THE SIX INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS; DEVELOPMENTAL EXPRESSION AND STRUCTURAL ASPECTS

DE ZES INSULINE-ACHTIGE GROEIFACTOR BINDENDE EIWITTEN; EXPRESSIE TIJDENS ONTWIKKELING EN STRUCTURELE ASPECTEN

PROEFSCHRIFT

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LIST OF ABBREVIATIONS

AD	androstenedione
AF	amniotic fluid
ALS	acid labile subunit
ATP	adenosine triphosphate
BMI	body mass index
bp	basepairs
cAMP	cyclic adenosine monophosphate
cDNA	copy DNA
DNA	deoxyribonucleic acid
E_2	17-ß-estradiol
FF	follicle fluid
FSH	follicle stimulating hormone
GH	growth hormone
hCG	human chorionic gonadotropin
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
kDa	kilo dalton
MAb	monoclonal antibody
mRNA	messenger RNA
PAGE	polyacrylamide gel electrophorese
PCOS	polycystic ovary syndrome
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcriptase PCR
SDS	sodium dodecyl sulphate
T	testosterone

CHAPTER 1

INTRODUCTION

1.1 THE INSULIN-LIKE GROWTH FACTORS.

Insulin-like growth factors (IGF-I and IGF-II) are single chain polypeptides mediating many of the growth promoting effects of growth hormone, as stated in the "somatomedin hypothesis" (Daughaday et al., 1972). IGF-I is a basic protein and consists of 70 AA with a predicted molecular weight of 7.6 kDa. IGF-II is a slightly acidic protein consisting of 67 AA from which 60% are identical to IGF-I. IGF-II has a predicted molecular weight of 7 kDa. Like proinsulin, the IGFs can be divided in a B-domain and an A-domain which are connected by a short C-domain. Three intrachain disulphide bonds are present in both IGF-I and IGF-II, two between the B and A domains and one within the A domain. Furthermore, a D-domain is found in the IGFs, which is absent in proinsulin. (Rinderknecht and Humbel 1978a, 1978b, Raschdorf et al., 1988). IGF-I and -II carry their names based on the structural and functional homology with insulin (Rinderknecht and Humbel 1976, Blundell and Humbel 1980, Blundell et al., 1983). Recently, 2-dimensional nuclear magnetic resonance techniques have revealed that the structure of IGF-I in solution is indeed similar to insulin, although some minor differences were observed (Cooke et al., 1991).

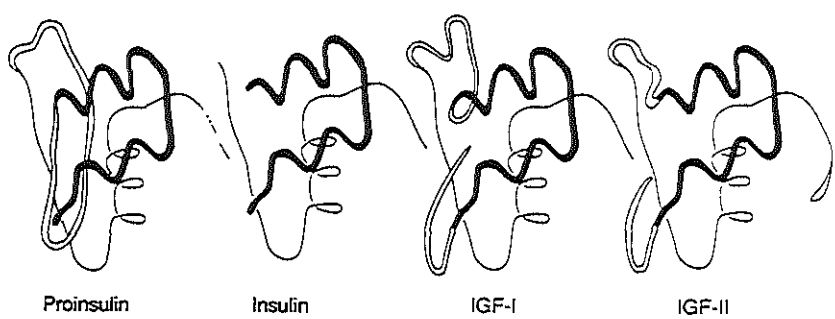

Fig.1. Schematic representation of the 3-dimensional structure of insulin and proposed conformation of proinsulin, IGF-I and IGF-II (Blundell and Humbel, 1980).

The physiological effects of the IGFs are diverse. IGFs stimulate DNA, RNA and protein synthesis and increase the proliferation rate of a wide variety of cells in vivo and in vitro (Froesch et al., 1985, Humbell 1990, Cohick and Clemmons 1993). An involvement of IGFs in cell differentiation has also been proposed, since they have been shown to play a role in neurite formation in isolated sensory and sympathetic ganglia and neuroblastoma cells, differentiation of preadipocytes into adipocytes, increase in type I procollagen mRNA in the bone derived cell line PyMS1 and differentiation of myoblasts into postmitotic contractile myotubes (Recio-Pinto et al., 1984, Smith et al., 1988, Schmid et al., 1989, Florini et al., 1991 and Ewton and Florini 1981). Finally, IGFs exert metabolic effects, like stimulation of glucose transport in muscle and adipose cells and stimulation of glycogen and protein synthesis as well as lipolysis in adipose cells (Froesch et al., 1985).

1.2 THE INSULIN-LIKE GROWTH FACTORS IN DEVELOPMENT.

Preimplantation.

IGFs in the ovary

Already before ovulation the developing oocyte is in contact with IGFs present in follicular fluid. Human follicular fluid contains both IGF-I and IGF-II, in concentrations similar to serum levels (Rasasharma et al., 1986, Eden et al., 1988). In rat, IGF-I gene expression has been localized predominantly to granulosa cells of developing follicles (Oliver et al., 1989), whereas expression of the IGF-II gene has been reported to be theca-interstitial cell exclusive (Hernandez et al., 1990). In humans, ovarian expression of the IGF-I and IGF-II genes has been reported to be the opposite. Here, mRNA expression of IGF-II has been found in granulosa cells and expression of IGF-I has been demonstrated in the theca-interstitial cell compartment (Ramasharma and Li 1987, Hernandez et al., 1991, Voutilainen and Miller 1987).

IGF-I has been reported to augment follicle stimulating hormone (FSH) induced progesterone and estrogen production as well as FSH induced expression of luteinizing hormone receptors in cultured rat granulosa cells, suggesting that IGF is involved in the amplification of gonadotropin hormonal action (Adashi et al., 1985a, Adashi et al., 1985b, Adashi et al., 1985c). Furthermore, IGF-I was found to stimulate basal, as well as FSH induced, proteoglycan synthesis, which has been implicated in follicular antrum formation and follicular atresia (Adashi et al., 1986). These results indicate that the IGFs present in follicular fluid are, at least partly, produced locally and that IGFs stimulate the growth of the developing ovarian follicle.

IGFs in the reproductive tract

Following ovulation and fertilization, the embryo is exposed to fluid from the oviduct and uterus. Porcine oviduct fluid has been reported to contain both IGF-I and IGF-II in concentrations ranging from 25% to 50% of that found in serum. Furthermore, primary cultures of porcine oviduct cells secreted both IGF-I and IGF-II in the culture medium, demonstrating that at least part of the IGF levels in oviduct fluid was due to local production

(Wiseman et al., 1992).

IGF-I has also been demonstrated in the rat fallopian tube. Transcripts for IGF-I were found in the luminal region of the fallopian tube and immunoreactive IGF-I was most abundant in the epithelial cells. IGF-I mRNA levels changed during the estrous cycle, being highest on the day of proestrus. Furthermore, IGF-I mRNA expression was increased dose-dependently after a subcutaneous injection of estradiol (Carlsson et al., 1993).

The presence of IGFs has also been described in uterine fluid (Murphy and Ghahary, 1990), of which at least some may be produced locally, since high expression of IGF-I and IGF-II was reported in rodent uterus and mRNA expression of IGF-I has been localized to stromal cells (Murphy et al., 1987, Croze et al., 1990). Abundant IGF-I mRNA expression has also been reported in the outer longitudinal smooth muscle layer of the uterus, being increased after 17 beta-estradiol administration (Ghahary et al., 1990).

Finally, IGF-I and IGF-II have been demonstrated in human seminal fluid in concentrations varying from 10% to 50% of concentrations found in serum (Baxter et al., 1984, Rasasharma et al., 1986). Although the role of IGFs in seminal plasma is not known, seminal plasma can reach the upper parts of the female reproductive tract and could contribute to the microenvironment of the preimplantation embryo.

IGF expression in preimplantation embryos

In addition to the extraembryonic IGF sources mentioned before, the preimplantation embryo can express IGFs itself, either by translation of residual mRNA from the oocyte or from transcription of its own genome. Using reverse transcriptase polymerase chain reaction (RT-PCR), mRNA expression of IGF-I has been shown as early as the eight-cell stage mouse embryo (Rappolee 1990). Transcripts for IGF-II were even detectable at the two-cell stage (Heyner et al., 1989, Schultz et al., 1992). Since transcription of the embryonic genome is at first thought to occur around the two-cell stage (Schultz, 1986, Telford et al., 1992), these IGF-II transcripts may be both from maternal origin and from transcription of the embryonic genome.

IGF actions in preimplantation embryo

Preimplantation embryos can be isolated from the reproductive tract and cultured in chemically defined culture media devoid of any growth factors. However, a serious lag in the development of isolated mouse embryos in culture was observed when compared with development in vivo (Bowman and McLaren 1970, Harlow and Quinn 1982). Furthermore, a significant percentage of the in vitro cultured embryos underwent maturation arrest, demonstrating that maternally derived factors are needed for optimal development of the preimplantation embryo.

It has been demonstrated that IGFs may have a direct effect on the preimplantation embryo. Addition of IGF-I to the culture medium of isolated two cell stage mouse embryos for 54 hours resulted in blastocysts showing an increase in the number of cells in the inner cell mass, with no effect on trophectoderm cell proliferation. The same stimulation of cell number was seen when the inner cell mass was isolated from blastocysts, prior to culture with IGF-I for 24 hours. In addition, the proportion of blastocysts was increased by IGF-I, while the number of embryos remaining in the early cleavage stages or as morulae decreased (Harvey and Kaye 1992). Addition of IGF-II to the culture medium of preimplantation mouse embryos has also been reported to increase the number of cells present in blastocysts and raise the number of embryos reaching the blastocyst stage (Rappolee et al., 1992). Since these studies were performed using 60 pM IGF-I (within the physiological range of 17-170 pM found in the reproductive tract), IGFs present in the reproductive tract can stimulate the growth and development of the preimplantation mouse embryo.

The physiological relevance of the IGFs expressed by the preimplantation embryo is not known. Recent gene disruption experiments indicated that loss of IGF-I or IGF-II genes resulted in severe growth retardation. The growth retardation became apparent at embryonal day 13½ and 11 respectively, suggesting that IGFs are first needed after embryonal day 11 (discussed further in chapter 1.2, postimplantation). However, in the preimplantation embryo, the loss of IGFs may partly be overcome by maternally derived IGFs, which can influence the growth and development of the preimplantation embryo.

Postimplantation.

At the time of implantation, maternally derived factors can be available to the embryo since there is an increased bloodflow and vascular permeability at the site of implantation. As soon as the placenta has developed, maternal growth factors can only reach the embryo via placental delivery. Although IGFs and IGFBPs are able to cross the endothelium of capillary vessels in the rat heart (Bar et al., 1990a, Bar et al., 1990b, Boes et al., 1992), the IGFs are reported not to cross the placenta (Underwood and D'Ercole 1984, Davenport et al., 1990).

IGF gene expression in postimplantation embryos

Messenger RNA expression of IGF-I has been shown in postimplantation rat embryos as early as rat embryonic day 11 by solution hybridization techniques. The expression was increased 8.6 fold at 13 days after which it seemed to reach a plateau. IGF-II mRNA was more abundant in all stages tested and remained constant between embryonal day 10-14 (Rotwein et al., 1987). In rat liver, levels of IGF-II mRNA were detected at embryonic day 11 and increased at day 12. The major IGF-II mRNAs (4 and 3.6 kb) remained constant between embryonic day 14 and birth (Gray et al., 1987).

The sites of IGF-I and IGF-II gene expression in postimplantation rat embryos have been studied extensively by Northern blotting, solution hybridization and in situ hybridization techniques (Brown et al., 1986, Lund et al., 1986, Gray et al., 1987, Beck et al., 1987, Stylianopoulou et al., 1988a, Bondy et al., 1990, Streck and Pintar 1992, Ferguson et al., 1992). A summary of these results is given in Table 1.

The earliest site of IGF-I mRNA expression in rat embryos, detectable by in situ hybridization techniques, has been reported to be the septum transversum. The mesenchymal cells of the septum transversum play a critical role in inducing proliferation and differentiation of liver endoderm (LeDouarin 1975). Most interesting, the onset of IGF-I mRNA expression has been demonstrated at the time that penetration by proliferating endodermal cells had just begun, suggesting that IGF-I is involved in the mesenchymal/endodermal interactions that induce liver development (Streck and Pintar 1992).

Table 1. Major sites of IGF-I and IGF-II mRNA expression in postimplantation embryos and fetuses.

	early gestation	*midgestation*	*late gestation*
IGF-I	septum transversum →	liver	liver
	endocardial cushion and truncus arteriosus	forming cardiac valves	
	undifferentiated mesenchyme in the vicinity of sprouting nerves and spinal ganglia		
	mesenchyme surrounding developing muscle	skeletal muscle	
	mesenchyme surrounding developing cartilage		
		brows, maxillary and mandibular prominence	
		perichondrium	
		ocular sclera	
		developing diaphragm	
		region of division of tongue from mandible	
			intestine
		lung	lung
			brain
IGF-II	septum transversum → hepatic bud →	liver	liver
	branchial arch, maxillary process		
	Rathke's pouch		
	developing vasculature	choroid plexus	choroid plexus
	cardiac mesoderm →	heart	heart
		urogenital system →	kidney
	dermatomyotomes	myoblasts and myotubes →	muscle
	sclerotomes	precartilaginous condensations,	
		immature chondrocytes and osteoblasts	
		tongue	
		horizontal prefusion palate	
		lung	lung
		forming of extraocular muscles	
		and ocular sclera	
			brain-stem
			cortex
			hypothalamus
			skin
			thymus
			intestine

During mouse palate development (embryonic day 12-15), developmentally regulated IGF-II mRNA expression was reported. IGF-II gene expression was absent in the vertical palatal shelves (embryonic day 12 to 13). On early embryonal day 14, IGF-II mRNA expression was found in mesenchyme of the horizontal prefusion palate, but not in epithelium. Once palatal fusion had occurred the mRNA expression decreased rapidly to undetectable levels. Since the IGF-II peptide was found predominantly in the epithelium, it was concluded that IGF-II may act in a paracrine way during murine palatogenesis (Ferguson et al., 1992).

IGF gene expression around birth

As shown in table 1, IGF-I mRNA expression has been reported in fetal rat tissues including the liver (Lund et al., 1986). IGF-I mRNA expression was still present in postnatal rat liver, with levels increasing up to 8 weeks (Albiston and Herington 1992). Transcripts for IGF-II have also been described in various tissues before birth (Table 1), however after birth the IGF-II mRNA levels were decreased, with persisting IGF-II mRNA expression only in adult cerebral cortex, hypothalamus and choroid plexus (Brown et al., 1986, Soares et al., 1986, Stylianopoulou et al., 1988b).

Consistent with the IGF-I and IGF-II mRNA expression during rat gestation, IGF-II levels in fetal serum exceed those of IGF-I throughout gestation. At late gestation, the IGF-II levels in fetal rat serum are even 20-100 times higher than in maternal serum, after which they decline to low levels by the time of weaning (Moses et al., 1980, Daughaday et al., 1982).

IGF action in cultured postimplantation embryos

IGF-I and IGF-II have been shown to stimulate growth and development of rat embryos cultured from the early head fold stage. However, the improvement of growth and development observed, did not match normal in vivo embryo development, indicating that other growth factors may also be required (Travers et al., 1992). In addition, studies using IGF inhibitors have suggested that IGF action is needed for normal embryonal development. Culturing of mouse embryos with inhibitors of IGF activity, isolated from streptozotocin-

induced diabetic rats, caused growth retardation and teratogenic effects. Addition of the IGF inhibitors during neurulation (3-5 somites) resulted in craniofacial and neural tube defects, whereas addition during early limb-but development (18-19 somites) resulted in abnormalities of the forebrain and face. In addition, embryos of both stages were growth retarded (Sadler et al., 1986).

IGF action in postimplantation embryos as determined by "gene knock-out" procedures

Gene disruption techniques recently have indicated that both IGF-I and IGF-II are essential for normal embryonic and fetal growth. Disruption of the IGF-I or IGF-II genes has been shown to result in severe growth retardation (birth weight 60% of normal littermates) (De Chiara et al., 1990, Liu et al., 1993). Double mutants carrying both disrupted IGF-I and IGF-II genes displayed an even more severe growth deficiency, reaching only 30% of the normal birth weight. In addition, the IGF-I/IGF-II double mutants died shortly after birth due to respiratory failure (Liu et al., 1993).

Comparison of embryonal growth kinetics revealed that the growth retardation induced by IGF-I gene disruption was first seen at embryonal day 13½, whereas that induced by IGF-II disruption was already seen at embryonal day 11. Furthermore, double mutants carrying disrupted genes of IGF-I and the type I IGF receptor were identical to mutants carrying only the disrupted type I IGF receptor genes, whereas double mutants carrying both the disrupted IGF-II and type I IGF receptor genes displayed a more severe growth retardation (30% of normal birth weight) than mutants carrying only the disrupted IGF-II genes (60% of normal birth weight) or IGF-I receptor genes (45% of normal birth weight) (Baker et al., 1993). Therefore, IGF-I action is probably mediated solely by the type I IGF receptor, whereas IGF-II may also act via another receptor.

Conclusions

The preimplantation embryo expresses IGF-I and IGF-II and is exposed to fluid containing both IGFs, which can stimulate the growth and development of the preimplantation embryo. The IGF-I and IGF-II genes are tissue specifically expressed and their expression is developmentally regulated during postimplantation embryogenesis.

Furthermore, IGF-II mRNA expression is decreased in many tissues after birth. Addition of IGF to cultured embryos stimulates growth and differentiation of the postimplantation embryo, whereas addition of inhibitors of IGF action results in growth retardation and teratogenic effects.

Gene disruption techniques have proven that both IGF-I and IGF-II are essential for normal development of the postimplantation embryo. IGF-II action, most likely, precedes that of IGF-I. In addition, IGF-I acts via the type I IGF receptor, whereas the IGF-II effects are probably mediated also by another receptor (further discussed in 1.4, postimplantation).

In contrast to the addition of inhibitors of IGF activity, disruption of the IGF genes did not lead to abnormalities in craniofacial and neural tube development. Since the specificity of the inhibitors of IGF activity is not known, it is possible that besides the IGF activity, also other growth factor actions are blocked which resulted in the embryopathy found.

Furthermore, it is striking that effects from the ablation of IGF-I or IGF-II genes are first observed at embryonal day 13½ and 11 respectively, especially since expression of both genes has been reported at earlier stages. The possibility remains that translatable IGF mRNA needs to reach a threshold as discussed by Baker et al. However, the loss of IGFs in the knock-out mice may also be partly compensated by either other growth factors or by maternally derived IGFs. Although, IGFs are reported not to cross the placental barrier (Underwood and D'Ercole 1984, Davenport et al., 1990), IGFs and IGFBPs have been shown to cross the endothelium of capillary vessels in the rat heart (Bar et al., 1990a, Bar et al., 1990b, Boes et al., 1992). Therefore, it is still not unlikely that IGFs can cross the placental barrier, either directly or transported by binding proteins. Also, IGF action during the development of the preimplantation embryo can not be excluded, since at that stage the embryo has free access to maternally derived IGFs.

Because of the possible involvement of maternally derived IGFs during embryonal development, it will be most interesting to study the effects of IGF gene disruptions in offspring of IGF knock-out mice. This second generation, will be devoid of both embryonic and extraembryonic IGF sources and therefore is a perfect model to study directly the effects of IGF gene disruptions in pre- and postimplantation embryos.

1.3 THE INSULIN-LIKE GROWTH FACTOR RECEPTORS.

The IGFs exert their biological effects upon binding to specific cell surface receptors. Two types of receptors have been identified for the IGFs: the type I IGF receptor and the IGF-II/ mannose-6-phosphate receptor.

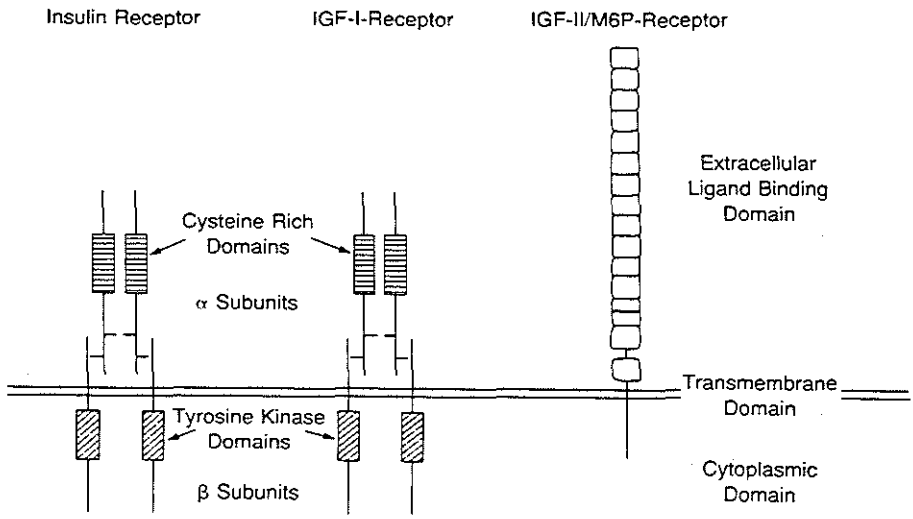

Fig. 2. Schematic representation of the insulin receptor, the type I IGF receptor and the IGF-II/mannose-6-phosphate receptor (from Bach et al., 1991).

The type I IGF receptor.

The type I IGF receptor is a glycosylated protein with a molecular weight of 350 kDa, which displays considerable homology to the insulin receptor (Chernausek et al., 1981, Kasuga et al., 1981, Duronio et al., 1986). The mature receptor consists of two α and two ß subunits together forming a heterotetrameric $\alpha_2ß_2$ structure. The type I IGF receptor has a higher affinity for IGF-I than for IGF-II and can bind insulin 100 times less efficiently than IGF-I (Steele-Perkins et al., 1988). The α subunits, which form the main part of the extracellular domain, contain a cysteine-rich region important for the binding of IGF (Gustafson and Rutter 1990, Kjeldsen et al., 1991). Since a specific antibody was able to block high affinity binding of IGF-I, but not of IGF-II, it has been suggested that the type I IGF receptor contains different binding sites for both IGFs (Casella et al., 1986, Lee et al.,

1986). A tyrosine kinase domain important for signal transduction is located in the large intracellular domain of the ß subunits (Yu et al., 1986). Specific antibodies against the type I IGF receptor have been reported to block the mitogenic effects of both IGF-I and IGF-II in cultured cells, showing that the type I IGF receptor plays an important role in transducing the mitogenic effects of both IGFs (Steele-Perkins and Roth 1990, Pietrzkowski et al., 1992).

Several variant forms of the type I IGF receptor have been described, which differ in their molecular weight, immunological detection, peptide maps and affinity for IGF-I, IGF-II and insulin (Moxham et al., 1989, Garofalo and Rosen 1989, Soos and Sidlle 1989, Treadway et al., 1989, Soos et al., 1990, Garofalo and Barenton 1992, Germain et al., 1992, Barenton et al., 1993, Soos et al., 1993). Some of these receptors were shown to be hybrid receptors containing an insulin receptor $\alpha ß$ dimer in association with a type I IGF receptor $\alpha ß$ dimer. The function of the variant type I IGF receptor forms and hybrid receptors is not clear.

The IGF-II/mannose-6-phosphate receptor.

The IGF-II/mannose-6-phosphate receptor is a glycosylated single chain protein with a molecular weight of approximately 265 kDa (Massague et al., 1981, August et al., 1983). The IGF-II/mannose-6-phosphate receptor is identical to the cation-independent mannose-6-phosphate receptor, which has an important intracellular role in that it directs lysosomal enzymes to the lysosomes (Morgan et al., 1987, Oshima et al., 1988). This receptor has the highest affinity for IGF-II, a much lower affinity for IGF-I and it does not bind insulin (Tong et al., 1988, Nissley and Kiess 1991). One high affinity binding site for IGF-II and two for mannose-6-phosphate-containing proteins have been described, which are located in the extracellular domain (Tong et al., 1988, Tong et al., 1989, Tong and Kornfeld 1989).

The role of the IGF-II/mannose-6-phosphate receptor in IGF signal transduction is unclear. The mitogenic effect of IGF-II on cultured cells could not be blocked by addition of antibodies against the IGF-II/mannose-6-phosphate receptor, whereas antibodies against the type I IGF receptor inhibited growth (Mottola and Czech 1984, Kadowaki et al., 1986, Furlanetto et al., 1987). Furthermore, mutant IGF-II, having a decreased affinity for the type I IGF receptor only, was not able to stimulate growth of mouse preimplantation embryos,

whereas wild type IGF-II did (Rappolee et al., 1992). Thus, the mitogenic effects of the IGFs are most likely mediated via the type I IGF receptor.

It has been proposed that the IGF-II/mannose-6-phosphate receptor may play a role in tissue remodelling, because lysosomal enzymes are involved in remodelling of bone and other tissues during development. The targeting of lysosomal enzymes to the lysosomes as directed by the IGF-II/mannose-6-phosphate receptor may be influenced by binding of IGF-II to the receptor (Dahms et al., 1989, Nissley and Kiess 1991). Furthermore, the IGF-II/mannose-6-phosphate receptor may play a role in the degradation of excess IGF, since this receptor binds and internalizes IGF-II and has a rapid turnover (Moxham and Jacobs, 1992). Therefore, a function of the IGF-II/mannose-6-phosphate receptor in IGF-II physiology and IGF-II signalling can not be excluded.

1.4 THE INSULIN-LIKE GROWTH FACTOR RECEPTORS IN DEVELOPMENT.

Preimplantation.

Cell surface binding of IGF-I and IGF-II was observed as early as the morula and blastocyst stages (Mattson et al., 1988). Using gold-labelled IGF-I and des[1-3]-IGF-I (displaying a decreased affinity for IGFBPs), beginning of specific IGF-I binding was even observed at the eight-cell stage. That IGF was bound to the IGF receptor was demonstrated by the finding that binding was inhibited by an excess of unlabelled IGF-I, but not by insulin. Furthermore, identical binding to the mouse embryos was observed when des[1-3]-IGF-I was used, demonstrating that IGF-I did not bind to IGF binding proteins, but was bound to specific IGF receptors (Smith et al., 1993). Both the type I and II IGF receptor mRNAs have been identified in preimplantation mouse embryos by RT-PCR. Type I IGF receptor protein and mRNA could be found as early as the eight cell stage and transcripts of the IGF-II/mannose-6-phosphate receptor were even detectable in two cell embryos (Rappolee et al., 1990, Schultz et al., 1992, Smith et al., 1993).

The findings that both the type I and IGF-II/mannose-6-phosphate receptors are expressed already in the early stages of the preimplantation embryo indicates that IGFs, either expressed by the embryo itself or maternally derived, can exert their actions in the preimplantation embryo and therefore may influence embryonal growth and development. However, recent gene disruption techniques indicated that disruption of the type I IGF receptor genes resulted in severe growth retardation, being first apparent at embryonal day 11 (further discussed in chapter 1.4, postimplantation), suggesting that the type I IGF receptor is not needed before embryonal day 11. This may mean that the type I IGF receptor is not functional in the preimplantation embryo. Alternatively, it may be that the type I IGF receptor displays important functional properties during preimplantation embryogenesis, but that its loss is compensated by other growth factor systems.

Table 2. Major sites of type I IGF receptor and IGF-II/mannose-6-phosphate receptor mRNA expression in postimplantation embryos and fetuses.

	early gestation	*midgestation*	*late gestation*
Type I IGF Receptor		tongue	
		myotomes	muscle
		sclerotomes	
		mesonephros	kidney
		bowel walls	
		liver	liver
		heart	heart
		pituitary	
		brain	brain
		floorplate of the hindbrain	
		spinal cord	
		spinal ganglia	
			stomach
			lung
IGF-II/M6P receptor	primary endoderm		
	mesoderm moving out from the primitive streak		
		endothelium of the heart tube	
		myo-epicardial mantle → cardiac muscle	heart
		endothelium and smooth muscles associated with developing muscular arteries and arterioles	
		epithelium constituting floor of foregut →	gut
		skeletal muscle	limb muscle
		perichondrium and periosteum	
		liver	liver
		mesenchyme of the lung	lung
			intestine
			kidney
			brain

Postimplantation.

Type I IGF receptor expression in the postimplantation embryo

In postimplantation rat embryos, mRNA expression of the type I IGF receptor has extensively been studied by in situ hybridization and solution hybridization/RNAse protection assays. A summary of these results is given in Table 2.

Widespread expression of the type I IGF transcript was observed, being abundant in tongue, myotomes, vertebral sclerotomes, mesonephros and bowel walls, and at lower levels in liver, heart and pituitary. In addition, mRNA expression of the type I IGF receptor was located in spinal cord, spinal ganglia and the brain, being very abundant in a specialized group of cells in the floorplate of the hind brain (Bondy et al., 1990). Also in fetal rat tissues mRNA expression of the type I IGF receptor has been reported. Using solution hybridization/RNAse protection assays, mRNA expression was observed in liver, brain, muscle, stomach, kidney, heart and lung. After birth, the steady state levels of type I IGF receptor mRNA decreased dramatically in all tissues tested, although the extent of the decrease differed among the various tissues (Werner et al., 1989).

These studies indicate that the type I IGF receptor is expressed in various tissues of the postimplantation embryo. Together with the observation that the mRNA levels decrease after birth, these results imply that the type I IGF receptor displays an important function during postimplantation embryogenesis.

Type I IGF receptor action in postimplantation embryos

The involvement of the type I IGF receptor in development has been proven by gene targeting experiments. Mice carrying a disrupted type I IGF receptor gene, exhibit a severe growth retardation (45% of normal size) and die at birth due to respiratory failure. The animals displayed a generalized organ hypoplasia, delayed ossification and abnormalities in the central nervous system and epidermis (Liu et al., 1993). Growth kinetics of the mouse embryos carrying the disrupted type I IGF receptor gene indicated that the growth retardation is first seen at embryonic day 11 (Baker et al., 1993). Examination of type I IGF receptor mutants, IGF-II mutants and double mutants carrying both the disrupted type I IGF receptor

genes and disrupted IGF-II genes indicated that the IGF-II/type I IGF receptor double mutant displayed a more severe growth retardation then either the IGF-II or type I IGF receptor mutant alone. This difference became apparent at embryonic day 13½, implying that between embryonic day 11 and 12½, IGF-II effects are mediated by the type I IGF receptor and after day 13½, IGF-II may also function through another receptor (Baker et al., 1993). This second class of receptors mediating part of the IGF-II effects was reported unlikely to be the IGF-II/mannose-6-phosphate receptor, since disruption of the type I IGF receptor in a mouse strain carrying the T^{hp} deletion mutant, (which lacks the IGF-II/mannose-6-phosphate receptor gene) did not result in the same phenotype as the IGF-II/type I IGF receptor double mutant (Baker et al., 1993).

As discussed for IGF-I and IGF-II gene disruption, it is most striking that an effect of type I IGF receptor gene disruption was first seen at embryonic day 11. If IGFs play a role at earlier stages of embryonal development, these actions may potentially be mediated by the newly proposed IGF receptor, which also exerts part of the IGF-II effects after embryonal day 13½. The nature of this new IGF receptor is unknown.

IGF-II/mannose-6-phosphate receptor expression in postimplantation embryos

Expression of the IGF-II/mannose-6-phosphate receptor has been extensively studied in rat embryos and fetuses between gestational day 5½ and 21½ by Northern blotting, in situ hybridization and immunocytochemistry (Senior et al., 1990). A summary of these results is given in Table 2.

Expression of the IGF-II/mannose-6-phosphate receptor was first apparent in the primitive embryo at gestational day 8½. At 9½ days, expression was found in the primary endoderm and in the mesoderm moving out from the primitive streak, whereas the ectoderm including the neural folds was negative. The most striking region of expression of the IGF-II/mannose-6-phosphate receptor was found in the cardiovascular system. Both mRNA and protein expression were already found at day 10½ in the endothelium of the heart tube, cells of the myo-epicardial mantle and the developing blood vessels and persisted throughout gestation in endothelial cells, the cardiac muscle and the smooth muscle cells associated with developing muscular arteries and arterioles.

IGF-II/mannose-6-phosphate receptor expression around birth

Transcripts of the IGF-II/mannose-6-phosphate receptor have been described by solution hybridization / RNase protection assays in rat fetal tissues (20 days gestation). The mRNA expression was most abundant in heart, being lower in limb muscle, lung, intestine, kidney and liver and lowest in brain (Sklar et al., 1992), which is in agreement with the protein levels of the IGF-II/mannose-6-phosphate receptor in these tissues (Sklar et al., 1989). In all tissues examined, the level of IGF-II/mannose-6-phosphate receptor mRNA decreases postnatally (Sklar et al., 1992). The widespread expression of the IGF-II/mannose-6-phosphate receptor and the decrease of the expression of this receptor in many tissues after birth, indicates the importance of this receptor in embryonal and fetal life. However, as mentioned before, the function of this receptor in transducing IGF signalling is not clear.

Soluble IGF-II/mannose-6-phosphate receptor

A soluble form of the IGF-II/mannose-6-phosphate receptor has been described, which is 10 kDa smaller than the membrane bound receptor and has been shown to be truncated near the carboxy-terminal end (Kiess et al., 1987, MacDonald et al., 1989). This receptor was found in fetal serum and in conditioned medium of fetal rat heart, skeletal muscle, kidney and liver explants. Fetal rat heart and muscle seamed to be the major source of the soluble IGF-II/mannose-6-phosphate receptor (Bobek et al., 1992).

The role of this IGF receptor is not known. It has been suggested that the soluble form is just a degradation product of the receptor (Clairmont and Czech 1991). Alternatively, it might be that the soluble IGF-II/mannose-6-phosphate receptor plays a role in stabilizing and transporting IGF-II and/or mannose-6-phosphate containing proteins.

Conclusions

The type I IGF receptor and the IGF-II/mannose-6-phosphate receptor are expressed by the preimplantation embryo. In the postimplantation embryo, expression of both IGF receptor genes is tissue specifically and developmentally regulated. After birth, the mRNA levels of the type I IGF receptor and IGF-II/mannose-6-phosphate receptor are decreased in many tissues. Taken together, these observations point towards a function for these receptors during embryonal and fetal development.

Gene disruption techniques have demonstrated that the type I IGF receptor is necessary for normal development of the postimplantation embryo. Most likely, the type I IGF receptor mediates the growth promoting effects of IGF-I, but only part of the IGF-II effects in postimplantation embryos. An effect from the ablation of type I IGF receptor genes was first observed at embryonal day 11, indicating that the type I IGF receptor is not needed before embryonal day 11. This may mean that the type I IGF receptor is not functional in the preimplantation embryo and early postimplantation embryo. Alternatively, the loss of the type I IGF receptor is compensated by other growth factor systems or by the newly proposed IGF receptor which also seems to exert part of the IGF-II effects after embryonal day 13½.

The role of the IGF-II/mannose-6-phosphate receptor in transducing IGF signalling is not clear.

1.5 THE INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS.

The IGFs are found in serum and extracellular fluids complexed to a specific group of proteins: the insulin-like growth factor binding proteins (IGFBPs). The IGFBPs bind IGFs with an affinity equal to the IGF receptors and play important roles in modulating IGF action. One of the functions of the IGFBPs is to prolong the half-life of the IGFs and to restrict the efflux of IGFs from the circulation (Zapf et al., 1986, Guler et al., 1989, Cascieri et al., 1988, Ballard et al., 1991). This has particularly been demonstrated for IGFBP-3, which, in the presence of IGF-I or IGF-II, forms a ternary complex with an approximately 85 kDa glycoprotein. This protein, known as the acid labile subunit, is not related to the IGFBPs or the IGF receptors (Baxter 1988, Baxter and Martin 1989, Leong et al., 1992, Dai and Baxter 1992). Another proposed role of the IGFBPs is to distribute IGFs over the various bodyfluid compartments and transport them to specific cell-types (Bar et al., 1990a, Bar et al., 1990b, Boes et al., 1992). Finally, the IGFBPs have been implicated in modulating IGF action at the cellular level either by inhibiting IGF-receptor interactions and thereby diminishing the IGF response or by facilitating IGF-receptor interactions and thereby stimulating IGF action (for review see: Clemmons 1993).

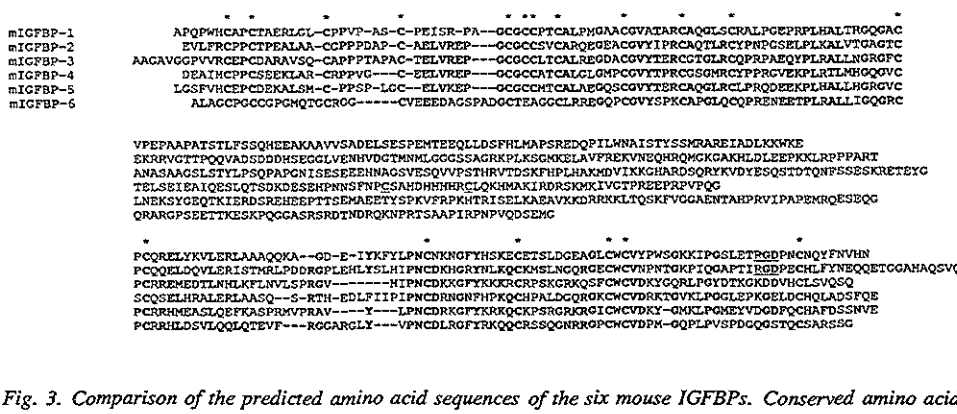

Fig. 3. Comparison of the predicted amino acid sequences of the six mouse IGFBPs. Conserved amino acids are shown bold and cysteine residues are indicated with an aterisk.

Structural aspects of the IGFBPs

At present cDNAs encoding six distinct human, rat and mouse IGFBPs have been isolated and characterized (Brinkman et al., 1988, Murphy et al., 1990, Binkert et al., 1989, Margot et al., 1989, Wood 1988, Shimasaki 1989, Shimasaki 1990, Shimasaki 1991a, Shimasaki et al., 1991b, Chapter 4). Comparison of the primary structure of the six IGFBPs revealed that although they are clearly distinct, they share regions with strong homology in both the cysteine rich N-terminal and C-terminal ends (Drop et al., 1992, Chapter 4). The middle part of the IGFBPs shows no obvious conservation between the six IGFBPs, but is conserved for each IGFBP in mouse, rat and human. This suggests that this region may enclose functional domains important for features unique for each of the IGFBPs (Chapter 4). Furthermore, 18 cysteine residues are spatially conserved across all IGFBPs, with the exception of IGFBP-6 which lacks two (human and mouse) or four (rat) cysteine residues and IGFBP-4, having two additional cysteines (Drop et al., 1992, Chapter 4).

IGFBP-1 and -2 contain an Arg-Gly-Asp (RGD) sequence at the C-terminal end which may be involved in binding to cell surface receptors or integrins (Ruaslathi and Pierschlaber 1987). IGFBP-1 has recently been shown to bind to the $\alpha_5\beta_1$ integrin (fibronectin receptor), whereas IGFBP-1 mutated in the RGD sequence (to Trp-Gly-Asp) did not. Furthermore, IGFBP-1 stimulated migration of Chinese hamster ovary (CHO) cells, which could be blocked by the addition of RGD-containing peptides. Mutant IGFBP-1 had no effect on the migration of CHO cells, demonstrating that IGFBP-1 binds to $\alpha_5\beta_1$ integrin receptors and stimulates CHO cell migration both via an RGD-dependent mechanism (Jones et al., 1993).

The IGF binding domain of the IGFBPs

The function that all six IGFBPs share is binding of IGF. Both the conserved N- and C- terminal ends have been thought to enclose the IGF binding domain since truncated IGFBPs, still capable of binding IGF, have been described, having either an intact N-terminus or C-terminus (Huthala et al., 1986, Wang et al., 1988, Sommer et al., 1991). However, site directed mutagenesis of well conserved regions of both the N- and C- terminal ends did not result in loss of IGF binding. Only mutations presumably leading to conformational changes (like Cys36, Cys226 and Gly222), resulted in loss of IGF binding

capability (Brinkman et al., 1991a, Brinkman et al., 1991b).

Monoclonal antibodies against IGFBP-1 have been described which interfered with the binding of IGF, suggesting that these antibodies bound to or sterically near the IGF binding domain (Rutanen et al., 1988, Chapter 2). Localization of the epitope of one of these monoclonal antibodies, revealed that two regions were needed for antibody recognition. One surrounding amino acids 188-196 and one spanning residue 222-227, both located in the C-terminal part of human IGFBP-1 (Chapter 2).

These findings indicate that the IGF binding domain of the IGFBPs has not yet been identified. The presence of many cysteine residues and the observation that in human IGFBP-1 most if not all are involved in disulphide bonds (Brinkman et al., 1991b), suggests that the IGFBPs are tightly folded proteins. Together with the finding that both C- and N-terminally truncated IGFBPs are capable of binding IGF and the fact that denatured IGFBPs hardly bind IGF, it is not likely that the IGF binding domain is a linear stretch of amino acids. More probably, the IGF binding domain is a three dimensional structure, to which both the N- and C-terminal domains of the IGFBPs contribute.

1.6 THE INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS IN DEVELOPMENT.

Preimplantation.

IGFBPs in the ovary

Before ovulation the developing oocyte bathes in follicular fluid which is rich in IGFBPs. Human follicular fluid has been shown to contain a variety of IGFBPs, including IGFBP-1, -2 and -3 (Giudice et al., 1990). The levels of IGFBPs in follicular fluid are dependent on the developmental stage of the follicles, since it has been shown that fluid obtained from dominant follicles contains decreased levels of IGFBP-2, 28 kDa and 24 kDa IGFBP (presumably IGFBP-4), as compared to fluid obtained from healthy non-dominant and atretic follicles (Chapter 3). In rat ovaries, mRNA expression of all IGFBPs except IGFBP-1 could be detected by in situ hybridization. IGFBP-2 mRNA expression was localized to theca and secondary interstitial cells, whereas IGFBP-3 mRNA was found in subtypes of corpora lutea. IGFBP-4 and -5 mRNAs were detected in granulosa cells of atretic follicles and mRNA expression of IGFBP-6 was found in smooth muscle cells of the theca externa and major blood vessels (Nakatani et al., 1991, Erickson et al., 1992a, Erickson et al., 1993). IGFBP-4 mRNA expression was detected in granulosa cells of atretic and not of healthy follicles. Furthermore, the levels of IGFBP-4 mRNA changes during the estrous cycle (Erickson et al., 1992b).

These observations not only show that the IGFBPs present in follicular fluid may be, at least in part, produced locally, but also raise the question as to what role the IGFBPs may play in ovarian function and folliculogenesis. Interestingly, a decrease in the 28 and 24 kDa IGFBP levels (presumably IGFBP-4) was found in fluid of dominant follicles and mRNA expression of IGFBP-4 in rat ovaries was found to be restricted to atretic follicles. This suggests that IGFBP-4 expression may determine whether the follicle will become dominant or whether it is directed to atresia.

IGFBPs in the reproductive tract

After ovulation and fertilization, the preimplantation embryo moves down the reproductive tract where it is exposed to oviduct and uterine fluid. Using western ligand blotting, the presence of IGFBPs in mouse and rat uterine luminal fluid has been studied, showing that all the major IGFBPs present in serum are also found in uterine fluid (Murphy and Ghahary 1990). Trophoderm isolated from pig blastocysts secrete IGFBPs ranging from 32-42 kDa into the culture medium. These IGFBPs may play a role in the interaction between in uterus-derived IGFs and the trophectoderm, but might also be secreted into the lumen of the blastocyst influencing embryonal development (Corps et al., 1990). Finally, seminal fluid may be an extraembryonic IGFBP source. In human semen the presence of IGFBP-2 and a 24 kD IGFBP (presumably IGFBP-4) has been described (Rosenfeld et al., 1990).

IGFBP expression in preimplantation embryos

In mouse preimplantation embryos, mRNAs for IGFBP-2, -3, -4 and -6 have been detected by RT-PCR (Schultz et al., 1993).

Postimplantation.

After implantation it is not clear whether maternally derived IGFBPs can reach the postimplantation embryo via the placenta. However, the observations that IGFBPs can cross the endothelium of capillary vessels in the rat heart (Bar et al., 1990a, Bar et al., 1990b, Boes et al., 1992), makes it a not unlikely possibility.

IGFBP gene expression in postimplantation embryos

IGFBP gene expression has been studied in whole mouse embryos by Northern blotting. IGFBP-2, -3, -4 and -5 mRNA expression was observed as early as embryonal day 11½, whereas IGFBP-1 mRNA expression was first seen at embryonic day 13½ (Chapter 4).

Table 3. Major sites of IGFBP mRNA expression in postimplantation embryos and fetuses.

	early gestation	*midgestation*	*late gestation*
IGFBP-1		liver	liver
IGFBP-2	egg cylinder surface ectoderm, especially of branchial arches, the lining of the oral cavity and ectodermal placodes neural ectoderm → rostral brain of primary neural tube → neuroepithelium formed in tail bud limb buds, especially the apical ectodermal ridge hindgut and dorsal region of foregut	esophagus stomach intestine	
	notochord anterior splanchnic mesodermal plate liver diverticulum →	liver	liver
	tongue		
		mesonephros	kidney
		choroid plexus	choroid plexus
		floor plate	
		infundibulum	
			brain
			vertebrae
		bronchial epithelium	lung
			skin
			heart
			muscle
IGFBP-3		urogenital tract →	kidney
		nasal epithelia	
		tongue	
		mesenchymal tissue in lower jaw and neck	
		heart	
		lung	
		liver	liver
		muscle	

Table 3. Major sites of IGFBP mRNA expression in postimplantation embryos and fetuses (continued).

	early gestation	*midgestation*	*late gestation*
IGFBP-4		mesencephalon, telencephalon	
		sclerotomes →	vertebrae
		liver	liver
		lung	lung
			kidney
			intestine
		precartilage layers of the sinus	
		precartilaginous wall of the middle nasal meatus	
		tongue	
		muscularis of the esophagus	
		mesenchymal periphery of the larynx	
		endothelial layers of major blood vessels	
IGFBP-5		tongue	
		nasal placode	
		sclerotomes →	vertebrae
			meninges
			lung
			kidney
			intestine
IGFBP-6		liver	liver
		nasal epithelia	
		cells surrounding developing cartilage	
			vertebrae
			lung

The sites of IGFBP mRNA expression during rat and mouse embryogenesis and fetal development have been studied extensively by Northern blotting and in situ hybridization (Binkert et al., 1989, Margot et al., 1989, Stiles and Moats-Staats 1989, Wood et al., 1990, Streck and Pintar 1992, Wood et al., 1992, Streck et al., 1992, Cerro et al., 1993, Green et al., 1993, Price et al., 1993 and Chapter 5, 6 and 7). A summary of these results is given in Table 3.

In rat, IGFBP-2 mRNA expression was detected by in situ hybridization as early as embryonic day 7 in the epiblast of the egg cylinder. Later, IGFBP-2 mRNA expression was found in various tissues, mainly in populations of rapidly dividing cells and in regions that direct the growth and differentiation of neighboring cells and tissues, suggesting that IGFBP-2 may have important roles during organogenesis and the development of fetal tissues. In all stages, the expression of IGFBP-2 was complementary to IGF-II (Wood et al., 1990, Wood et al., 1992). Around embryonic day 9½, mRNA expression of IGFBP-2 was reported in both the endodermal cells still in contact with the lumen of the liver diverticulum and in the endodermal derivatives after invasion of the septum transversum. Taken together with the pattern of IGF-I mRNA expression (discussed further in 1.2), the results suggested that IGF-I and IGFBP-2 are involved in the induction and early growth of the liver (Streck and Pintar 1992).

A potential role for IGFBP-2 in rat limb outgrowth and patterning has also been suggested. Expression of IGFBP-2 was abundant in an anterior-posterior strip of ectoderm along the distal edge of the limb bud (the progenitor of the apical ectodermal ridge) as early as limb stage 1 and continued to characterize the apical ectodermal ridge throughout its existence. Since both IGF-I and -II mRNAs were also abundantly expressed in developing limbs, it was postulated that the IGFs and IGFBP-2 contribute to limb outgrowth and patterning (Streck et al., 1992).

In mice, the mRNA expression of the six IGFBP genes during mid- and late gestation (11-18 days) has been studied by in situ hybridization. Expression of IGFBP-1 was restricted to the liver, whereas transcripts for IGFBP-2, -4 and -5 were detected in various tissues. IGFBP-3 and -6 mRNAs were weakly expressed, only in fetal stages. Comparison of the expression of IGFBP-2, -4 and -5 revealed complementary expression patterns in several

tissues. In embryonal tongue, IGFBP-2 and -5 transcripts were found mainly in the ectodermal layer, whereas mRNA expression of IGFBP-4 was restricted to the mesodermal part of the tongue. A similar expression pattern was found in fetal lung. Here, mRNA expression of IGFBP-2 and -5 where found mainly in respiratory epithelium, whereas IGFBP-4 was found in interstitium (Chapter 5 and 6). Thus, the expression of the six IGFBP genes is tissue specifically and developmentally regulated.

The IGFBPs around birth

The mRNA expression of the IGFBPs has been studied in perinatal mouse liver. IGFBP-1 mRNA levels were detectable before birth (embryonic day 18), increased dramatically the first days after birth, after which they decreased to low levels. IGFBP-2 mRNA expression also increased after birth but remained abundant until adulthood. IGFBP-3 and -4 mRNAs were detected in fetal mouse liver, increased slowly after birth, reaching maximum levels 1 week postnatally (Chapter 4).

In rat, abundant mRNA expression of IGFBP-1 and -2 has been reported in liver around birth, decreasing to low levels in adult liver (Ooi et al., 1990). Run-on experiments have indicated that the transcriptional activity of the IGFBP-1 and -2 genes in liver is increased 25 and 5 times respectively at birth, as compared to 16 day fetal liver. IGFBP-1 and -2 mRNA levels were increased 50 times and 5-10 times respectively at birth, after which IGFBP-1 mRNA levels decreased, whereas the levels of IGFBP-2 mRNA remained constant (Babajko et al., 1993). IGFBP-3 mRNA expression has been reported in livers of 1 day old rats. Levels were increased in livers of 1 week old rats, remaining constant thereafter (Albiston and Herington 1992).

IGFBP-2 mRNA expression has also been reported in various other fetal rat tissues. In the first weeks after birth IGFBP-2 mRNA expression decreases to low levels in at least kidney, intestine and lung (Brown et al., 1989, Orlowski et al., 1990).

Conclusions

All six IGFBP genes are expressed in postimplantation rodent embryos, IGFBP-2, -3, -4 and -5 before or at embryonic day 11½ and IGFBP-1 and -6 at least at 13½-14 days

gestation. This is of special interest since gene disruption techniques have indicated that both IGFs are essential for normal embryonic and fetal development. The effects of IGF-I and IGF-II gene disruption were first seen at gestational day 11 for IGF-II and 13½ for IGF-I, times when at least four, if not all, IGFBP genes are expressed. Therefore, the IGFBPs may mediate IGF action in the postimplantation embryo.

The six IGFBP genes are developmentally regulated in a tissue specific manner. The expression patterns are partly overlapping, but distinct. In general, the IGF-II and IGFBP-2 genes are complementarily expressed in the postimplantation embryo. This is, at least in some tissues, also true for the IGFBP-4 and -5 genes.

The expression of IGFBP-2 in rapidly dividing cells and in cell populations that direct the growth and differentiation of other cells, implicates that IGFBP-2 is involved in the development of several embryonic and fetal tissues. The decrease of IGFBP-2 mRNA expression in non-neural tissues after birth suggests a more pronounced function for this IGFBP before birth. Although this may be the case, several adult mouse tissues still express the IGFBP-2 gene (Chapter 4). Furthermore, metabolic changes like fasting, protein deprivation and diabetes result in increased serum IGFBP-2 levels and hepatic IGFBP-2 mRNA levels (Orlowski et al., 1990, Ooi et al., 1990, Straus and Takemoto 1990, Boni-Schnetzler et al., 1989). Therefore, IGFBP-2 may fulfil its action in adult life as well.

The high hepatic IGFBP-1 mRNA levels during the first 3 days after birth suggests that this IGFBP has a function during the first days of life either in the liver itself or in the circulation.

The physiological role of the six IGFBPs in pre- and postimplantation embryos is still unclear. They presumably prolong the half-life of the IGFs, protect the embryo from hypoglycemic effects, transport IGFs to their target tissues and play an important role in mediating IGF action at the cellular level. The findings that IGFBPs are assessable to the embryo at all stages of development shows that this group of proteins can mediate IGF action during embryonal growth and differentiation. Therefore, the six IGFBPs, different in respect to expression, IGF affinity, cell specific attachment and sensitivity to proteases may determine the specificity of IGF activity and may be necessary for controlled IGF action.

1.7 SCOPE OF THE THESIS.

The IGFs are important mitogens involved in embryonal growth and development. The expression of the IGF and IGF receptor genes during embryogenesis is developmentally regulated in a tissue specific fashion. Recent gene disruption strategies have confirmed the importance of IGF-I, IGF-II and the type I IGF receptor for normal embryonal growth and development.

In this thesis a region potentially involved in the interaction of IGFBP-1 with IGF is described. Monoclonal antibodies specific for human IGFBP-1 were raised, their influence on the binding of IGF to IGFBP-1 was determined, and their epitopes were established (Chapter 2). When it became evident that the IGFBP family consists of six structurally related, but distinct, proteins, we hypothesized that the six IGFBPs might each have a discrete function. To address this question, we concentrated on the possible involvement of the IGFBPs in development and studied the developmentally regulated expression of the IGFBPs and their tissue specificity. The expression of IGFBPs during folliculogenesis was studied in human follicular fluid obtained from follicles in different stages of maturation (Chapter 3). The cDNAs encoding the six mouse IGFBPs have been isolated and characterized and the mRNA expression of the six mouse IGFBPs has been studied in whole embryos, perinatal liver and several adult tissues (Chapter 4). In order to study the localization of the six mouse IGFBP mRNAs during mouse embryonal and fetal development and to investigate whether the IGFBP genes were coexpressed or complementary expressed, *in situ* hybridization techniques were used (Chapter 5). In addition, the mRNA expression of the mouse IGFBPs was studied in relation to the expression of the IGFs and the type I IGF receptor during lung and kidney development (Chapter 6 and 7).

1.8 REFERENCES.

1 Adashi, E.Y., Resnick, C.E., D'Ercole, J., Svoboda, M.E. and Van Wyk, J.J. (1985a) Somatomedin-C-mediated potentiation of follicle-stimulating hormone-induced aromatase activity of cultured rat granulose cells. Endocrinology 117, 2313-2320.

2 Adashi, E.Y., Resnick, C.E., Brodie, A.M., Svoboda, M.E. and Van Wyk, J.J. (1985b) Somatomedin-C enhances induction of luteinizing hormone receptors by follicle-stimulating hormone in cultured rat granulosa cells. Endocrinology 116, 2369-2375.

3 Adashi, E.Y., Resnick, C.E., Brodie, A.M. and Van Wyk, J.J. (1985c) Somatomedin-C synergizes with follicle stimulating hormone in the acquisition of progesterone biosynthetic capacity by cultured rat granulosa cells. Endocrinology 116, 2135-2142.

4 Adashi, E.Y., Resnick, C.E., Svoboda, E.M., Van Wyk, J.J., Hascall, V.C. and Yanagishita, M. (1986) Independent and synergistic actions of somatomedin-C in the stimulation of proteoglycan biosynthesis by cultured rat granulosa cells. Endocrinology 118, 456-458.

5 August, G.P., Nissley, S.P., Kasuga, M., Lee, L., Greenstein, L. and Rechler, M.M. (1983). Purification of an insulin-like growth factor II receptor from rat chondrosarcoma cells. J. Biol. Chem. 258, 9033-9036.

6 Albiston, A.L. and Herington, A.C. (1992). Tissue distribution and regulation of insulin-like growth factor (IGF)-binding protein-3 messenger ribonucleic acid (mRNA) in the rat: Comparison with IGF-I mRNA expression. Endocrinology 130, 497-502.

7 Babajko, S., Hardouin, S., Segovia, B., Groyer, A. and Binoux, M. (1993). Expression of insulin-like growth factor binding protein-1 and -2 genes through the perinatal period in the rat. Endocrinology 132, 2586-2592.

8 Baker, J., Liu, J-P., Robertson, J.E. and Efstratiadis, A. (1993). Role of insulin-like growth factors in embryonic and postnatal growth. Cell 75, 73-82.

9 Ballard, F.J., Knowles, S.E., Walton, P.E., Edson, K., Owens, P.C., Mohler, M.A. and Ferraiolo, B.L. (1991). Plasma clearance and tissue distribution of labelled IGF-I, -II and des(1-3) IGF in rats. J. Endocrinol. 128: 197-204.

10 Bar, R.S., Boes, M., Clemmons, D.R., Busby, W.H., Sandra, A., Dake, B.L. and Booth, B.A. (1990a). Insulin differentially alters transcapillary movement of intravascular IGFBP-1, IGFBP-2 and endothelial cell IGF binding protein in the rat heart. Endocrinology 127: 497-499.

11 Bar, R.S., Clemmons, D.R., Boes, M., Busby, W.H., Booth, B.A., Dake, B.L. and Sandra, A. (1990b). Transcapillary permeability and subendothelial distribution of endothelial and amniotic fluid insulin-like growth factor binding proteins in the rat heart. Endocrinology 127, 1078-1086.

12 Barenton, B., Domeyne, A., Garandel, V. and Garofalo, R.S. (1993) A developmentally regulated form of insulin-like growth factor receptor beta-subunit in C2 myoblasts exhibiting altered requirements for differentiation. Endocrinology 133, 651-660.

13 Baxter, R.C., Martin, J.L. and Handelsman, D.J. (1984). Identification of human semen insulin-like growth factor-I/somatomedin-C immunoreactivity and binding protein. Acta Endocrinol. 106, 420-427.

14 Baxter, R.C. (1988). Characterization of the acid-labile sub-unit of the growth hormone dependent insulin-like growth factor binding protein. J. Clin. Endocrinol. Metab.67, 265-272.

15 Baxter, R.C. and Martin, J.L. (1989) Structure of the Mr 140,000 growth hormone dependent insulin-like growth factor binding complex: Determination by reconstruction and affinity labeling. Proc. Natl. Acad. Sci. USA. 86, 6898-6902.

16 Beck, F., Samani, N.J., Penschow, J.D., Thorley, B., Tregear, G.W. and Coghlan, J.P. (1987). Histochemical localization of IGF-I and -II mRNA in the developing rat embryo. Development 101, 175-184.

17 Binkert, C., Landwehr, J., Mary, J.L., Schwander, J. and Heinrich, G. (1989). Cloning, sequence analysis, and expression of a cDNA encoding a novel IGF binding protein (IGFBP-2). EMBO J 8: 2497-2502.

18 Blundell, T.L. and Humbel, R.E. (1980) Hormone families: pancreatic hormones and homologous growth factors. Nature 287, 781-787.

19 Blundell, T.L., Bedarkar, S. and Humbel, R.E. (1983) Tertiairy structures, receptor binding and antigenicity of insulin-like growth factors. Federation Proc. 42, 2592-2597.

20 Bobek, G., Scott, C.D. and Baxter, R.C. (1992). Radioimmunoassay of soluble insulin-like growth factor-II/mannose 6-phosphate receptor: Developmental regulation of receptor release by rat tissues in culture. Endocrinology 130, 3387-3394.

21 Boes, M., Booth, B.A., Sandra, A., Dake, B.L., Bergold, A. and Bar, R.S. (1992). Insulin-like growth factor binding protein (IGFBP)4 accounts for the connective tissue distribution of endothelial cell IGFBPs perfused through the isolated heart. Endocrinology 131, 327-330.

22 Bondy, C.A., Werner, H., Roberts Jr., C.T. and LeRoith D. (1990). Cellular pattern of insulin-like growth fact-I (IGF-I) and type I IGF receptor gene expression in early organogenesis: Comparison with IGF-II gene expression. Mol. Endocrinol. 4, 1386-1398.

23 Boni-Schnetzler, M., Binz, K., Mary, J-L., Schmid, C., Schwander, J. and Froesch, E.R. (1989) Regulation of hepatic expression of IGF-I and fetal IGF binding protein mRNA in streptozotocin-diabetic rats. FEBS Lett. 251, 253-256.

24 Bowman, P. and McLaren, A. (1970). Cleavage of rat and mouse embryos in vivo and in vitro. J. Embryol. Expl. Morphol. 24,203-207.

25 Brinkman, A., Groffen, C., Kortleve, D.J., Geurts van Kessel, A. and Drop, S.L.S. (1988). Isolation and characterization of a cDNA encoding the low molecular weight insulin-like growth factor-binding protein (IBP-1). EMBO. J. 7: 2417-2423.

26 Brinkman, A., Kortleve, D.J., Zwarthoff, E.C. and Drop, S.L.S. (1991a) Mutations in the C-terminal part of IGF binding protein-1 result in dimer formation and loss of IGF binding capacity. Mol. Endo. 5, 987-994.

27 Brinkman, A., Kortleve, D.J., Schuller, A.G.P., Zwarthoff, E.C. and Drop, S.L.S. (1991b) Site-directed mutagenesis of the N-terminal region of IGF binding protein 1; analysis of IGF binding capacity. FEBS letters 291, 264-268.

28 Brown, A.L., Graham, D.E., Nissley, S.P., Hill, D.J., Strain, A.J. and Rechler, M.M. (1986). Developmental regulation of insulin-like growth factor II mRNA in different rat tissues. J. Biol. Chem. 261, 13144-13150.

29 Brown, A.L., Chiariotti, L., Orlowski, C.C., Mehlman, T., Burgess, W.H., Ackerman, E.J., Bruni, C.B. and Rechler, M.M. (1989) Nucleotide sequence and expression of a cDNA clone encoding a fetal rat binding protein for insulin-like growth factors. J. Biol. Chem. 264, 5148-5154.

30 Carlsson, B., Hillensjö, Nilsson, A., Törnell, J. and Billig, H. (1993) Expression of insulin-like growth factor-I (IGF-I) in the rat fallopian tube: Possible autocrine and paracrine action of fallopian tube-derived IGF-I on the fallopian tube and on the preimplantation embryo. Endocrinology 133, 2031-2039.

31 Cascieri, M.A., Saperstein, R.S., Hayes, N.S., Green, B.G., Chicchi, G.G., Applebaum, J. and Bayne, M.L. (1988). Serum half-life and biological activity of mutants of human IGF-I which do not bind to serum binding proteins. Endocrinology 123: 373-381.

32 Casella, S.J., Han, V.K., D'Ercole, J., Svoboda, M.E. and Van Wyck, J.J. (1986) Insulin-like growth factor II binding to type I somatomedin receptor: evidence for two high affinity binding sites. J. Biol. Chem. 261, 9268-9273.

33 Cerro, J.A., Grewal, A., Wood, T.L. and Pintar, J.E. (1993). Tissue-specific expression of the insulin-like growth factor binding protein (IGFBP) mRNAs in mouse and rat development. Regulatory Peptides 48, 189-198.

34 Chernausek, S.K., Jacobs, S. and Van Wyk, J.J. (1981). Structural similarities between human receptors for somatomedin C and insulin: analysis by affinity labelling. Biochem. 20, 7345-7350.

35 Cheung, P.T., Smith, E.P., Shimasaki, S., Ling, N. and Chernausek, S.D. (1991) Characterization of an insulin-like growth factor binding protein (IGFBP-4) produced by the B104 rat neuronal cell line: chemical and biological properties and differential synthesis by sublines. Endocrinology 129, 1006-1015.

36 Clairmont, K.B. and Czech, M.P. (1991). Extracellular release as the major degradative pathway of the insulin-like growth factor-II/mannose 6-phosphate receptor. J. Biol. Chem. 266, 12131-12134.

37 Clemmons, D.R. (1993). IGF binding proteins: Regulation of cellular actions. Growth Regulation 2, 80-87.

38 Cohick, W.S. and Clemmons, D.R. (1993). The insulin-like growth factors. Ann. Rev. Physiol. 55, 131-153.

39 Cooke, R.M., Harvey, T.S. and Campbell, I.D. (1991) Solution structure of human insulin-like growth factor I: a nuclear magnetic resonance and restrained molecular dinamics study. Biochemistry 30, 5484-5491.

40 Corps, A.N., Brigstock, D.R., Littlewood, C.J. and Brown, K.D. (1990). Receptors for epidermal growth factor and insulin-like growth factor-I on preimplantation trophoderm of the pig. Development 110, 221-227.

41 Croze, F., Kennedy, T.G., Schroedter, I.C., Friesen, H.G. and Murphy, L.J. (1990). Expression of insulin-like growth factor-I and insulin-like growth factor binding protein-1 in the rat uterus during decidualization. Endocrinology 127, 1995-2001.

42 Dahms, N.M., Lobel, P. and Kornfeld, S. (1989). Mannose 6-phosphate receptors and lysosomal enzyme targeting. J. Biol. Chem. 264, 12115-12118.

43 Dai, J. and Baxter, R.C. (1992) Molecular cloning of the acid-labile subunit of the rat insulin-like growth factor binding protein complex. Bioch. Bioph. Res. Comm. 188, 304-309.

44 Daughaday, W.H., Hall, K., Raben, M.S., Salmon Jr, W.D., Van den Brande, J.L. and Van Wyk J.J. (1972). Somatomedin: proposed designation for sulphation factor. Nature 235, 107.

45 Daughaday, W.H., Parker, K.A., Borowsky, S., Trivedi, B. and Kapadia, M. (1982). Measurement of somatomedin-related peptides in fetal, neonatal and maternal rat serum by IGF-I radioimmunoassay, IGF-II radioreceptor assay (RRA) and multiplication stimulating activity RRA after acid-ethanol extraction. Endocrinology 110, 575-581.

46 Davenport, M.L., Clemmons, D.R., Miles, M.V., Camacho-Hubner, C., D'Ercole, J.A. and Underwood, L.E. (1990) Regulation of serum insulin-like growth factor-I (IGF-I) and IGF binding proteins during rat pregnancy. Endocrinology 127, 1278-1286.

47 DeChiara, T.M., Efstratiadis, A. and Robertson, E.J. (1990) A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. Nature 345, 78-80.

48 Drop, S.L.S., Schuller, A.G.P., Lindenbergh-Kortleve, D.J., Groffen, C., Brinkman, A. and Zwarthoff, E.C. (1992). Structural aspects of the IGFBP family. Growth Regulation 2, 69-79.

49 Duronio, V., Jacobs, S. and Cuatrecasas, P. (1986). Complete glycosilation of the insulin and insulin-like growth factor I receptors is not necessary for their biosynthesis and function: the use of swainsonine as an inhibitor in IM-9 cells factor I. J. Biol. Chem. 261, 970-975.

50 Eden, I.A., Jones, J., Carter, G.D. and Alaghband-Zadeh, J. (1988).A comparison of follicular fluid levels of insulin-like growth factor-I in normal dominant and cohort follicles, polycystic and multicystic ovaries. Clin. Endocrinol. 29, 327-336.

51 Erickson, G.F., Nakatani, A., Ling, N. and Shimasaki, S. (1992a). Localization of insulin-like growth factor binding protein-5 (IGFBP-5) mRNA in adult rat ovaries. Endocrinology 130, 1867-1878.

52 Erickson, G.F., Nakatani, A., Ling, N. and Shimasaki, S. (1992b). Cyclic changes in insulin-like growth factor binding protein-4 (IGFBP-4) mRNA in the rat ovary. Endocrinology 130, 625-636.

53 Erickson, G.F., Nakatani, A., Liu, X.-J., Shimasaki, S. and Ling, N. (1993). The role of IGF-I and IGFBPs in folliculogenesis. In: Findlay, J.K. (ed) Cellular and molecular mechanisms in female reproduction. Academic Press.

54 Ewton, D.Z. and Florini, J.R. (1981). Effects of somatomedins and insulin on myoblast differentiatin in vitro. Developmental Biology 56, 31-39.

55 Ferguson, M.W., Sharpe, P.M., Thomas, B.L. and Beck, F. (1992) Differential expression of insulin-like growth factors I and II (IGF I and II), mRNA, peptide and binding protein 1 during mouse palate development: comparison with TGF beta peptide distribution. J. Anat. 181, 219-238.

56 Fielder, P.J., Thordarson, G., Talamantes, F. and Rosenfeld, R. (1990) Characterization of insulin-like growth factor binding proteins (IGFBPs) during gestation in mice: effects of hypophysectomy and an IGFBP-specific serum protease activity. Endocrinology 127, 2270-2280.

57 Furlanetto, R.W., DiCarlo, J.N. and Wisehart, C. (1987) The type II insulin-like growth factor receptor does not mediate deoxyribonucleic acid synthesis in human fibroblasts. J. Clin. Endocrinol. Metab. 64, 1142-1149.

58 Florini, J.R., Ewton, D.Z. and Roof, S.L. (1991). Insulin-like growth factor-I stimulates terminal myogenic differentiation by induction of myogenin gene expression. Mol. Endocrinol. 5, 718-724.

59 Froesch, E.R., Schmid, C., Schwander, J. and Zapf, J. (1985). Actions of insulin-like growth factors. Ann. Rev. Physiol. 47,443-467.

60 Garofalo, R.S. and Rosen, O.M. (1989) Insulin and insulin-like growth factor (IGF) I receptors during central nervous system development: expression of two immunologically distinct IGF I receptor beta subunits. Mol. Cell. Biol. 9, 2806-2817.

61 Garofalo, R.S. and Barenton, B. (1992) Functional and immunological distinction between insulin-like growth factor I receptor subtypes in KB cells. J. Biol. Chem. 267, 11470-11475.

62 Germain-Lee, E.L., Janicot, M., Lammers, R., Ullrich, A. and Casella, S.J. (1992) Expression of a type I insulin-like growth factor receptor with low affinity for insulin-like growth factor II. Biochem. J. 218, 413-417.

63 Ghahary, A., Chakrabarti, S. and Murphy, L.J. (1990) Localization of the sites of synthesis and action of insulin-like growth factor-I in the rat uterus. Mol. Endo. 4, 191-195.

64 Giudice, L.C., Farrell, E.M., Pham, H. and Rosenfeld, R.G. (1990). Identification of insulin-like growth factor binding protein-3 (IGFBP-3) and IGFBP-2 in human follicular fluid. J. Clin. Endocrinol. Metab. 71, 1330-1338.

65 Gray, A., Tam, A.W., Dull, T.J., Hayflick, J., Pintar, J., Cavenee, W.K., Koufos, A. and Ullrich, A. (1987). Tissue-specific and developmentally regulated transcription of the insulin-like growth factor 2 gene. DNA 6, 283-295.

66 Green, B., Jones, S.B., Streck, R., Rotwein, P. and Pintar, J.E. (1994). Distinct expression patterns of insulin-like growth factor binding proteins-2 and -5 during fetal and postnatal development. Endocrinology, 134, 954-962.

67 Guler, H.P., Zapf, J., Schmid, C. and Froesch, E.R. (1989). IGF-I and -II in healthy man; estimation of half-lives and production rates. Acta Endocrinol 121: 753-758.

68 Gustafson, T.A. and Rutter, W.J. (1990). The cysteine-rich domains of the insulin-like growth factor I receptors are primary determinants of hormone binding specificity: evidence from receptor chimeras. J. Biol. Chem. 265, 18663-18667.

69 Harlow, G.M. and Quinn, P. (1982). Development of preimplantation mouse embryo in vivo and in vitro. Aust. J. Biol. Sci. 35, 187-193.

70 Harvey, M.B. and Kaye, P.L. (1992). Insulin-like growth factor-I stimulates growth of mouse preimplantation embryo in vitro. Mol. Reprod. Dev. 31, 195-199.

71 Hernandez, E.R., Roberts, C.H., Hurwitz, A., LeRoith, D. and Adashi, E. Y. (1990). Rat ovarian insulin-like growth fgactor II gene expression is theca-interstitial cell-exclusive: Hormonal regulation and receptor distribution. Endocrinology 127, 3249-3251.

72 Hernandez, E.R., Hurwitz, A., Vera, A., Pellicer, A., Adashi, E.Y., LeRoith, D. and Roberts, C.T. Jr. (1992). Expression of the genes encoding the insulin-like growth factors and their receptors in the human ovary. J. Clin. Endocrinol. Metab. 74, 419-425.

73 Heyner, S., Smith, R.M. and Schultz, G.A. (1989). Temporally regulated expression of insulin and insulin-like growth factors and their receptors in early mammalian development. Bioessays 11, 171-176.

74 Humbel, R.E. (1990). Insulin-like growth factors I and II. Eur. J. Biochem. 190, 445-462.

75 Huthala, M.L., Koistinen, R., Palomäki, P., Partanen, P., Bohn, H. and Seppälä, M. (1986) Biological active domain in somatomedin-binding protein. Biochem. Biophys. Res. Commun. 141, 263-270.

76 Jones, J.I., Gockerman, A., Busby, W.H.Jr., Wright G. and Clemmons, D.R. (1993) Insulin-like growth factor binding protein 1 stimulates cell migration and binds to the $\alpha_5\beta_1$ integrin by means of its Arg-Gly-Asp sequence. Proc. Natl. Acad. Sci. USA 90, 10553-10557.

77 Kadowaki, T., Koyasu, S., Nishida, E., Sakai, H., Takaku, F., Yahara, I. and Kasuga, M. (1986). Insulin-like growth factors, insulin, and epidermal growth factor cause rapid cytoskeleton reorganization in KB cells. J. Biol. Chem. 261, 16141-16147.

78 Kasuga, M., Van Obberghen, E., Nissley, S.P. and Rechler, M.M. (1981). Demonstration of two subtypes of insulin-like growth factor receptors by affinity crosslinking. J. Biol. Chem. 256, 5305-5308.

79 Kiess, W., Greenstein, L.A., White, R.M., Lee, L., Rechler, M.M. and Nissley, S.P. (1987). Type II insulin-like growth factor receptor is present in rat serum. Proc. Natl. Acad. Sci. USA. 84, 7720-7724.

80 Kjeldsen, T., Andersen, A.S., Wilberg, F.C., Rasmussen, J.S., Schäffer, L., Baldschmidt, P., Möller, K.B. and Möller, N.P.H. (1991). The ligand specificities of the insulin receptor and the insulin-like growth factor I receptor recide in different regions of a common binding site. Proc. Natl. Acad. Sci. USA. 88, 4404-4408.

81 Lee, P.D.K., Rosenfeld, R.G., Hintz, R.L. and Smith, S.D. (1986) Characterization of insulin, insulin-like growth factors I and II and growth hormone receptors on human leukemic lymphoblasts. J. Clin. Endocrinol. Metab. 62, 28-35.

82 LeDouarin, N. (1975) An experimental analysis of liver development. Med. Bio. 53, 427-455.

83 Leong, S.R., Baxter, R.C., Camerato, T., Dai, J. and Wood, W.I. (1992) Structure and functional expression of the acid-labile subunit of the insulin-like growth factor-binding protein complex. Mol. Endocrinol. 6, 870-876.

84 Liu, J-P., Baker, J., Perkins, A.S., Robertson, J.E. and Efstratiadis, A. (1993). Mice carrying nul mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell 75, 59-72.

85 Lund, P.K., Moats-Staats, B.M., Hynes, M.A., Simmons, J.G., Jansen, M., D'Ercole, A.J. and Van Wyk, J.J. (1986). Somatomedin-C/insulin-like growth factor-I and insulin-like growth factor II mRNAs in rat fetal and adult tissues. J. Biol. Chem. 261, 14539-14544.

86 MacDonald, R.G., Tepper, M.A., Clairmont, K.B., Perregaux, S.B. and Czech, M.P. (1989). Serum form of the rat insulin-like growth factor-II/mannose 6-phosphate receptor is truncated in the carboxyl-terminal domain. J. Biol. Chem. 264, 3256-3261.

87 Margot, J.B., Binkert, C., Mary, J.-L., Landwehr, J., Heinrich, G. and Schwander, J. (1989). A low molecular weight insulin-like growth factor binding protein from rat: cDNA cloning and tissue distribution of its messenger RNA. Mol. Endocrinol. 3, 1053-1060.

88 Massague, J., Guillette, B.J. and Czech, M.P. (1981). Affinity labeling of multiplication stimulating activity receptors in membranes from human and rat tissues. J. Biol. Chem. 256, 2122-2125.

89 Mattson, B.A., Rosenblum, I.Y., Smith, R.M. and Heyner, S. (1988). Autoradiographic evidence for stage specific insulin binding to mouse embryo development. Diabetes 37, 585-590.

90 Morgan, D.O., Edman, J.C., Standing, D.N., Fried, V.A., Smith, M.C., Roth, R.A. and Rutter, W.J. (1987). Insulin-like growth factor II receptor as a multifunctional binding protein. Nature 329, 301-307.

91 Moses, A.C. et al., (1980). Elevated levels of multiplication-stimulated activity, an insulin-like growth factor, in fetal rat serum. Proc. Natl. Acad. Sci. USA. 77, 3649-2653.

92 Mottola, C. and Czech, M.P. (1984). The type II IGF receptor does not mediate increased DNA synthesis in H-35 hepatoma cells. J. Biol. Chem. 259, 12705-12713.

93 Moxham, C.P., Duronio, V. and Jacobs, S. (1989) Insulin-like growth factor I receptor ß-subunit heterogeneity. Evidence for hybrid tetramers composed of insulin-like growth factor I and insulin heterodimers. J. Biol. Chem. 264, 13238-13244.

94 Moxham, C. and Jacobs, S. (1992) Insulin-like growth factor receptors. In: Schofield, P.N. (ed) The insulin-like growth factors. Oxford University Press, Oxford, pp 80-109.

95 Murphy, L.J., Bell, G.I. and Friesen, H.G. (1987). Tissue distribution of insulin-like growth factor I and II mesenger ribonucleic acids in the adult rat. Endocrinology 120, 1279-1281.

96 Murphy, L.J. and Ghahary, A. (1990). Uterine insulin-like growth factor-I: Regulation of expression and its role in estrogen-induced uterine proliferation. Endocrin. Rev. 11, 443-453.

97 Murphy, L.J., Seneviratne, C., Ballejo, G., Croze, F. and Kennedy, T.G. (1990). Identification and characterization of a rat decidual IGF binding protein complementary DNA. Mol. Endocrinol. 4: 329-336.

98 Nakatani, A., Shimasaki, S., Erickson, G.F. and Ling, N. (1991). Tissue specific expression of four insulin-like growth factor binding proteins (1, 2, 3 and 4) in the rat ovary. Endocrinology 129, 1521-1529.

99 Nissley, S.P. and Kiess, W. (1991). Binding of insulin-like growth factor-II (IGF-II) and lysosomal enzymes to the IGF-II/Mannose-6-phosphate receptor. In: Modern concepts of insulin-like growth factors. E.M. Spencer (ed). Elsevier Science Publishing Co., Inc. New York, 419-430.

100 Oliver, J.E., Aitman, T.J., Powell, J.F., Wilson, C.A. and Clayton, R.N. (1989). Insulin-like growth factor gene expression in rat ovary is confined to the granulosa cells of developing follicles. Endocrinology 124, 267-279.

101 Ooi, G.T., Orlowski, C.C., Brown, A.L., Becker, R.E., Unterman, T.G. and Rechler, M.M. (1990). Different tissue distribution and hormonal regulation of messenger RNAs encoding rat insulin-like growth factor-binding proteins-1 and -2. Mol. Endocrinol. 4, 321-328.

102 Orlowski, C.C., Brown, A.L., Ooi, G.T., Yang, W.-H., Tseng, L.Y.-H. and Rechler, M.M. (1990). Tissue, developmental, and metabolic regulation of messenger ribonucleic acid encoding a rat insulin-like growth factor-binding protein. Endocrinology 126, 644-652.

103 Oshima, A., Molan, C.M., Kyle, J.W., Grubb, J.H. and Sly, S.W. (1988). The human cation-independent mannose-6-phosphate receptor. Cloning and sequence of the full-lenght cDNA and expression of functional receptor in COS cells. J. Biol. Chem. 263, 2553-2562.

104 Pietrzkowski, Z., Sell, C., Lammers, R., Ullrich, A. and Baserga, R. (1992). Roles of insulin-like growth factor I (IGF-I) and the IGF-I receptor in epidermal growth factor-stimulated growth of 3T3 cells. Mol. Cell. Biol. 12, 3883-3889.

105 Price, W.A., Moats-Staats, B.M., D'Ercole, A.J. and Stiles, A.D. (1993) Insulin-like growth factor binding protein production and regulation in fetal rat lung cells. Am. J. Respir. Cell Mol. Biol. 8, 425-432.

106 Rappolee, D.A., Sturm, K., Schultz, G.A., Pedersen, R.A. and Werb, Z. (1990). The expression of growth factor ligands and receptors in preimplantation mpouse embryos. In: S. Heyer, L. Wiley (eds): "Early Embryo Development and Paracrine Relationships." UCLA Symposia on Molecular and Cellular Biology, New Series, Vol. 117. New York: Wiley-Liss, pp 11-25.

107 Rappolee, D.A., Sturm, K.S., Behrendtsen, O., Schultz, G.A., Pedersen, R.A. and Werb, Z. (1992). Insulin-like growth factor II acts through an endogenous growth pathway regulated by imprinting in early mouse embryos. Genes Dev. 6, 939-952.

108 Ramasharma, K., Cabrera, C.M. and Li, C.H. (1986). Identification of insulin-like growth factor-II in human seminal and follicular fluids. Biochem. Biophys. Res. Commun. 140, 536-542.

109 Ramasharma, K. and Li, C.H. (1987). Human pituitary and placental hormones control human insulin-like growth factor II secretion in human granulosa cells. Proc. Natl. Acad. Sci. USA. 84, 2643-2647.

110 Raschdorf, F., Dahinden, R., Maerki, W., Richter, W.J. and Merrywater, J.P. (1988) Localization of disulphide bonds in insulin-like growth factors (IGFs) synthesized by recombinant DNA technology. Biomed. Env. Mass Spec. 16, 3-8.

111 Recio-Pinto, E., Lang, F.L. and Ishii, D.N. (1984). Insulin and insulin-like growth factor II permit nerve growth factor binding and the neurite formation response in cultured human neuroblastoma cells. Proc. Nat. Acad. Sci. USA. 81, 2562-2566.

112 Reeve, J.G., Morgan, J., Schwander, J. and Bleehen, N.M. (1993) Role for membrane and secreted insulin-like growth factor-binding protein-2 in the regulation of insulin-like growth factor action in lung tumors. Cancer Research 53, 4680-4685.

113 Rinderknecht, E. and Humbel, R.E. (1976). Amino-terminal sequences of two polypeptides from human serum with nonsuppressible insulin-like and cell-growth-promoting activities: Evidence for structural homology with insulin B chain. Proc. Natl. Acad. Sci. USA. 73, 4379-4381.

114 Rinderknecht, E. and Humbel, R.E. (1978a). The amino-acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. J. Biol. Chem. 253, 2769-2775.

115 Rinderknecht, E. and Humbel, R.E. (1978b). Primary structure of human insulin-like growth factor II. FEBS Lett. 89, 283-286.

116 Rosenfeld, R.G., Pham, H., Oh, Y., Lamson, G. and Guidice, L.C. (1990). Identification of insulin-like growth factor-binding protein-2 (IGFBP-2) and a low molecular weight IGF-BP in human seminal plasma. J. Clin. Endocrinol. Metab. 70, 551-553.

117 Rotwein, P., Pollock, K.M., Watson, M. and Milbrandt, J.D. (1987). Insulin-like growth factor gene expression during rat embryonic development. Endocrinology 121, 2141-2144.

118 Ruoslathi, E. and Pierschlaber, M.D. (1987). New perspectives in cell adhesion: RGD and integrins. Science 238, 491-497.

119 Rutanen, E.M., Kärkkäinen, T., Lundqvist, C., Pekonen, F., Ritvos, O., Tanner, P., Welin, M. and Weber, T. (1988) Monoclonal antibodies to the 27-34K insulin-like growth factor binding protein. Biochem. Biophys. Res. Commun. 152, 208-215.

120 Sadler, T.W., Phillips, L.S., Balkan, W. and Goldstein, S. (1986). Somatomedin inhibitors from diabetic rat serum alter growth and development of mouse embryos in culture. Diabetes 35, 861-865.

121 Schmid, C., Guler, H.P., Rowe, D. and Froesch, E.R. (1989). Insulin-like growth factor I regulates type I procollagen messenger ribonucleic acid steady state levels in bone of rats. Endocrinology 125, 1575-1580.

122 Schuller, A.G.P., Lindenbergh-Kortleve, D.J., de Boer, W.I., Zwarthoff, E.C. and Drop, S.L.S. (1993) Localization of the epitope of a monoclonal antibody against human insulin-like growth factor binding protein-1, functionally interfering with insulin-like growth factor binding. Growth Regulation 3, 32-34.

123 Schuller, A.G.P., Zwarthoff, E.C. and Drop, S.L.S. (1993) Gene expression of the six insulin-like growth factor binding proteins (IGFBPs) in the mouse conceptus during mid- and late gestation. Endocrinology 132, 2544-2550.

124 Schuller, A.G.P., Lindenbergh-Kortleve, D.J., Pache, T.D., Zwarthoff, E.C., Fauser, B.C.J.M. and Drop, S.L.S. (1993) Insulin-like growth factor binding protein-2, 28 kDa and 24 kDa insulin-like growth factor binding protein levels are decreased in fluid of dominant follicles, obtained from normal and polycystic ovaries. Regulatory Peptides 48, 157-163.

125 Schuller, A.G.P., Groffen, C., van Neck, J.W., Zwarthoff, E.C. and Drop, S.L.S. (1994) cDNA cloning and mRNA expression of the six mouse insulin like growth factor binding proteins. submitted.

126 Schuller, A.G.P., Beukenholdt, R.W., van Neck, J.W., Zwarthoff, E.C. and Drop, S.L.S. (1994) Insulin-like growth factor (IGF), type I IGF receptor and IGF binding protein mRNA expression in the developing mouse lung. submitted.

127 Schuller, A.G.P., Lindenberg-Kortleve, D.J., van Neck, J.W., Zwarthoff, E.C. and Drop, S.L.S. (1994) Insulin-like growth factor (IGF), type I IGF receptor and IGF binding protein mRNA expression in pre- and poistnatal mouse kidneys. submitted.

128 Schultz, G.A. (1986) Utilization of genetic information in the preimplantation mouse embryo. In: Experimental approaches to mammalian embryogenic development. Eds: J. Rossant and R.A. Pedersen. Cambridge University Press, Camebridge, pp. 239-265.

129 Schultz, G.A., Hogan, A., Watson, A.J., Smith, R.M. and Heyner, S. (1992) Insulin, insulin-like growth factors and glucose transporters: Temporal patterns of gene expression in early murine and bovine development. Reprod. Fertil. Dev. 4, 361-371.

130 Schultz, G.A., Hahnel, A., Arcellana-Panlilio, M., Wang, L., Goubau, S., Watson, A. and Harvey, M. (1993) Expression of IGF ligand and receptor genes during preimplantation mammalian development. Mol. Reprod. Dev. 35, 414-420.

131 Senior, P.V., Byrne, S., Brammar, W.J. and Beck, F. (1990). Expression of the IGF-II/mannose-6-phosphate receptor mRNA and protein in the developing rat. Development 109, 67-73.

132 Shimasaki, S., Koba, A., Mercado, M., Shimonaka, M. and Ling, N. (1989). Complementary DNA structure of high mol weight rat IGF binding protein and tissue distribution of its mRNA. Biochem Biophys Res Comm 165: 907-912.

133 Shimasaki, S., Uchiyama, F., Shimonaka, M. and Ling, N. (1990). Molecular cloning of the cDNAs encoding a novel IGF binding protein from rat and human. Mol Endocrinol 4: 1451-1458.

134 Shimasaki, S., Shimonaka, S., Zhang, H.P. and Ling, N. (1991a). Identification of five different IGFBPs from adult rat serum and molecular cloning of a novel IGFBP-5 in rat and human. J. Biol. Chem. 266: 10646-10653.

135 Shimasaki, S., Gao, L., Shimonaka, M. and Ling, N. (1991b). Isolation and molecular cloning of IGF binding protein 6. Mol. Endocrinol. 5:938-948.

136 Sklar, M.M., Kiess, W., Thomas, C.L. and Nissley, S.P. (1989). Developmental expression of the tissue insulin-like growth factor II/mannose 6-phosphate receptor in the rat. Measurement by quantitative immunoblotting. J. Biol. Chem. 264, 16733-16738.

137 Sklar, M.M., Thomas, C.L., Municchi, G., Roberts Jr., C.T., LeRoith, D., Kiess, W. and Nissley, S.P. (1992). Developmental expression of rat insulin-like growth factor-II/mannose 6-phosphate receptor messenger ribonucleic acid. Endocrinology 130, 3484-3491.

138 Smith, P.J., Wise, L.S., Berkowitz, R., Wan, C. and Rubin, C.S. (1988). Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes. J. Biol. Chem. 263, 9402-9408.

139 Smith, R.M., Garside, W.T., Aghayan, M., Shi, C.-Z., Shah, N., Jarret, L. and Heyner, S. (1993). Mouse preimplantation embryos exhibit receptor-mediated binding and transcytosis of maternal insulin-like growth factor I. Biol. Reprod. 49, 1-12.

140 Soares, M.B., Turken, A., Ishii, D., Mills, L., Episcopou, V., Cotter, S., Zeitlin, S. and Efstratiadis, A. (1986) Rat insulin-like growth factor II gene: A single copy gene with two promoters expressing a multitranscript family. J. Molec. Biol. 192, 737-752.

141 Soos, M.A. and Siddle, K. (1989) Immunological relationships between receptors fgor insulin and insulin-like growth factor I. Evidence for structural heterogeneity of insulin-like growth factor I receptors involving hybrids with insulin receptors. Biochem. J. 263, 553-563.

142 Soos, M.A., Whittaker, J. Lammers, R. Ullrich, A. and Siddle, K. (1990) Receptors for insulin and insulin-like growth factor I can form hybrid dimers: Characterization of hybrid receptors in transfected cells. Biochem. J. 270, 383-390.

143 Soos, M.A., Field, C.E. and Siddle, K. (1993) Purified hybrid insulin/insulin-like growth factor-I receptors bind insulin-like growth factor-I, but not insulin, with high affinity. Biochem. J. 290, 419-426.

144 Sommer, A., Maack, C.A., Spratt, S.K., Hunt, T.K. and Spencer, E.M. (1991) Molecular genetics and actions of recombinant IGFBP-3. In: Modern concepts of IGFs. Ed. M.E. Spencer. Elsevier Sci. Publ. Co. New York. 715-728.

145 Steele-Perkins, G., Turner, J., Edman, J.C., Hari, J., Pierce, S.B., Stover, C., Rutter, W.J. and Roth, R.A. (1988). Expression and characterization of a functional human insulin-like growth factor I receptor. J. Biol. Chem. 263, 11486-11492.

146 Steele-Perkins, G. and Roth R.A. (1990). Monoclonal antibody αIR-3 inhibits the ability of insulin-like growth factor II to stimulate a signal from the type I receptor without inhibiting its binding. Biochem. Biophys. Res. Commun. 171, 1244-1251.

147 Stiles, A.D. and Moats-Staats, B.M. (1989) Production and action of insulin-like growtyh factor I/somatomedin C in primary cultures of fetal lung fibroblasts. Am. J. Respir. Cell Mol. Biol. 1, 21-26.

148 Straus, D.S. and Takemoto, C.D. (1990) Effect of dietary protein deprivation on insulin-like growth factor (IGF)-I and -II, IGF binding protein-2, and serum albumine gene expression in rat. Endocrinology 127, 1849-1860.

149 Streck, R.D. and Pintar, J.E. (1992). The embryonic pattern of rat insulin-like growth factor-I gene expression suggests a role in induction and early growth of the liver. Endocrinology 131, 2030-2032.

150 Streck, R.D., Wood, T.L., Hsu, M.-S. and Pintar, J.E. (1992). Insulin-like growth factor I and II and insulin-like grwoth factor binding protein-2 RNAs are expressed in adjacent tissues within rat embryonic limbs. Developmental Biology 151, 586-596.

151 Stylianopoulou, F., Efstratiadis, A., Herbert, J. and Pintar, J. (1988a). Pattern of the insulin-like growth factor II gene expresion during rat embryogenesis. Development 103, 497-506.

152 Stylianopoulou, F., Herbert, J., Soares, M.B. and Efstratiadis, A. (1988b). Expression of the insulin-like growth factor II gene in the choroid plexus and leptomeninges of the adult rat central nervous system. Proc. Natl. Acad. Sci. USA. 85, 141-145.

153 Telford, N.A., Watson, A.J. and Schultz, G.A. (1990) Transition of maternal to embryonal control in early mammalian development: a comparison of several species. Mol. Reprod. Dev. 26, 90-100.

154 Tong, P.V., Tollefsen, S.E. and Kornfeld, S. (1988). The cation-independent mannose-6-phosphate receptor binds insulin-like growth factor II. J. Biol. Chem. 263, 2585-2588.

155 Tong, P.V. and Kornfeld, S. (1989) Ligand interactions of the cation-dependent mannose 6-phosphate receptor. Comparison with the cation-independent mannose 6-phosphate receptor. J. Biol. Chem. 15, 7970-7975.

156 Tong, P.V., Gregory, W. and Kornfeld, S. (1989) Ligand interactions of the cation-independent mannose 6-phosphate receptor. The stoichiometry of mannose 6-phosphate binding. J. Biol. Chem. 264, 7962-7969.

157 Travers, J.P., Exell, L., Huang, B., Town, E., Lammiman, M.J. Pratten, M.K. and Beck, F. (1992) Insulin and insulin-like growth factors in embryonic development. Effects of a biological inert insulin (guinea pig) on rat embryonic growth and development in vitro. Diabetes 41, 318-324.

158 Treadway, J.L., Morrison, B.D., Goldfine, I.D. and Pessin, J.E. (1989) J. Biol. Chem. 264, 21450-21453.

159 Underwood,L.E. and D'Ercole, A.J. (1984). Insulin and somatomedin/insulin-like growth factors in fetal and neonatal development. Clin. Endocrinol. Met. 13, 69-89.

160 Voutilainen, R. and Miller, W.L. (1987). Coordinate tropic hormone regulation of mRNAs for insulin-like growth factor II and the cholesterol side-chain-cleavage enzyme, P450ssc, in human steroidogenic tissues. Proc. Natl. Acad. Sci. USA. 84, 1590-1594.

161 Wang, J.F., Hampton, B., Mehlman, T., Burgess, W.H. and Rechler, M.M. (1988) Isolation of a biological active fragment from the carboxy terminus of fetal rat binding protein for insulin-like growth factors. Biochem. Biophys. Res. Commun. 157, 718-726.

162 Werner, H., Woloschak, M., Adamo, M., Shen-Orr, Z., Roberts Jr., C.T. and LeRoith, D. (1989). Developmental regulation of the rat insulin-like growth factor I receptor gene. Proc. Nat. Acad. Sci. USA. 86, 7451-7455.

163 Wiseman, D.L., Henricks, D.M., Eberhardt, D.M. and Bridges, W.C. (1992). Identification and content of insulin-like growth factors in porcine oviductal flui. Biol. Reprod. 47, 126-132.

164 Wood, T.L., Brown, A.L., Rechler, M.M. and Pintar, J.E. (1990). The expression pattern of an insulin-like growth factor (IGF)-binding protein gene is distinct from IGF-II in the midgestational rat embryo. Mol. Endocrinol. 4, 1257-1263.

165 Wood, T.L., Streck, R.D. and Pintar, J.E. (1992). Expression of the IGFBP-2 gene in post-implantation rat embryos. Development 114, 59-66.

166 Yu, K.T., Peters, M.A. and Czech, M.P. (1986). Similar control mechanisms regulate the insulin and type I insulin-like growth factor receptor kinases. J. Biol. Chem. 261, 11341-11349.

167 Zapf J, Hauri C, Waldvogel M and Froesch ER. (1986). Acute metabolic effects and half-lives of intravenously administered insulin-like growth factors I and II in normal and hypophysectomized rats. J. Clin. Invest. 77, 1768-1775.

CHAPTER 2

LOCALIZATION OF THE EPITOPE OF A MONOCLONAL ANTIBODY AGAINST HUMAN INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-1, FUNCTIONALLY INTERFERING WITH INSULIN-LIKE GROWTH FACTOR BINDING.

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Growth Regulation (1993) 3, 32-34

SUMMARY

In order to identify regions in insulin-like growth factor binding protein-1 involved in the binding of IGFs, we tested three monoclonal antibodies, designated MAb A, B and C on their interference with IGF-binding. Monoclonal A, interfered with the binding of IGF to IGFBP-1 as determined by immunoprecipitation whereas monoclonal B and C did not. Furthermore MAb A was found to abolish IGFBP-1 inhibition of IGF stimulation in an in vitro proliferation assay. The epitopes of all three monoclonal antibodies were found to be located within the C-terminal part of IGFBP-1. The regions surrounding residue 188-196 and 222-227 are especially important for antibody recognition. These results indicate that MAb A functionally interferes with the binding of IGF to IGFBP-1. Furthermore, we suggest that part of the epitope of MAb A is located at or sterically near the IGF binding domain of IGFBP-1.

INTRODUCTION

Insulin like growth factors (IGFs) are important regulators of cell growth and differentiation. In serum and extracellular fluids, the IGFs are complexed to specific IGF binding proteins (IGFBPs). The IGFBPs are thought to modulate the biological actions of the IGFs.[1] To date the complete primary structure of six distinct human IGFBPs has been determined from cDNA clones, and the proteins have been named IGFBP-1 to -6.[2] In view of the conservation of the N- and C-terminal region of the IGFBPs, it has been suggested that these regions contain the binding site for IGFs. In a previous study we demonstrated that deletion of the C-terminal 17 amino acids of IGFBP-1 resulted in loss of IGF binding.[3] However, site directed mutagenesis of the C-terminal region revealed that substitution of several amino acids in the C-terminal part of IGFBP-1 did not dramatically alter the IGF binding capacity. Only when Cys-226 was mutated or when a negatively charged or hydrophobic residue was introduced at position 222, IGF binding was lost. This suggested that disruption of the intramolecular disulfide bond formed by Cys-226 and its putative partner abolishes IGF binding.[3]

In order to investigate the role of the C-terminal region of IGFBP-1 in IGF binding in more detail, we tested several monoclonal antibodies against IGFBP-1. Here we present three monoclonal antibodies of which one functionally interferes with the binding of IGFs to IGFBP-1. The epitopes of all three monoclonal antibodies are located in the C-terminal region of IGFBP-1.

MATERIALS AND METHODS

Purification of IGFBP-1 and production of monoclonal antibodies

IGFBP-1 was purified from human midterm amniotic fluid (AF) as described previously.[4] Immunization was carried out according to standard procedures. After immunization, the spleen was removed for fusion with a P3-myeloma cell line essentially as described by Köhler.[5] Three independent hybrid cell lines were used to produce ascites. When necessary, antibodies were purified using a HITrap protein G column (Pharmacia, Uppsala, Sweden) according the manufacturers guidelines.

Interference with IGF binding

Interference with IGF-binding was assayed by preincubation of IGFBP-1 before cross-linking to iodinated IGF and by immunoprecipitation of the IGF/IGFBP-complex essentially as described by Rutanen et al.[6] For the in vitro proliferation assay a mouse urothelial cell line (NUC-1) was used.[7]

Construction and analysis of fusion proteins

Complementary DNA fragments with an increasing length of the IGFBP-1 C-terminal coding region were isolated and inserted into the *Escherichia coli* expression vector pEX2.[8] After expression of ß-galactosidase/IGFBP-1 fusion proteins in pop 2136 cells, the proteins were subjected to SDS-PAGE followed by immunoblotting.

RESULTS

Out of 87 growing hybridomas, 8 independent colonies were found to secrete antibodies against IGFBP-1. After subcloning by limited dilution, 3 of these, designated MAb A, B and C were used to produce ascites. The titre of the ascites used for immunoblots varied between the different monoclonal antibodies. MAb A, B and C detected IGFBP-1 on a Western blot at a dilution of 1:5000, 1:200000 and 1:1000 respectively. No cross reactivity was seen with other proteins when human midterm amniotic fluid or adult human serum was separated on a SDS-polyacrylamide gel. Moreover mouse IGFBP-1 did not react with either of the three monoclonal antibodies as assayed by immunoblots of mouse amniotic fluid

(results not shown). Surprisingly, when amniotic fluid was separated under reducing conditions, IGFBP-1 could be detected only at a much lower dilution of 1:125, 1:250 and 1:125 for MAb A, B and C respectively.

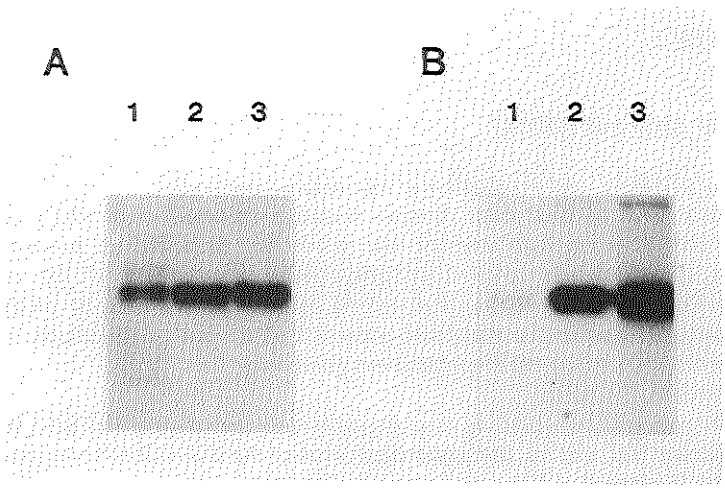

Fig. 1 Interference of three monoclonal antibodies with the binding of iodinated IGF-I to IGFBP-1. (A) IGFBP-1 was first preincubated with MAb A (1), B (2) and C (3), followed by incubation with iodinated IGF-I and cross-linking. (B) IGFBP-1 was cross-linked to iodinated IGF-I followed by immunoprecipitation using MAb A (1), B (2) and C (3). The samples were analyzed by SDS-PAGE and autoradiography.

To test whether the monoclonal antibodies could interfere with IGF-binding, AF was preincubated with MAb A, B or C followed by incubation with radiolabeled IGF-I and cross-linking with disuccinimidyl suberate. As shown in Figure 1a, only a small amount of IGFBP-1 was found to be cross-linked to IGF-I when IGFBP-1 was preincubated with MAb A. The IGF-IGFBP-1 complex was clearly detectable when MAb B or C were used. In order to examine the capacity of the monoclonal antibodies in precipitating IGFBP-1 complexed to IGF, radiolabeled IGF-I was incubated with AF and cross-linked. Immunoprecipitation revealed that MAb B and C were capable of precipitating IGFBP-1 when bound to IGF-I, while MAb A was not (Fig. 1b). Incubation with radiolabeled IGF-II gave similar results (results not shown). These results suggested that MAb A, but not B or C, interfered with the binding of IGF to IGFBP-1.

In order to investigate whether MAb A also functionally interferes with the binding of IGF to IGFBP-1 a mouse urothelial cell line (NUC-1) was used. Incubation of the NUC-1 cells with 10 ng/ml IGF-1 resulted in a 3.5 fold increase in [^{3}H]-thymidine incorporation.[7] Addition of increasing amounts of IGFBP-1 resulted in a dose dependent inhibition of the IGF-I stimulated proliferation of NUC-1 cells, with no effect on the basal level of proliferation. A 50% inhibition of IGF-I (10 ng/ml) stimulation was achieved with approximately 100 ng/ml IGFBP-1 (Fig. 2a). When NUC-1 cells were incubated with increasing amounts of MAb A in the presence of 100 ng/ml IGFBP-1, no effect on the proliferation was observed. However, when NUC-1 cells were stimulated with IGF-I in the presence of 100 ng/ml IGFBP-1, addition of MAb A resulted in a dose dependent stimulation of the proliferation (Fig. 2b), indicating that MAb A functionally interfered with the binding of IGF to IGFBP-1.

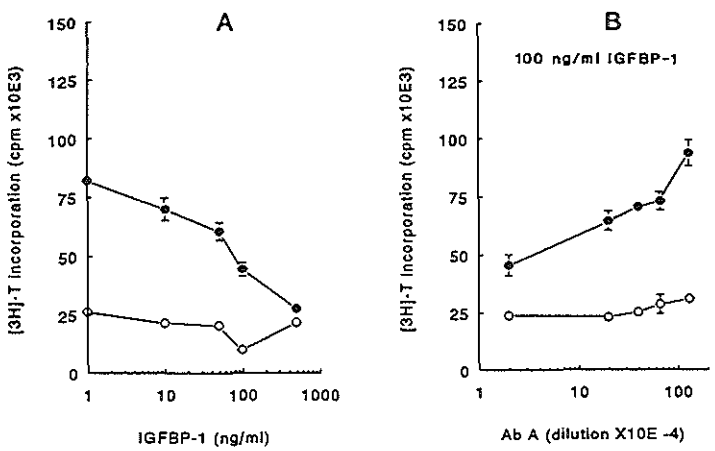

Fig.2 Interference of MAb A with the binding of IGF-I to IGFBP-1 in an in vitro proliferation assay. (A) NUC-1 cells were incubated with increasing amounts of IGFBP-1 in the absence (open circles) or presence (closed circles) of 10 ng/ml IGF-I. (B) NUC-1 cells were incubated with increasing amounts of MAb A in the presence of 100 ng/ml IGFBP-1 and in the absence (open circles) or presence (closed circles) of 10 ng/ml IGF-I.

To determine the epitope of the monoclonal antibodies, several deletion mutants of IGFBP-1 were tested under reducing conditions for antibody recognition. Deletion of the C-terminal 21 amino acids of IGFBP-1 (clone pSV19Ba[3]) resulted in a protein which could not

be detected by any of the monoclonal antibodies (results not shown). In order to investigate this region in more detail several point mutants were tested under reducing conditions. Mutations resulting in a substitution of the glycine, proline or cysteine residue at position 222, 224 and 226 respectively resulted in a protein which could not be detected by any of the monoclonals. Substitution of a proline (218), arginine (221), methionine (228) and valine (232) was without effect on the recognition by MAb A, B or C (Fig. 3). These results suggested that the epitopes of the three monoclonals were located between amino acid position 222-227.

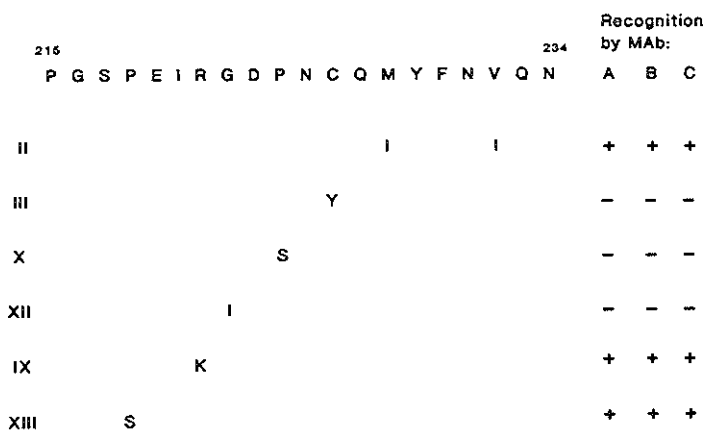

Fig.3 Recognition of IGFBP-1 point mutants by three monoclonal antibodies. Mutated cDNA was transfected into COS-1 cells and the mutant protein tested by immunoblotting using MAb A, B and C.

To show that expression of this region was sufficient for antibody recognition we inserted and expressed the 21 C-terminal amino acids of hIGFBP-1 in the *E. coli* expression vector pEX2. The ß-galactosidase/IGFBP-1 fusion protein formed was not recognized by any of the three monoclonal antibodies. Even expression of the 39 C-terminal amino acids did not result in a fusion protein detectable by MAb A, B or C, whereas the fusion proteins were clearly detectable by a polyclonal antibody against IGFBP-1. Only when the 47 C-terminal amino acids of IGFBP-1 were expressed was the fusion-protein detected by MAb A, B and C (Fig. 4). These results indicate that part of the epitope of MAb A, B and C is located between residues 222-227 and another part is located around residues 188-196.

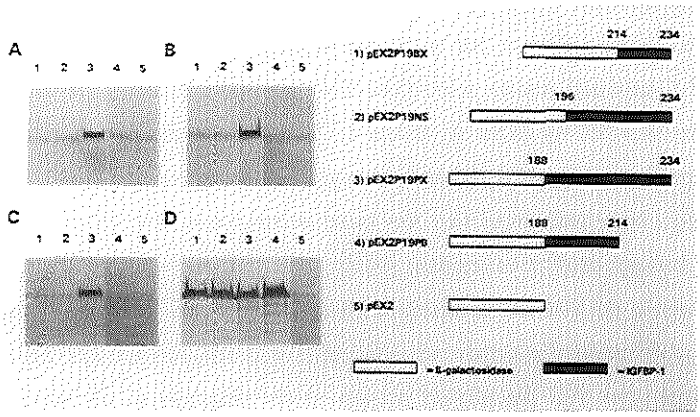

Fig.4 Recognition of IGFBP-1 fusion proteins by three monoclonal antibodies. IGFBP-1/β-galactosidase fusion proteins were produced and tested by immunoblotting using MAb A (A), B (B), C (C) and a polyclonal antibody (D). The numbers refer to the amino acid stretches of IGFBP-1 expressed.

DISCUSSION

The three monoclonal antibodies described are highly specific for IGFBP-1 since no cross-reactivity with other proteins was found in human adult serum and midterm AF. Moreover, mouse IGFBP-1 did not react with either of the three monoclonal antibodies as assayed by immunoblots of mouse AF. Furthermore, it was found that the three monoclonal antibodies preferentially interacted with non-reduced IGFBP-1. This observation was most obvious for MAb B, which only detected reduced IGFBP-1 at a concentration a thousand fold higher than needed for detection of non-reduced IGFBP-1. It is likely that destruction of the three-dimensional structure of IGFBP-1 results in a lower affinity for the monoclonal antibodies, especially for MAb B.

The three monoclonal antibodies did not recognize mutant IGFBP-1 in which the 21 C-terminal amino acids (residue 214-234) were deleted. Moreover substitution of the glycine, proline or cysteine residues at position 222, 224, or 226 (clone XII, X and III[3]) resulted in a mutant protein not recognized by MAb A, B or C, whereas substitution of several of the

surrounding amino acids was without effect. However, expression of the 39 C-terminal amino acids also resulted in a protein that could not be detected by any of the monoclonals. Only when the 47 C-terminal amino acids (188-234) were expressed could the fusion protein synthesized be detected by MAb A, B and C. These observations suggested that both the region surrounding residue 188-196 and the region spanning residue 222-227 are needed for antibody recognition, indicating that the three monoclonals are directed against a large epitope. The average amount of residues present in an epitope is approximately 15.[9] Another explanation would be that the epitopes of MAb A, B and C are discontinuous and may consist of a combination of residues between amino acid 188-196 and 222-227. This tentative conclusion implicates that the IGFBP protein partly renatures to its native configuration during gel electrophoresis.

MAb A clearly interfered with both IGF-I and IGF-II binding as shown by immunoprecipitation. Addition of this monoclonal antibody in a mouse urothelial cell proliferation assay completely abolished the IGFBP-1 mediated inhibition of IGF-I induced proliferation, showing that MAb A also functionally interfered with IGF binding. These results suggest that the binding domains for IGF-I and IGF-II are either the same or in close vicinity. Furthermore, the results suggest that the epitope of MAb A is at or near the IGF binding domain of IGFBP-1. Since no cross-reactivity with other IGFBPs, not even to mouse IGFBP-1, is found, it is not likely that MAb A is solely directed against the IGF binding domain of IGFBP-1. Nevertheless, the possibility that those residues unique for the epitope of MAb A contribute to the IGF-binding domain can not be ruled out. Another possibility would be that MAb A sterically interferes with the binding of IGF to IGFBP-1. Therefore, further studies are needed to understand the conformation of IGFBP-1 and the role of the epitope of MAb A in the binding of IGF to IGFBP-1.

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REFERENCES

1 Clemmons D R. IGF binding proteins: Regulation of cellular actions. Growth Regulation 1992; 2: 80-87.

2 Drop S L S, Schuller A G P, Lindenbergh-Kortleve D J, Groffen C, Brinkman A, Zwarthoff E C. Structural aspects of the IGFBP family. Growth Regulation 1992; 2: 69-79.

3 Brinkman A, Kortleve D J, Zwarthoff E C, Drop S L S. Mutations in the C-terminal part of IGF binding protein 1 result in dimer formation and loss of IGF binding capacity. Mol Endocrinol 1991; 5: 987-994.

4 Liu L, Brinkman A, Blat C, Harel L. IGFBP-1, an insulin-like growth factor-binding protein, is a cell growth inhibitor. Biochem Biophys Res Commun 1991; 174: 673-679.

5 Köhler G. The technique of Hybridoma Production. In: Lefkovits I, Pernis B (eds) Immunological Methods II. New York, Academic Press. 1981: 285-298.

6 Rutanen E M, Kärkkäinen T, Lundqvist C, Pekonen F, Ritvos O, Tanner P, Welin M, Weber T. Monoclonal antibodies to the 27-34K insulin-like growth factor binding protein. Biochem Biophys Res Commun 1988; 152: 208-215.

7 de Boer W I, Rebel J M J, Foekens J A, Vermey M, van der Kwast T H. Growth factors affect proliferation of three mouse urothelial cells differentially in vitro, but not differentiation. 1992: in preparation.

8 Stanley K K, Luzio J P. Construction of a new family of high efficiency bacterial expression vectors: identification of cDNA clones coding for human liver proteins. EMBO J 1984; 3:1429-1434.

9 Janin J, Chothia C. The structure of protein-protein recognition sites. J Biol Chem 1990; 265: 16027-16030.

CHAPTER 3

INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-2, 28 kDa AND 24 kDa INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN LEVELS ARE DECREASED IN FLUID OF DOMINANT FOLLICLES, OBTAINED FROM NORMAL AND POLYCYSTIC OVARIES.

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Regulatory Peptides (1993) 48, 157-163

SUMMARY

In order to investigate potential changes in insulin-like growth factor binding proteins (IGFBPs) during human follicle maturation, we examined the IGFBP profiles in follicular fluid from follicles in different stages of maturation. Samples were obtained from ovaries of women with regular menstrual cycles and of subjects with cycle abnormalities and polycystic ovaries (diagnosed as polycystic ovary syndrome (PCOS)) and analyzed by Western ligand blotting. IGFBPs of 43 kDa, 37 kDa, 31 kDa, a doublet around 28 kDa and a minor band of 24 kDa were detected in follicle fluid of normal non-dominant (size < 10 mm) and atretic (androstenedione/estradiol ratio > 4) follicles of both regularly menstruating women and PCOS patients. The 43 and 37 kDa IGFBPs could be identified as IGFBP-3 and the 31 kDa IGFBP as IGFBP-2, whereas the 28 kDa IGFBP could not be identified as IGFBP-1, all by immunoblotting techniques. A dramatic decrease in IGFBP-2, the 28 kDa and 24 kDa IGFBPs was observed in follicular fluid of dominant follicles (size > 10 mm) of both regular menstruating individuals and one PCOS patient as compared with follicular fluid of normal non-dominant or atretic follicles. These observations indicate that the PCOS follicle may not be different from normal with respect to IGFBP profiles. Furthermore, these results suggest that at least one of these IGFBPs might be involved in human folliculogenesis.

INTRODUCTION

The insulin-like growth factors (IGFs) are low molecular weight peptides with both metabolic and mitogenic properties believed to play a role in ovarian function (1, 2). In the human preovulatory granulosa cell, expression of IGF-II mRNA but not IGF-I mRNA has been detected (3). Human granulosa cells contain transcripts for both the type I and type II IGF receptor (4) and contain the type I IGF receptor on their surface (5). In human granulosa cells, isolated from polycystic ovaries, IGF-I synergizes with follicle-stimulating hormone (FSH) and human chorionic gonadotropin (hCG) in increasing 17ß-estradiol (E_2) production, suggesting a role for IGF-I in enhancing gonadotropin action in human folliculogenesis (6).

In serum and extracellular fluids, the IGFs are present bound to high affinity binding proteins (IGFBPs). These IGFBPs comprise a family of six structurally related proteins (7), which modulate IGF action (8). In human follicular fluid several of these IGFBPs have been found and one such species, IGFBP-1, was localized in luteinized granulosa cells (9). Human granulosa cells express mRNA for IGFBP-1 (10). In addition, IGFBP-1 was reported to inhibit IGF induced proliferation of human granulosa cells, suggesting that IGFBP-1 is one of the endogenous factors regulating growth and differentiation of human granulosa cells (11). IGFBP-2, -4 and -5 mRNA expression was found mainly in granulosa of atretic follicles and IGFBP-4 and -5 gene expression was located primarily in theca and stromal cells. Gene expression of IGFBP-3 was detected in theca of all stages tested and was selectively expressed in granulosa cells of dominant follicles (12). IGFBP-3 has been shown to inhibit IGF-I and FSH induced estrogen production by cultured human granulosa cells (13), suggesting that this binding protein may modify FSH actions.

Since there is mounting evidence that the IGF system, complete with ligands, IGF-receptors and IGFBPs, is present in the human ovary, we decided to investigate potential changes in IGFBPs during follicle maturation. Therefore, we examined the IGFBP profiles in fluid from follicles in different stages of maturation obtained from ovaries of women with regular menstrual cycles and of PCOS patients presenting with cycle abnormalities.

MATERIALS AND METHODS

Follicular fluid samples

16 women volunteered to participate in this study, which was approved by the local Ethics Review Committee. Their mean age was 33 years (range 27-44) and they all were regularly menstruating with a mean cycle length of 28 $\pm$ 2 days (mean $\pm$ SD). Follicular fluid samples were collected through puncture of individual follicles as described previously (14). In addition, follicle fluid was obtained from five infertile PCOS women. Four patients presented with amenorrhea and one with severe oligomenorrhea (cycle length 72-84 days). All five fulfilled our criteria for transvaginal sonographic diagnosis of polycystic ovaries; (1) ovarian volume above 8.0 ml, (2) > 11 follicles between 2 and 10 mm in size in each ovary, and (3) increased ovarian stroma echogenecity (15). The mean age of these patients was 28 years, 2 women were obese (BMI > 25 kg/m^2) and hirsute, 1 woman was hirsute only. Serum hormone concentrations were 10.6 (range 7.9 to 12.1) IU/l for luteinizing hormone (LH), and 6.9 (range 3.9 to 8.3) IU/l for follicle stimulating hormone (FSH) estimated by immunoradiometric assays. Radioimmunoassay serum determinations for serum testosterone were 2.9 (range 1.6 to 7.2) nmol/l. All FF samples were divided into three groups

(depending on their androstenedione/estradiol (AD/E_2) ratio as described by McNatty et al (16)) and pooled per individual. Group A (normal non-dominant follicles): AD/E_2 ratio < 4 and a diameter < 10 mm, Group B (atretic follicles): AD/E_2 ratio > 4 and Group D (dominant follicles): AD/E_2 ratio < 4 and a size > 10 mm.

Western ligand blotting

Western ligand blots were prepared essentially as described by Hossenlopp et al. (17). FF samples (3 μl) were applied to a 9% sodium dodecylsulfate (SDS)-polyacrylamide gel, run under non-reducing conditions, and the separated proteins were transferred onto nitrocellulose filters by electroblotting. The filters were washed with 3% NP-40 in 100 mM Tris pH 7.5/0.9% NaCl (TBS), 3% BSA in TBS, and then incubated with 500,000 cpm [^{125}I]IGF-II (kindly provided by Dr. S. van Buul-Offers, Wilhelmina Childrens Hospital, Utrecht The Netherlands) for 14 hrs. at 4°C. Subsequently, filters were washed and exposed to Kodak X-Omat AR film with intensifying screen at -70 °C. for up to 14 days.

Immunoblotting

After autoradiography, filters were immunostained using either a monoclonal antibody against IGFBP-1 (18), a polyclonal antibody against IGFBP-2 (kindly provided by Dr. J. Schwander, Kantonspital Basel, Switzerland) or a polyclonal antibody against IGFBP-3 (kindly provided by Dr. R. Rosenfeld, Stanford University Stanford, USA) using standard procedures. Briefly, filters were incubated with 3% BSA in TBS followed by incubation with an IGFBP specific antibody. Filters were washed in 0.1% NP-40 in TBS and incubated with an alkaline-phosphatase coupled second antibody. Finally, filters were washed and stained with 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer, Mannheim).

Quantification of IGFBP levels was performed by densitometric scanning of autoradiograms using an image analyzer (IBAS 2000 Zeiss Kontron, Oberkochen, FRG) and the Kontron IBAS1 version 4.4 software program supplied. Mean intensities were calculated from measurements of autoradiograms of at least four samples and statistical confidence of differences calculated
using Student's *t*-test.

RESULTS

Follicle fluid (FF) samples obtained from individuals with regular cycles were subjected to SDS-PAGE and Western ligand blotting using iodinated IGF-II. As shown in Fig. 1, two major bands of 43 and 37 kDa, corresponding to the glycosylated forms of IGFBP-3, were detected. In addition a band of 31 kDa, a doublet around 28 kDa and a minor band of approximately 24 kDa (corresponding to the molecular mass of IGFBP-2, IGFBP-1 and IGFBP-4, respectively) were seen (Fig. 1). Using a polyclonal antibody specific for

IGFBP-3, we were able to show that the 43 and 37 kDa bands were indeed isoforms of IGFBP-3 (Fig. 2a). The 31 kDa band was identified as IGFBP-2 by immunoblotting (Fig. 2b). However, the 28 kDa IGFBP doublet did not react with a specific antibody against IGFBP-1, whereas the antibody clearly detected the positive control (IGFBP-1 in amniotic fluid), indicating that this 28 kDa IGFBP was not IGFBP-1 (Fig. 2c).

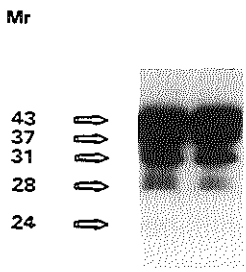

Fig. 1. Autoradiograph of a representative Western ligand blot of equal volumes (3 µl) of follicular fluid from normal non-dominant follicles (left lane) and atretic follicles (right lane) obtained from one individual. Molecular weight markers (in kDa) are indicated by arrows.

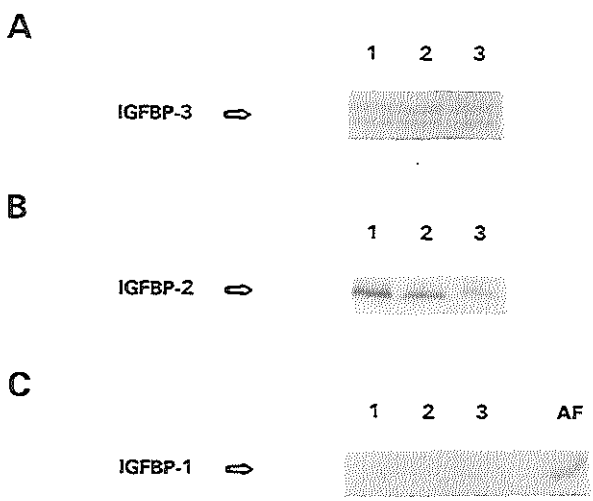

Fig. 2. Immunostaining of a representative Western blot of equal volumes (3 µl) of follicular fluid from normal non-dominant (lanes 1), atretic (lanes 2) and dominant (lanes 3) follicles obtained from one regularly menstruating individual. Filters are stained using a polyclonal antibody against IGFBP-3 (A), IGFBP-2 (B) and a monoclonal antibody against IGFBP-1 (C). Amniotic fluid (AF) was used as a positive control for IGFBP-1.

Comparison of the Western ligand blot profile of the IGFBPs in FF of follicles in different stages of development, revealed that in all dominant follicles, IGFBP-2, the 28 kDa and the 24 kDa IGFBP were strongly decreased, as compared to normal follicles (Fig. 3). Most atretic follicles, diagnosed based on Ad/E_2 ratio > 4, showed no difference in IGFBP profiles as compared to normal follicles (Fig. 3). Only in two samples classified as FF of atretic follicles, a decrease in IGFBP-2 and the 28 kDa and 24 kDa IGFBPs was observed (results not shown).

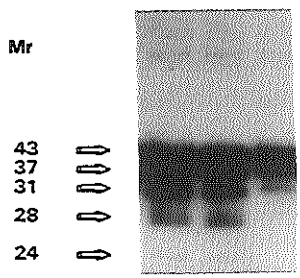

Fig. 3. Autoradiograph of a representative Western ligand blot of equal volumes (3 µl) of follicular fluid from normal non-dominant (left lane), atretic (middle lane) and dominant (right lane) follicles obtained from a single regularly menstruating individual. Molecular weight markers (in kDa) are indicated by arrows.

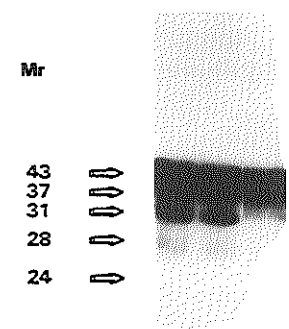

Fig. 4. Autoradiograph of a representative Western ligand blot of equal volumes (3 µl) of follicular fluid from normal non-dominant (left lane), atretic (middle lane) and dominant (right lane) follicles obtained from a single PCOS patient. Molecular weight markers (in kDa) are indicated by arrows.

In FF samples obtained from five PCOS patients IGFBP profiles were also determined. When compared to FF of normal and atretic follicles of regular menstruating women, no dramatic difference in IGFBP profiles could be observed (Fig. 4). One of the PCOS patients developed a dominant follicle. In FF of this follicle the same decrease in

IGFBP-2, 28 kDa IGFBP and 24 kDa IGFBP was observed as seen in the dominant follicles of regular menstruating individuals (Fig. 4), showing no difference in IGFBP profile between FF of follicles obtained from PCOS patients or regularly menstruating women.

In order to quantify differences in IGFBP levels, autoradiograms were analyzed by densitometric scanning. The mean relative IGFBP-2 levels in fluid of normal non-dominant (100 $\pm$ 52%) and atretic follicles (50 $\pm$ 23%, n=6) was not significantly different ($p > 0.05$). Also no significant difference between the relative 24 kDa IGFBP levels could be observed (100 $\pm$ 39%, n=6 for normal non-dominant and 69 $\pm$ 21%, n=6 for atretic follicles). Valid quantification of the differences in IGFBP-2, the 28 kDa IGFBP and the 24 kDa IGFBP levels between follicle fluid of normal non-dominant and dominant follicles could not be made due to the dramatic decrease in the intensities of these IGFBPs in fluid of the dominant follicles.

DISCUSSION

Results presented show that follicle fluid of healthy follicles obtained from regularly menstruating women contain IGFBPs with a molecular mass of 43, 37, 31, 28 and 24 kDa. Using standard immunoblotting techniques, we were able to identify the 43 and 37 kDa bands as IGFBP-3 and the 31 kDa protein as IGFBP-2. The 28 kDa doublet, however, could not be identified as IGFBP-1. Recently, Cataldo and Giudice also reported the presence of a 28 and 24 kDa band in follicular fluid. The 28 kDa IGFBP was shown to be glycosylated, and upon deglycosylation this protein most likely comigrated with 24 kDa IGFBP, because no additional band was found. Since indeed a glycosylated variant of IGFBP-4 was reported (19, 20), the authors suggested that the 28 kDa and 24 kDa IGFBPs found in follicular fluid were the glycosylated and non-glycosylated forms of IGFBP-4 (21). Alternatively, the 28 kDa glycosylated IGFBP could be IGFBP-6. This IGFBP has been shown to contain a potential Asn-linked glycosylation site, but is up to now only detected in porcine follicular fluid (22). Furthermore, no IGFBP-6 mRNA expression could be observed in any cell type of the human ovary (12). Finally, since the 28 kDa IGFBP is a doublet, the possibility exists that this doublet represents both IGFBP-4 and IGFBP-6.

Comparison of the IGFBP profiles in follicular fluid samples of follicles in different stages of development revealed that their was no dramatic change in IGFBPs in follicular fluid of normal healthy non-dominant follicles as compared to atretic follicles. Only in two out of 12 pools of atretic follicles a decrease in IGFBP-2, the 28 kDa and 24 kDa IGFBP (presumably IGFBP-4), was observed. Furthermore, no differences in IGFBP profiles were observed between follicular fluid samples obtained from PCOS patients and regularly menstruating women. From one PCOS patient follicular fluid was obtained from a dominant follicle, showing the same decrease in IGFBP-2, the 28 kDa and 24 kDa IGFBPs as seen in regularly menstruating women, indicating that with respect to the IGFBPs, follicular fluid of PCOS patients is not different from normal. These observations are in contrast with previous findings. Cataldo and Giudice described 3-, 6- and 19-fold higher amounts of IGFBP-2, 28 kDa and 24 kDa IGFBPs respectively, in follicular fluid of atretic compared to healthy follicles and these changes in IGFBP levels were also observed in follicular fluid obtained from three PCOS patients (21; 23). Some of this disagreement might be explained by the way atretic and healthy follicles are defined. We have classified the follicles according to AD/E_2 ratios as validated previously (16), whereas E_2:T was used by Cataldo and Giudice. Secondly, our group of PCOS patients is defined according to rigid clinical (obesity, hirsutism), endocrine (hyperandrogenemia) and sonographic (polycystic ovaries) criteria, as described previously (15). Finally, the most striking differences between atretic and healthy non-dominant follicles noted (21) might be found only in a subgroup of these follicles (namely those with a E_2:T ratio of 136-500).

A dramatic decrease in IGFBP-2, the 28 kDa and 24 kDa IGFBPs was observed in all samples of dominant follicles tested as compared to healthy developing non-dominant follicles. This finding seems of special interest since several studies indicate that the IGFBPs may play a role in regulating the potential of IGF to enhance gonadotropin action and subsequent follicle development (24-26). Both IGFBP-1 and IGFBP-3 have been shown to inhibit IGF-I and FSH induced estrogen production by cultured human granulosa cells. These IGFBPs were also capable of inhibiting the IGF-I, but not FSH induced progesterone response (13), demonstrating the inhibitory effect of these IGFBPs on the IGF-I-stimulated granulosa cell steroidogenesis. Furthermore, IGFBP-1 has been shown to inhibit the DNA

amplification induced by IGFs in human granulosa-luteal cells (11). Also a role for the IGFBPs not directly related to their IGF binding capacity has been proposed. Bicsak et al showed that, like an IGF-I antiserum, IGFBP-2 and -3 were capable of inhibiting steroid production by granulosa cells, but that IGFBP-3 was 2-3 fold more potent then IGFBP-2. Furthermore, the IGFBPs had no effect on the IGF-I stimulated cAMP production induced by FSH, suggesting that IGFBPs may have alternative ways of action, other than IGF sequestration (27).

In summary, it may be concluded from this study that IGFBP-2, the 28 kDa and 24 kDa IGFBPs are decreased in the dominant follicles as compared to normal healthy non-dominant and atretic follicles. A decrease of the inhibitory IGFBPs may result in an increase in free IGFs resulting in a stimulation of the mitogenic response of granulosa cells and an amplified steroidogenic response to FSH stimulation. This in turn facilitates the follicle to gain dominance. Furthermore, no differences in the IGFBP follicle fluid profile between PCOS and regularly menstruating women could be observed, which may suggest that with respect to intra-ovarian IGFBPs, PCOS patients are not different from normal.

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REFERENCES

1 Adashi, E.Y., Resnick, C.E., Hurwitz, A., Ricciarelli, E., Hernandez, E.R., Roberts, C., Leroith, D. and Rosenfeld, R., Insulin-like growth factors: the ovarian connection, Hum. Reprod., 6 (1991) 1213-1219.

2 Giudice, L., Insulin-like growth factors and ovarian follicular development, Endocrine Rev., 13 (1992) 641-669.

3 Geisthovel, F., Moretti-Rojas, I., Asch, R.H. and Rojas, F.J., Expression of insulin-like growth factor-II (IGF-II) messenger ribonucleic acid (mRNA), but not IGF-I mRNA, in human preovulatory granulosa cells, Hum. Reprod., 4 (1989) 899-902.

4 Hernandez, E.R., Hurwitz, A., Pellicer, A, et al., Human ovarian insulin-like growth factor receptor gen expression, Thirty-seventh Annual Meeting, Society for Gynecologic Investigation, St. Louis, March 1990 (Abstract 414).

5 Gates, G.S., Bayer, S., Seibel, M., Poretsky, L., Flier, J.S. and Moses, A.C., Characterization of insulin-like growth factor binding to human granulosa cells obtained during *in vitro* fertilization, J. Recept. Res., 7 (1987) 885-902.

6 Erickson, G.F., Magoffin, D.A., Cragun, J.R. and Chang, R.J., The effects of insulin and insulin-like growth factor-I and -II on estradiol production by granulosa cells of polycystic ovaries, J. Clin. Endocrinol. Metab., 70 (1990) 894-902.

7 Drop, S.L.S., Schuller, A.G.P., Lindenbergh-Kortleve, D.J., Groffen, C., Brinkman. A. and Zwarthoff. E.C., Structural aspects of the IGFBP family, Growth Regulation, 2 (1992) 69-79.

8 Clemmons, D.R., IGF binding proteins: regulation of cellular actions, Growth Regulation, 2 (1992) 80-87.

9 Seppälä, M., Wahlstrom, T., Koskimies, A.I., Tenhunen, A., Rutanen, E.M., Koistinen, R., Huhtaniemi, I., Bohn, H. and Stenman, U.H., Human preovulatory follicular fluid, luteinized cells of hyperstimulated preovulatory follicles, and corpus luteum contain placental protein 12, J. Clin. Endocrinol. Metab., 58 (1984) 505-510.

10 Koistinen, R., Suikkari, A.M., Tiitinen, A., Kontula, K. and Seppälä, M., Human granulosa cells contain insulin-like growth factor-binding protein (IGF BP-1) mRNA, Clin. Endocrinol., 32 (1990) 635-640.

11 Angervo, M., Koistinen, R., Suikkari, A.M. and Seppälä, M., Insulin-like growth factor binding protein-1 inhibits the DNA amplification induced by insulin-like growth factor I in human granulosa-luteal cells, Hum. Reprod., 6 (1991) 770-773.

12 El-Roeiy, A., Roberts, V.J., Shimasaki, S., Ling, N. and Yen, S.S.C., Localization, expression of insulin-like growth factor binding proteins (IGFBPs) 1-6 in normal, polycystic (PCO) human ovaries, Program of the 48th Annual Meeting of the American Fertility Society, New Orleans, LA, (1992) pS9 (Abstract 0-020).

13 Mason, H.D., Willis, D., Holly, J.M.P., Cwyfan-Hughes, S.C., Seppälä, M. and Franks, S., Inhibitory effects of insulin-like growth factor-binding proteins on steroidogenesis by human granulosa cells in culture, Mol. Cell. Endocrinol., 89 (1992) R1-R4.

14 Pache, T.D., Hop, W.C.J., de Jong, F.H., Leerentveld, R.A., v Geldrop, H., van de Kamp, T.M.M., Gooren, L.J.G. and Fauser, B.C.J.M., 17-ß estradiol, androstenedione, and inhibin levels in fluid from individual follicles of normal and polycystic ovaries, and in ovaries from androgen treated female to male transsexuals, Clin. Endocrinol. (Oxf), 36 (1992) 565-571.

15 Fauser, B.C.J.M., Pache, T.D., Lamberts, S.W.J., Hop, W.C.J., de Jong, F.H. and Dahl, K.D., Serum bioactive and immunoreactive luteinizing hormone and follicle-stimulating hormone levels in women with cycle abnormalities, with or without polycystic ovarian disease, J. Clin. Endocrinol. Metab., 73 (1991) 811-817.

16 McNatty, K.P., Moore Smith, D., Makris, A., Osathanondh, R. and Ryan, K.J., The microenvironment of the human follicle: inter-relationships among the steroid levels in antral fluid, the population of granulosa cells and the status of the oocyte in vivo and vitro, J. Clin. Endocrinol. Metab., 49 (1979) 851-860.

17 Hossenlopp, P., Seurin, D., Segovia-Quinson, B., Hardouin, S. and Binoux, M., Analysis of serum insulin-like growth factor binding proteins using Western blotting: use of the method for titration of the binding proteins and competitive binding studies, Anal. Biochem., 154 (1986) 138-143.

18 Schuller, A.G.P., Lindenbergh-Kortleve, D.J., de Boer, W.I., Zwarthoff, E.C. and Drop S.L.S., Localization of the epitope of a monoclonal antibody against human insulin-like growth factor binding protein-1, functionally interfering with insulin-like growth factor binding, Growth Regulation, (1993) in press.

19 Fielder, P.J., Thordarson, G., Talamantes, F. and Rosenfeld, R., Characterization of insulin-like growth factor binding proteins (IGFBPs) during gestation in mice: effects of hypophysectomy and an IGFBP-specific serum protease activity, Endocrinology, 127 (1990) 2270-2280.

20 Cheung, P.T., Smith, E.P., Shimasaki, S., Ling, N. and Chernausek, S.D., Characterization of an insulin-like growth factor binding protein (IGFBP-4) produced by the B104 rat neuronal cell line: chemical and biological properties and differential synthesis by sublines, Endocrinology, 129 (1991) 1006-1015.

21 Cataldo, N.A. and Giudice, L.C., Insulin-like growth factor binding protein profiles in human ovarian follicular fluid correlate with follicular functional status, J. Clin Endocrinol. Metab., 74 (1992) 821-829.

22 Shimasaki, S., Gao, L., Shimonaka, M. and Ling, N., Isolation and molecular cloning of insulin-like growth factor binding protein-6, Mol. Endocrinol., 5 (1991) 938-948.

23 Cataldo, N.A. and Giudice, L.C., Follicular fluid insulin-like growth factor binding protein profiles in polycystic ovary syndrome, J. Clin Endocrinol. Metab., 74 (1992) 695-697.

24 Barreca, A., Minuto, F., Volpe, A., Cecchelli, E., Cella, F., Del Monte, P., Artini, P. and Giordano, G., Insulin-like growth factor-I (IGF-I) and IGF-I binding protein in the follicular fluids of growth hormone treated patients, Clin. Endocrinol., 32 (1990) 497-505.

25 Holly, J.M.P., Eden, J.A., Alaghband-Zadeh, J., Carter, G.D., Jemmott, R.C., Cianfarani, S., Chard, T. and Wass, Insulin-like growth factor binding proteins in follicular fluid from normal dominant and cohort follicles, polycystic and multicystic ovaries, Clin. Endocrinol., 33 (1990) 53-64.

26 Bicsak, T., Ling, N. and DePaolo, L.V., Ovarian intrabursal administration of insulin-like growth factor-binding protein inhibits follicle rupture in gonadotropin-treated immature female rats, Biol. Reprod., 44 (1991) 599-603.

27 Bicsak, T.A., Shimonaka, M., Malkowski, M. and Ling, N., Insulin-like growth factor-binding protein (IGF-BP) inhibition of granulosa cell function: effect on cyclic adenosine 3',5'-monophosphate, deoxyribonucleic acid synthesis, and comparison with the effect of an IGF-I antibody, Endocrinology, 126 (1990) 2184-2189.

CHAPTER 4

cDNA CLONING AND mRNA EXPRESSION OF THE SIX MOUSE INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS.

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submitted (1994)

SUMMARY

The insulin-like growth factor binding proteins (IGFBPs) comprise a family of six distinct proteins which modulate insulin-like growth factor action. We have isolated cDNAs encoding the six mouse IGFBPs (mIGFBPs). In addition, we studied the mRNA expression of the six mIGFBPs during development and in various adult tissues. Our results show that each of the six mIGFBPs is highly homologous to their human and rat counterparts, whereas only the N and C terminal ends are conserved between the six mIGFBPs. Northern blotting revealed that mIGFBP-2, -3, -4 and -5 genes are already expressed at gestational day 11½, suggesting a role for these mIGFBPs in embryonal development. In liver, a peak of mIGFBP-1 mRNA expression was found around birth, suggesting a function for mIGFBP-1 in the newborn mouse. Finally, tissue-specific expression of the six mouse IGFBP genes was observed in adult tissues suggesting different roles or modes of actions in adult life.

INTRODUCTION

Insulin-like growth factors (IGFs) are peptides displaying important functions in regulating cell proliferation, differentiation and metabolism (Sara and Hall, 1990). The IGFs are synthesized and secreted by many tissues and interact with the cell by binding to specific membrane receptors (Daughaday and Rotwein, 1989; Rosenfeld and Hintz, 1986). In the body fluids, the IGFs are found in complex with a specific group of proteins, the IGF binding proteins (IGFBPs). These IGFBPs have an affinity for IGFs equal to that of IGF-receptors and are involved in the modulation of IGF action in different tissues (Hardouin et al., 1987; Baxter and Martin, 1989). The IGFBPs belong to a family of at least six different proteins, designated IGFBP-1 to -6. At present, the cDNAs encoding the six rat and human IGFBPs have been isolated and characterized revealing that the six IGFBPs are clearly distinct, but share regions with strong homology (Drop et al., 1992). Furthermore, IGFBP gene expression is tissue specific and developmentally regulated (Rechler and Brown, 1992).

Proposed roles of the IGFBPs are to distribute the IGFs over the various body fluid compartments and to protect the body from the hypoglycemic effects of IGFs. Another

function of the IGFBPs appears to be the modulation of IGF binding to their receptors, thereby playing an important role in mediating IGF action (Clemmons, 1992). However, the precise role of the six different IGFBPs is still poorly understood. Using in situ hybridization techniques, we recently have shown that the six mouse IGFBPs are expressed in a tissue and cell specific manner during development, suggesting distinct roles for the six mouse IGFBPs in embryonal and fetal life (Schuller et al., 1993). Here we describe the isolation and characterization of the cDNAs encoding the six mouse IGFBPs. We have studied the mRNA expression of the six mouse IGFBPs during the second half of gestation and postnatally by examining total RNA extracted from whole conceptuses (day 11-18) and from pre- and postnatal liver. In addition we determined the mRNA expression in various adult mice tissues.

MATERIALS AND METHODS

Isolation of cDNA clones

For isolation of mouse IGFBP-1 cDNA clones, 1 x 10^6 phage plaques of a liver cDNA library in lambda gt11 (Promega, Madison, USA) were lifted onto nitrocellulose filters and hybridized with a [^{32}P]-labelled Pst1-Xba1 (490 bp) fragment of a human IGFBP-1 cDNA clone, p19 (Brinkman et al., 1988) at 59^{0}C according to standard procedures (Maniatis et al., 1982). For isolation of the cDNAs encoding mouse IGFBP-2, -3, -4, -5 and -6, mouse liver and kidney cDNA libraries in lambda gt11 and a mouse liver cDNA library in lambda zap (kindly provided by Dr. Leuven, University of Leuven, Belgium) were screened. Complementary DNA fragments specific for each of the IGFBPs were amplified by PCR as described before (Schuller et al., 1993), [^{32}P]-labelled and used as probe at 65^{0}C. After washing, filters were exposed to X-Ray film with an intensifying screen at -80^{0}C for 3 days. Positive phage plaques were purified by rescreening and DNA isolated according to standard procedures (Maniatis et al., 1982). All cDNA inserts were excised with EcoR1/Not1, inserted into pBluescript KS$^+$ (Stratagene) and sequenced according to the dideoxy chain termination method (Sanger et al., 1977) using the M13 forward and reverse primers or specifically designed oligonucleotide primers, synthesized on a DNA Synthesizer (Applied Biosystems).

Northern analysis

Tissues (liver, kidney, lung, heart, spleen, brain, muscle, testis and ovary) were obtained from adult BALB/c mice, frozen in liquid nitrogen and stored at -80^{0}C until use. Likewise, mouse conceptuses (11-18 days gestation) were frozen in liquid nitrogen and stored. In addition, liver was isolated from 16 and 18 day old mouse fetuses and from mice 1, 3, 7, 14 and 28 days postnatally. Total RNA was extracted by the guanidinium thiocyanate method as described (Chomczynski and Sacchi, 1989). RNA was denatured with

dimethylsulfoxide and glyoxal, electrophoresed in 1% agarose and transferred to nylon membranes (GeneScreen, Du Pont de Nemours, Germany). Hybridization was performed with [^{32}P]-labelled mIGFBP cDNA fragments at 42°C in a RNA hybridization buffer containing 50% formamide, 10% dextran sulphate, 100 μg/ml ssDNA, 10x Denhardt's, 0.1% $Na_4P_2O_7$, 50 mM Tris pH 7.5 and 0.1% SDS.

Quantification of blots

Autoradiograms were quantified by densitometric scanning using a Bio-Rad video densitometer model 620.

RESULTS

Isolation of mIGFBP cDNA clones.

Screening of mouse liver and kidney cDNA libraries using IGFBP specific probes resulted in the isolation of several independent clones. Sequencing of the inserts and comparison of the nucleotide sequence with that of the six rat IGFBPs revealed that we had isolated several cDNA clones for each of the six mouse IGFBPs. Furthermore, for all six mouse IGFBPs, at least one complete cDNA was isolated. The nucleotide sequences and predicted amino acid sequences of the six mouse IGFBP cDNAs are shown in Fig. 1. Comparison of the amino acid sequence of the six mouse IGFBPs showed conservation of 18 cysteine residues in all mIGFBPs, with the exception of mIGFBP-4 which has two additional cysteines and mIGFBP-6 which only contained 16 cysteine residues. Furthermore, a high degree of homology between the six mIGFBPs was observed in both the N-terminal and C-terminal regions, whereas the middle part of the IGFBPs is less well conserved. Both mIGFBP-1 and -2 contain an Arg-Gly-Asp sequence at their C-terminal end (Fig. 2). Comparison of the predicted amino acid sequences of mIGFBP-1, -2, -3, -4, -5 and -6 with the corresponding human IGFBP showed a homology of respectively 73%, 89%, 81%, 93%, 97% and 73%. The predicted amino acid sequences showed an even higher degree of homology with the rat IGFBPs (94%, 96%, 93%, 99%, 99.6% and 94% respectively). In mIGFBP-2 an additional Glycine at position 118 was found and mIGFBP-3 lacked an Alanine at position 7, when comparing them with their respective rat counterparts (Margot et al., 1989; Shimasaki et al., 1989). Furthermore, mIGFBP-6 has an insertion of 9 amino acids (residues 14-22), which is not found in rat IGFBP-6 (Shimasaki et al., 1991) (Fig. 2).

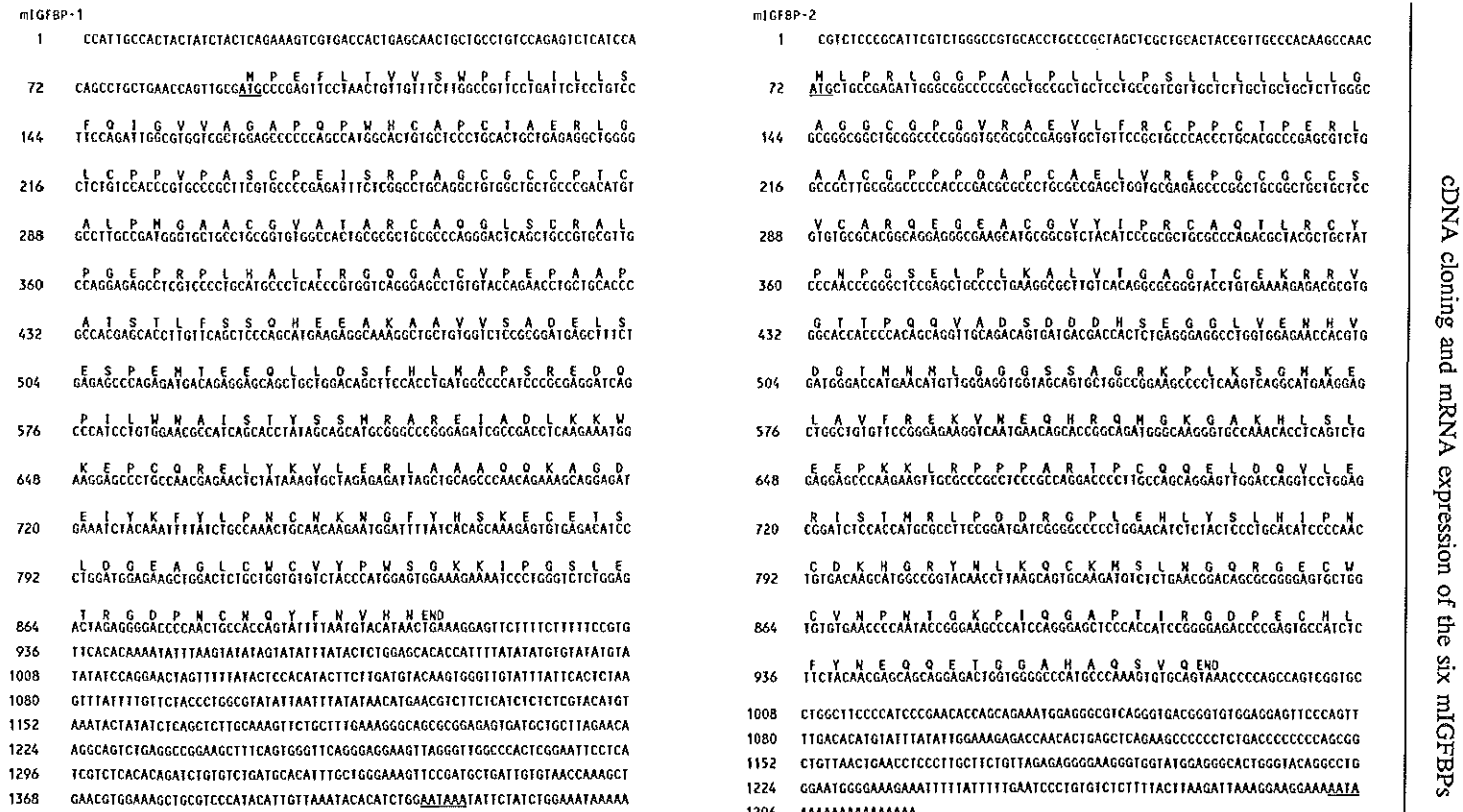

Fig. 1a. Nucleotide sequence and predicted amino acid sequence of mIGFBP-1 and -2. Putative initiation codons and polyadenylation signals are underlined.

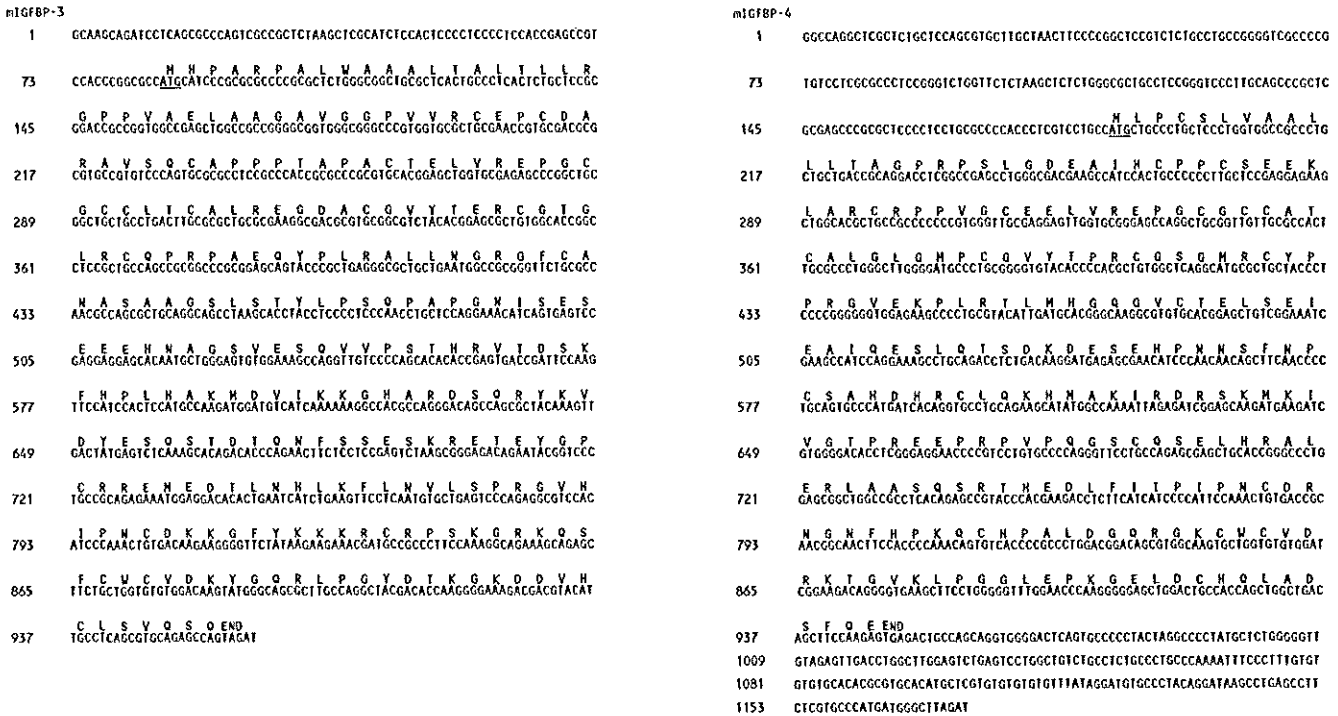

Fig. 1b. Nucleotide sequence and predicted amino acid sequence of mIGFBP-3 and -4. Putative initiation codons and polyadenylation signals are underlined.

Fig. 1c. Nucleotide sequence and predicted amino acid sequence of mIGFBP-5 and -6. Putative initiation codons and polyadenylation signals are underlined.

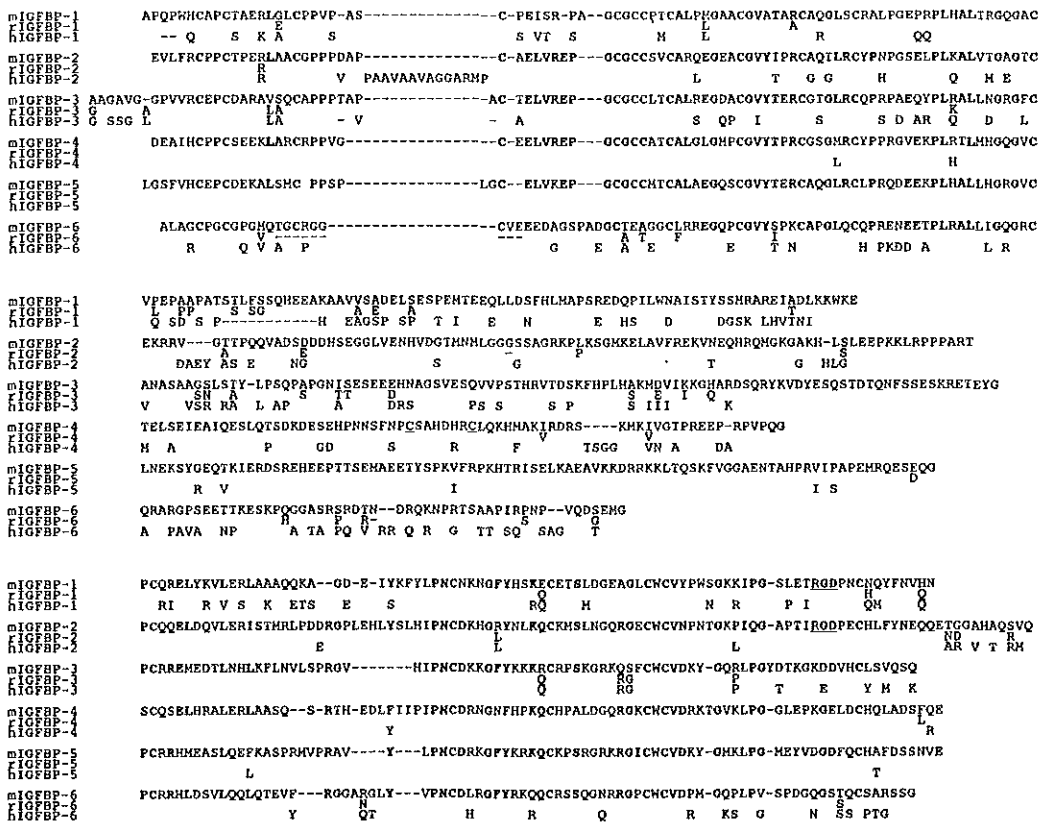

Fig. 2. Comparison of the predicted amino acid sequences of the six mouse, rat and human IGFBPs. The human and rat amino acid sequences are reproduced with kind permission from Shimasaki et al. (27). Residues shared by at least four of the six IGFBPs are printed in bold letters. Only the residues that differ from the mouse sequence are shown for the human and rat IGFBPs. A dash denotes the absence of a specific residue.

Developmental mIGFBP mRNA expression.

The mRNA expression of the six mIGFBP genes during development was examined in total RNA extracted from whole conceptuses (11-18 days gestation) and in total RNA extracted from pre- and postnatal liver. As is shown if Fig. 3, mRNA expression of mIGFBP-2, -3, -4 and -5 was already detectable in embryos of day 11½. Expression of mIGFBP-1 mRNA was hardly detectable and first seen in 13½ day embryos (not shown). Transcripts for mIGFBP-6 were not detected even after an exposure time of two weeks (not shown).

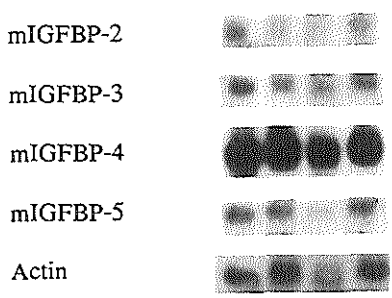

Fig. 3. Messenger RNA expression of mIGFBP-2, -3, -4 and -5 in total RNA isolated from mouse embryos. 20 μg RNA was applied to each lane. The RNA samples were obtained from embryos at 11½ (lane 1), 12½ (lane 2), 13½ (lane 3) and 14½ (lane 4) days of gestation.

In total RNA extracted from fetal and postnatal livers (Fig 4a), expression of mIGFBP-1 was detectable at day 16½ of gestation and showed a dramatic increase at the first day after birth. Expression of mIGFBP-1 was still high at day 3 postnatally, but decreased to lower levels thereafter. Expression of mIGFBP-2 was also detectable in fetal livers and increased dramatically after birth. However, the expression of mIGFBP-2 decreased only slightly after postnatal day 3 and remained abundant thereafter. Expression of mIGFBP-3 transcripts was observed from day 18½ of gestation. The levels of mIGFBP-3 mRNA expression slowly increased to a maximum at 1 week postnatally. Expression of mIGFBP-4 was observed from fetal day 16½ and did not change dramatically over the period examined. An interpretation of these results based on densitometric measurements is given in Fig.4b.

Expression of mIGFBP-5 and -6 mRNA was not observed, even after an exposure time of 2 weeks. Finally, ß-actin mRNA expression was found to decrease after birth. This observation has also been made during brain development in mouse (Lazarini et al., 1991), demonstrating that ß-actin expression can not always be used to show equality of the amount of RNA applied.

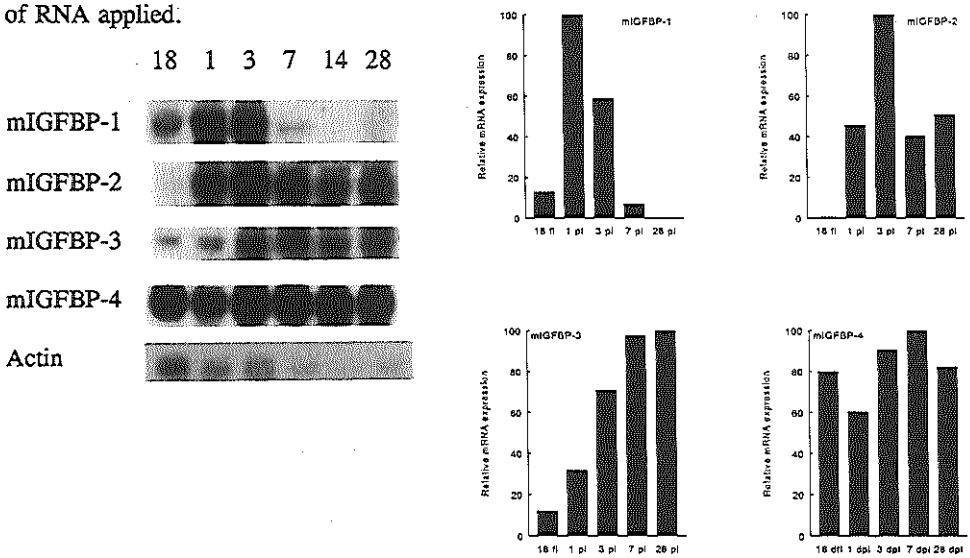

Fig. 4. a. Messenger RNA expression of mIGFBP-1, -2, -3 and -4 in fetal and postnatal mouse liver. In each lane 20 µg total RNA was applied isolated from 18 days fetal liver (18) and from liver obtained 1, 3, 7, 14 and 28 days after birth (1, 3, 7, 14 and 28 respectively). b. Relative hybridization of mIGFBP-1, -2, -3 and -4 based on densitometric measurements. Highest mRNA expression level of each mIGFBP in liver was set to 100%. Results are presented as a percentage of the maximum expression level. 18 fl represents 18 days fetal liver; 1, 3, 7, 14 and 28 pl represent 1, 3, 7, 14 and 28 days postnatal liver.

Tissue specific mIGFBP mRNA expression in adult mice.

Total RNA from different organs of adult BALB/c mice was examined for IGFBP gene expression by Northern blot analysis. A summary of these results is given in Table 1. Expression of IGFBP-1 was detected solely in liver. High expression of mIGFBP-2 was found in liver, but also in kidney, lung, brain, spleen, testis and ovary. Transcripts for mIGFBP-3 were detected mainly in kidney, liver, lung, heart, spleen and muscle. Expression of mIGFBP-4 was highest in liver, kidney and spleen, but also detectable in lung, heart,

brain and muscle. Expression of mIGFBP-5 was abundant in kidney, muscle and ovary, being lower in lung, heart, brain and testis, whereas mIGFBP-6 was expressed mainly in lung, heart, muscle, ovary and testis (Table 1).

Table 1. Messenger RNA expression of the six mIGFBPs in adult mouse tissues.

	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6
Liver	+	++	+	++	-	+/-
Kidney	-	++	++	++	++	-
Lung	-	+	+	+	+	+
Heart	-	+/-	+	+	+	+
Spleen	-	+	+	++	+/-	+/-
Brain	-	+	+/-	+	+	+/-
Muscle	-	-	+	+	++	+
Testis	-	+	-	-	+	+
Ovary	-	+	+/-	+/-	++	+

+, detected; -, not detected; +/-, detected after exposure times of > 10 days

DISCUSSION

Our data show that the six mIGFBPs are highly homologous in both the C-terminal and N-terminal region. Furthermore, 18 cysteine residues were found spatially conserved in all mIGFBPs, with the exception of mIGFBP-4 which has two additional cysteines and mIGFBP-6 which lacks two cysteine residues. Thus we conclude that the distribution of the cysteine residues in the mIGFBPs is completely analogous to that of the human IGFBPs (Drop et al., 1992). In addition, every murine IGFBP is highly homologous to its human and rat counterpart. This also holds true for the central part of the IGFBPs where no obvious conservation is found between different IGFBPs from one species. In relation to the functions of the mIGFBPs, these observations may indicate that the N- and C- terminal regions contain domains important for all six mIGFBPs, for instance binding of IGF, whereas the middle part may represent a region which is important for the unique features of each of the mIGFBPs, such as cell specific attachment, sensitivity to proteases and protein interactions. Recently, the isolation of a mIGFBP-2 genomic clone has been reported (Landwehr et al., 1993). The predicted amino acid sequence of this clone was identical to the mIGFBP-2 cDNA clone reported here, with the one exception being a His residue at position 99, where we found an Asp residue. Whether this difference represents a cloning artefact or a natural polymorphism remains to be investigated.

In whole embryos, transcripts for mIGFBP-2, -3, -4 and -5 were already detectable at 11½ days gestation, whereas low levels of mIGFBP-1 mRNA were first found in 13½ day old embryos and expression of mIGFBP-6 was below detection limits. Using in situ hybridization, we have shown that mRNAs for mIGFBP-2, -4 and -5 are expressed in various tissues of mid gestational mouse embryos and mRNA expression of mIGFBP-1 was detected in liver. Expression of the mIGFBP-3 and -6 genes was found in fetal stages (Schuller et al., 1993). Furthermore, Cerro et al., recently showed by in situ hybridization techniques that IGFBP-1, -3, -4 and -6 were expressed at least as early as rat embryonic day 14 (Cerro et al., 1993). Our finding that mRNA expression of mIGFBP-6 was below detection limits in 20 μg total RNA extracted from whole embryos suggests that the mRNA expression of IGFBP-6 observed in rat embryonal liver and a previously unrecognized cell population surrounding developing cartilage (Cerro et al., 1993) is either at a low level or that these cells contribute to only a small percentage of the embryo and therefore the expression of mIGFBP-6 mRNA is not detectable in total RNA extracted from a whole embryo. Taken together, these observations indicate that all IGFBPs are expressed in mid gestational embryos.

Using gene disruption techniques, it has recently been shown that both IGF-I and IGF-II are essential for normal embryonic and fetal mouse development. Homozygous disruption of the IGF-I or IGF-II genes resulted in severe growth retardation (birth weight 60% of normal littermates). Double mutants carrying both disrupted IGF-I and IGF-II genes displayed an even more severe growth deficiency, reaching only 30% of the normal birth weight (DeChiara et al., 1990; Liu et al., 1993). Comparison of embryonal growth kinetics revealed that the growth retardation induced by IGF-I gene disruption was first seen at embryonal day 13.5, whereas that induced by IGF-II disruption was already seen at embryonal day 11.5 (Baker et al., 1993). Thus, these observations indicate that all six mIGFBP genes are expressed at the stage of development when IGF action is essential. Therefore, the IGFBPs may play a significant role in mediating the IGF effects in embryonal growth and development.

In fetal and postnatal liver, a striking mRNA expression pattern was observed for mIGFBP-1, being highest at day 1 and 3 after birth. Expression of mIGFBP-2 mRNA also increases after birth and decreases after postnatal day 3, but the expression stays high thereafter. These findings confirm the observations of Babajko et al., who showed that the transcriptional activity of the rat IGFBP-1 and -2 genes in liver are 25 times and 5 times greater at birth than at 16 days gestation. At birth, hepatic mRNA levels of rIGFBP-1 and -2 were increased approximately 50 fold and 5-10 fold, decreasing to approximately 20% by day 6 for rIGFBP-1, whereas the levels of rIGFBP-2 mRNA remained constant (Babajko et al., 1993). These observations suggest a distinct role for mIGFBP-1 produced by the liver during the first days after birth. A possible role for transiently high levels of IGFBP-1 in early postnatal life was suggested to be the modulation of the growth promoting activities of IGFs, prior to growth hormone sensitivity (Cerro et al., 1993). Although this possibility exists, transcripts for the growth hormone receptor have been detected at earlier stages in rat liver (Berry et al., 1993). Furthermore, mRNA of the growth hormone-responsive serpin gene Spi 2.1 is expressed in fetal rat liver. In addition, the same deoxyribonuclease I hypersensitive sites were present in the 5' flanking region of the Spi 2.1 gene in DNA isolated from fetal and postnatal rat livers, suggesting that the gene is transcriptional competent in fetal life (Berry et al., 1993). Therefore, the possibility exists that the growth promoting effects of IGFs are also already under growth hormone control in late fetal and early postnatal life. Another possibility is that the high IGFBP-1 mRNA levels reflect the nutritional changes of the newborn mice, since for instance fasting has also been shown to induce both IGFBP-1 and -2 mRNA expression in rat liver (Ooi et al., 1990; Murphy et al., 19990; Orlowski et al., 1990).

The mRNA expression of the six mIGFBP mRNAs in various adult mouse tissues, as studied by Northern blotting, revealed tissue specific expression patterns for each of the six mIGFBPs, which were partly overlapping, but distinct. In general, the mRNA expression patterns of the six mIGFBPs as described here, are in agreement with previous observations for the human and rat IGFBPs (Rechler and Brown 1992), although additional sites of low IGFBP mRNA expression have been reported.

In summary, we have isolated and characterized the cDNAs encoding the six mIGFBPs. Northern blotting revealed that mIGFBP-2, -3, -4 and -5 genes are already expressed at gestational day 11½ and that at least 5 of the six mIGFBP genes are expressed at 13½ days gestation, suggesting roles for the IGFBPs in organogenesis and embryonal development. Furthermore, the peak in especially mIGFBP-1 mRNA expression found in liver shortly after birth suggests an important role for this protein in the newborn mouse. Finally, the tissue specific expression patterns of the six mIGFBP genes in adult mice, suggest distinct roles or modes of actions for the different mIGFBPs in adult life.

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REFERENCES

1 Babajko, S., Hardouin, S., Segovia, B., Groyer, A. and Binoux, M. (1993) Endocrinology 132, 2586-2592.
2 Baker, J., Liu, J.-P., Robertson, J.E. and Efstratiadis, A. (1993) Cell 75, 73-82.
3 Baxter, R.C. and Martin, J.L. (1989) Prog. Growth Factor Res. 1, 49-68.
4 Berry, S.A., Bergad, P.L. and Bundy, M.V. (1993) Am. J. Physiol. 264, 973-980.
5 Brinkman, A., Groffen, C., Kortleve, D.J., Geurts van Kessel, A. and Drop, S.L.S. (1988) EMBO J. 7, 2417-2423.
6 Cerro, J.A., Grewal, A., Wood, T.L. and Pintar, J.E. (1993) Reg. Peptides 48, 189-198.
7 Chomczynski, P. and Sacchi, N. (1989) Anal. Biochem. 162, 156-159.
8 Clemmons, D.R. (1992) Growth Regulation 2, 80-87.
9 Daughaday, W.H. and Rotwein, P. (1989) Endocr. Rev. 10, 68-91.
10 DeChiara, T.M., Efstratiadis, A. and Robertson, E.J. (1990) Nature 345, 78-80.
11 Drop, S.L.S., Schuller, A.G.P., Lindenbergh-Kortleve, D.J., Groffen, C., Brinkman, A. and Zwarthoff, E.C. (1992) Growth Regulation 2, 69-79.
12 Hardouin, S., Hossenlopp, P., Segovia, B., Seurin, D., Portolan, G., Landwehr, J., Kaupmann, K., Heinrich, G. and Schwander, J. (1993) Gene 124, 281-286.
13 Lassarre, C. and Binoux, M. (1987) Eur. J. Biochem. 170, 121-132.
14 Lazarini, F., Deslys, J.P. and Dormont, D. (1991) Brain Res. Mol. Brain Res. 10, 343-346.
15 Liu, J.-P., Baker, J., Perkins, A.S., Robertson, J.E. and Efstratiadis, A. (1993) Cell 75, 59-72.

16 Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
17 Margot, J.B., Binkert, C., Mary, J.-L., Landwehr, J., Heinrich, G. and Schwander, J. (1989) Mol. Endocrinol. 3, 1053-1060.
18 Murphy, L.J., Seneviratne, C., Ballejo, G., Croze, F. and Kennedy, T.G. (1990) Mol. Endocrinol. 4, 329-336.
19 Ooi, G.T., Orlowski, C.C., Brown, A.L., Becker, R.E., Unterman, T.G. and Rechler, M.M. (1990) Mol. Endocrinol. 4, 321-328.
20 Orlowski, C.C., Brown, A.L., Ooi, G.T., Yang, Y.W.-H., Tseng, L.Y.-H. and Rechler, M.M. (1990) Endocrinology 126, 644-652.
21 Rechler, M.M. and Brown, A.L. (1992) Growth Regulation 2, 55-68.
22 Rosenfeld, R.G. and Hintz, R.L. (1986) in The Receptors (Cohn, P.M. ed.) vol 3, pp. 281-329, Acadamic Press, New York.
23 Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA. 74, 5463-5467.
24 Sara, V.R. and Hall, K. (1990) Physiol. Rev. 70, 591-614.
25 Schuller, A.G.P., Zwarthoff, E.C. and Drop, S.L.S. (1993) Endocrinology 132, 2544-2550.
26 Shimasaki, S., Koba, A., Mercado, M., Shimonaka, M., Ling, N. (1989) Biochem. Biophys. Res. Comm. 165, 907-912.
27 Shimasaki, S., Gao, L., Shimonaka, M. and Ling, N. (1991) Mol. Endocrinol. 5, 938-948.

CHAPTER 5

GENE EXPRESSION OF THE SIX INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS (IGFBPs) IN THE MOUSE CONCEPTUS DURING MID- AND LATE GESTATION.

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ABSTRACT

The insulin-like growth factor binding proteins (IGFBPs) comprise at least six distinct species that may modulate the action of IGFs. IGFs are important regulators of fetal growth and differentiation. To define sites of IGFBP mRNA synthesis, we have used *in situ* hybridization techniques in mouse conceptuses of different gestational ages (11-18 days).

Expression of mouse (m) IGFBP-1 was detected in mouse conceptuses after day 12 of gestation and was restricted to the liver. Transcripts for mIGFBP-2, -4 and -5 were detected in various tissues and were found in all stages tested. In contrast, expression of mIGFBP-3 and -6 could be only weakly detected in late gestational conceptuses. Comparison of the expression patterns of mIGFBP-2, -4 and -5, which were found widely distributed in mouse conceptuses, revealed that mIGFBP-2 was expressed in the mesoderm-derived part of the tongue (day 13.5), but mainly in the ectodermal layer. Transcripts for mIGFBP-4, however, were detected only in the mesodermal part, whereas expression of mIGFBP-5 was restricted to the ectodermal layer. A similar distribution pattern was observed in the lung (day 18). In general, expression of mIGFBP-2 and -5 was detected in the same cells, whereas mIGFBP-4 and -5 were expressed mainly in different cell types. These data suggest that the different mIGFBPs might play distinct roles in mouse embryonal and fetal life.

INTRODUCTION

The insulin-like growth factors (IGF-I and -II) are peptides with both mitogenic and metabolic properties. The IGFs are thought to be important regulators of fetal growth and differentiation. In the rat the gene for IGF-II is highly expressed in a variety of fetal tissues and high levels of IGF-II are found in fetal serum (1, 2). After birth, IGF-II expression rapidly decreases in all tissues except the brain, suggesting a role for IGF-II primarily as a fetal growth factor (1, 3). Expression of IGF-I is also found in a variety of fetal tissues, although to a lesser extent (4, 5).

Both pre- and postnatally, IGFs occur in plasma and other biological fluids bound to IGF binding proteins (IGFBPs). These IGFBPs are thought to modulate IGF action (6).

Recently, cDNAs encoding for six distinct IGFBPs (designated IGFBP-1 to -6) have been isolated and characterized (for review, see Ref. 7). At least two IGFBPs are expressed in fetal tissues. IGFBP-1 is most abundantly expressed in fetal liver of both humans (8) and rats (9). Expression of IGFBP-2 has been found in term gestation rat embryos, especially in liver, stomach, brain, kidney, and lung (9, 10). More specifically, IGFBP-2 mRNA has been detected in many cells types derived from ectoderm and endoderm, and the distribution of IGFBP-2 expression in mid gestational rat embryos was, in general, distinct from that of IGF-II (11). The specific cellular sites of IGFBP expression during embryogenesis have only partly been determined. In this report, we used *in situ* hybridization techniques to document more precisely the mRNA expression of the six mouse (m) IGFBPs in mid- and late gestational mouse conceptuses (day 11-18).

MATERIALS AND METHODS

Isolation of tissues and sectioning

Pregnant BALB/c mice were killed, and conceptuses of 11 to 18 days gestation were collected, staged according to Theiler (12), frozen in liquid nitrogen and stored at -80°C. In addition, at least 5 conceptuses for each time point were fixed in 4% paraformaldehyde, dehydrated in successive baths containing increasing amounts of ethanol and embedded in paraffin according to standard procedures. Ten-micron cryosections were cut on a cryostat microtome and mounted onto poly-L-lysine-coated slides. Six micron paraffin sections were cut on a microtome and mounted onto gelatin-coated slides on a prewarmed drop of 10% ethanol. Sections were stretched and dried on a 45°C heating plate for 1 day.

Preparation of cRNA probes

cDNA fragments specific for mIGFBP-1, -2, -3, -4, -5 and -6 were amplified from a mouse liver and kidney cDNA library (Promega, Madison, WI) by standard polymerase chain reaction techniques. Synthetic oligonucleotide primers (21 nucleotides in length) were generated based on known nucleotide sequences of the six rat IGFBPs (7). Using these primers, mouse IGFBP cDNA fragments, corresponding to amino acid position 82-152, 98-258, 137-204, 131-205, 88-182 and 83-140 of rat IGFBP-1 to -6, respectively, were amplified. The cDNA fragments were ligated into pTZ18R and pTZ19R (Pharmacia, Uppsala, Sweden) and sequenced according to the method of Sanger *et al.* (13). ^{35}S-Labeled RNA transcripts were generated from linearized plasmid using T7 RNA polymerase in the presence of 50 μCi [^{35}S]UTP (Amersham, Aylesbury, Buckinghamshire, United Kingdom). The DNA templates were removed by digestion with DNase-I, and the labeled RNA probes were purified by phenol-chloroform extraction and ethanol precipitation.

In situ hybridization

In situ hybridization was carried out essentially as previously described (14). Briefly, paraffin wax was removed from the sections using three successive baths of xylene, followed by two baths of 100% ethanol. Subsequently, sections were rehydrated and immersed in 0.2 N HCl for 20 min. Sections were rinsed in PBS, incubated in 2xSSC (0.3 M NaCl, 0.03 M Na citrate) for 15 min at 70 °C, and postfixed for 5 minutes in 4% paraformaldehyde. Sections were treated to block non-specific binding by rinsing in PBS and 10 mM dithiothreitol (DTT) and in PBS, 10 mM DTT, 10 mM iodoacetamide, and 10 mM *N*-ethylmaleimide, both for 15 min, at 45 °C, followed by two rinses of PBS and incubation with 0.1 M triethanolamine and 0.25 % acetic anhydride for 10 min. Sections were rinsed in 2xSSC and stored in 70% ethanol for 14 h. The next day sections were dehydrated, dried, and hybridized with ^{35}S-labeled RNA probes for 4 h at 66 °C in a solution containing 50% formamide, 4xSSC, 1x Denhardt's solution, 1mg/ml transfer RNA, 10% dextransulfate, 10mM DTT, and 0.2 mg/ml salmon sperm DNA. After hybridization, the sections were washed at 56 °C in successive baths containing 55% formamide, 2xSSC, and 20 mM ß-mercaptoethanol (twice for 15 min each); and 55% formamide, 2xSSC, 20 mM ß-mercaptoethanol, and 0.5% triton X-100 (twice for 15 min each); and 55% formamide, 2xSSC, and 20 Mm ß-mercaptoethanol (twice for 15 min each). Subsequently, sections were rinsed in 2xSSC, dehydrated, and dried before to exposure to Kodak AR x-ray film (Eastman Kodak, Rochester, NY) for 1-4 days. Finally, sections were exposed to Kodak NTB2 photographic emulsion for 3-35 days, developed, and stained with nuclear fast red for microscopic evaluation. The level of non-specific binding was determined using sense probes for each of the six mIGFBPs.

RESULTS

A first evaluation of the hybridization patterns of the six mIGFBPs on autoradiogram of tissue sections revealed that mIGFBP-2, -4 and -5 are highly expressed and widely distributed in the mouse conceptuses (Fig. 1, B-D). Expression of mIGFBP-1 could only be detected in cryosections of conceptuses after day 12 of gestation and was restricted to the liver (Fig. 1A). Transcripts of mIGFBP-3 and -6 were very weakly expressed and could not or only slightly be detected on the autoradiograms after a 4-day exposure (results not shown).

Microscopic evaluation revealed that expression of mIGFBP-2 was detected in telencephalon, mesencephalon, and tongue as early as day 11 of gestation. From day 13, the mIGFBP-2 transcript was detected in differentiating sclerotomes, tongue, esophagus, nasal placode, lung, and liver. After day 14, the expression of mIGFBP-2 was also found in the choroid plexus, meninges, cornea, sclera, submaxillary gland, thymus, vertebrae, kidney, intestine, bladder, and hair follicles.

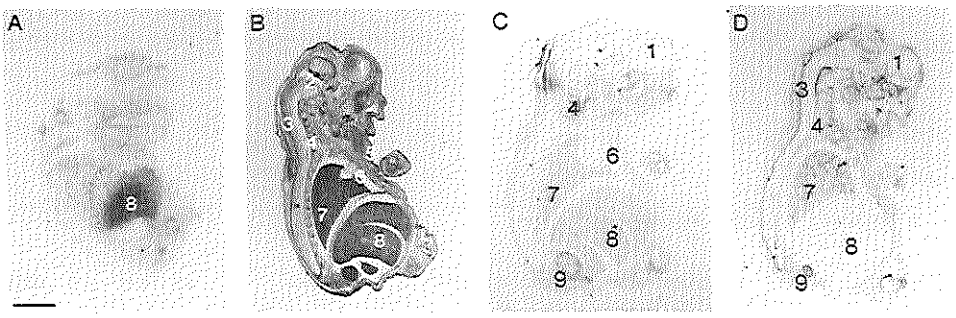

Fig.1. In situ hybridization to sagittal sections of mouse conceptuses. Sections were hybridized with ^{35}S-labeled RNA probes for mIGFBP-1, -2, -4 and -5, followed by autoradiography. The autoradiogram shown in A is from a freeze section of a 13.5-day-old mouse embryo hybridized with a probe for mIGFBP-1. B, C, and D show autoradiographic images of paraffin sections of an 18-day-old mouse fetus hybridized with probes for mIGFBP-2 (B), mIGFBP-4 (C), and mIGFBP-5 (D). The autoradiogram of mIGFBP-2 was exposed for 1 day, and the others for 3 days. 1, Brain; 2, Choroid plexus; 3, Meninges; 4, Vertebrae; 5, Skin; 6, Heart; 7, Lung; 8, Liver; 9, Kidney. Scale bar = 0.8 mm for A and 2 mm for B, C, and D.

Expression of mIGFBP-3 could be detected in the liver and in vertebrae of conceptuses of 16-18 days gestation, but only after an exposure of 3-5 weeks.

Transcripts of mIGFBP-4 were detected as early as day 11 in telencephalon, mesencephalon, snout, tongue, and differentiating sclerotomes. After day 14, mIGFBP-4 expression was undetectable in the brain areas. In contrast, mIGFBP-4 transcripts were clearly detectable in lung, liver, kidney, intestine, vertebrae, ribs, and incissivus.

Expression of mIGFBP-5 was also detectable as early as day 11 of gestation in differentiating sclerotomes and the ectodermal layer of the tongue. Furthermore, expression of mIGFBP-5 in 14 day old embryos was found in nasal placodes, pharynx, and esophagus. After day 14 of gestation, expression of mIGFBP-5 was found in the cornea and sclera of the eye, meninges, lung, kidney, intestine, bladder, vertebrae, and ribs. Expression of mIGFBP-5 was not above background level in the liver.

TABLE 1. Major tissues in which mIGFBP-1, -2, -3, -4, -5 and -6 mRNA expression in the midgestational mouse embryo (day 12) was detected.

	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6
Mesencephalon	-	+	-	+	-	-
Telencephalon	-	+	-	+	-	-
Tongue	-	+	-	+	+	-
Liver	+	+	-	+	-	-
Sclerotomes	-	+	-	+	+	-
Snout	-	-	-	+	-	-
Nasal Placode	-	+	-	-	+	-

+, detected; -, not detected.

TABLE 2. Major tissues in which mIGFBP-1, -2, -3, -4, -5 and -6 mRNA expression in the late gestational mouse fetus (day 18) was detected.

	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6
Choroid Plexus	-	+	-	-	-	-
Meninges	-	+	-	-	+	-
Vertebrae	-	+	+	+	+	+
Heart	-	+	-	-	-	-
Lung	-	+	-	+	+	+
Liver	+	+	+	+	-	+
Kidney	-	+	-	+	+	-
Intestine	-	+	-	+	+	-

+, detected; -, not detected.

Expression of mIGFBP-6 could be detected on day 18 in lung, liver, vertebrae and ribs, but only after a prolonged exposure time.

The major tissues in which mRNA expression of the six mIGFBPs was detected in mid- and late gestational mouse conceptuses are summarized in table 1 and 2, respectively.

Comparison of the hybridization patterns revealed that several tissues were found to express more than one mIGFBP. For instance, in the tongue of a mouse embryo of 13 days gestation, expression of mIGFBP-2, -4 and -5 was found. The highest expression of mIGFBP-2 was found in the ectodermal layer, with moderate expression in the mesodermal derived part of the tongue (Fig. 2, A and B). Transcripts for mIGFBP-4 were only expressed in the mesoderm-derived part (Fig. 2, C and D), whereas mIGFBP-5 was expressed solely in the ectodermal layer of the developing tongue (Fig. 2, E and F).

A similar distribution between mIGFBP-2, -4 and -5 was seen in the lung. Here, expression of mIGFBP-2 was highest in or around respiratory epithelium, but also detectable in interstitium (Fig. 3, A and B). Transcripts for mIGFBP-4, however, were found mainly

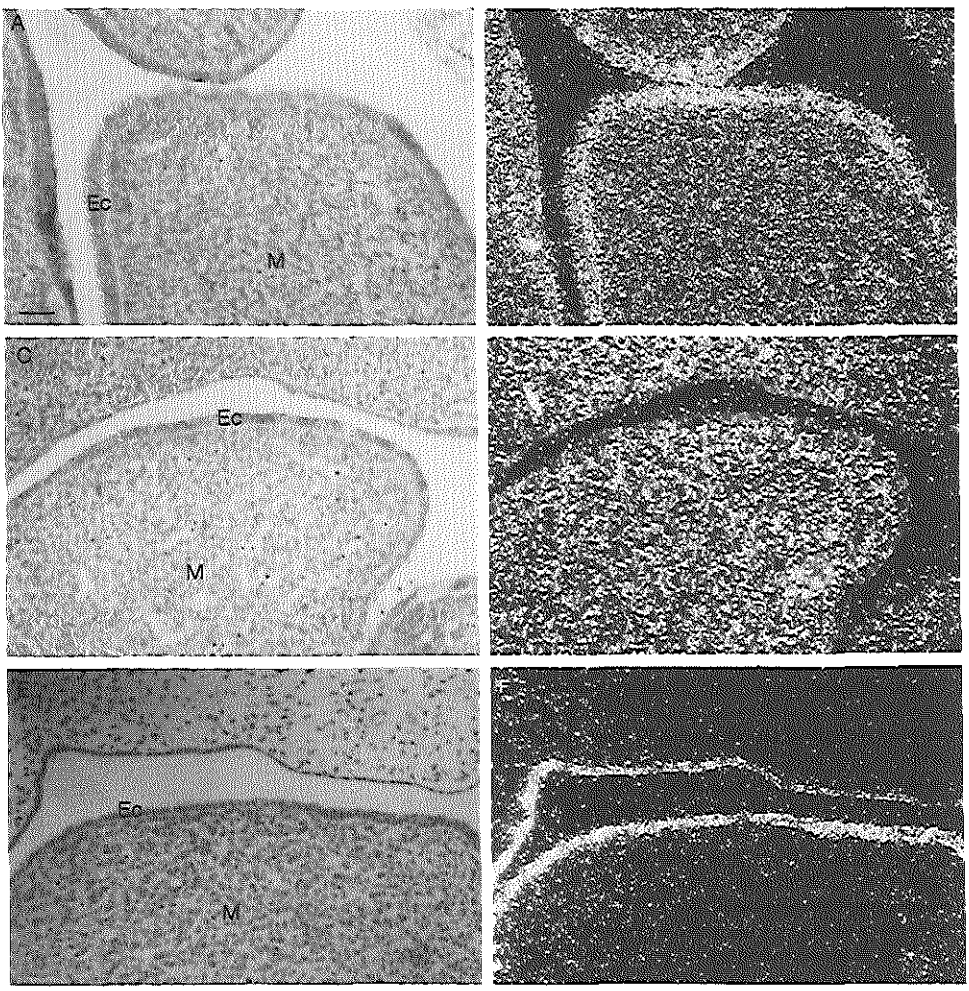

Fig.2. Brightfield (A, C, and E) and darkfield (B, D, and F) images of a section of the tongue of a 13.5 day mouse embryo. Sections were hybridized with probes for mIGFBP-2 (A and B), mIGFBP-4 (C and D), and mIGFBP-5 (E and F). Ec, Ectodermal layer; M, mesoderm-derived part. Scale bar = 70 μm.

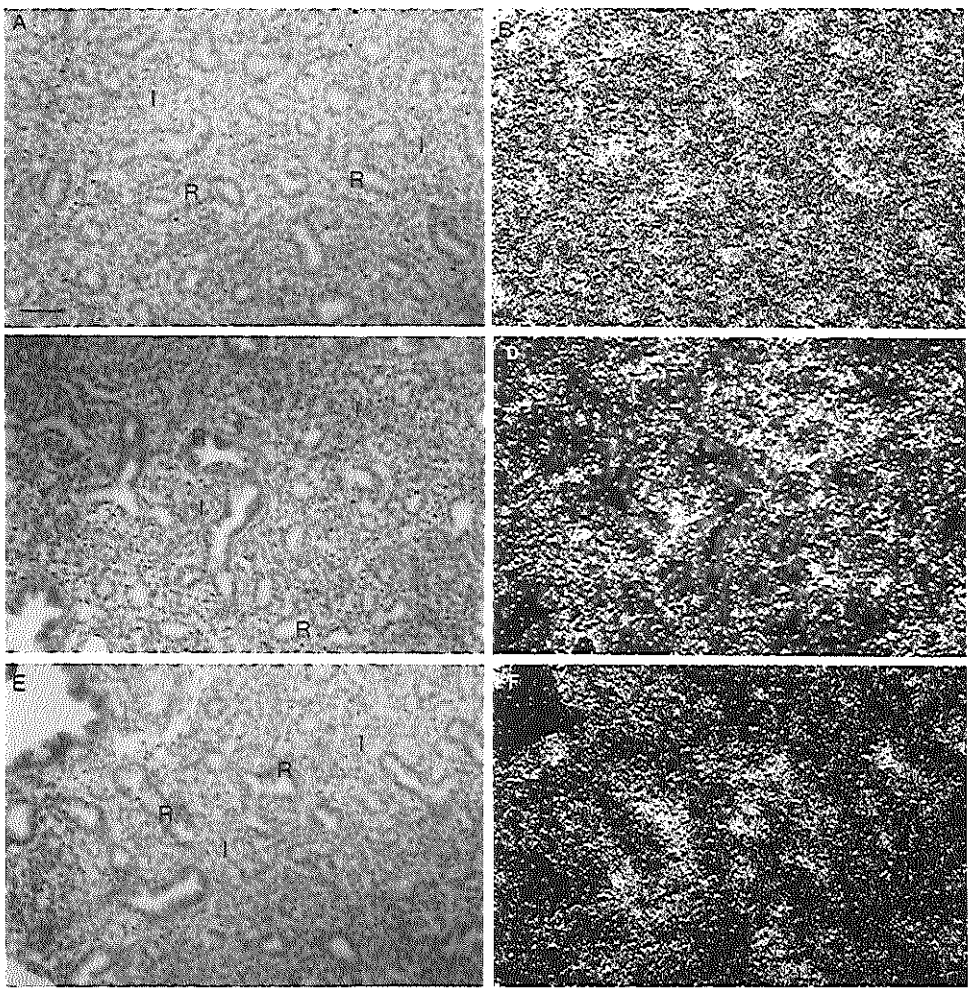

Fig.3. Brightfield (A, C, and E) and darkfield (B, D, and F) images of a section of the lung of an 18 day mouse fetus. Sections were hybridized with probes for mIGFBP-2 (A and B), mIGFBP-4 (C and D), and mIGFBP-5 (E and F). I, Interstitium; R, Respiratory epithelium. Scale bar = 70 μm.

in the interstitium (Fig. 3, C and D), whereas highest expression of mIGFBP-5 was located in or around the respiratory epithelium (Fig. 3, E and F).

In the eye (18 days gestation), transcripts for mIGFBP-2 and -5 were visualized. Expression of mIGFBP-2 was mainly located in the developing cornea (Fig. 4A and B), whereas mIGFBP-5 expression was equally distributed between the cornea (Fig. 4, C and D) and sclera.

DISCUSSION

In this study we showed that mIGFBP-1, -2, -4 and -5 are highly expressed in mouse conceptuses. Transcripts for mIGFBP-1 were restricted to the liver, whereas expression of mIGFBP-2, -4 and -5 was found in various tissues in all stages tested. Expression of mIGFBP-3 and -6 could only be detected after a long exposure time.

IGFBP-3 is the major carr r of IGFs in adult serum. One of its proposed functions is to restrict IGF efflux from the vascular space (15). During embryogenesis one might expect that the IGFs are required within the tissues to mediate growth and differentiation. Therefore, storage of IGFs in serum in the high mol wt complex, which is not able to cross the capillary barrier, could be a disadvantage to the embryo. This could explain the low expression of mIGFBP-3 in the fetus. Indeed, early studies have indicated that the 150 kDa IGFBP-3 complex was not present in fetal mouse serum (16), and that in rats, expression of IGFBP-3 increases with postnatal age (17).

Comparison of the expression patterns of the mIGFBPs revealed that they were clearly distinct, although several tissues were found to express more than one mIGFBP. For instance, mRNA expression of mIGFBP-1, -2, and -4 was high in fetal liver as early as day 11 of embryogenesis. Indeed, in rat fetal serum high levels of these low mol wt IGFBPs were found (18). Although synthesis of IGF-II has been demonstrated in a variety of fetal rat tissues (19), the major source of IGF-II in fetal serum is thought to be the liver. Taken together, these findings suggest that mIGFBP-1, -2, and -4 are synthesized and secreted by the liver and that they may play a role in transporting IGFs in fetal serum. Furthermore, the finding that these mIGFBPs are all expressed in the liver raises the question whether these

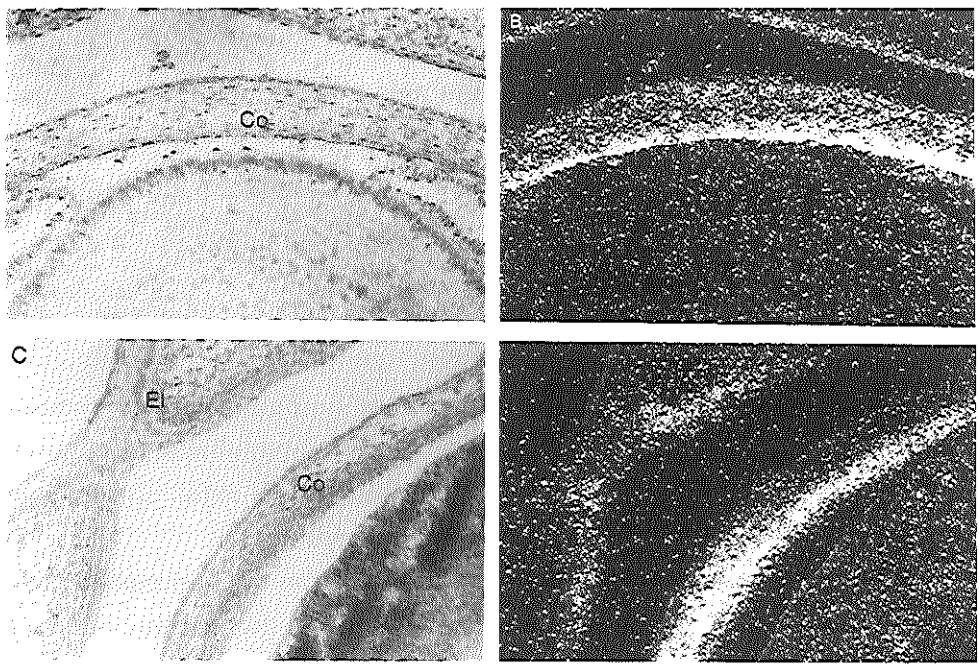

Fig.4. Brightfield (A and C) and darkfield (B and D) images of a section of the eye of a 18 day mouse fetus. Sections were hybridized with probes for mIGFBP-2 (A and B) and mIGFBP-5 (C and D). Co, Cornea; El, Eyelid. Scale bar = 70μm.

mIGFBPs might have distinct effects. Also, in the epithelium of the choroid plexus high levels of mIGFBP-2 transcripts were found. Recently, it was shown that in midgestational rat brain, IGFBP-2 mRNA was expressed in epithelium of the choroid plexus, whereas IGF-II was expressed in adjacent mesenchymal cells. It was suggested that epithelial derived IGFBP-2 might mediate the delivery of mesenchymally synthesized IGF-II to the cerebrospinal fluid (11).

Expression of several mIGFBPs in the same tissue was also observed in the developing lung and tongue. However, microscopic evaluation revealed that in the lung, highest expression of mIGFBP-2 was found in or around respiratory epithelium, but was also detectable in interstitium. In contrast, expression of mIGFBP-4 was located mainly in the interstitium, whereas mIGFBP-5 was concentrated in or around the respiratory epithelium.

Earlier studies have shown that mRNA expression of IGF-II was present in lung of human conceptuses, but could not be detected in respiratory epithelium (20). In contrast, immunohistochemical studies showed that the IGF peptides were present in the epithelium of the airways, with only slight immunostaining of the pulmonary interstitium (21, 22). Since mRNA expression was limited to fibroblasts and mesenchymal cells, these immunoreactive cells were thought not to be the primary sites of IGF synthesis. Therefore, it was suggested that these cells may define sites of IGF action (21). All these findings may suggest that in the lung expression of mIGFBP-4 and IGF-II may take place in the same cell types, whereas mIGFBP-5 might be expressed in cells that do not synthesize IGF-II, but may be target cells of IGFs. A similar expression pattern of mIGFBP-2, -4, and -5 was found in the developing tongue. Here, the highest expression of mIGFBP-2 was found in the ectodermal layer, but expression was also detectable in the mesoderm-derived part. However, mIGFBP-4 transcripts could only be detected in the mesoderm-derived part of the tongue, whereas mIGFBP-5 expression was restricted to the ectodermal layer. Recently, in midgestational rat embryos, it was shown that expression of IGF-II was also restricted to the mesoderm-derived part of the tongue (11). Furthermore, transcripts for the type I IGF receptor were found. Although it is not known whether this includes the ectodermal layer of the tongue, these results indicated that IGFs may act locally in tongue development (5). These findings suggest that in the developing tongue, IGF-II, mIGFBP-4 and mIGFBP-5 mRNA may be expressed compartmentalized and that expression of IGF-II and mIGFBP-4 may occur in the same compartment, whereas mIGFBP-5 may be expressed mainly in another compartment. Furthermore, the distinct expression patterns of mIGFBP-2, -4 and -5 in both the lung and tongue may indicate different functions or modes of action of these mIGFBPs.

In the eye of the mouse fetus (18 days gestation) it has been reported that expression of both IGF-II and the type II IGF receptor could be visualized in the developing sclera, but not in the cornea (23, 24). However, transcripts of mIGFBP-2 and -5 were easily detected in both the sclera and the cornea at this stage. These findings tempt one to speculate about functions of mIGFBP-2 and -5 not directly related to IGF-binding. However, the presence of the type I IGF receptor in eye development is unknown. Therefore, the sites of IGF action have not yet been established, making it impossible to exclude IGF action on the cornea.

In summary, we have shown the expression patterns of the six mIGFBPs in mid- and late gestational mouse conceptuses. The results obtained show that in several tissues expression of mIGFBP-2 and -5 seems to occur in the same cell types, whereas expression of mIGFBP-4 and -5 is found mainly in different cell types. Although further studies are needed to understand the role of this distribution pattern and to investigate whether this distribution is a general phenomenon, these data suggest that the different mIGFBPs may play distinct roles in fetal development.

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REFERENCES

1 Daughaday WH, and Rotwein P 1989 Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and genestructures, serum, and tissue concentrations. Endocr Rev 10:68-91

2 Stylianopoulou F, Efstratiadis A, Herbert J, Pintar J 1988a Pattern of the insulin-like growth factor II gene expression during rat embryogenesis. Development 103:497-506

3 Stylianopoulou F, Herbert J, Soares MB, Efstratiadis A 1988b Expression of the insulin-like growth factor II gene in the choroid plexus and the leptomeninges of the adult rat central nervous system. Proc Natl Acad Sci USA 85:141-145.

4 Han VK, Lund PK, Lee DC, D'Ercole AJ 1988 Expression of somatomedin/insulin-like growth factor messenger ribonucleic acids in the human fetus: identification, characterization, and tissue distribution. J Clin Endocrin Metab 66:422-429

5 Bondy CA, Werner H, Roberts Jr. CT, LeRoith D 1990 Cellular pattern of IGF-I, and type I IGF receptor gene expression in early organogenesis: comparison with IGF-II gene expression. Mol Endocrinol 4:1386-1398

6 Rechler MM, Nissley SP 1990 Insulin-like growth factors. In: Sporn MB, Roberts AB (eds) Handbook of experimental pharmacology, vol 95/I Springler-Verlag, Berlin pp, 263-267

7 Drop SLS, Schuller AGP, Lindenbergh-Kortleve DJ, Groffen C, Brinkman A, Zwarthoff EC 1992 Structural aspects of the IGFBP family. Growth Regulation 2:80-87

8 Brinkman A, Groffen C, Kortleve DJ, Geurts van Kessel A, Drop SLS 1988 Isolation and characterization of a cDNA encoding the low molecular weight insulin-like growth factor-binding protein (IBP-1). EMBO J 7:2417-2423

9 Ooi GT, Orlowski CG, Brown AL, Becker RE, Unterman TG, Rechler MM 1990 Different tissue distribution and hormonal regulation of mRNAs encoding rat IGF binding proteins 1 and 2. Mol Endocrinol 4:321-328

10 Brown AL, Chiariotti L, Orlowski CC, Mehlman T, Burgess WH, Ackerman EJ, Bruni CB, Rechler MM 1989 Nucleotide sequence and expression of a cDNA clone encoding a fetal rat binding protein for insulin-like growth factors. J Biol Chem 264:5148-5154

11 Wood TL, Brown AL, Rechler MM, Pintar JE 1990 The expression pattern of an insulin-like growth factor (IGF)-binding protein gene is distinct from IGF-II in the midgestational rat embryo. Mol Endocrinol 4:1257-1263

12 Theiler, K 1972 The House Mouse. Springler-Verlag, Berlin

13 Sanger F, Nicklen S, Coulson AR 1977 DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74:5463-5467

14 Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Strahl K 1991 Current protocols in molecular biology Vol 2

15 Clemmons DR 1992 IGF binding proteins: regulation of cellular actions. Growth Regulation 2:80-87

16 D'Ercole AJ, Underwood LE 1980 Ontogeny of somatomedin during development in the mouse. Dev Biol 79:33-45

17 Donovan SM, Oh Y, Pham H, Rosenfeld RG 1989 Ontogeny of serum IGF binding proteins in the rat. Endocrinology 125:2621-2627

18 Fielder PJ, Thordarson G, Talamantes F, Rosenfeld RG 1990 Characterization of IGF binding proteins during gestation in mice: effects of hypophysectomy and an specific serum protease activity. Endocrinology 127:2270-2280

19 Romanus, JA, Yang, YWH, Adams SO, Sofair AN, Tseng LYH, Nissley P, Rechler MM 1988 Synthesis of insulin-like growth factor II (IGF-II) in fetal rat tissues: translation of IGF-II ribonucleic acid and processing of pre-pro-IGF-II. Endocrinology 122:709-716

20 Han VKM, D'Ercole AJ, Lund PK 1987a Cellular localization of somatomedin (insulin-like growth factor) messenger RNA in the human fetus. Science 236:193-197

21 Han VKM, Hill DJ, Strain AJ, Towle AC, Lauder JM, Underwood LE, D'Ercole AJ 1987b Identification of somatomedin/insulin-like growth factor immunoreactive cells in the human fetus. Pediatr Res 22:245-249

22 Hill DJ, Clemmons DR, Wilson S, Han VKM, Strain AJ, Milner RDG 1989 Immunological distribution of one form of insulin-like growth factor (IGF)-binding protein and IGF peptides in human fetal tissues. J Mol Endocrinol 2:31-38

23 Beck F, Samani NJ, Byrne S, Morgan K, Gebhard R, Brammar WJ 1988 Histochemical localization of IGF-I and IGF-II mRNA in the rat between birth and adulthood. Development 104:29

24 Cuthbertson RA, Beck F, Senior PV, Haralambidis J, Penschow JD, Coghlan JP 1989 Insulin-like growth factor II may play a local role in the regulation of ocular size. Development 107:123-130

CHAPTER 6

INSULIN-LIKE GROWTH FACTOR (IGF), TYPE I IGF RECEPTOR AND IGF BINDING PROTEIN mRNA EXPRESSION IN THE DEVELOPING MOUSE LUNG.

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ABSTRACT

The insulin-like growth factors (IGFs) are important mitogens, thought to be involved in lung growth and development. The regulation of IGF action depends not only on the expression of IGFs and IGF receptors, but also on the modulation of IGF activity by IGF binding proteins. In this study, we describe the mRNA expression of IGF-I, IGF-II, type I IGF receptor, IGFBP-2, -4 and -5 during mouse lung development as studied by *in situ* hybridization techniques. The IGF, type I IGF receptor and IGFBP-2, -4 and -5 genes were expressed in developing lung as early as embryonal day 12½ and the expression continued over the examined period. IGF-I and IGF-II mRNAs were expressed both in mesenchymal and epithelial cells. Type I IGF receptor transcripts were also observed throughout the developing lung, with the exception of the epithelial cells of the bronchi after embryonal day 15. Furthermore, mRNA expression of IGFBP-4 and -5 was noted in neighboring cell types and after embryonal day 15, coexpression of the type I IGF receptor and IGFBP-4 transcripts. The observed expression patterns imply that the IGFBP-2, -4 and -5 genes are differentially regulated and suggest that each may have a discrete function in mouse lung development. A possible role for IGFBP-2, -4 and -5 is to participate in the regulation of cell specific IGF responses during mouse lung development.

INTRODUCTION

Insulin like growth factors, IGF-I and IGF-II, are single chain polypeptides involved in cell growth and differentiation (Daughaday and Rotwein, 1989). The IGFs are thought to exert their mitogenic effects primarily through activation of the type I IGF receptor (Steele-Perkins and Roth, 1990, Pietrzowski et al., 1992). An important role of the IGFs has been implicated in lung development. In developing rat lung, mRNA expression of IGF-I, IGF-II and the type I IGF receptor has been reported (Stylianopoulou et al., 1988a, Werner et al., 1989, Bondy et al., 1990, Wood et al., 1990). In addition, gene disruption strategies have demonstrated that both IGFs and the type I IGF receptor are indispensable for normal embryonic and postnatal growth (Liu et al., 1993, Baker et al., 1993). Moreover, type I IGF

receptor mutants and IGF-I/IGF-II double mutants died at birth of respiratory failure (Liu et al., 1993).

In extracellular fluids, IGFs are complexed to IGF binding proteins (IGFBPs), which modulate the availability and action of IGFs (reviewed by Clemmons 1992). A family of six distinct IGFBPs has been characterized in human, rat (reviewed by Drop et al., 1992) and mouse (Schuller et al., 1994). Expression of all six IGFBP genes has been described during mouse and rat embryonal development, each having a unique pattern of expression (Wood et al., 1990, Wood et al., 1992, Schuller et al., 1993, Cerro et al., 1993, Green et al., 1994). In fetal mouse lung, we recently have shown that of the six IGFBP genes, the IGFBP-2, -4 and -5 genes are abundantly expressed and that IGFBP-4 and -5 mRNAs are localized in different cell types (Schuller et al., 1993).

Since IGFs, the type I IGF receptor and IGFBPs all are expressed in fetal lung, it is likely that these proteins participate in lung development in a paracrine or autocrine way. In this study we examined in further detail the mRNA expression of IGFBP-2, -4 and -5 during mouse lung development, in relation to IGF-I, IGF-II and type I IGF receptor mRNA expression using *in situ* hybridization techniques.

MATERIALS AND METHODS

Isolation of tissues and sectioning.

Pregnant BALB/c mice were sacrificed, conceptuses of 12-16 days gestation were collected and staged according to Kaufman (Kaufman, 1992). In addition, lungs were dissected from fetuses (embryonal day 16 and 18) and from mice 1, 3 and 7 days after birth. Tissues were fixed in 4% paraformaldehyde, dehydrated in successive baths containing increasing amounts of ethanol and embedded in paraffin according to standard procedures. 6 μm sections were cut on a microtome and mounted onto 3-aminopropyl-trioxysilane-coated slides on a drop of 10% ethanol. Sections were stretched and dried on a 45^{0}C heating plate for 1 day.

Preparation of RNA probes.

cDNAs encoding mouse IGF-I and -II were kindly provided by Dr. G.I. Bell (Howard Hughes Medical Institute, Chicago, USA). Fragments (EcoR1 for IGF-I and EcoR1/Sac-1 for IGF-II) were subcloned in pBluescript (Stratagene, La Jolla, USA). A 265 bp EcoR1/Sma1 fragment of the rat type I IGF receptor cDNA clone ligated in pGEM3 was kindly provided by Dr. H. Werner and Dr. D. LeRoith (National Institutes of Health,

Bethesda, USA). As templates for IGFBP-specific RNA probes, plasmids containing cDNA fragments corresponding to mouse IGFBP-2, -4 and -5 were used as described before (Schuller et al., 1993). ^{35}S-labelled RNA transcripts were generated from linearized plasmids using SP6, T3 or T7 RNA polymerase in the presence of 50 μCi [^{35}S]UTP (Amersham, UK). The DNA templates were removed by digestion with DNase-I and the labelled RNA probes were purified by phenol/chloroform extraction and ethanol precipitation.

In situ hybridization.

In situ hybridization was carried out as described (Schuller et al., 1993). Briefly, paraffin wax was removed from the sections using successive baths of xylene. Sections were rehydrated and immersed in 0.2N HCl for 20 minutes. Subsequently, sections were rinsed in PBS, incubated in 2xSSC for 15 minutes at 70^{0}C and postfixed for 5 minutes in 4% paraformaldehyde. Sections were treated to block non-specific binding by rinsing in PBS/10mM DTT, followed by two rinses of PBS and incubation with 0.1M triethanolamine/0.25% acetic anhydride for 10 minutes. Sections were rinsed in 2xSSC and stored in 70% ethanol for 14 hrs. The next day sections were dehydrated, dried and hybridized with ^{35}S-labelled RNA probes for 4 hours at 66^{0}C in a solution containing 50% formamide, 4xSSC, 1x Denhardt's, 1mg/ml tRNA, 10% dextransulphate, 10mM DTT and 0.2 mg/ml ssDNA. After hybridization the sections were washed at 56^{0}C in successive baths containing 55% formamide/2xSSC/20mM ß-mercaptoethanol (2x 15 minutes), 55% formamide/2xSSC/20mM ß-mercaptoethanol/0.5% triton X-100 (2x 15 minutes) and 55% formamide/2xSSC/20mM ß-mercaptoethanol (2x 15 minutes). Subsequently, sections were rinsed in 2xSSC, dehydrated and dried prior to exposure to Kodak AR X-ray film for 1-3 days. Finally, sections were exposed to Kodak NTB2 photographic emulsion for 3-21 days, developed and stained with nuclear fast red for microscopic evaluation. The level of non-specific binding was determined using sense probes for each of the IGFs, type I IGF receptor and IGFBPs.

RESULTS

At embryonal day 12½-13, mRNA expression of IGF-I, IGF-II, the type I IGF receptor and IGFBP-2, -4, and -5 was observed in the developing lung. As shown in figure 1, in all cells a weak expression of IGF-I was found, which was more pronounced in the cells that surround the epithelial cells of the lobar bronchus (Fig. 1a+b). IGF-II and type I IGF receptor mRNA expression was found in all cells of the lung bud, with no apparent variation between the different regions (Fig. 1c-f). IGFBP-2 transcripts were abundantly expressed in epithelial cells, but were also found in mesenchyme (Fig. 1g+h). IGFBP-4 transcripts were detected in the developing lung, but were less obvious in the epithelial lining of the lobar bronchi (Fig. 1i+j). In contrast, IGFBP-5 mRNA expression was concentrated

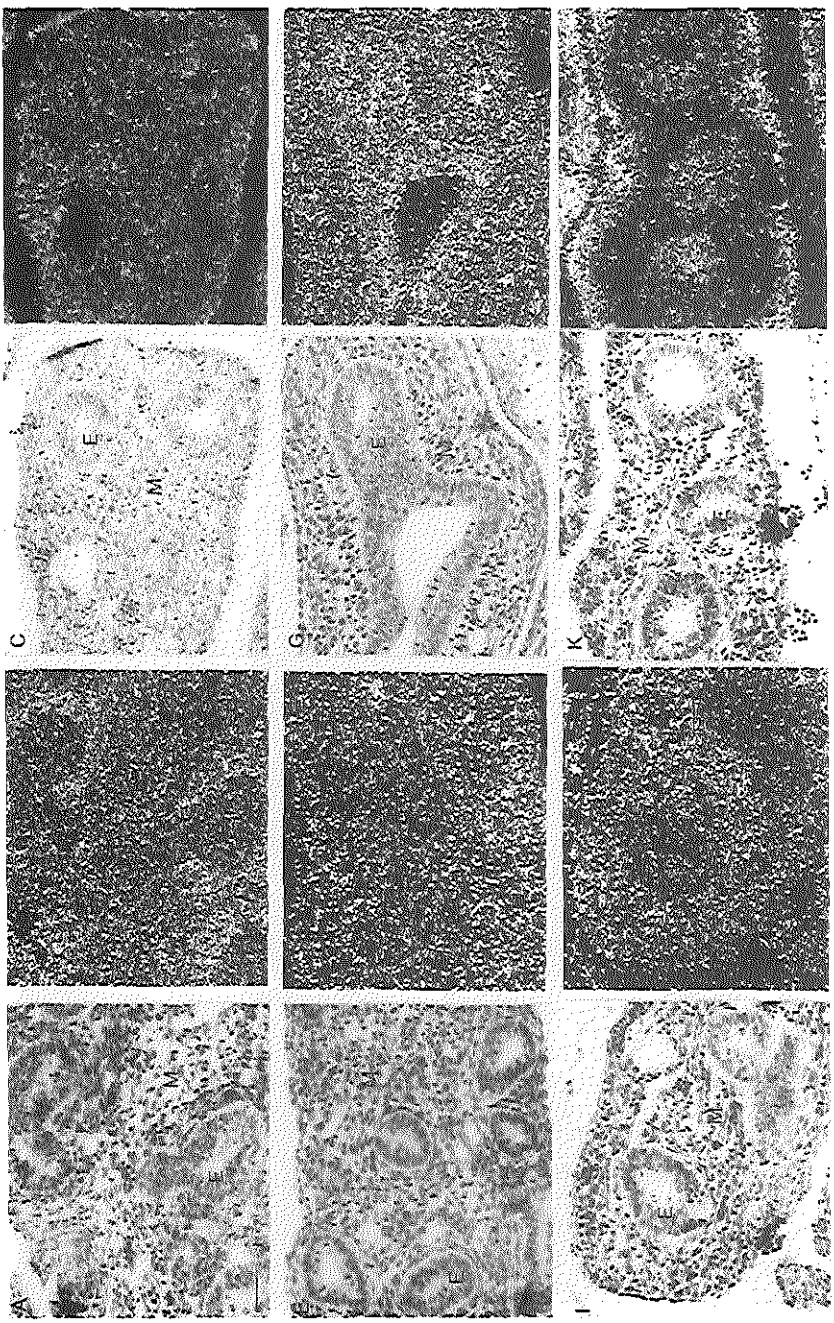

Fig. 1. Brightfield (A, C, E, G, I and K) and darkfield (B, D, F, H, J and L) images of sections of a 12½ day-old embryonal lung hybridized with probes for IGF-I (A and B), IGF-II (C and D), type I IGF receptor (E and F), IGFBP-2 (G and H), IGFBP-4 (I and J) and IGFBP-5 (K and L). M, mesenchyme; E, epithelial cells. scale bar = 100 μm.

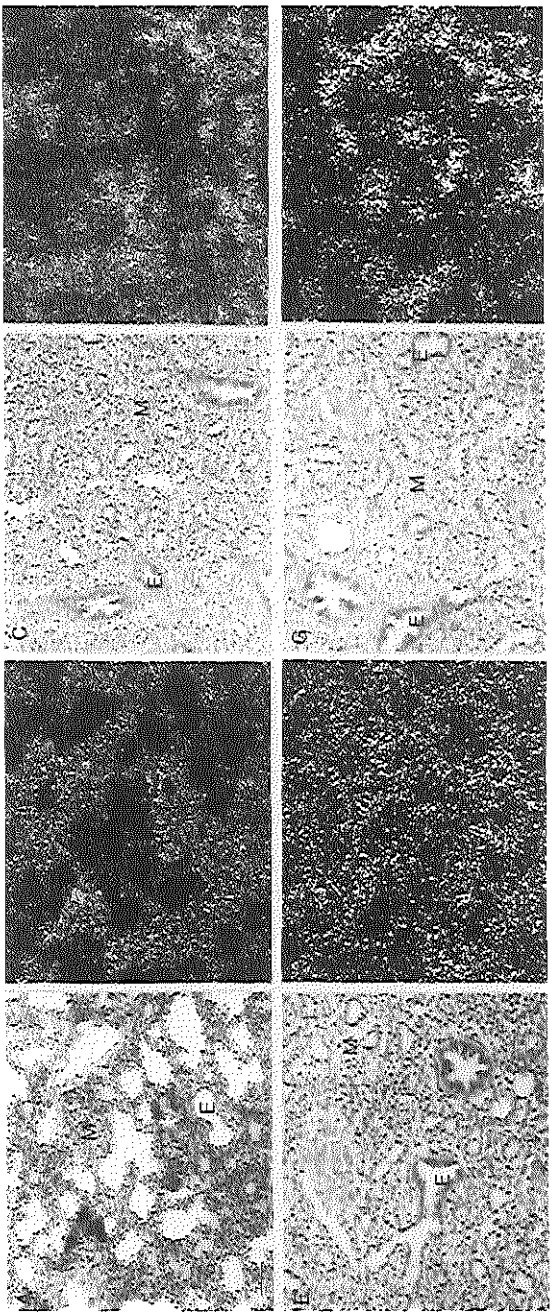

Fig. 2. Brightfield (A, C, E and G) and darkfield (B, D, F and H) images of sections of 16½-day-old fetal lung hybridized with probes for type I IGF receptor (A and B), IGFBP-2 (C and D), IGFBP-4 (E and F) and IGFBP-5 (G and H). M, mesenchyme; E, epithelial cells. scale bar = 100 μm.

in these epithelial cells, as well as in a region of cells covering the lung buds, including the primordium of the visceral layer of the pleura (Fig. 1k+l).

Around embryonal day 15, both IGF-I and IGF-II gene expression was equally abundant in all regions of the lung (not shown). In contrast, type I IGF receptor mRNA expression was predominantly found in the mesenchyme, with only little if any expression in epithelial cells of terminal bronchi (figure 2a+b). IGFBP-2 mRNA expression was found mainly in epithelial cells, but the expression was clearly detectable in mesenchymal cells as well (Fig. 2c+d). Expression of IGFBP-4 was still observed primarily in mesenchyme, whereas IGFBP-5 transcripts were located in the epithelial cells of terminal bronchi (figure 2e-h).

Around birth, localization of the transcripts became more difficult due to expansion of the alveolar lumen. However, surrounding the bronchi, it was still apparent that type I IGF receptor and IGFBP-4 transcripts were hardly expressed in the epithelial cells and that the mRNA expression of IGFBP-2 and -5 in epithelial cells remained (figure 3a-f). Transcripts of IGF-I and IGF-II were also detectable in late fetal lung. As shown in earlier stages, no variation between the different regions was observed (not shown).

One week after birth, the expression patterns observed were similar to those found in late fetal lung. If any change occurred, it was a decrease in the expression levels of the IGFs and the type I IGF receptor (not shown).

Together with the lung sections, sagittal sections of midgestational mouse embryos were hybridized to determine the specificity of the various probes used. Hybridization with an anti-sense IGF-I probe showed only a faint signal on autoradiograms exposed for three days (not shown). In contrast, IGF-II and the type I IGF receptor mRNA expression was easily detected (Fig. 4). The expression patterns of the IGF and type I IGF receptor genes observed were very similar to those already described in rat embryos (Bondy et al., 1990, Wood et al., 1990). Anti-sense IGFBP-2, -4 and -5 RNA probes revealed expression patterns identical to those we described previously (Schuller et al., 1993). Furthermore, no signal was observed after hybridization with sense RNA probes (not shown).

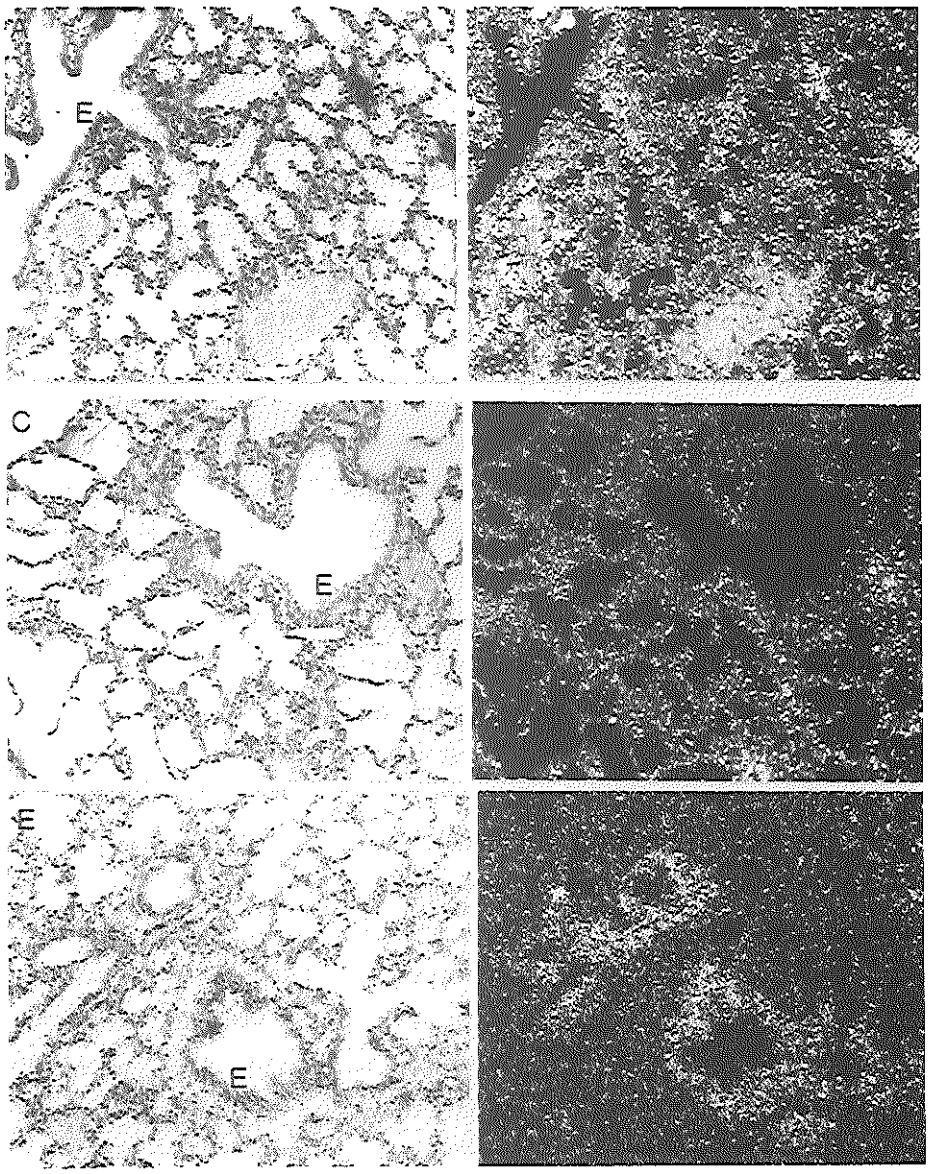

Fig. 3. Brightfield (A, C and E) and darkfield (B, D and F) images of sections of 18½ day-old fetal lung hybridized with probes for type I IGF receptor (A and B), IGFBP-4 (C and D) and IGFBP-5 (E and F). M, mesenchyme; E, epithelium. scale bar = 100 μm.

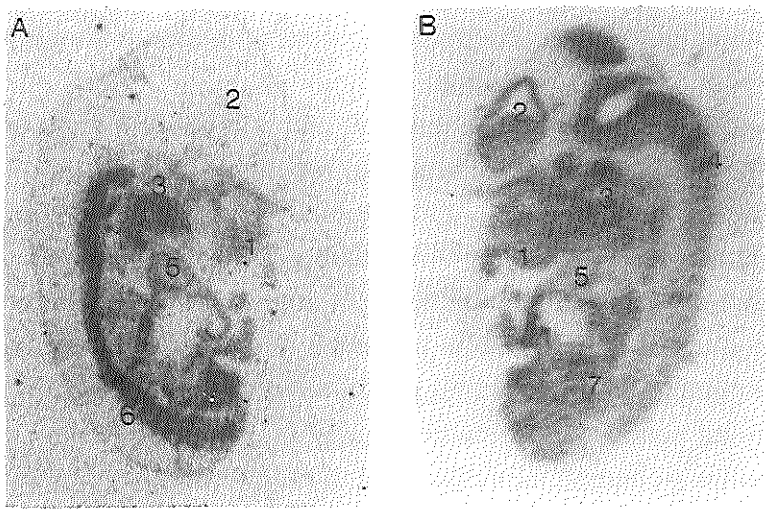

Fig. 4. Autoradiograms of midgestational mouse embryo sections hybridized with ^{35}S-labeled RNA probes for IGF-II (A) and type I IGF receptor (B). 1, snout; 2, brain; 3, tongue; 4, spinal cord; 5, heart; 6, somites; 7, stomach.

DISCUSSION

In this study, we have shown that the genes for both IGFs, the type I IGF receptor and IGFBP-2, -4 and -5 are expressed in the developing mouse lung. Transcripts were detected as early as embryonal day 12½ and were continuously expressed over the period examined (embryonal day 12½ to postnatal day 7). In two previous articles in which IGF-II mRNA expression was studied, specific mesenchymal expression in human embryonal lung was observed by Han et al (Han et al., 1987) and in contrast specific epithelial expression was found in embryonic rat lung by Stylianopoulou et al. (Stylianopoulou et al., 1988b). However, our results show that IGF-II mRNA in the mouse lung is expressed equally in mesenchymal and epithelial cells between embryonal day 12½ and postnatal day 7. Furthermore, the overall mRNA expression pattern of IGF-II found in midgestational mouse embryos (see fig. 4) was very similar to that reported for rat embryos (Bondy et al., 1990, Wood et al., 1990). This indicates that their are no great discrepancies between IGF-II

mRNA expression in mouse and rat. Thus, we suggest that IGF-II is ubiquitously expressed in the developing mouse lung and that it is unlikely that the expression switches from one cell type to the other during lung development.

Transcripts for the type I IGF receptor were detected in the developing lung, but after embryonal day 15, mRNA expression in the epithelial cells ceased. These cells do contain high concentrations of IGF-II mRNA. This implies that in these stages, IGF-II expressed in the epithelial cells of bronchi may only activate the type I IGF receptor expressed by other cell types. We can only speculate about the functional significance of the disappearance of type I IGF receptor mRNA expression in epithelial cells of the developing lung. It is conceivable that this decrease in type I IGF receptor mRNA expression results in insensitivity of the epithelial cells to IGF stimulation. Alternatively, if IGFs still would affect the epithelial cells of the bronchi, these effects must be mediated via another receptor than the type I IGF receptor after embryonal day 15. Gene disruption strategies have indicated that after embryonal day 13½, at least part of the IGF-II effects is mediated via an as yet unknown receptor (Baker et al., 1993). In addition, the mRNA expression of the IGFs and type I IGF receptors, suggests that the IGFs can participate in lung development both via paracrine and autocrine mechanisms.

In sections of lungs obtained from animals after birth, the quantities of the IGF and type I IGF receptor transcripts were decreased. This is in agreement with other studies in which it has been reported that mRNA levels of IGF-II and type I IGF receptor in fetal rat lung are higher than in adult lung (Stylianopoulou et al., 1988b, Werner et al., 1989). A decrease in IGF and type I IGF receptor mRNA expression in lung after birth is consistent with the idea that IGFs participate in prenatal lung development.

Recent gene targeting experiments have shown that mutant mice carrying disrupted IGF-I genes (Powell-Braxton et al., 1993), disrupted type I IGF receptor genes or both disrupted IGF-I and IGF-II genes (Liu et al., 1993, Baker et al., 1993), are not only growth retarded, but die at birth due to respiratory failure. The primary cause of this respiratory failure is at present unknown. It has been suggested that the failure of mutants to breathe was due to muscle hypoplasia (Liu et al., 1993). However, besides muscle hypoplasia, Powell-Braxton et al., demonstrated that the lungs of their IGF-I deficient mice were poorly

developed and less organized (Powell-Braxton et al., 1993). These results underline the importance of IGF action for normal lung development.

The IGFBP-2 genes were expressed both in mesenchymal and epithelial cells, being more prominent in epithelial cells. Transcripts for IGFBP-4 and -5 were expressed in neighboring cell types, IGFBP-4 being expressed mainly in mesenchyme and IGFBP-5 in epithelial cells surrounding the bronchi and in the primordium of the visceral layer of the pleura. This was already apparent around embryonal day 12½, and continued up to early fetal stages. It is of interest to note that those cells that express IGFBP-5, also contain high levels of IGF-II mRNA, and after embryonal day 15 no transcripts for the type I IGF receptor. Furthermore, cells that express the type I IGF receptor gene also contain IGFBP-4 transcripts. The distinct expression patterns of the IGFBP-2, -4 and -5 genes not only show that they are regulated differentially, but suggest that IGFBP-2, -4 and -5 each may have a discrete role in lung development.

Primary cultures of 19 day fetal rat lung mesenchymal and epithelial cells were observed to express and secrete IGFBP-2, -4 and -5 (Price et al., 1993). This is in agreement with our observations, since IGFBP-2 mRNA expression was observed in both cell types over the examined period, and the mRNA expression of IGFBP-4 and -5 in late fetal stages only differed around the major bronchi. More interestingly, IGFBP-4 production was reported to be influenced by agents known to affect lung cell growth and differentiation, indicating that changes in IGFBP production may influence lung development (Price et al., 1993). In addition, both SCLC and NSCLC cell lines express IGFBPs, including IGFBP-2, -4 and -5 (Reeve et al., 1992, Reeve et al., 1993, Wegmann et al., 1993). In SCLC cells, but not in NSCLC cells, IGFBP-2 was associated with the cell membrane. Both soluble and membrane-associated IGFBP-2 were shown to compete with IGF receptors for ligand binding and thus may influence IGF responsiveness (Reeve et al., 1993). IGFBP-1 and -2 both contain an Arg-Gly-Asp (RGD) sequence at their C-terminal end. For IGFBP-1 this sequence has been shown to be essential for binding to $\alpha_5\beta_1$ integrin (fibronectin receptor) (Jones et al., 1993a). Fibronectin is a 500 kDa glycoprotein, forming high molecular weight polymers which interact via integrin receptors with embryonic cells during migration, differentiation and organogenesis. Three discrete domains in fibronectin are essential for its assembly into the

insoluble form, including the RGD-containing integrin binding domain. Addition of RGD peptides inhibits fibronectin assembly and branching morphogenesis of embryonic mouse lung in culture (reviewed by Warburton et al., 1993). Therefore, IGFBP-2 may potentially inhibit fibronectin function and thereby influence lung development.

IGFBP-4, isolated and cloned from human osteosarcoma cells, has been reported to inhibit IGF action (LaTour et al., 1990). In contrast, a human bone derived IGFBP, with an amino-terminal sequence highly homologous to that of IGFBP-5, potentiates IGF action in cultured mouse bone cells (Bautista et al., 1991). In addition, IGFBP-5 has been found in the extracellular matrix of human fetal fibroblasts where it could potentiate the biological actions of IGF-I (Jones et al., 1993b). These results suggest that IGFBP-4 and -5 may differently affect IGF responsiveness. Furthermore, the effect of these IGFBPs may depend on their capability to bind to certain lung cells. Together with the distinct mRNA expression of IGFBP-2, -4 and -5, a regulatory system may exist to determine cell specific IGF responses during lung development.

In summary, we have shown that IGF-I, IGF-II, the type I IGF receptor, IGFBP-2, -4 and -5 mRNAs are expressed in developing mouse lung. Based on the mRNA expression patterns observed, we suggest that IGFBP-2, -4 and -5 may each have a discrete function during lung development. A possible function of IGFBP-2, -4 and -5 would be to participate in the regulation of cell specific IGF responsiveness.

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REFERENCES

1 Daughaday, W.H. and Rotwein, P. (1989) Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum and tissue concentrations. Endocrine reviews, 10, 68-91.

2 Steele-Perkins, G. and Roth R.A. (1990). Monoclonal antibody αIR-3 inhibits the ability of insulin-like growth factor II to stimulate a signal from the type I receptor without inhibiting its binding. Biochem. Biophys. Res. Commun. 171, 1244-1251.

3 Pietrzowski, Z., Sell, C., Lammers, R., Ullrich, A. and Baserga, R. (1992). Roles of insulin-like growth factor I (IGF-I) and the IGF-I receptor in epidermal growth factor-stimulated growth of 3T3 cells. Mol. Cell. Biol. 12, 3883-3889.

4 Stylianopoulou, F., Efstratiadis, A., Herbert, J. and Pintar, J. (1988a). Pattern of the insulin-like growth factor II gene expression during rat embryogenesis. Development 103, 497-506.

5 Werner, H., Woloschak, M., Adamo, M., Shen-Orr, Z., Roberts Jr., C.T. and LeRoith, D. (1989). Developmental regulation of the rat insulin-like growth factor I receptor gene. Proc. Nat. Acad. Sci. USA. 86, 7451-7455.

6 Bondy, C.A., Werner, H., Roberts Jr., C.T. and LeRoith D. (1990). Cellular pattern of insulin-like growth fact-I (IGF-I) and type I IGF receptor gene expression in early organogenesis: Comparison with IGF-II gene expression. Mol. Endocrinol. 4, 1386-1398.

7 Wood, T.L., Brown, A.L., Rechler, M.M. and Pintar, J.E. (1990). The expression pattern of an insulin-like growth factor (IGF)-binding protein gene is distinct from IGF-II in the midgestational rat embryo. Mol. Endocrinol. 4, 1257-1263.

8 Liu, J-P., Baker, J., Perkins, A.S., Robertson, J.E. and Efstratiadis, A. (1993). Mice carrying nul mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell 75, 59-72.

9 Baker, J., Liu, J-P., Robertson, J.E. and Efstratiadis, A. (1993). Role of insulin-like growth factors in embryonic and postnatal growth. Cell 75, 73-82.

10 Clemmons, D.R. (1992) IGF binding proteins: Regulation of cellular actions. Growth Regulation 2, 80-87.

11 Drop, S.L.S., Schuller, A.G.P., Lindenbergh-Kortleve, D.J., Groffen, C., Brinkman, A. and Zwarthoff, E.C. (1992). Structural aspects of the IGFBP family. Growth Regulation 2, 69-79.

12 Schuller, A.G.P., Groffen, C., van Neck, J.W., Zwarthoff, E.C. and Drop, S.L.S. (1994). cDNA cloning and mRNA expression of the six mouse insulin-like growth factor binding proteins. submitted.

13 Wood, T.L., Streck, R.D. and Pintar, J.E. (1992). Expression of the IGFBP-2 gene in post-implantation rat embryos. Development 114, 59-66.

14 Schuller, A.G.P., Zwarthoff, E.C. and Drop, S.L.S. Gene expression of the six insulin-like growth factor binding proteins (IGFBPs) in the mouse conceptus during mid- and late gestation. Endocrinology, 1993, 132, 2544-2550.

15 Cerro, J.A., Grewal, A., Wood, T.L. and Pintar, J.E. (1993). Tissue-specific expression of the insulin-like growth factor binding protein (IGFBP) mRNAs in mouse and rat development. Regulatory Peptides 48, 189-198.

16 Green, B., Jones, S.B., Streck, R., Rotwein, P. and Pintar, J.E. (1994). Distinct expression patterns of insulin-like growth factor binding proteins 2 and 5 during fetal and postnatal development. Endocrinology, 134, 954-962.

17 Kaufman, M.H. The atlas of mouse development. 1992, Academic Press, London.

18 Han, V.K.M., D'Ercole, A.J. and Lund, P.K. (1987) Cellular localization of somatomedin (insulin-like growth factor) messenger RNA in the human fetus. Science, 236, 193-197.

19 Stylianopoulou, F., Herbert, J., Soares, M.B. and Efstratiadis, A. (1988b) Expression of the insulin-like growth factor II gene in the choroid plexus and the leptomeninges of the adult rat central nervous system. Proc. Natl. Acad. Sci. USA, 85, 141-145.

20 Powell-Braxton, L., Hollingshead, P., Warburton, C., Dowd, D., Pitts-Meek, S., Dalton, D., Gillet, N. and Stewart, T.A. (1993) IGF-I is required for normal embryonic growth in mice. Genes and Development 7, 2609-2617.

21 Price, W.A., Moats-Staats, B.M., D'Ercole, A.J. and Stiles, A.D. (1993) Insulin-like growth factor binding protein production and regulation in fetal rat lung cells. Am. J. Respir. Cell Mol. Biol. 8, 425-432.

22 Reeve, J.G., Brinkman, A., Hughes, S.A., Mitchell, J., Schwander, J. and Bleehen, N.M. (1992) Expression of insulin-like growth factor (IGF) and IGF-binding protein genes in human lung tumor cell lines. J. Natl. Cancer Inst. 84, 628-634.

23 Reeve, J.G., Morgan, J., Schwander, J. and Bleehen, N.M. (1993) Role for membrane and secreted insulin-like growth factor-binding protein-2 in the regulation of insulin-like growth factor action in lung tumors. Cancer Research 53, 4680-4685.

24 Wegmann, B.R., Schöneberger, H.J., Kiefer, P.E., Jaques, G., Brandscheid, D. and Havemann, K. 1993 Molecular cloning of IGFBP-5 from SCLC cell lines and expression of IGFBP-4, IGFBP-5 and IGFBP-6 in lung cancer cell lines and primary tumours. Eur. J. Cancer, 29, 1578-1584.

25 Jones, J.I., Gockerman, A., Busby, W.H.Jr., Wright, G. and Clemmons, D.R. (1993a) Insulin-like growth factor binding protein 1 stimulates cell migration and binds to the $\alpha_5\beta_1$ integrin by means of its Arg-Gly-Asp sequence. Proc. Natl. Acad. Sci. USA 90, 10553-10557.

26 Warburton, D., Lee, M., Berberich, M.A. and Bernfield, M. (1993) Molecular embryology and the study of lung development. Am. J. Respir. Cell Moll. Biol. 9, 5-9.

27 LaTour, D., Mohan, S., Linkhart, T.A., Baylink, D.J. and Strong, D.D. (1990) Inhibitory insulin-like growth factor binding protein: Cloning, complete sequence, and physiological regulation. Mol. Endocrinol. 4, 1806-1814.

28 Bautista, C.M., Baylink, D.J. and Mohan, S. (1991) Isolation of a novel insulin-like growth factor (IGF) binding protein from human bone: A potential candidate for fixing IGF-II in human bone. Bioch. Bioph. Res. Commun. 176, 756-763.

29 Jones, J.I., Gockerman, A., Busby, W.H., Camacho-Hubner, C. and Clemmons, D.R. (1993b) Extracellular matrix contains insulin-like growth factor binding protein-5: Potentiation of the effects of IGF-I. J. Cell. Biol. 121, 679-687.

CHAPTER 7

INSULIN-LIKE GROWTH FACTOR (IGF), TYPE I IGF RECEPTOR AND IGF BINDING PROTEIN mRNA EXPRESSION IN PRE- AND POSTNATAL MOUSE KIDNEYS.

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ABSTRACT

The insulin-like growth factors (IGFs) are important mitogens, thought to be involved in kidney growth and development. IGF action can be modulated by specific IGF binding proteins (IGFBPs). In this study, we describe the mRNA expression of IGF-I, IGF-II, the type I IGF receptor and the six IGFBPs in fetal and postnatal mouse kidneys as studied by *in situ* hybridization techniques. The IGF genes were mainly expressed in the undifferentiated metanephric cap tissue and in developing and mature glomeruli. The type I IGF receptor mRNAs were also detected in the immature and mature glomeruli. Like the IGFs, IGFBP-1 and -2 were abundantly expressed in the undifferentiated metanephric cap tissue and in developing glomeruli. Only, little mRNA expression was observed in mature glomeruli. In contrast, IGFBP-5 mRNA expression was not detected in immature glomeruli, but was abundantly expressed in the mature glomeruli. IGFBP-3, -4 and -6 were expressed both in immature and mature glomeruli. The mRNA expression patterns found, suggest that IGFs and IGFBPs may participate in early glomerular development. In addition, we suggest that IGFBPs, especially IGFBP-5, might play a role in glomerular function.

INTRODUCTION

Insulin-like growth factors (IGF-I and IGF-II) are small peptides displaying both mitogenic and metabolic actions (Bang and Hall 1992). The mitogenic effects of the IGFs are mediated predominantly by the type I IGF receptor (Steele-Perkins and Roth, 1990, Pietrzowski et al., 1992). In fetal rat kidney, the presence of mRNAs for IGF-I, IGF-II and the type I IGF receptor has been reported. After birth, IGF-I expression was increased, whereas the levels of IGF-II and type I IGF receptor mRNAs decreased (Adamo et al., 1989, Brown et al., 1986, Werner et al., 1989). In addition, gene disruption strategies have indicated that both IGF-I and IGF-II are indispensable for normal embryonic and postnatal development (Baker et al., 1993, Liu et al., 1993, Powell-Braxton et al., 1993).

IGF action and availability can be modulated by specific IGF binding proteins (reviewed by Clemmons 1992). Six distinct IGFBPs have been characterized in human, rat

(reviewed by Drop et al., 1992) and mouse (Schuller et al., 1994a). In mouse fetal kidney, we recently have shown that of the six IGFBPs, IGFBP-2, -4 and -5 are abundantly expressed. In addition, IGFBP-1 gene expression has been described in human fetal kidney (Suikkari et al., 1992) and IGFBP-3 mRNAs in the developing rat urogenital system (Cerro et al., 1993).

The expression of IGFs, the type I IGF receptor and several IGFBPs in fetal kidneys implies that the IGF system is involved in kidney growth and development. Therefore, we have examined in detail the mRNA expression of the six IGFBPs during fetal and postnatal kidney development in relation to IGF-I, IGF-II and type I IGF receptor mRNA expression using *in situ* hybridization techniques.

MATERIALS AND METHODS

Isolation of tissues and sectioning.

Pregnant BALB/c mice were sacrificed, conceptuses of 17-19 days gestation were collected and staged according to Kaufman (Kaufman, 1992). Kidneys were dissected from fetuses (embryonal day 17 and 19) and from mice 0, 2, 7 and 28 days after birth. Tissues were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin according to standard procedures. 6 μm sections were cut on a microtome and mounted onto 3-aminopropyl-trioxysilane-coated slides on a drop of 10% ethanol. Sections were stretched and dried on a 45^{0}C heating plate for 1 day.

Preparation of RNA probes.

cDNAs encoding mouse IGF-I and -II were kindly provided by Dr. G.I. Bell (Howard Hughes Medical Institute, Chicago, USA). Fragments (EcoR1 for IGF-I and EcoR1/Sac-1 for IGF-II) were subcloned in pBluescript (Stratagene, La Jolla, USA). A 265 bp EcoR1/Sma1 fragment of the rat cDNA clone ligated in pGEM3 was kindly provided by Dr. H. Werner and Dr. D. LeRoith (National Institutes of Health, Bethesda, USA). As templates for IGFBP-specific RNA probes, plasmids containing cDNA fragments corresponding to mouse IGFBP-1 to -6 were used as described before (Schuller et al., 1993). ^{35}S-labelled RNA transcripts were generated from linearized plasmids using SP6, T3 or T7 RNA polymerase in the presence of 50 μCi [^{35}S]UTP (Amersham, UK). The DNA templates were removed by digestion with DNase-I and the labelled RNA probes were purified by phenol/chloroform extraction and ethanol precipitation.

In situ hybridization.

In situ hybridization was carried out as described (Schuller et al., 1993). Briefly, paraffin wax was removed from the sections using successive baths of xylene. Sections were rehydrated and immersed in 0.2N HCl for 20 minutes. Subsequently, sections were rinsed in PBS, incubated in 2xSSC for 15 minutes at 70^{0}C and postfixed for 5 minutes in 4%

paraformaldehyde. Sections were treated to block non-specific binding by rinsing in PBS/10mM DTT, followed by two rinses of PBS and incubation with 0.1M triethanolamine/0.25% acetic anhydride for 10 minutes. Sections were rinsed in 2xSSC and stored in 70% ethanol for 14 hrs. The next day sections were dehydrated, dried and hybridized with ^{35}S-labelled RNA probes for 4 hours at 66^{0}C in a solution containing 50% formamide, 4xSSC, 1x Denhardt's, 1mg/ml tRNA, 10% dextransulphate, 10mM DTT and 0.2 mg/ml ssDNA. After hybridization the sections were washed at 56^{0}C in successive baths containing 55% formamide/2xSSC/20mM ß-mercaptoethanol (2x 15 minutes), 55% formamide/2xSSC/20mM ß-mercaptoethanol/0.5% triton X-100 (2x 15 minutes) and 55% formamide/2xSSC/20mM ß-mercaptoethanol (2x 15 minutes). Subsequently, sections were rinsed in 2xSSC, dehydrated and dried prior to exposure to Kodak AR X-ray film for 1-3 days. Finally, sections were exposed to Kodak NTB2 photographic emulsion for 3-21 days, developed and stained with nuclear fast red for microscopic evaluation. The level of non-specific binding was determined using sense probes for each of the IGFs, type I IGF receptor and IGFBPs.

RESULTS

In fetal and postnatal kidneys mRNAs for both IGFs, the type I IGF receptor and all six IGFBPs were found. At embryonal day 17, mRNA expression of IGF-II was detected in the renal capsule, the undifferentiated metanephric cap tissue and in cells of developing and mature glomeruli. IGF-II mRNA was weakly expressed in cortical (proximal and distal) tubules (Fig. 1a+b). IGF-I mRNA was found less abundantly expressed in the same regions as IGF-II (not shown). Transcripts for the type I IGF receptor were found in the developing and mature glomeruli, but were undetectable in the cortical tubules (Fig. 1c+d).

The genes for IGFBP-1 and -2 were mainly expressed in undifferentiated metanephric cap tissue and in developing glomeruli. Both IGFBP-1 and -2 mRNAs were weakly expressed in mature glomeruli. Furthermore, little, if any, IGFBP-1 and -2 mRNA was found in cortical tubules (Fig. 1e-h). Transcripts for IGFBP-5 were also observed in undifferentiated metanephric cap tissue, but no expression was found in the developing glomeruli. In contrast, IGFBP-5 mRNAs were detected in mature glomeruli (Fig. 1k+l). IGFBP-3 mRNA was also expressed in fetal kidney, but no apparent differences between the various regions were observed (not shown).

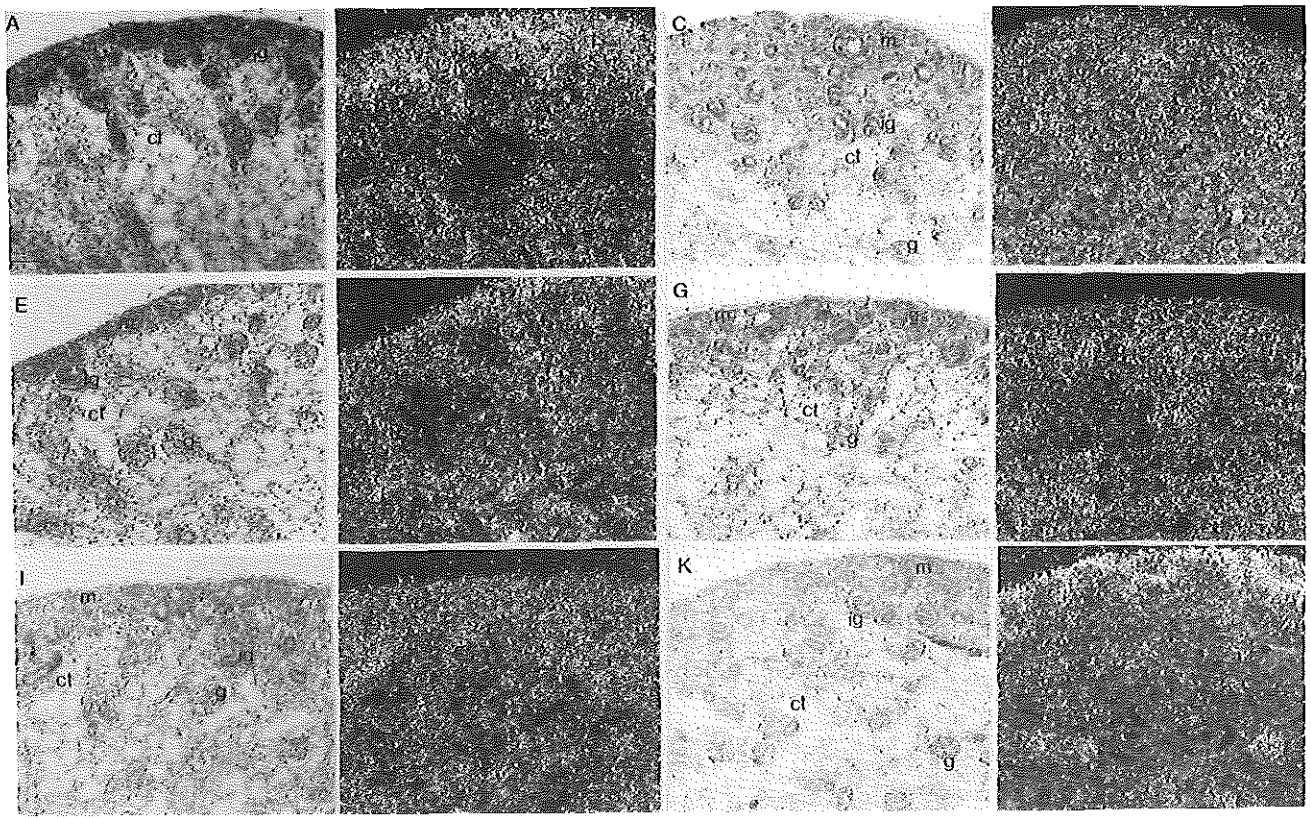

Fig. 1. Brightfield (A, C, E, G, I and K) and darkfield (B, D, F, H, J and L) images of sections of a 17 day-old fetal kidney hybridized with probes for IGF-II (A and B), type I IGF receptor (C and D), IGFBP-1 (E and F), IGFBP-2 (G and H), IGFBP-4 (I and J) and IGFBP-5 (K and L). M, undifferentiated metanephric cap tissue; IG, immature glomerulus; G, glomerulus, CT, cortical tubule. scale bar = 100 μm.

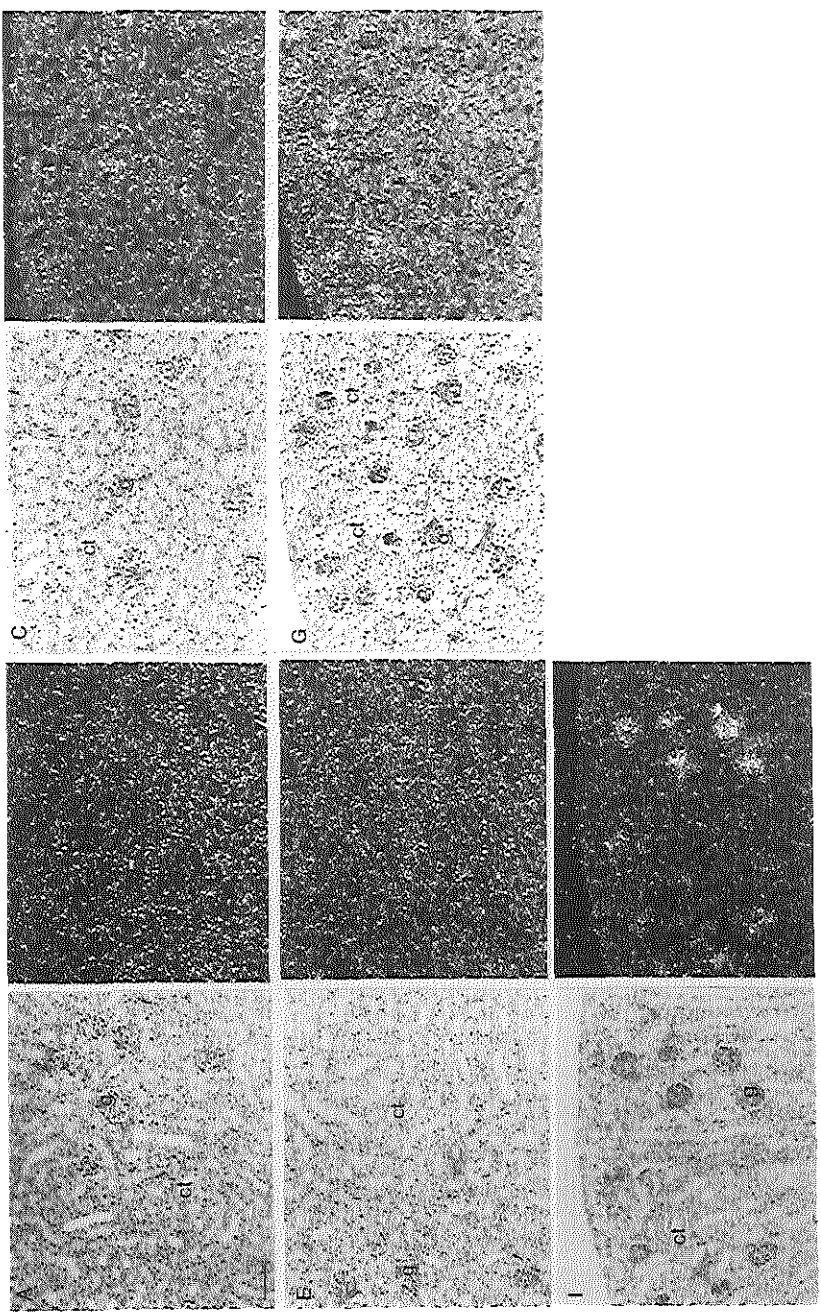

Fig. 2. Brightfield (A, C, E, G and I) and darkfield (B, D, F, H and J) images of sections of a 1 week old kidney hybridized with probes for IGF-II (A and B), IGFBP-1 (C and D), IGFBP-2 (E and F), IGFBP-4 (G and H) and IGFBP-5 (I and J). G, glomerulus, CT, cortical tubule. scale bar = 100 μm.

IGFBP-4 mRNA expression was detected in the cortex, including the developing and mature glomeruli. No expression was found in the cortical tubules (Fig. 1i+j). IGFBP-6 mRNAs were expressed in all regions of the fetal kidney except cortical tubules (not shown).

Between embryonal day 17 and birth, no obvious changes in the expression patterns occurred. However, 2 days after birth, the high expression of IGF-II, IGFBP-1, IGFBP-2 and IGFBP-5 mRNAs in the cortical region was no longer observed, which is consistent with the almost complete disappearance of undifferentiated metanephric cap tissue. This was even more pronounced 1 week after birth (Fig. 2). At that time continuing up to 4 weeks after birth, IGF-I and II transcripts were expressed in the kidney with no apparent variation between the different regions (Fig. 2a+b). Type I IGF receptor mRNAs were mainly observed in glomeruli (not shown). IGFBP-1 mRNAs were only weakly expressed at this time. Some expression remained in glomeruli and in the mesenchymal cells located between the cortical tubules (Fig. 2c+d). Transcripts for IGFBP-2 were found in all regions of the kidney (Fig. 2e+f). In contrast, IGFBP-5 mRNA was exclusively expressed in glomeruli (Fig. 2i+j). IGFBP-4 mRNA expression was found in the glomeruli and in some cortical tubules (Fig. 2g+h). The patterns of IGFBP-3 and -6 mRNA expression did not differ from those described in fetal stages (not shown).

DISCUSSION

In this study, we have shown that the genes for both IGFs, the type I IGF receptor and all six IGFBPs are expressed in fetal and postnatal mouse kidneys. In the fetal stages, IGF-I, IGF-II and the type I IGF receptor mRNAs were expressed in both developing and mature glomeruli. In adult rats, glomerular expression of IGF-I mRNA and protein has been reported (Matejka et al., 1992, Bortz et al., 1988) and iodinated IGF-I binding to glomeruli of fetal and adult human kidneys (Gröne et al., 1992). IGF-I stimulates DNA synthesis of rat glomerular mesangial cells in culture (Arnqvist et al., 1988). Furthermore, enlargement of glomeruli is seen in mice transgenic for IGF-I (Doi et al., 1990). These results show that at least IGF-I can stimulate the growth of the glomeruli and suggest that both IGFs are involved in glomerular development. IGF-II mRNAs were abundantly expressed in the

undifferentiated metanephric cap tissue. Since the metanephric cap tissue gives rise to the developing glomeruli, the abundant IGF-II mRNA expression may indicate that IGF-II plays a role in the early development of the glomeruli.

In human adults, subcutaneous infusion of IGF-I results in an increased creatinine clearance and glomerular filtration rate (Guler et al., 1989). In rats, IGF-I infusion enhanced total and single nephron glomerular filtration rate without altering filtration pressure (Hirschberg and Kopple 1989, Hirschberg et al., 1990). In mouse kidneys 1 and 4 weeks after birth, IGF-I, IGF-II and type I IGF receptor mRNAs were still expressed in the glomeruli. This implies that the effects of IGFs on glomerular filtration rate can also be mediated by locally produced IGFs.

All six IGFBP genes were expressed in immature and/or mature glomeruli. The IGFBP-1 and -2 mRNAs were only weakly expressed in mature glomeruli, and the IGFBP-5 gene was not expressed in the developing glomeruli. Furthermore, IGFBP-1, -2 and -5 mRNAs were abundantly expressed in the undifferentiated metanephric cap tissue. The localization of IGFBP-1 mRNAs is in agreement with previous observations in human fetal kidney (Suikkari et al., 1992). These observations suggest that IGFBPs participate in the growth and development of the glomeruli and that IGFBP-1, -2 and -5 may be involved in the early processes initiating glomerular development. Recently, we have shown that IGFBP-4 and -5 are expressed in neighboring cell types during mouse lung development and in embryonic tongue (Schuller et al., 1994b, Schuller et al., 1993). Here we show that in the mouse kidney IGFBP-4 and -5 mRNAs are both located in the mature glomeruli, indicating that expression of IGFBP-4 and -5 in adjacent cell types exists in some, but not all tissues.

We can only speculate about the functional significance of the IGFBP mRNA expression patterns found. It is reasonable to assume that the IGFBPs will influence IGF action during kidney growth and development. IGFBP-1, -2 and -3 have both inhibitory and stimulatory effects on IGF actions, whereas IGFBP-4 inhibits IGF actions. For IGFBP-1 and IGFBP-3 the stimulatory effects most likely depend on their capability to adhere to the cell surface (reviewed by Clemmons 1992). IGFBP-5 has been found in the extracellular matrix

of human fetal fibroblasts where it could potentiate the biological responses of IGF-I (Jones et al., 1993a). In baby hamster kidney (BHK) cells, two unidentified cell-associated IGFBPs with a molecular weight of 30 and 25 kDa have been reported. When high levels of IGF-I were present in the medium, IGFBP secretion was increased and cell surface IGFBPs were decreased. In contrast, when the IGF-I concentration in the medium was low, IGFBP secretion diminished and the amount of cell attached IGFBPs increased (Hsu and Olefsky 1992). These results indicate that the amount of IGFBPs attached to cells might regulate the availability of IGFs. Therefore, the capability of IGFBPs to bind to specific kidney cells may influence IGF responsiveness during kidney growth and development.

In addition, IGFBPs may influence kidney growth and development independently of IGFs. IGFBP-1 and -2 both contain an Arg-Gly-Asp (RGD) sequence at their C-terminal end. For IGFBP-1 this sequence has been shown to be essential for its stimulating effect on the migration of Chinese hamster ovary (CHO) and its binding to $\alpha_5\beta_1$ integrin (fibronectin receptor). Since addition of IGFs had no effect on cell migration and no endogenous IGF-I or IGF-II mRNA was detectable, these results suggest that IGFBP-1 stimulates CHO cell migration independently of IGF (Jones et al., 1993b). Likewise, IGFBP-2 might affect cell migration. Secondly, fibronectin forms high molecular weight polymers which interact via integrin receptors with embryonic cells during migration, differentiation and organogenesis. Three discrete domains are essential for the assembly into the insoluble form, including the RGD-containing integrin binding domain. Addition of RGD peptides inhibits fibronectin assembly and branching morphogenesis of embryonic mouse lung in culture (reviewed by Warburton et al., 1993). Therefore, IGFBP-1 and -2 may potentially inhibit fibronectin action and thereby affect kidney growth and development.

In one week old kidneys, all six IGFBP genes are expressed in the mature glomeruli. This was most obvious for IGFBP-5 mRNAs. The finding that IGFBP-5 mRNA was exclusively expressed in glomeruli points towards a discrete role in mature glomerular function. A possible role of IGFBP-5, and the other IGFBPs expressed in mature glomeruli, may be to participate in the regulation of IGF-I enhanced glomerular filtration rate.

In summary, we have shown that IGF-I, IGF-II, the type I IGF receptor and all six IGFBP mRNAs are expressed in fetal and postnatal mouse kidneys. Based on the mRNA expression patterns observed we suggest that the IGFBPs participate in early glomerular development. Secondly, we speculate that IGFBPs, especially IGFBP-5, might play a role in glomerular function.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. G.I. Bell for kindly providing the mouse IGF-I and IGF-II cDNAs, Dr. H. Werner and Dr. D. LeRoith for kindly providing the type I IGF receptor cDNA probe, M. Vermey for collecting the kidney samples and F.L. van der Panne for photography. This work was supported by grants from the Sophia Foundation for Medical Research, the Netherlands and Novo-Nordisk Insulin Laboratories, Denmark.

REFERENCES

1 Bang, P. and Hall, K. (1992) Insulin-like growth factors as endocrine and paracrine hormones. In Schofield, P.N. (ed): The insulin-like growth factors. Oxford University Press, Oxford pp. 151-177.

2 Steele-Perkins, G. and Roth R.A. (1990). Monoclonal antibody αIR-3 inhibits the ability of insulin-like growth factor II to stimulate a signal from the type I receptor without inhibiting its binding. Biochem. Biophys. Res. Commun. 171, 1244-1251.

3 Pietrzowski, Z., Sell, C., Lammers, R., Ullrich, A. and Baserga, R. (1992). Roles of insulin-like growth factor I (IGF-I) and the IGF-I receptor in epidermal growth factor-stimulated growth of 3T3 cells. Mol. Cell. Biol. 12, 3883-3889.

4 Adamo, M., Lowe Jr, W.L., LeRoith, D. and Roberts Jr, C.T. (1989) Insulin-like growth factor I messenger ribonucleic acids with alternative 5'-untranslated regions are differentially expressed during development of the rat. Endocrinology 124, 2737-2744.

5 Brown, A.L., Graham, D.E., Nissley, S.P., Hill, D.J., Strain, A.J. and Rechler, M.M. (1986) Developmental regulation of insulin-like growth factor II mRNA in different rat tissues. J. Biol. Chem. 261, 13144-13150.

6 Werner, H., Woloschak, M., Adamo, M., Shen-Orr, Z., Roberts Jr., C.T. and LeRoith, D. (1989). Developmental regulation of the rat insulin-like growth factor I receptor gene. Proc. Nat. Acad. Sci. USA. 86, 7451-7455.

7 Liu, J-P., Baker, J., Perkins, A.S., Robertson, J.E. and Efstratiadis, A. (1993). Mice carrying nul mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell 75, 59-72.

8 Baker, J., Liu, J-P., Robertson, J.E. and Efstratiadis, A. (1993). Role of insulin-like growth factors in embryonic and postnatal growth. Cell 75, 73-82.

9 Powell-Braxton, L., Hollingshead, P., Warburton, C., Dowd, D., Pitts-Meek, S., Dalton, D., Gillet, N. and Stewart, T.A. (1993) IGF-I is required for normal embryonic growth in mice. Genes and Development 7, 2609-2617.

10 Clemmons, D.R. (1992) IGF binding proteins: Regulation of cellular actions. Growth Regulation 2, 80-87.

11 Drop, S.L.S., Schuller, A.G.P., Lindenbergh-Kortleve, D.J., Groffen, C., Brinkman, A. and Zwarthoff, E.C. (1992). Structural aspects of the IGFBP family. Growth Regulation 2, 69-79.

12 Schuller, A.G.P., Groffen, C., van Neck, J.W., Zwarthoff, E.C. and Drop, S.L.S. (1994a). cDNA cloning and mRNA expression of the six mouse insulin-like growth factor binding proteins. submitted.

13 Suikkari, A.M., Leivo, I., Kämäräinen, M., Holthöfer, H., Seppälä, M., Julkunen, M. and Koistinen, R. (1992) Expression of insulin-like growth factor binding protein-1 mRNA in human fetal kidney. Kidney International 42, 749-754.

14 Cerro, J.A., Grewal, A., Wood, T.L. and Pintar, J.E. (1993). Tissue-specific expression of the insulin-like growth factor binding protein (IGFBP) mRNAs in mouse and rat development. Regulatory Peptides 48, 189-198.

15 Kaufman, M.H. The atlas of mouse development. 1992, Academic Press, London.

16 Schuller, A.G.P., Zwarthoff, E.C. and Drop, S.L.S. Gene expression of the six insulin-like growth factor binding proteins (IGFBPs) in the mouse conceptus during mid- and late gestation. Endocrinology, 1993, 132, 2544-2550.

17 Matejka, G., Eriksson, P., Carlsson, B. and Jennische, E. (1992) Distribution of IGF-I mRNA and IGF binding sites in the rat kidney. Histochem. 97, 173-180.

18 Bortz, J., Rotwein, P., DeVol, D., Bechtel, P., Hansen, V. and Hammerman, M. (1988) Focal expression of insulin-like growth factor I in rat kidney collecting duct. J. Biol. Chem. 107, 811-819.

19 Arnqvist, H.J., Ballerman, B.J. and King, G.K. (1988) Receptors for and effects of insulin and IGF-I in rat glomerular mesangial cells. Am. J. Physiol. 254, C411-416.

20 Doi, T., Striker, L.J., Gibson, C.C., Agodoa, L.Y.C., Brinster, R.L. and Striker, G.E. (19990) Glomerular lesions in mice transgenic for growth hormone and insulin-like growth factor-1. I. Relationship between increased glomerular size and mesangial sclerosis. Am. J. Pathol. 137, 541-552.

21 Guler, H.P., Smid, C., Zapf, J. and Froesch, E.R. (1989) Effects of recombinant IGF-I on insulin secretion and renal function in normal human subjects. Proc. Natl. Acad. Sci. USA 86, 2868-2872.

22 Hirschberg, R. and Kopple, J.D. (1989) Evidence that insulin-like growth factor I increases renal plasma flow and glomerular filtration rate in fasted rats. J. Clin. Invest. 83, 326-330.

23 Hirschberg, R., Kopple, J.D., Blantz, R. and Tucker, B. (1990) Effects of IGF-I on glomerular dynamics in rats (abstract). J. A. Soc. Nephrol. 1, 666.

24 Schuller, A.G.P., Beukenholdt, R.W., van Neck, J.W., Zwarthoff, E.C. and Drop, S.L.S. (1994b) Insulin-like growth factor (IGF), type I IGF receptor and IGF binding protein mRNA expression in the developing mouse lung. Submitted.

25 Jones, J.I., Gockerman, A., Busby, W.H., Camacho-Hubner, C. and Clemmons, D.R. (1993b) Extracellular matrix contains insulin-like growth factor binding protein-5: Potentiation of the effects of IGF-I. J. Cell. Biol. 121, 679-687.

26 Hsu, D. and Olefsky, J.M. (1992) Characterization of insulin-like growth factor (IGF) binding proteins and their role in modulating IGF-I action in BHK cells. J. Biol. Chem. 267, 25576-25582.

27 Jones, J.I., Gockerman, A., Busby, W.H.Jr., Wright, G. and Clemmons, D.R. (1993a) Insulin-like growth factor binding protein 1 stimulates cell migration and binds to the $\alpha_5\beta_1$ integrin by means of its Arg-Gly-Asp sequence. Proc. Natl. Acad. Sci. USA 90, 10553-10557.

28 Warburton, D., Lee, M., Berberich, M.A. and Bernfield, M. (1993) Molecular embryology and the study of lung development. Am. J. Respir. Cell Moll. Biol. 9, 5-9.

CHAPTER 8

SUMMARY AND CONCLUDING REMARKS

IGFs are single chain polypeptides with both mitogenic and metabolic properties. The IGF system plays an important role during embryogenesis and is essential for normal embryonal and fetal development. In sera and extracellular fluids, the IGFs are complexed to a specific group of proteins: the IGFBPs. These IGFBPs comprise a family of six structurally related peptides, which modulate IGF action.

The raising and characterization of monoclonal antibodies specific for human IGFBP-1 is described in Chapter 2. One of these monoclonal antibodies (MAb A) interferes functionally with the binding of both IGF-I and IGF-II to IGFBP-1 as determined by immunoprecipitation and in vitro proliferation assays, suggesting that the IGF-I and IGF-II binding domains of IGFBP-1 are either the same or in close vicinity. The epitope of MAb A was found in the C-terminal part of IGFBP-1. Two other monoclonal antibodies, which do not influence the binding of IGF to IGFBP-1, were found to recognize the same regions in IGFBP-1 as MAb A. Therefore, it is unlikely that the complete epitope of MAb A is involved in IGF binding. However, the possibility that those residues unique for the epitope of MAb A contribute to the IGF binding domain of IGFBP-1 can not be ruled out. Alternatively, the epitope of MAb A is located sterically near the IGF binding domain and therefore MAb A interferes with the binding of IGF to IGFBP-1.

The IGF binding domain of IGFBP-1, or any other IGFBP, has not yet been characterized. As discussed in the introduction, it is not likely that the IGF binding domain is a linear stretch of amino acids. Probably, the IGF binding domain is a three dimensional structure, to which both the N- and C-terminal domains of the IGFBPs contribute. Therefore, the most promising approach to study the IGF binding domain will be by resolving the three dimensional structure of the IGFBPs, alone or bound to IGF. Although large amounts of purified IGFBPs may be needed, this should not be a problem. IGFBP-1 can be purified in large amounts easily from human mid-term amniotic fluid. In addition, it is possible to synthesize recombinant IGFBPs.

The expression of the IGFBPs during follicle maturation is reported in Chapter 3. Using Western ligand blotting, IGFBPs of 43 kDa, 37 kDa, 31 kDa, a doublet around 28 kDa and 24 kDa were found in fluid obtained from follicles in different stages of maturation. The 43 and 37 kDa IGFBPs were identified as IGFBP-3 and the 31 kDa IGFBP as IGFBP-2, both by immunoblotting techniques, whereas the 28 kDa IGFBP did not react with an antibody specific for IGFBP-1. A dramatic decrease in IGFBP-2 and the 28 kDa and 24 kDa IGFBPs was observed in follicular fluid of dominant follicles as compared to normal non-dominant or atretic follicles. This decrease in IGFBP-2 and the 28 kDa and 24 kDa IGFBPs may result in an increased IGF stimulation, resulting in a mitogenic response of granulosa cells and an amplified steroidogenic response to FSH stimulation. Since this, in turn, facilitates the follicle to gain dominance, it might be that IGFBP-2, 28 kDa and 24 kDa IGFBPs are involved in human folliculogenesis.

The identity of the 28 kDa and 24 kDa IGFBP, decreased in follicular fluid of dominant follicles has not been established. According to the molecular weight, the 24 kDa IGFBP most likely represents IGFBP-4. The 28 kDa IGFBP may be a glycosylated variant of IGFBP-4 (Fielder et al., 1990, Cheung et al., 1991), but could also be IGFBP-6. Since the band migrating at 28 kDa is a doublet, the possibility exists that this doublet represents both IGFBP-4 and IGFBP-6. The decrease in presumably IGFBP-4 levels in follicular fluid of dominant follicles is most interesting, since IGFBP-4 mRNA expression changes during the estrous cycle of the rat, and is exclusively found in atretic follicles. Therefore, the loss of IGFBP-4 may facilitate the follicle to gain dominance, whereas follicles expressing IGFBP-4 may be directed to atresia.

The isolation and characterization of cDNAs encoding the six mouse IGFBPs (mIGFBPs), together with the mRNA expression of the six mIGFBPs in whole embryos, perinatal liver and several adult tissues is presented in Chapter 4. The amino acid sequences of the six mIGFBPs are clearly distinct, but share regions with strong homology in both the N-terminal and C-terminal ends. 18 cysteine residues are found, spatially conserved in all mIGFBPs, with the exception of mIGFBP-6, which lacks two cysteine residues and mIGFBP-4 which contains two additional cysteines. Every mIGFBP is highly homologous to its human

and rat counterpart. This also holds true for the central part where no obvious conservation is found between different IGFBPs from one species. In relation to the functions of the IGFBPs, this may mean that the N- and C-terminal ends contain domains important for all six IGFBPs, for instance IGF binding, whereas the middle part may represent a region which is important for the unique features of each of the IGFBPs, such as cell specific attachment, sensitivity to proteases and protein interactions. In whole embryos, mIGFBP-2, -3, -4 and -5 mRNAs were detected as early as embryonal day 10½, whereas expression of mIGFBP-1 was first seen at day 13½. Also in liver, mRNA expression of the mIGFBPs is developmentally regulated. IGFBP-2, -3 and -4 mRNAs increased in time, reaching maximal levels around 1 week after birth. In contrast, a peak of mIGFBP-1 mRNA expression was observed between 1 and 3 days after birth. In adult mouse tissues, expression of the six mIGFBP genes was partly overlapping, but distinct. Thus, the expression of the six mIGFBP genes is developmentally regulated in a tissue specific manner.

Now it is evident that the six IGFBP genes are tissue specifically expressed and that their expression is developmentally regulated, it will be interesting to study the factors determining the expression of the six IGFBP genes. Cell lines expressing various IGFBPs will be of help not only to understand the regulation of IGFBP expression by various hormones, but also to identify the transcription factors responsible for the specificity of IGFBP gene transcription.

In mid and late gestational mouse conceptuses, the localization of the six mIGFBP mRNAs has been studied by *in situ* hybridization (Chapter 5). Expression of mIGFBP-1 was detected after embryonal day 12 and was restricted to the liver. In contrast, widespread expression of mIGFBP-2, -4 and -5 mRNAs was observed in all stages tested. Transcripts for mIGFBP-3 and -6 were only detected in fetal stages. Complementary expression of mIGFBP-4 and -5 was observed in some tissues, whereas several cell types were observed to coexpress IGFBP-2 and -5. In the embryonal tongue for instance, mRNA expression of IGFBP-4 was located in the mesodermally derived part, whereas IGFBP-5 mRNA was restricted to the ectodermal layer. Also in fetal lung, IGFBP-4 and -5 were expressed in different cell types. Here, IGFBP-4 mRNA was predominantly found in interstitium, whereas

expression of IGFBP-5 was restricted to the respiratory epithelium. These data show that also in mid and late gestational conceptuses, the expression of the six mIGFBPs is tissue specifically and developmentally regulated.

The mRNA expression of IGFBP-4 and -5 in neighboring cell types and the coexpression of IGFBP-2 and -5 observed in some tissues is interesting, especially since IGF-II and IGFBP-2 are complementary expressed during embryonal development. This may mean that in some cell systems IGF-II and IGFBP-4 are coexpressed and that IGF-II and IGFBP-5 may be expressed in adjacent cell types.

The mRNA expression of IGFBP-2, -4 and -5 has been studied in detail during mouse lung development in relation to the expression of IGF-I, IGF-II and the type I IGF receptor (Chapter 6). The IGF, type I IGF receptor and IGFBP-2, -4 and -5 genes were expressed in developing lung as early as embryonal day 12½ and the expression continued over the examined period (from embryonal day 12½, up to 4 weeks postnatally). IGF-I and IGF-II mRNAs were expressed both in mesenchymal and epithelial cells. Type I IGF receptor transcripts were also observed throughout the developing lung, with the exception of the epithelial cells of the bronchi after embryonal day 15. IGFBP-4 and -5 mRNA expression was noted in neighboring cell types, IGFBP-4 mRNA was predominantly found in mesenchyme, whereas IGFBP-5 mRNA expression was mainly detected in epithelial cells. Furthermore, coexpression of the type I IGF receptor and IGFBP-4 transcripts was observed after embryonal day 15. The observed expression patterns imply that the IGFBP-2, -4 and -5 genes are differentially regulated and suggest that each may have a discrete function in mouse lung development.

Before embryonal day 15, all cells of the developing lung express IGF-I, IGF-II and type I IGF receptor mRNAs. Assuming that these mRNAs are translated into functional IGFs and type I IGF receptors, all cells of the developing lung can respond to IGF stimulation. At present, it is not known whether cell type specific IGF responses are needed during lung development or that all cells respond equally to IGF stimulation. However, if cell type specific IGF responses are needed, this may be encountered by the expression of IGFBP-2, -4 and -5. IGFBP-4 and -5 are expressed in adjacent cell types. Furthermore, IGFBP-2 may

preferentially bind to certain lung cells, since it has been demonstrated that IGFBP-2 associates with the cell membrane of small cell lung cancer cells, but not of non small cell lung cancer cells. Finally, IGFBP-2, -4 and -5 may affect IGF action distinctly via either inhibiting (IGFBP-2 and -4) or enhancing (IGFBP-2 and -5) IGF stimulation. Therefore, a possible role for IGFBP-2, -4 and -5 is to participate in the regulation of cell specific IGF responses during lung development.

IGFBP-1 can bind to the $\alpha_5\beta_1$ integrin (fibronectin receptor) and stimulate the migration of Chinese hamster ovary cells in culture, both via its Arg-Gly-Asp (RGD) sequence. Since IGFBP-2 also contains an RGD sequence at its C-terminal end, IGFBP-2 may potentially stimulate cell migration as well, and thereby influence lung development.

In Chapter 7, the mRNA expression of the IGFs, the type I IGF receptor and the six IGFBPs in fetal and postnatal mouse kidneys, is described as determined by *in situ* hybridization techniques (Chapter 7). Both IGF genes were mainly expressed in the undifferentiated metanephric cap tissue and in developing and mature glomeruli. The type I IGF receptor mRNAs were also expressed in both immature and mature glomeruli. Like the IGFs, IGFBP-1 and -2 were abundantly expressed in the undifferentiated metanephric cap tissue and in immature glomeruli. However, weak mRNA expression was observed in mature glomeruli. In contrast, IGFBP-5 mRNA expression was not detected in immature glomeruli, but was abundantly expressed in the mature glomeruli. IGFBP-3, -4 and -6 mRNAs were expressed both in mature and immature glomeruli. The mRNA expression patterns found, suggest that IGFs and IGFBPs may participate in early glomerular development.

As mentioned before, the IGFBPs could distinctly affect IGF action, might participate in the regulation of cell specific IGF responses and, in the case of IGFBP-1 and -2, may potentially stimulate cell migration. The restrictive expression of IGFBP-5 in mature glomeruli may point toward a discrete role in glomerular function. In that respect, IGFBP-5 might be involved in the IGF mediated enhancement of the glomerular filtration rate.

Besides studying the mRNA expression of the IGFBPs, it is important to investigate where the resulting proteins will be localized. Some IGFBPs are able to bind to the extracellular matrix or cell membrane. For instance, IGFBP-1 has been demonstrated to bind to $\alpha_5\beta_1$ integrin (the fibronectin receptor) and to stimulate migration of Chinese hamster ovary cells. Also, membrane associated IGFBP-2 has been demonstrated to compete with IGF receptors for ligand binding in small cell lung cancer cells. Therefore, IGF action may not only be determined by the amount of IGFs, IGFBPs and IGF receptors, but may also depend on the capability of IGFBPs to bind to certain cells. As a consequence, it will be interesting to study IGF/IGFBP actions, not only in cells expressing IGFBPs, but also in cells to which a certain IGFBP can bind.

Another promising approach to study the functions of the IGFBPs is to apply gene disruption strategies. It is possible that the function of the deleted IGFBP will be compensated by one of the other IGFBPs. However, expression of the IGFBP genes is tissue specific and developmentally regulated. In addition, many physiological and pathological conditions have been described in which the levels of only certain IGFBPs change. Furthermore, the six IGFBPs are distinct in respect to cell specific attachment, sensitivity to proteases, IGF affinity, phosphorylation, glycosylation and protein interactions. Finally, IGF independent effects have been suggested for some of the IGFBPs. Therefore, it is not unlikely that an effect of IGFBP gene disruption will be observed, if not obvious, it might be found under specific physiological or pathological conditions.

SAMENVATTING

Insuline-achtige groeifactoren (IGFs) zijn kleine eiwitten met zowel mitogene als metabole eigenschappen. Het IGF systeem speelt een belangrijke rol tijdens de embryogenese en is essentieel voor normale embryonale en foetale ontwikkeling. In serum en extracellulaire vloeistoffen zijn de IGFs gebonden aan een specifieke groep eiwitten: de IGF bindende eiwitten (IGFBPs). Deze IGFBPs vormen een familie van zes structureel verwante eiwitten die in staat zijn de IGF actie te moduleren.

Het opwekken en karakteriseren van monoclonale antilichamen specifiek voor humaan IGFBP-1 is beschreven in Hoofdstuk 2. Eén van deze monoclonale antilichamen (MAb A) interfereert functioneel met de binding van zowel IGF-I als IGF-II aan IGFBP-1, zoals aangetoond met immuunprecipitatie en in vitro proliferatie experimenten. Dit suggereert dat de bindingsplaatsen op IGFBP-1 voor IGF-I en IGF-II of hetzelfde zijn of vlak bij elkaar in de buurt liggen. Het epitoop van MAb A werd gevonden in het C-terminale deel van IGFBP-1. Twee andere monoclonale antilichamen, die geen effect op de IGF binding aan IGFBP-1 hebben, herkenden dezelfde regio als MAb A. Vandaar dat het onwaarschijnlijk is dat het gehele epitoop van MAb A betrokken is bij de binding van IGF. De mogelijkheid dat de residuen uniek voor het epitoop van MAb A betrokken zijn bij de binding van IGF kan echter niet worden uitgesloten. Als alternatief bestaat de mogelijkheid dat het epitoop van MAb A sterisch in de nabijheid van de bindingsplaats voor IGF ligt en zodoende interfereert met de binding van IGF aan IGFBP-1.

De IGF bindingsplaats van IGFBP-1, of elk ander IGFBP, is nog niet gekarakteriseerd. Zoals bediscussieerd in de introductie, is het onwaarschijnlijk dat de bindingsplaats voor IGF bestaat uit een lineaire keten aminozuren. Meer waarschijnlijk is de IGF bindingsplaats een drie-dimensionale structuur, waartoe zowel het N-terminale als ook het C-terminale deel bijdragen. De meest veelbelovende aanpak om achter de IGF bindingsplaats te komen, is dan ook het bepalen van de drie-dimensionale structuur van de IGFBPs, vrij of gebonden aan IGF. Hoewel grote hoeveelheden gezuiverd IGFBP nodig kunnen zijn, mag dat geen probleem opleveren. IGFBP-1 kan makkelijk in grote hoeveelheden gezuiverd worden uit humaan vruchtwater. Daarnaast is het mogelijk om recombinant IGFBP te synthetiseren.

De expressie van de IGFBPs tijdens de ontwikkeling van het follikel is gerapporteerd in Hoofdstuk 3. Met behulp van ligand-blotten werden in vocht verkregen uit follikels van verschillende rijpingstadia IGFBPs gevonden met een moleculair gewicht van 43 kDa, 37 kDa, 31 kDa, een doublet rond 28 kDa en 24 kDa. De 43 en 37 kDa IGFBPs werden geïdentificeerd als IGFBP-3 en het 31 kDa IGFBP als IGFBP-2, beiden mbv immunoblot technieken. Het 28 kDa IGFBP reageerde echter niet met een antilichaam specifiek voor IGFBP-1. Een dramatische afname in IGFBP-2 en de 28 kDa en 24 kDa IGFBPs werd waargenomen in follikel vocht van dominante follikels, vergeleken met follikel vocht van normale niet-dominante of atretische follikels. Deze afname in IGFBP-2 en de 28 kDa en 24 kDa IGFBPs zou kunnen resulteren in een verhoogde IGF stimulatie, wat weer leidt tot een mitogene respons van de granulosa cellen en een versterkte respons op FSH stimulatie. Aangezien dit het follikel de mogelijkheid geeft dominant te worden, kan het zijn dat IGFBP-2, het 28 kDa IGFBP en het 24 kDa IGFBP betrokken zijn bij de humane follikel rijping.

De identiteit van het 28 kDa en het 24 kDa IGFBP, afgenomen in follikel vocht van dominante follikels, werd niet bepaald. Vanwege het moleculair gewicht, is het 24 kDa IGFBP hoogst waarschijnlijk IGFBP-4. Het 28 kDa IGFBP zou een geglycosyleerde variant van IGFBP-4 kunnen zijn (Fielder et al., 1990, Cheung et al., 1991), maar kan ook IGFBP-6 zijn. Aangezien de band migrerend rond 28 kDa een doublet is, zou het ook kunnen zijn dat deze band zowel IGFBP-4 als IGFBP-6 representeert. De afname van, hoogst waarschijnlijk, IGFBP-4 is erg interessant aangezien de IGFBP-4 mRNA expressie veranderd tijdens de cyclus van de rat, en exclusief gevonden wordt in atretische follikels. Het verlies van IGFBP-4 zou ertoe kunnen leiden dat het follikel dominant wordt, terwijl follikels die IGFBP-4 tot expressie brengen in de richting van atresie gestuurd worden.

De isolatie en het karakteriseren van de cDNAs coderend voor de zes muis IGFBPs (mIGFBPs) wordt gepresenteerd in Hoofdstuk 4, samen met de mRNA expressie van de zes mIGFBPs in hele embryo's, in lever voor en na geboorte, en in verschillende volwassen organen. De aminozuur sequentie van de zes mIGFBPs zijn duidelijk verschillend, maar bevatten in zowel het N-terminale als ook in het C-terminale deel regio's met sterke gelijkenis. Een conservering van de positie van 18 cysteïne residuen wordt gevonden in alle

mIGFBPs met de uitzondering van mIGFBP-6, met slechts 16 cysteïnes, en mIGFBP-4, met twee extra cysteïnes. Elk mIGFBP vertoont een hoge mate van gelijkenis met het humane en rat IGFBP. Dit geld ook voor het midden gedeelte, waar geen opvallende overeenkomst tussen de verschillende IGFBPs van één specie bestaat. Met betrekking tot de functie van de IGFBPs, zou dit kunnen betekenen dat de N- en C-terminale gedeeltes van belang zijn voor gemeenschappelijke functies van alle zes de IGFBPs, zoals het binden van IGF, terwijl het middelste gedeelte belangrijk is voor eigenschappen uniek voor elk IGFBP, zoals celbinding, gevoeligheid voor proteases en eiwit interacties. In hele embryo's werden mRNAs voor mIGFBP-2, -3, -4 en -5 gevonden vanaf dag 11½ van de embryogenese, terwijl IGFBP-1 mRNA expressie voor het eerst gezien werd rond day 13½. Ook in de lever wordt de mRNA expressie van de mIGFBPs ontwikkelings afhankelijk gereguleerd. De mRNA expressie van IGFBP-2, -3 en -4 nam geleidelijk toe in de tijd en bereikte een maximum 1 week na geboorte. Daarentegen werd een piek in de mRNA expressie van mIGFBP-1 waargenomen tussen dag 1 en 3 na geboorte. De zes mIGFBP mRNAs werden tevens gedetecteerd in volwassen organen. Hoewel enig overlap aanwezig was, waren de expressie patronen verschillend. Deze gegevens laten zien dat de zes mIGFBPs weefselspecifiek tot expressie komen en dat deze expressie ontwikkelings afhankelijk gereguleerd wordt.

Aangezien het duidelijk is dat de zes IGFBP genen weefselspecifiek tot expressie komen en dat deze expressie ontwikkelings afhankelijk is, is het interessant om de factoren te identificeren en te bestuderen die de expressie van de IGFBP genen bepalen. IGFBP-producerende cellijnen, zullen van belang zijn, niet alleen om de regulatie van IGFBPs door verschillende hormonen te bepalen, maar ook om de transcriptiefactoren te identificeren die verantwoordelijk zijn voor de specificiteit van de IGFBP gen transcriptie.

De lokalisatie van de zes mIGFBP mRNAs in muis embryo's en foeten werd bestudeerd met *in situ* hybridisatie technieken (Hoofdstuk 5). Expressie van mIGFBP-1 werd gevonden na ontwikkelingsdag 12 en alleen in de lever. De mIGFBP-2, -4 and -5 mRNAs werden gedetecteerd in verschillende weefsels en in alle stadia bestudeerd. Transcripten voor mIGFBP-3 en -6 werden alleen gevonden in foetale stadia. In sommige weefsels was de

mRNA expressie van mIGFBP-4 en -5 in verschillende celtypen gelokaliseerd, terwijl mIGFBP-2 en -5 mRNAs regelmatig in dezelfde celtypen werden gedetecteerd. In de embryonale tong was de mIGFBP-4 expressie gelegen in het mesodermale deel, terwijl mIGFBP-5 mRNAs alleen in de ectodermale laag tot expressie kwamen. Ook in de foetale long kwamen de mIGFBP-4 en -5 mRNAs in verschillende celtypen tot expressie. Hier werden mIGFBP-4 transcripten gevonden in het interstitium, terwijl mIGFBP-5 mRNA alleen in het respiratoir epitheel tot expressie kwam. Deze resultaten laten zien dat ook tijdens de embryonale en foetale ontwikkeling de expressie van de zes mIGFBPs weefselspecifiek is en ontwikkelings afhankelijk wordt gereguleerd.

De mRNA expressie van mIGFBP-4 en -5 in naburige celtypen en de coexpressie van IGFBP-2 en -5 is voornamelijk interessant, omdat IGF-II en IGFBP-2 complementair tot expressie komen tijdens de embryonale ontwikkeling. Dit zou kunnen betekenen dat in sommige celtypen zowel IGF-II als IGFBP-4 tot expressie komen en dat IGF-II en IGFBP-5 in naastliggende celtypen tot expressie worden gebracht.

De mRNA expressie van IGFBP-2, -4 en -5 is in meer detail bestudeerd tijdens de long ontwikkeling, in relatie tot de expressie van IGF-I, IGF-II en de type I IGF receptor (Hoofdstuk 6). Expressie van de IGF, type I IGF receptor en IGFBP-2, -4 en -5 genen was detecteerbaar in de ontwikkelende long vanaf embryonale dag 12½ tot aan het eind van de bestudeerde periode (vanaf ontwikkelingsdag 12½ tot 4 weken postnataal). IGF-I en IGF-II mRNAs werden gevonden in zowel het epitheel als het mesenchyme. De type I IGF receptor transcripten werden ook overal gevonden in de ontwikkelende long, behalve in de epitheliale cellen na ontwikkelingsdag 15. IGFBP-4 en -5 mRNA expressie werd gevonden in naastliggende celtypen, IGFBP-4 mRNA voornamelijk in het mesenchyme en IGFBP-5 mRNA in het epitheel. Na embryonale dag 15 kwamen IGFBP-4 en de type I IGF receptor beiden in mesenchymale cellen tot expressie. Deze expressie patronen impliceren dat de IGFBP-2, -4 en -5 genen verschillend gereguleerd zijn en suggereren dat elk IGFBP een discrete rol kan vervullen tijdens de longontwikkeling van de muis.

Voor ontwikkelingsdag 15 brengen alle cellen van de long mRNAs voor IGF-I, IGF-II en de type I IGF receptor tot expressie. Er vanuit gaande dat deze transcriptie ook

daadwerkelijk leidt tot functionele IGFs en IGF receptoren, is elke cel van de ontwikkelende long gevoelig voor IGF stimulatie. Op dit moment is het niet bekend of een cel specifieke IGF respons nodig is voor de long ontwikkeling, of dat alle cellen gelijk reageren op IGF stimulatie. Echter, wanneer cel specifieke IGF acties nodig zijn, kan de expressie van IGFBP-2, -4 en -5 hier zorg voor dragen. IGFBP-4 en -5 komen in verschillende celtypen tot expressie. Daarnaast zou IGFBP-2 een voorkeur kunnen hebben om aan specifieke long cellen te binden aangezien het beschreven is dat IGFBP-2 is geassocieerd met klein-cellig longkanker cellen en niet met niet-klein-cellig longkanker cellen. Tenslotte, kunnen IGFBP-2, -4 en -5 de IGF actie verschillend beïnvloeden door de IGF stimulatie te remmen (IGFBP-2 en -4) of te versterken (IGFBP-2 en -5). Vandaar dat het mogelijk is dat IGFBP-2, -4 en -5 participeren in de regulatie van cel specifieke IGF response tijdens de long ontwikkeling.

IGFBP-1 bindt aan de $\alpha_5\beta_1$ integrine (fibronectine receptor) en stimuleert de migratie van Chinese hamster ovarium cellen in kweek, beide via de Arg-Gly-Asp (RGD) sequentie. Aangezien ook IGFBP-2 een RGD sequentie bevat, is IGFBP-2 een potentiële kandidaat om cel migratie te stimuleren en zou daardoor de long ontwikkeling kunnen beïnvloeden.

In foetale en postnatale nieren werd de mRNA expressie van de IGFs, de type I IGF receptor en de IGFBPs bestudeerd met *in situ* hybridisatie technieken (Hoofdstuk 7). Beide IGF mRNAs kwamen voornamelijk voor in het ongedifferentieerde metanephros weefsel en in ontwikkelende en volwassen glomeruli. De type I IGF receptor mRNAs werden ook in onvolwassen en volwassen glomeruli gemaakt. Net als voor de IGFs, werden de IGFBP-1 en -2 mRNAs hoog tot expressie gebracht in het ongedifferentieerde metanephros weefsel en in ontwikkelende glomeruli. Echter, slechts weinig mRNA was detecteerbaar in volwassen glomeruli. Daarentegen werd IGFBP-5 mRNA niet gedetecteerd in ontwikkelende glomeruli, maar hoog tot expressie gebracht in volwassen glomeruli. De mRNAs voor IGFBP-3, -4 en -6 kwamen zowel in ontwikkelende als in volwassen glomeruli voor. Deze mRNA patronen suggereren dat de IGFs en IGFBPs kunnen participeren in de vroege ontwikkeling van de glomerulus.

Zoals al eerder vermeld, kunnen de IGFBPs, de IGF actie zowel positief als negatief beïnvloeden, participeren in de regulering van cel specifieke IGF response, en, in het geval van IGFBP-1 en -2 mogelijk de cel migratie stimuleren. De vinding dat IGFBP-5 in de volwassen nier alleen in de glomeruli tot expressie komt zou kunnen duiden op een discrete rol in de functie van de glomerulus. Een mogelijkheid zou kunnen zijn dat IGFBP-5 invloed uitoefent op de IGF gemedieerde verhoging van de glomerulaire filtratie snelheid.

Naast het bestuderen van de mRNA expressie van de IGFBPs, is het van belang om te bepalen waar de resulterende eiwitten gelokaliseerd zijn. Sommige IGFBPs zijn in staat om aan de extracellulaire matrix of celmembraan te binden. Bijvoorbeeld, IGFBP-1 bind aan de $\alpha_5\beta_1$ integrine (fibronectine receptor) en stimuleert de migratie van Chinese hamster ovarium cellen. Daarnaast is aangetoond dat membraan geassocieerd IGFBP-2 competeert met IGF receptoren voor ligand binding in klein-cellig long kanker cellen. Vandaar dat de IGF actie niet alleen bepaald hoeft te worden door de hoeveelheid IGF en IGF receptor, maar ook afhankelijk kan zijn van de mogelijkheid van IGFBPs om aan bepaalde cellen te binden. Het zal dan ook interessant zijn om de IGF/IGFBP actie te bestuderen, niet allen in cellen die IGFBPs maken, maar ook in cellen waaraan een specifiek IGFBP kan binden.

Een andere veel belovende aanpak om de functie van de IGFBPs te bestuderen is door gebruik te maken van gen-disruptie strategieën. Het is mogelijk dat de functie van het gedeleteerde IGFBP gecompenseerd wordt door één (of meer) van de andere IGFBPs. Echter, de expressie van de IGFBP genen is weefselspecifiek en ontwikkelings afhankelijk gereguleerd. Verder zijn er verschillende fysiologische en pathologisch condities beschreven waarin de concentraties van slechts enkele IGFBPs veranderen. Daarnaast zijn de zes IGFBPs verschillend qua cel specifieke binding, gevoeligheid voor proteases, IGF affiniteit, fosforylering, glycosylering en eiwit interacties. Tenslotte zijn er effecten van sommige IGFBPs gesuggereerd die onafhankelijk zijn van IGF. Vandaar dat het niet onwaarschijnlijk is dat disruptie van een IGFBP gen een effect laat zien. Wanneer dit effect niet opvallend is, zou het gevonden kunnen worden onder specifieke fysiologische of pathologische omstandigheden.

APPENDIX

The *in situ* hybridization technique, as applied in Chapter 5, 6 and 7, is a relative sensitive method to detect mRNAs. A disadvantage of using ^{35}S-labeled RNA probe is that the signal is detected in a photographic emulsion lying on top of the sections. Consequently, in some cases the resolution is not sufficient to identify, without doubt, the cell expressing a certain mRNA. This problem may be overcome by using non-radioactively labeled RNA probes, for these probes can visualized directly, or by immunocytochemistry, using a color reaction within the cell expressing the mRNA. One should keep in mind that it is generally thought that the sensitivity of the *in situ* hybridization is lower when non-radioactively labeled RNA probes are used, although some investigators have shown similar results and sensitivities using both non-radioactively and ^{35}S-labeled probes. In mouse fetal lung sections, we were able to detect IGFBP-4 mRNAs using digoxigenin-labeled RNA probes (Boehringer, Mannheim). The IGFBP-4 mRNA expression was located in mesenchymal cells and the expression pattern was identical to that described in Chapter 5 and 6. This indicates that non-radioactively labeled RNA probes can be used successfully to detect at least some of the IGFBP mRNAs in certain tissues.

Non-radioactively labeled RNA probes also allows one to apply whole mount *in situ* hybridization techniques. This technique is most suitable for small tissues, like early gestational mouse embryos and, to our opinion, will complement the "traditional" *in situ* hybridization techniques. We performed the whole mount *in situ* hybridization as described by Joosen et al. (ref. in the legend of Fig. 1) using digoxigenin-labeled IGFBP-5 RNA probes. As shown on the cover page and in figure 1 and 2, was IGFBP-5 mRNA easily detected in the eye, ear, branchial arches (surface ectoderm), in or near somites and in the apical ectodermal ridge of the limb buds of a 11-12 day-old mouse embryo. In Chapter 5, we have shown that IGFBP-5 mRNA is expressed in the fetal eye and in the ectodermal layer of the embryonal tongue. Furthermore, Green et al. (ref. 66 in Chapter 1) have shown that IGFBP-5 mRNA is expressed in the apical ectodermal ridge of the limb bud and in myotome cells derived from the somite, using ^{35}S labeled RNA probes. Therefore, it may be concluded that the whole mount *in situ* hybridization can be used successfully to detect IGFBP-5 mRNAs.

Since in our experience, IGFBP-2 and -4 are as abundantly expressed in early- and midgestational mouse embryos, it is only logical to assume that IGFBP-2 and -4 mRNAs can be detected by whole mount *in situ* hybridization techniques as well.

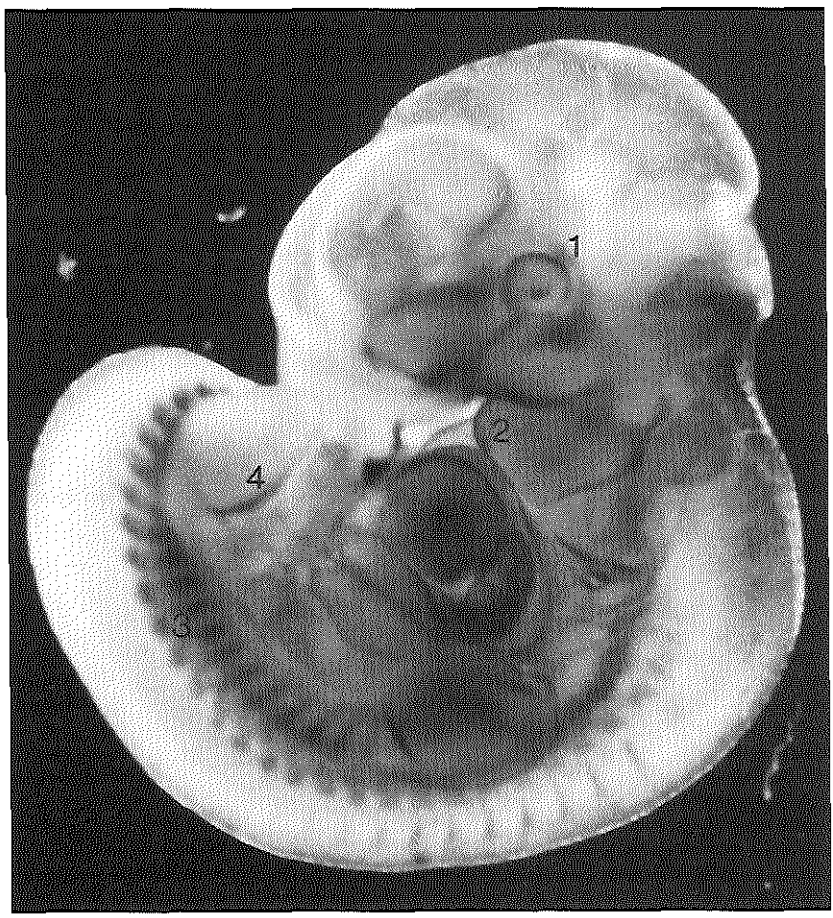

Fig. 1. IGFBP-5 mRNA expression in a 11 day-old mouse embryo as determined by whole mount in situ hybridization techniques. The whole mount in situ hybridization was performed as described by: Joore et al. (J. Joore, G.B.L.J. van der Lans, P. Lanser, J.M.A. Vervaart, D. Zivkovic, J.E. Speksnijder en W. Kruijder. Effects of retinoic acid on the expression of retinoic acid receptor during zebrafish embryogenesis. 1994, Mech. of Development, in press.) 1, eye; 2, branchial arch; 3, somite;, 4, apical ectodermal ridge.

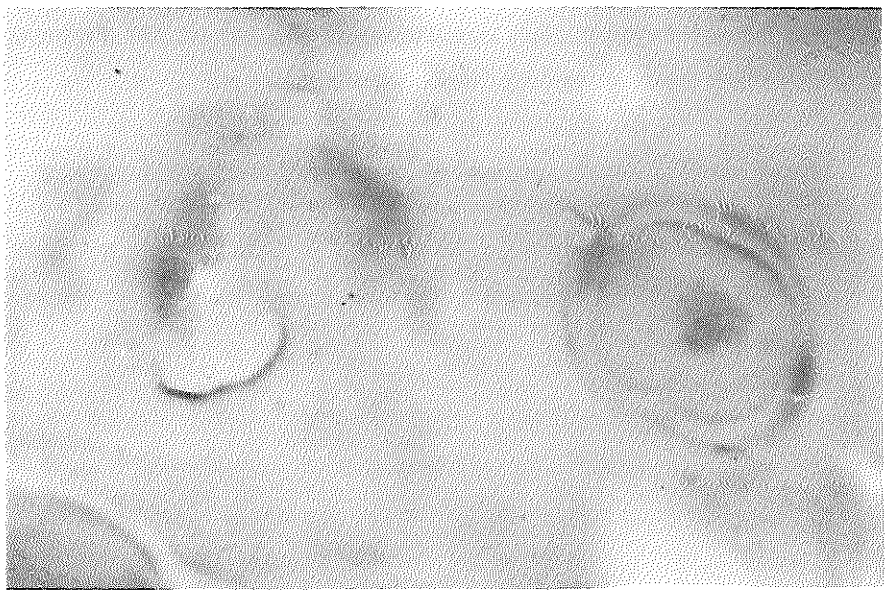

Fig. 2. IGFBP-5 mRNA expression in the eye and ear of a 12½ day-old embryo as determined by whole mount in situ hybridization techniques.

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- Brinkman, A., Kortleve, D.J., Schuller, A.G.P., Zwarthoff, E.C. and Drop, S.L.S. Site-directed mutagenesis of the N-terminal region of IGF binding protein 1; analysis of IGF binding capacity. FEBS letters, 1991, 291, 264-268.

- Drop, S.L.S., Brinkman, A., Schuller, A.G.P., Kortleve, D.J., Groffen, C. and Zwarthoff, E.C. The evolution of the IGFBP family. In: Modern concepts of IGFs. E.M. Spencer Ed. Elsevier Amsterdam - New-York, 1991, p311-328.

- Drop, S.L.S., Schuller, A.G.P., Lindenbergh-Kortleve, D.J., Groffen, C., Brinkman, A. and Zwarthoff, E.C., Structural aspects of the IGFBP family. Growth Regulation, 1992, 2, 69-79.

- Schuller, A.G.P., Lindenbergh-Kortleve, D.J., de Boer, W.I., Zwarthoff, E.C. and Drop, S.L.S. Localization of the epitope of a monoclonal antibody against human insulin-like growth factor binding protein-1, functionally interfering with insulin-like growth factor binding. Growth Regulation, 1993, 1, 32-43.

- Schuller, A.G.P., Zwarthoff, E.C. and Drop, S.L.S. Gene expression of the six insulin-like growth factor binding proteins (IGFBPs) in the mouse conceptus during mid- and late gestation. Endocrinology, 1993, 132, 2544-2550.

- Schuller, A.G.P., Lindenbergh-Kortleve, D.J., Pache, T.D., Zwarthoff, E.C., Fauser, B.C.J.M. and Drop, S.L.S. Insulin-like growth factor binding protein-2, 28 kD and 24 kD insulin-like growth factor binding protein levels are decreased in fluid of dominant follicles, obtained from normal and polycystic ovaries. Regulatory Peptides, 1993, 48, 157-163.

- Hoekman, K., van Doorn, J., Gloudemans, T., Maassen, T.A., Schuller, A.G.P. and Pinedo, H.M. A patient with hypoglycemia due to a sarcoma producing IGF-II and IGFBP-6. 1993, submitted.

- Schuller, A.G.P., van Neck, J.W., Lindenbergh-Kortleve, D.J., Groffen, C., de Jong, I., Zwarthoff, E.C. and Drop S.L.S. Gene expression of the IGF binding proteins during post-implantation embryogenesis of the mouse; comparison with the expression of IGF-I and -II and their receptors in rodent and human. In: Advances in experimental medicine and biology, vol. 343, Current directions in insulin-like growth factor research, D. LeRoith and M. K. Raizada Eds. 1993, pp. 267-277.

- Schuller, A.G.P., Groffen, C., van Neck, J.W., Zwarthoff, E.C. and Drop, S.L.S. cDNA cloning and mRNA expression of the six mouse insulin-like growth factor binding proteins. 1994, submitted.

- Schuller, A.G.P., Flyvbjerg, A., Groffen, C., Dørup, I., van Neck, JW, Zwarthoff, E.C. and Drop, S.L.S. Insulin-like growth factor binding protein mRNA expression in potassium depleted rats infused with IGF-I. 1994, submitted.

- Schuller, A.G.P., Beukenholdt, R.W., van Neck, J.W., Zwarthoff, E.C. and Drop, S.L.S. Insulin-like growth factor (IGF), type I IGF receptor and IGF binding protein mRNA expression in the developing mouse lung. 1994, submitted.

- de Boer, W.I., Schuller, A.G.P., Vermey, M., Heldin, C.H. and van der Kwast, T.H. Expression of growth factors and receptors during specific phases in regenerating urothelium in vivo: correlation with their function in vitro. 1994, submitted.

- Schuller, A.G.P., Lindenbergh-Kortleve, D.J., van Neck, J.W., Zwarthoff, E.C. and Drop, S.L.S. Insulin-like growth factor (IGF), type I IGF receptor and IGF binding protein mRNA expression in pre- and postnatal mouse kidneys. 1994, submitted.

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