mouse SM-α-actin to the rIGFBP-4 cDNA followed by the SV40 early polyadenylation signal fragment. SMP8 contains −1074 bp of the 5′-flanking region, 63 bp of 5′-UT, and the 2.5-kb first intron of SM-α-actin. A 0.24-kb Sau3A1 fragment of SV40-pA from pSMP2 was cloned into pRBP-4–503 at the BamHI site, resulting in plasmid pRBP-4–5SV. A 3.6-kb SMP8 fragment, released from pRBP8 by digestion with BamHI and filled in by Klenow, was partially digested with HindIII and cloned into pRBP-4–5SV at the HindIII and EcoRI sites, so that rat IGF-II fused to SV40 early polyadenylation signal is driven by SMP8.

Generation of transgenic mice

The SMP8-BP-4 fusion gene was released from pSMP8-BP-4 by XhoI and BamHI restriction before microinjection, and that fragment was isolated and purified as previously described (10). The male pronuclei of fertilized eggs from FVB-N mouse strains were microinjected with 2 pl linearized DNA at the transgenic mouse facility of the University of Cincinnati. Microinjected eggs were implanted into the oviduct of pseudopregnant female mice and carried to term. Positive founders were

**Fig. 1.** Linear map of the SMP2-BP-4 and the SMP8-BP-4 fusion genes. SMP2-BP-4, A −724 bp fragment of the mouse SM-α-actin promoter (*light gray box*) was cloned upstream of rat IGFBP-4 cDNA (*dark gray box*) containing the entire coding sequence as well as 184 bp 5′-UT and 318 bp 3′-UT, followed by 0.6 kb of the small T intron, and a 240 bp of SV40 early polyadenylation signal sequence (*open box*). SMP8-BP-4, The SMP8 mouse SM-α-actin promoter fragment consisted of −1074 bp of 5′-flanking region (*light gray box*), the transcription start site, 48 bp of exon 1 (*black box*), the 2.5-kb intron 1 of the SM-α-actin gene (*line*), and 15 bp of exon 2 (*black box*) of SM-α-actin fused to the IGFBP-4 cDNA. The small T intron distal to the IGFBP-4 cDNA was removed. The following riboprobes were used for *in situ* hybridization: A, complementary to SV40 polyadenylase signal sequence (specific for all transgenes); B, complementary to rat IGFBP-4 mRNA, cross-hybridizes with mouse IGFBP-4 mRNA.
identified by Southern blotting and bread to wild-type FVB-N mice for propagation of the line. Heterozygotes and nontransgenic progeny from F1 and subsequent generations were selected by Southern blotting of EcoRI-restricted genomic DNA, as previously described (10). Hybridization was performed with a rat IGBP-4 cDNA labeled by random priming (Prime-It II kit, Stratagene, La Jolla, CA). The transgene was identified as a unique 1.3-kb band. Transgene copy number was calculated by quantitative Southern blotting, using known amounts of the SMPS-BP-4 construct added to nontransgenic mouse genomic DNA as a standard according to the following formula: 8100 bp (size of SMPS-BP-4) £ 5 µg/6 £ 10°6 bp/copy = 6.75 £ 10°6 µg/copy = 6.75 pg/copy.

To examine the effects of overexpression of IGBP-4 in SMC-rich tissues, where IGBP-4 levels were also increased, the SMPS-BP-4 mouse line f23928 was crossed with the recently characterized SMPS-IGBP-4 transgenic mouse line f23988 (10). The wet weights of SMC tissues of specific age and sex-matched controls, as described below.

RNA isolation and Northern blot analysis

Total RNA was isolated from tissues by a single step, acid guanidium thiocyanate-phenol-chloroform extraction method. Northern blots were previously blocked with 5× SSC, 0.5% SDS, and 30% deionized sucrose before hybridization. A section of the arterial vessel, from the aortic arch to the level of femoral fork, was excised and placed in PBS. Adhering fat and connective tissue from the adventitia were scraped off under surgical microscope, and the vessel was rinsed with PBS to remove residual blood. Organs were immediately placed on dry ice and stored at −80°C until use.

Tissue extraction and RIA for IGBP-1

Tissues were placed in 1 ml ice-cold 1 M acetic acid and immediately homogenized for 1 min on ice using a Polytron PT3000 (Brinkmann Instruments, Westbury, NY) at full speed. After standing on ice for 2 h, the tissue extracts were centrifuged in siliconized microcentrifuge tubes at 18,000 £ g for 1 h at 4°C. The supernatants were then concentrated to 2–3 µg/ml protein through Centricon-3 devices (Amicon, Beverly, MA) and then hybridized with random primed rat IGBP-4 cDNA. For standardization, blots were rehybridized with either human glyceraldehyde-3-phosphate dehydrogenase cDNA, for comparisons within the same tissue type, or 18S ribosomal RNA, for comparisons between different tissues.

Allometry of SMP8-BP-4 transgenic mice

Transgenic mice and their nontransgenic littermates were killed by CO2 asphyxiation. After determining the body weight, blood was collected by cardiac puncture, and serum was stored at −80°C until use. Organs of interest, except the aorta, were dissected, rinsed in ice-cold PBS, tissue-blotted, weighed, and immediately frozen in dry ice. The contents of the stomach and small intestine were flushed out with PBS before weighing. A section of the arterial vessel, from the aortic arch to the level of femoral fork, was excised and placed in PBS. Adhering fat and connective tissue from the adventitia were scraped off under surgical microscope, and the vessel was rinsed with PBS to remove residual blood. Organs were immediately placed on dry ice and stored at −80°C until use.

In situ hybridization

In situ hybridization was performed as previously described (10). Tissues dissected from animals at the indicated ages were fixed in 4% paraformaldehyde, saturated overnight with 30% sucrose in PBS, and frozen in OCT (Miles, Elkhart, IN). Cryostat sections (7 µm) were mounted on silane-coated slides. Sense and antisense complementary RNA probes for rat IGBP-4, which hybridizes to both endogenous mouse and transgenic rat IGBP-4 messenger RNA (mRNA), and for a transgene-specific SV40 3′UT/polyadenylase signal sequence, which recognizes only the exogenous mRNA, were labeled with [35S]UTP, using a commercially available kit (Stratagene, La Jolla, CA). For generation of the antisense IGBP-4 riboprobe, the pRBP-4-SH (gift from Dr. Shinmasaki) plasmid was linearized with SmaI and transcribed with T7 RNA polymerase. The product was 493 bases long, 449 of which were complementary to transgenic mRNA (Fig. 1, probe A). The sense IGBP-4 riboprobe was 491 bases long and was obtained after linearizing the antisense IGBP-4 riboprobe, the pRBP-4-SH (gift from Dr. Shinmasaki) plasmid was linearized with SmaI and transcribed with T7 RNA polymerase.

Tissue histomorphometry

Morphometry was performed using NIH Image V1.61, an image-processing and analysis program for the Macintosh. The arterial section images of SMPS-BP-4 transgenic mice (line 23942) and their age-matched nontransgenic controls were color-captured into the computer from Trichrome-stained sections through the microscope. After adjusting the

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image contrast, the area of interest was auto-outlined, and the regions outside and inside the area were cleared. The lumen area of the aorta and the thickness of the aortic media were calculated from a hypothetical perfect circle. The formulas are as follows: Do = P/π, Ai = P/2/4π - A, Di = 2(Ai/π)0.5, T = (Do - Di)/2, where Do is the outer diameter, P is the perimeter, Ai is the lumen area, A is the measured area, Di is the inner diameter, and T is the thickness of the muscular area.

**Determination of myocyte number and tissue DNA and RNA contents**

RNA and DNA were extracted from bladder, intestine, stomach, and brain tissues of SMP8-BP-4 transgenic mice and their nontransgenic littermates as previously described (10). The concentrations of the extracted nucleic acids were measured by UV absorption at 260 nm. To verify RNA/DNA data, the myocyte number per surface area was directly counted on hematoxylin- and eosin-stained slides using a grid as a reference point. For each tissue examined, a minimum of five fields (×40) of five sections were counted.

**Statistics**

Statistical analysis was performed using either Student's t-test or, when more than two groups were compared, one-way ANOVA. Differences between any two groups selected by the ANOVA were then analyzed using the Walker-Duncan adaptive procedure.

**Results**

The first construct we studied consisted of a ~724 bp mouse SM-α-actin 5′-flanking region (RSP2), containing a single E box, upstream of rat IGFBP-4 cDNA (Fig. 1). This choice was made based on data reported by Foster et al. (11), indicating that the most proximal E box is sufficient to evoke maximal transcriptional activity in BC3H1 myoblasts in vitro. However, SMP2-BP-4 mice had only a very modest increase in IGFBP-4 mRNA levels (at best 2-fold) that was not specifically targeted to SMC-rich tissues (data not shown). We, therefore, generated new fusion genes containing a more extended 5′-flanking region of mouse SM-α-actin (Fig. 1). Within the ~1074 bp region of SM-α-actin, there is an evolutionary conserved motif that represses expression in nonmyogenic fibroblast cells as well as six E box motifs mediating high level expression in postconfluent BC3H1 myoblasts (11, 13, 14). In addition, the new constructs included the first intron of SM-α-actin, which has been proposed to contain additional motifs important for SMC-specific transcription (14).

**Generation of SMP8-BP-4 mice and examination of transgene expression**

Six SMP8-IGFBP-4 founder animals were obtained. The percentage of transgenic offspring in the F1 generation indicated that three of the six founders were mosaics. In subsequent generations, matings of hemizygous transgenic mice with controls produced about 50% transgenic offspring with equal sex distribution. Six transgenic lines were further propagated for more complete analysis.

The tissue distribution of endogenously produced and transgenic IGFBP-4 mRNA is shown in Fig. 2 (left panel). Endogenous IGFBP-4 mRNA was abundant in the liver, kidney, and uterus of nontransgenic mice and was expressed at relatively low levels in other SM-rich tissues. The IGFBP-4 transgene, identified because of its lower size, was detected at very high levels in bladder and aorta and to a lesser degree in stomach and uterus. Note that although expression of native IGFBP-4 mRNA was readily observed in aorta, stomach, and bladder, the abundance of the transgenic IGFBP-4 mRNA was severalfold higher than that of the endogenous transcript in these tissues. Exogenous IGFBP-4 mRNA was observed almost exclusively in smooth muscle-rich tissues of transgenic mice in all founder lines tested, although the levels of expression varied between the lines (Fig. 2, right panel). Western ligand blotting demonstrated a marked increase in IGFBP-4 protein in bladder and aorta of transgenic mice from several lines (Fig. 3). Glycosylated as well as deglycosylated forms of IGFBP-4 were present in greater abundance, as confirmed by N-glycanase digestion of bladder and aortic extracts of SMP8-BP-4 mice (Fig. 3D). The overexpression of IGFBP-4 in bladder and aorta was also confirmed by Western blotting (Fig. 3E). These data document selective overexpression of IGFBP-4 in SMC-rich tissues. Increased biosynthesis of...
the binding protein in SM-rich tissues did not result in a concomitant rise in serum IGFBP-4 levels, as determined by Western ligand blotting (Fig. 3C).

Endogenous expression of IGFBP-4 in nontransgenic mouse was most prominent in the kidney cortex and the stroma of the stomach and intestine, as demonstrated by in situ hybridization histochemistry with an IGFBP-4 riboprobe (Fig. 4, A–C, and E). By contrast, expression of the SMP8-BP-4 transgene mRNA, detected with the same probe, was targeted to medial aortic SMC and the SMC layer of the small intestine (Fig. 4, D and F). When examined with a transgene-specific riboprobe complementary to vector-derived sequences from the SV40 3′-UT region of the construct, expression was entirely confined to smooth muscle, further confirming the cell specificity of transcription directed by this promoter. Figure 5 shows examples of transgene expression in the SMC layers of the uterus, stomach, ureter, vena cava, aorta, and urinary bladder.

**SMP8-BP-4 mice have decreased SMC mass.** There was no difference in total body weight of SMP8-BP-4 transgenic mice compared with that of their nontransgenic littermates. However, there was a significant decrease in the wet weights of aorta, bladder, and stomach of the transgenic mice (Fig 6). The surface area and thickness of the aortic medium were also significantly lower in SMP8-BP-4 transgenic mice compared with those in age- and sex-matched nontransgenic controls (Table 1). The SMP8-BP-4 transgenic line 23942 had low expression of transgenic IGFBP-4 in the intestine (Fig. 2, left panel), and correspondingly, there was no change in length or wet weight of small bowel. Line 23928 did have significant transgene expression in the bowel as well as marginally decreased intestinal weight (not shown). The lower SMC mass in the SMP8-BP-4 mice is due to a decrease in cell number and not to hypotrophy, as demonstrated by RNA/DNA ratios (Table 2). However, the admixture of other cell types in the tissues we examined may skew these ratios toward normality (this is less likely to be a factor in the artery wall, as these were stripped of adventitia). Additional evidence was, therefore, obtained by histomorphometry. Myocyte counts of ×40 magnified sections of bladder and aorta of transgenic and nontransgenic mice were not significantly different in the IGFBP-4 transgenics (five fields of five sections from the respective tissues of four separate transgenic and nontransgenic mice were examined). As the total mass of SMC tissue was decreased in SMP8-BP-4 mice (Table 1), this indicates that there are fewer cells overall.

Thus, the SMP8-BP-4 mice have a reciprocal phenotype of that of their SMP8-IGF-I counterparts and develop hypopla-
FIG. 4. In situ hybridization of nontransgenic and SMP8-BP-4 mice with a rat IGFBP-4 riboprobe recognizing both endogenous and transgenic IGFBP-4 mRNA. A, Stomach; nontransgenic; signal in stromal cells of the mucosa; no signal in epithelial or smooth muscle cells. B, Kidney; nontransgenic; strong signal in renal cortex, primarily in proximal tubules; no signal in glomeruli (arrow) or renal medulla (me). C, Aorta; nontransgenic; weak signal in aortic SMC, with moderate hybridization in adventitia. D, Aorta; SMP8-BP-4; strong signal in the media. E, Small intestine; nontransgenic; signal in stromal cells of mucosal layer. F, Small intestine; SMP8-BP-4 mice; localization to the SMC cell layer.
sia of smooth muscle tissue beds. In a companion paper, we report the generation of transgenic mice overexpressing rat IGF-I under control of the SMP8 α-actin promoter (SMP8-IGF-I mice). These animals exhibited remarkable SMC hyperplasia, with a distinct pattern of tissue remodeling (10). This manifested in marked thickening of the SMC layers of the bladder and artery wall, whereas the uterine horns and small bowel grew primarily in length. To determine whether overexpression of IGFBP-4 can antagonize these effects, heterozygous SMP8-IGF-I and SMP8-IGFBP-4 mice were crossed, and the double transgenics were analyzed. These animals had overexpression of both IGF-I and IGFBP-4 in

Fig. 5. In situ hybridization of SMP8-BP-4 transgenic mice. All tissues were hybridized with the transgene-specific riboprobe complementary to the SV40 3’-UT region of the transcript (see Fig. 1), except panel L, which was hybridized with the SV40 riboprobe sense control. G. Uterus; strong signal in SMC of wall; no signal in mucosa. H. Stomach; signal in SMC of wall (arrowhead); no signal in mucosa. I. Ureter; strong signal in SMC of wall of ureter; none in mucosal epithelium. J. Aorta, vena cava; signal in SMC of wall of vena cava (arrowhead); signal in aorta is obscured by refraction from elastin layer. K. Bladder; strong signal in SMC of bladder wall; none in mucosa. L. Bladder; SV40 sense control; no background signal evident.
smooth muscle, to a similar degree as that found in their respective single transgenic parents (not shown). However, the wet weight of SMC-rich tissues in the double transgenics was only modestly decreased and was significant only in aorta of males and in uterus and intestine of females (Table 3; as determined by ANOVA). Moreover, as the estrous cycle of the females was not synchronized, we cannot assume that changes in uterine weight were directly due to overexpression of IGFBP-4.

The mechanism of the growth inhibitory effect exerted by the overexpression of IGFBP-4 is unclear. The radioimmunoassayable IGF-I content of bladder and aorta was not significantly different between transgenic and nontransgenic bladder and aorta. Furthermore, there was no change in IGF-I mRNA levels (Fig. 7). Thus, the higher tissue IGFBP-4 levels decrease SMC mass without interfering with IGF-I gene expression or tissue IGF-I abundance.

**Discussion**

A poorly understood aspect of IGF action is the roles of the various IGFBPs in modifying the cellular response to IGF-I and IGF-II. There are at least six high affinity IGFBP, which are products of different genes (2). Each tissue environment appears to contain a particular set of these proteins that is bound to the extracellular matrix (i.e. IGFBP-5) (15, 16), to the cellular membrane (i.e. IGFBP-3) (17, 18), or, in the case of IGFBP-4, to an unknown site(s) in the extracellular milieu. IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-5 are, in turn, cleaved by specific proteases that degrade the respective binding proteins to fragments with low or absent affinity for the IGFs (19). The expression of the individual binding proteins is subject to regulation, as is the activity of the IGFBP3, IGFBP-4, and IGFBP-5 proteases (2, 19). The precise purpose of this multiprotein system, apparently designed to modulate access of IGFs to cells, remains quite puzzling. These events also apply to SMC, where IGFBP-4, IGFBP-5, and IGFBP-4, to an unknown site(s) in the extracellular milieu. Therefore, there is still considerable uncertainty about their mode of action, which can be clarified best by exploring their function in vivo.

To examine the function of IGFBP-4 in SMC in vivo, we used the SM-α-actin promoter to target its expression. As SM-α-actin gene expression is primarily restricted to SMC of adult rodents and rabbits (19–21), its promoter seemed well suited to the task. We first focused on the SMP2 and SMP8-α-actin promoter constructs to direct expression to SMC of the different tissue beds. Although the SMP2 fragment contained regulatory regions capable of directing high levels of expression to SMC in vitro (11), it was almost without effect in vivo. By contrast, the SMP8 promoter directed robust levels of transgene expression specifically to SMC, as reported here and in a companion paper (10). This indicates that regulatory regions between –724 and –1074 bp of SM-α-actin are important for SMC-specific expression in vivo. Alternatively, critical sequences may lie within the first intron of the SM-α-actin gene that were not present in SMP2 but were in-

**TABLE 2.** RNA/DNA ratio of tissues of SMP8-BP-4 transgenic mice and their nontransgenic controls

<table>
<thead>
<tr>
<th></th>
<th>Bladder</th>
<th>Intestine</th>
<th>Stomach</th>
<th>Brain</th>
</tr>
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<tbody>
<tr>
<td>NT</td>
<td>3.02 ± 0.43</td>
<td>0.75 ± 0.05</td>
<td>1.15 ± 0.25</td>
<td>0.86 ± 0.03</td>
</tr>
<tr>
<td>TG</td>
<td>3.12 ± 0.37</td>
<td>0.74 ± 0.08</td>
<td>1.01 ± 0.18</td>
<td>0.97 ± 0.16</td>
</tr>
<tr>
<td>TG/NT (%)</td>
<td>100.3</td>
<td>98.7</td>
<td>87.8</td>
<td>112.8</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

NT, Nontransgenic mice (n = 4); TG, SMP8-BP-4 transgenic mice (line 23928; n = 4). The RNA/DNA ratio is the mean ± sd.
OVEREXPRESSION OF IGFBP-4 IN SMOOTH MUSCLE CELLS

TABLE 3. Effects of IGFBP-4 overexpression on IGF-I-induced SMC hyperplasia: wet weight of SMC-rich tissues in SMP8-IGF-1, SMP8-IGFBP4, and double transgenic mice

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Aorta</th>
<th>Bladder</th>
<th>Intestine</th>
<th>Uterus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT</td>
<td>11</td>
<td>6.5 ± 0.4</td>
<td>10.7 ± 1.0</td>
<td>1302.7 ± 36.1</td>
<td>79.8 ± 4.1</td>
</tr>
<tr>
<td>IGF-I</td>
<td>4</td>
<td>8.6 ± 0.6*</td>
<td>20.2 ± 1.3*</td>
<td>2450.7 ± 72.7*</td>
<td>178.7 ± 2.2*</td>
</tr>
<tr>
<td>BP4/IGF-I</td>
<td>18</td>
<td>7.0 ± 0.6</td>
<td>17.3 ± 1.4</td>
<td>2185.5 ± 60.5b</td>
<td>125.4 ± 7.9b</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT</td>
<td>13</td>
<td>7.9 ± 0.3</td>
<td>27.4 ± 3.2</td>
<td>1395.8 ± 34.3</td>
<td></td>
</tr>
<tr>
<td>IGF-I</td>
<td>12</td>
<td>10.8 ± 0.8a</td>
<td>51.1 ± 5.1a</td>
<td>2320.8 ± 62.9a</td>
<td></td>
</tr>
<tr>
<td>BP4/IGF-I</td>
<td>13</td>
<td>9.0 ± 1.6b</td>
<td>49.1 ± 5.8</td>
<td>2136.2 ± 109.3</td>
<td></td>
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All organ wet weights are from 15-week-old mice and measured in milligrams (mean ± SEM).

* Significantly different between NT and IGF-I (P < 0.05).

b Significantly different between IGF-I and BP4/IGF-I (P < 0.01).

Fig. 7. IGF-I gene expression in SMP8-IGFBP-4 transgenic mice. Left panel, Northern blot of 25 μg RNA from aorta and bladder from 6- to 8-week-old female nontransgenic (NT) and the indicated SMP8-IGFBP4 transgenic lines hybridized with rat IGF-I cDNA (top panel) or 18S ribosomal RNA (bottom panel) probes. Liver, Five-microgram RNA positive control. Right panel, RIA of IGF-I from HPLC fractions of bladder tissue extracts of nontransgenic (NT; n = 3) and SMP8-IGFBP4 transgenic (TG; n = 3) mice.

including the SMP8 construct. The phenotypic changes in the SMP8-IGF-I (10) and SMP8-IGFBP4-IGF-I were confined to SMC. During development, SMC-actin is transiently expressed in skeletal and cardiac muscle and only becomes restricted to SMC during late fetal maturation (19, 23, 24). The lack of effect of overexpression of IGFBP-4 on cardiac or skeletal muscle mass indicates either that the SMP8 promoter does not entirely recapitulate the pattern of expression of the endogenous gene product during fetal life or that the binding protein is without measurable effect when overexpressed during that discrete period of time (embryonic day 11.5 to birth). Notably, the type I IGF receptor is expressed in skeletal muscle and heart at the appropriate period of rat embryogenesis (25).

The IGFBP-4 overexpressed in SMC-rich tissues remained entirely paracrine, as plasma levels of IGFBP-4 were not increased in the transgenic animals. Both glycosylated and unglycosylated forms were overproduced. SMP8-BP-4 mouse exhibited decreased weight of smooth muscle-containing tissues: i.e. aorta, bladder, stomach, uterus, and intestine. There was no alteration of expression of other IGFBPs in these tissues (based on Western ligand blotting) or of IGF-I itself, indicating that the observed changes were due to the individual action of increased IGFBP-4 and not to compensatory changes in expression or abundance of other members of the IGF system. The effects on smooth muscle were selective, as total body weight and those of other organs were unaffected. Overexpression of IGF-I by SMC in vivo is associated with increased cell mass, with characteristics dependent on the tissue microenvironment. IGF-I regulates SMC number through its mitogenic effects as well as by serving as an inhibitor of apoptosis (1). It is likely that the effects of IGFBP-4 were due to its IGF-binding activity and consequent prevention of ligand activation of the SMC type I IGF receptor. However, preliminary data on IGFBP-4 null mice indicate that, contrary to expectation, disruption of IGFBP-4 function leads to lower (10–15%) postnatal weight, an effect that becomes apparent by postnatal day 7 (26). The precise mechanisms of this paradoxical finding are still under investigation. Furthermore, although the effect of IGFBP-4 deletion on total body weight has been reported, information on its impact on expression of other binding proteins and on growth of individual organs and tissues is still pending.

When SMP8-IGF-I and SMP8-IGFBP4 were crossed, the double transgenic animals overexpressed both gene products in smooth muscle, i.e. IGF-I and IGFBP4, to a similar degree as in their single transgenic ancestors. However, there was only a modest inhibition of SMC mass that was only significant in selected tissues. Thus, IGFBP-4 cannot completely overcome the effects of very high levels of tissue IGF-I. This may be due to the stoichiometry of these two compounds in the tissues of this particular cross. Alternatively, we cannot exclude that IGFBP-4 may have effects that are independent of its interaction with IGF-I, although to our knowledge, there is no evidence of such an effect in vitro. It is more likely that the modest phenotype may be due to activation of the IGFBP-4 protease, which is known to be IGF-I dependent (7–9, 12, 27), leading to increased proteolysis of IGFBP-4 and partial negation of its presumed inhibitory properties. We have been unable to obtain information on whether the protease is more active in the double transgenics, because of nonspecific cross-reactivity of an abundant 16-kDa mouse protein with the IGFBP-4 antibody that prevents visualization of the cleavage products by Western blotting. Nevertheless, the role of this IGF-I-dependent protease remains an important issue, as tissue IGF-I levels increase remarkably in certain pathophysiological situations, such as vascular injury and bladder hypertrophy. It remains to be
seen whether overexpression of a cleavage-resistant form of IGFBP-4 results in a more powerful antagonism of tissue IGF-I action.

References


