THE BRAIN DEVELOPMENT RETARDATION

IN INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-1

TRANSGENIC MICE

BY

WEIMIN NI

A Thesis
submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Physiology
Faculty of Medicine
University of Manitoba
Winnipeg, Manitoba

(c) January 1997

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-23442-8

Canada

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES
*****
COPYRIGHT PERMISSION PAGE

THE BRAIN DEVELOPMENT RETARDATION IN INSULIN-LIKE

GROWTH FACTOR BINDING PROTEIN-1 TRANSGENIC MICE

BY

WEIMIN NI

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University

of Manitoba in partial fulfillment of the requirements of the degree

of

MASTER OF SCIENCE

Weimin Ni   1997 (c)

Dedicated to my grandmother with love

TABLE OF CONTENTS

Page

ACKNOWLEDGMENTS

I wish to acknowledge all those who have given me their support, encouragement and assistance in helping me to complete this thesis.

First, I would like to thank my advisor Dr. Liam Murphy for his constant guidance, encouragement and constructive criticism throughout this study. This thesis would not have been possible without his support. My sincere appreciation is extended to Dr. Jim Nagy for his precious time and endless patience and most of all for teaching me the logical and analytical approach to problem solving and the scientific method.

I further wish to thank my committee member Dr. Marc Del Bigio for his helpful advice throughout the course of this research.

My thanks must also be given to the former and present members of Dr. Nagy's and Dr. Murphy's laboratories for making my graduate studies a pleasant and memorable experience.

Finally, I would like to express my heartfelt appreciation to my family. I am forever grateful to my parents and my husband, Yi Dong, whose unconditional love, emotional support and understanding has made this thesis possible.

II. ABSTRACT

In order to determine the effects of IGF-I in brain development *in vivo*, we studied the brain development of transgenic mice that overexpress rat IGFBP-1 under the control of PGK promoter. The gross morphology of the brain in the transgenic mice was not different from those wild type control mice. Brains from the IGFBP-1 transgenic mice had reduction in weight owning to decreased total brain cell numbers. Retardation of brain growth was observed at the age as early as P0 and thereafter. The markedly reduced corpus callosum thickness in transgenic mice indicates decreased axons due to the expression of transgene. The area of hippocampus and dentate gyrus were reduced in excess of the decrease in brain weight. The distribution of cholinergic neurons, astroglia and microglia were not affected by transgene expression. The cell proliferation study showed reduced proliferating brain cells in the ventricular zone (VZ), the subventricular zone (SVZ) and the developing hippocampus, and increased apoptotic cells in neonatal transgenic mice. To further examine cell proliferation in transgenic mice, we introduced a stab wound to induce a brain lesion in adult transgenic mice and wild type control. The number of reactive astroglia was significantly reduced in transgenic mice, the immunostaining intensity for glial fibrillary acidic protein (GFAP) was also decreased in transgenic mice. These data demonstrate that the overexpression of IGFBP-1 via its inhibitory effect on IGF-I action selectively inhibit brain development. Our findings also suggest an *in vivo* promoting role of IGF-I on brain response to injury.

III. INTRODUCTION

III.1. Insulin -like growth factor-I

Insulin-like growth factor (IGF)-I and II are mitogenic peptides structurally related to insulin (Humbel, 1984; Sara et al., 1990). IGFs were discovered on the basis of their ability to stimulate sulfate uptake in cartilage (Salmon et al., 1957). Growth hormone (GH) does not directly stimulate the synthesis of matrix proteins of hypophysectomized (hypox) rats, the actions are mediated by so called somatomedins that are stimulated by GH. This has led to the "somatomedin hypothesis" (Daughaday et al., 1972). Analysis of amino acid sequence of these GH-dependent growth factors in serum revealed homology to proinsulin. The name insulin-like growth factor (IGF)-I and II was applied to avoid confusion in nomenclature (Daughaday et al., 1987).

IGFs circulate in blood and are present in a variety of tissues including muscle lung, liver, kidney, and brain (Tollefsen et al., 1989; Lund et al., 1986). Since the IGFs are synthesized and secreted by multiple different tissues and since the IGF receptors are similarly widespread, IGFs may exert autocrine or paracrine actions beside their endocrine modality action (Sara et al., 1990). IGF-II is expressed highest in the fetal period and expression is low after birth. IGF-I is expressed at relatively low levels during fetal growth, and rises during postnatal life. IGF-I has insulin-like actions and growth-promoting action *in vivo*. It mediates many of the somatic growth-promoting actions of GH postnatally (Jones and Clemmons; 1995). IGF-I also exerts growth-promoting actions

in brain which is independent of GH (Behringer et al., 1990). Besides the postnatal growth-promoting role of IGF-I, it is also important in embryogenesis and in growth and differentiation (Liu et al., 1993). IGF-I acts as both a mitogen and a differentiation factor *in vitro* in many cell types. Molecular characteristic, regulation and biologic actions of IGF-I are reviewed below.

III.1.1 Molecular characteristic of IGF-I

IGFs are well conserved during evolution. All mammalian IGF-I consists of 70 amino acids. IGF-I is a single-chain polypeptide with three intra-chain disulfide bridges. The molecular weight of IGF-I is 7649 dalton. IGF-II is a 7471 dalton single chain polypeptide with 62% homology in the amino acid sequences. Both IGF-I and IGF-II are related closely to proinsulin in structure. IGF-I is 43% and IGF-II is 41% homologous with proinsulin. The computer-aided modeling analysis of the IGFs indicate that the IGFs and proinsulin have related three-dimensional structures. Like proinsulin, the IGFs have an A domain, an amino-terminal B domain and a connecting peptide which is quite different in sequence compare with proinsulin. In contrast to proinsulin, the C domain does not undergo cleavage. Unlike proinsulin, the IGFs also contain an D-domain which is connected to C-terminal (Sara and Hall, 1990).

A single gene codes for IGF-I. The human IGF-I gene localized on the long arm of chromosome 12 and it has five exons (Brissenden et al., 1984). The human IGF-I gene is transcribed into two different mRNAs (IGF-Ia and IGF-Ib). Exon 4 is used for transcription of the C-terminal part in IGF-Ib, whereas exon 5 is used in IGF-Ia. The rat

2

IGF-I has at least 6 exons and the gene transcript is more complicated. In human, the use of exons 4 and 5 is mutually exclusive, while in the rat and mouse, both exons 4 and 5 are in the same mRNA. The differential splicing of the IGF-I mRNA results in either IGF-Ia or IGF-Ib which contains a "mini-exon" of 52 bp. Therefore, the C-terminal sequences of rat or mouse IGF-Ib are totally different from human's.

III.1.2 Regulation of gene expression

Development regulation

Previous studies have demonstrated that IGF-I is synthesized in multiple tissues and organs by localizing or measuring mRNAs and by assaying IGFs. The liver is the main organ producing IGF-I in humans and rodents, and accounts for the major part of serum IGF-I content. Most tissues express IGF-I at some stage of prenatal or postnatal development (Han et al., 1988). IGF-I mRNA is present at relatively low levels in the prenatal and neonatal period both in rodents and humans (Beck et al., 1988; Daughaday et al., 1989). For example, rat IGF-I mRNA was detectable in rat intestine, liver, lung and brain in fetal period (Lund et al., 1986), but both liver IGF-I mRNA and serum IGF-I level are lower in fetal than those in adulthood. After birth , liver gene expression of IGF-I rises and is the main source of IGF-I in the circulation and result in the increase in serum IGF-I. In human, serum IGF-I increases progressively until just before puberty (Sibergeld et al., 1986). During puberty, serum IGF-I rises to peak levels in boys and girls due to the increased secretion of growth hormone or steroids (Hall & Sara, 1983; Luna et al.,

3

1983; Harris et al., 1985). After puberty, the serum IGF-I levels slowly declines due to the decreased GH secretion, and remains relatively constant in adulthood (Humbel, 1990).

Growth hormone regulation

The predominant regulator of IGF-I production is GH (Mathews et al., 1986). GH increase IGF-I production in a variety of tissues and cell culture. GH treatment increases IGF-I mRNA level in various tissues (Mathews et al., 1986; Roberts et al., 1987). The increase in IGF-I mRNAs was least marked in the brain and most marked in skeletal and cardiac muscles. This indicates GH regulation is tissue- specific (Murphy et al., 1987a). The concentrations of IGF-I generally reflect the concentrations of GH. Serum concentrations of IGF-I are about 4 times higher in acromegaly and about 20 times lower in hypopituitary children than in normal individuals (Rieu et al., 1984). IGF-I is produced by multiple tissues, the most important source of serum IGF-I appears to be liver. GH has been shown to regulate liver IGF-I mRNA. The liver IGF-I mRNA levels was remarkable low in hypophysectomized rats and administration of GH to hypophysectomized rat restores liver IGF-I mRNA levels.

Estrogen

Estrogen has been found to stimulate IGF-I expression in the rat uterus (Murphy et al., 1987b). E2 administration increased uterine IGF-I mRNA by 14-20 fold in ovariectomized or hypophysectomized, ovariectomized rats (OVX-hypophysectomized), whereas GH elevated uterine IGF-I mRNA only by 4 fold in OVX-hypophysectomized rat. E2 also increase the steady state levels of IGF-I mRNA 2-2.5 fold in osteoblast which plays important role in bone formation (Ernst et al., 1989).

Insulin

Insulin enhances IGF-I transcript in the rat liver (Johnson et al, 1989). Serum IGF-I levels are low in diabetic rats in association with growth retardation (Maes et al., 1986). Similarly, IGF-I mRNA decrease in various tissues of streptozotocin (STZ) induced diabetic rats (Goldstein et al., 1987). The growth retardation observed in STZ-diabetic rats may be associated with the reduced tissue availability of IGF-I. Insulin administration restored IGF-I mRNA levels in STZ-induced diabetic rats. It has been reported that insulin directly stimulates IGF-I mRNA in cultured hepatocytes. In contrast to the decreased IGF-I levels in liver, IGF-I levels increased by 69% in the pituitary in STZ-induced diabetic rats, but there is no change in IGF-I mRNA. Insulin treatment completely reversed the enhanced pituitary IGF-I level (Olchovsky et al., 1991).

Other hormones also influence IGF-I level. Glucocorticoid has been shown to decrease IGF-I mRNA levels in bone cell cultures (McCarthy et al., 1990). Parathyroid hormone stimulates IGF-I production in cultured osteoclast cells (Canalis et al., 1989). Nutritional status also appears to play a role in IGF-I gene expression. Upon fasting, IGF-I mRNA levels decrease and return to normal after hours of refeeding in rat (Lowe et al., 1989).

III.1.3 IGF-I receptor

The biological actions of IGFs are mediated by specific cell membrane receptors. The IGF-I receptor (type 1 IGF receptor) has a high degree of homology with the insulin receptor, and each of which can cross react at lower affinity with the heterologous ligand.

IGF-I receptors are present in a variety of cell types and tissues and control the extent of cell proliferation by different ways: it is mitogenic, it causes transformation and it protects cells from apoptosis (Li et al., 1996). IGF-I receptors have IGF-mediated signaling functions and mediate most of the effects of IGF-I and IGF-II, whereas IGF-II receptors (type 2 IGF receptors) are identical to mannose-6-phosphate and structurally distinct from the IGF-I receptor. The IGF-II receptor is supposed to have a role in the targeting of lysosomal enzymes, it has no known IGF signaling functions (Morgan et al., 1987).

Genomic mapping revealed that the genes for the human insulin receptor, the IGF-I receptor and the IGF-II receptor are mapped to short arm of chromosome 19, long arm of chromosome 15 and long arm of chromosome 6 respectively. The IGF-I receptor is $\alpha_2\beta_2$-heterotetramers. It is synthesized in the ribosome as a precursor of 1367 amino acid residues, a 30-residue signal is removed during translocation into the endoplasmic reticulum (ER) and is cleaved into the glycosylated subunits, two extracellular α subunits (130 kD) and two transmembrane β subunits (90 kD) linked by disulfide bonds. The α subunit contains 706 amino acid and β subunit contains 626 amino acid. The α subunit confers ligand-binding specificity. The cysteine rich domain in α subunit is required for recognition of ligand binding by the IGF-I receptor. The transmembrane portion of the β-subunit contains a tyrosine kinase catalytic domain and tyrosine residues which are targets for the tyrosine kinase (Czech, 1989). IGF-I receptors demonstrate high affinity for IGF-I with approximately 100-1000-fold lower affinity for insulin and 2-15 fold lower affinity for IGF-II (Steele et al., 1988; Germain et al., 1992). Similarly, insulin receptors have high affinity for insulin and 100-fold lower affinity for IGF-I.

III.1.4 Actions of IGF-I

In vitro

Insulin-like metabolic effect

The insulin-like metabolic effect of IGF-I has been demonstrated in many tissues. In adipose tissue, the insulin-like effects of IGF-I include stimulation of glucose uptake, glycogen synthesis, lipogenesis via the insulin receptor in adipose tissue. Insulin, IGF-I, IGF-II can substitute for each other in stimulating all the effects, but insulin is more potent in stimulating the metabolic effect compared with IGFs (Van Wyk 1984).

Mitogenic effect

IGFs are more potent in stimulating mitogenic effect compared with insulin. The most widely studied effect of IGFs *in vitro* is the stimulation of DNA synthesis and cell replication in a variety of cell lines, primary cultures and tissue implants (Sara and Hall, 1990). Previous studies have shown that IGF-I can promote the proliferation of chondrocytes, osteoblasts, keratinocytes, thyroid follicular cells, smooth muscle cells, skeletal muscle cells, neuronal cells, mammary epithelial cells, mesangial cells, erythroid progenitor cells, astroglia, thymic epithelium, granulosa cells, oocytes, spermatogonia and several cancer cell lines (Johns and Clemmons, 1995). In the studies of BALB/C3T3 cells, quiescent cells arrested in G_0 phase were induced to G_1 phase by "progression factors" including IGF-I and epidermal growth factor (EGF), and competence factors such as platelet-derived growth factor (PDGF). PDGF acts to stimulate transition from the G_1 to the S phase of the cell cycle (Pledger et al., 1978). An optimal concentration of IGF-I and

7

the competence factors mediate cell progression through G_1, entry into the S Phase and results in DNA synthesis and cell proliferation. Cell proliferation was also stimulated by IGF-I in the absence of other competence factors. It has been demonstrated that IGF-I can stimulate chick chondrocytes in primary cultures under serum-free conditions. IGF-I receptor may play a critical role in regulating the progression of cells through the cell cycle. The effects of IGF-I on cell cycle progression have been shown to involve the tyrosine phosphorylation of IRS-1 and in turn to activate Ras (Lu and Campisi, 1992). In cells overexpressing the IGF-I receptor, basic fibroblast grow factor (bFCF) can stimulate DNA synthesis either by an unknown mechanism or through transphosphorylation of the IGF-I receptor.

IGF-I has been also demonstrated to inhibit apoptosis in certain cell types *in vitro*. Apoptosis (programmed cell death) is a selective process of physiological cell deletion. it plays a critical role in the development of normal tissue and in regressing tumors (Cotter et al., 1990). The biochemical feature of apoptosis is the specific fragmentation of nuclear DNA into oligonucliosomal DNA ladders by endonucleases (McConkey et al., 1988). The signaling process in apoptosis is not clear at present time. To study IGF-I effect on the survival and proliferation, IL-3-dependent hemopoietic cell lines and in IL-3-responsive primary cultures of bone marrow derived mast cells were employed. It has been shown that in IL-3-depleted cultures, IGF-I prevented DNA breakdown or apoptotic cell death (Rodriguez et al., 1992). Similarly, IGF-I can suppress DNA fragmentation in human erythroid progenitor cells due to serum deprivation (Muta & Krantz, 1993). It has also been reported that IGF-I is a survival factor for oligodendrocytes and oligodendrocyte

precursors (Barres et al., 1992). The mechanism of the inhibitory effect of IGF-I on apoptosis remains unclear. It is supposed to be mediated by IGF-I receptor.

Effect on cell differentiation

Previous ¯studies have revealed that IGF-I has distinct effects on cell differentiation. IGF-I has been demonstrated to promote the differentiation of myoblast, osteoclasts, chondrocytes, oligodendrocyte, neural cells, adipocytes, and osteoblasts (Florini et al., 1989; Mochizuki et al., 1992; McMorris et al., 1986; Geduspan et al., 1993; Pahlman et al., 1990). For example, IGF-I induces myoblast terminal differentiation by stimulating expression of the myogenin gene (Florini et al., 1991). This stimulation action of IGF-I is inhibited by an antisense oligodeoxyribonucleotide to myogenin mRNA (Florini et al., 1990). Oligodendrocytes are responsible for production and maintenance of the myelin in the central nervous system. IGF-I was found to be a potent inducer of oligodendrocyte differentiation. In serum-free medium, 100 ng/ml of IGF-I increased the oligodendrocyte numbers 60-fold. IGF-I increases the survival and myelin synthesis of cultured oligodendrocytes by inducing maturation of the oligodendrocyte-type-2 astrocyte progenitor cells which can differentiate into either oligodendrocytes or astrocytes (McMorris and Dubois-Dalcq, 1988; McMorris et al., 1986). The effects of IGF-I on oligodendrocytes development are mediated by type 1 IGF receptors, which are present on oligodendrocytes and their precursors (Mozell and McMorris, 1991).

In vivo

Metabolic effects

Previous studies have been done to investigate the effects of IGF-I by administration of the peptide at high concentrations that exceed the binding capacity of the IGF binding protein *in vivo*. Hypoglycemia was observed after 15-30 min IGF-I infusion in normal rats. The fall of blood glucose was the result of stimulated peripheral glucose uptake by IGF-I (Zapf et al., 1986). The effects of IGF-I infusion in normal healthy human volunteers was similar to the effects seen in normal rats, a single bolus injection of 100 µg/kg caused a pronounced fall of blood glucose and was equipotent to 0.15 IU/kg of insulin (Guler et al., 1987). IGF-I infusion enhances glucose uptake and reduce free fatty acid (FFA), triglyceride and protein breakdown (Turkalj et al., 1992; Mauras et al, 1992). However, IGF-I is much less potent than insulin at decreasing FFA levels. IGF-I infusion in insulin-deficient diabetic rats stimulates peripheral glucose uptake and glycogen synthesis (Rosetti et al., 1991). IGF-I administration to diabetic patients with extreme insulin resistance caused a reduction in glucose levels and decreased insulin levels. The insulin resistance in these patients is due to the dysfunction of insulin receptors. IGF-I acts effectively as an insulin surrogate, probably through the IGF-I receptor actions (Schoenle et al., 1991; Kuzuya et al, 1993). In fact, IGF-I can be more effective than insulin in controlling blood glucose in patient with insulin receptor defects.

Growth promoting effects

The growth effect in terms of gain in body weight was first demonstrated by Schoenle et al (1982) in hypophysectomized rats after infusion of IGF-I. The administration of IGF-I in Snell dwarf mice and in normal rats showed similar growth stimulating effects (Hizuka et al., 1986; Van Wyk et al., 1986). To directly investigate the

actions of IGF-I *in vivo*, transgenic (Tg) animal have been developed. In a transgenic mouse line that carries a human IGF-Ia cDNA sequence under the transcription control of a mouse metallothionin promoter, the postnatal growth increased to about 30% over the normal body weight (Mathews et al., 1988a). Growth promoting effect has also been demonstrated in GH transgenic mice. Although the growth-promoting effects in mice of GH transgene were partly due to its induction of IGF-I over expression (Mathews et al., 1988b), differences of the growth-promoting patterns were observed in mice with the GH and IGF-I transgene overexpression. In GH transgenic mice, GH induced a significant increase of overall length and liver weight, whereas in IGF-I transgene mice, the weight gain was more obvious in brain which showed 50% increase. Also, the circulating IGF-I levels were lower in IGF-I transgenic mice over GH transgenic mice and this may account for the lower growth rate in IGF-I transgenic mice. The generation of mice which IGF-I overexpressed and were GH deficient enabled the distinguish of IGF-I effects in IGF-I transgenic mice from the effects of endogenous GH and IGF-I expression (Behringer et al., 1990). The disproportional gain of brain weight but loss of liver weight suggest that local expression of IGF-I stimulate specific tissues like brain via autocrine or paracrine mechanisms. Previous studies on IGF-I gene expression during rodent embryogenesis have indicated that IGF-I is important in prenatal development (Bondy et al., 1990). Similarly, the postnatal reduction of expression of IGF-I receptor mRNA in various rat embryonic tissue indicate that IGF-I receptor is involved in prenatal development (Werner et al., 1989b). To further determine the roles of IGF-I and IGF-I receptor during fetal development, IGF-I and IGF-I receptor null mutations have been generated by

11

homologous recombination in the genes encoding IGF-I and the IGF-I receptor (Liu et al., 1993; Baker et al., 1993). The embryonic growth was unaffected in any mutant strain until embryonic day 11 (E11). Mutation of IGF-I had no effect until E13.5. After E13.5, the body weight gradually decreased and was 60% of normal at birth. Postnatally, the body weight of IGF-I null mutant mice was reduced from 60% at birth to 30% of normal after 8 weeks (Baker et al., 1993). This study demonstrates that IGF-I is essential for prenatal growth. Similar effect of IGF-I in mice embryonic development was also demonstrated in IGF-I knock out mice (Powell et al., 1993). Some of the IGF-I (-/-) dwarfs die shortly after birth, while others survive and reach adulthood. In contrast, IGF-I receptor (-/-) knock out mice die invariably at birth of respiratory failure and exhibit a more severe growth deficiency compared with IGF-I(-/-) mice. Furthermore, mice with mutations on both IGF-I and IGF-I receptor showed identical phenotype to those lacking receptor only. These studies indicate that IGF-I signaling is exclusively through the IGF-I receptor (Liu et al., 1993).

III.2. Insulin-like growth factor-binding protein-1

The insulin-like growth factors, IGF-I and II are present in extracellular fluids bound to specific high affinity binding proteins. Insulin-like growth factor binding proteins (IGFBPs) are a group of discrete but structurally related polypeptides. Six IGFBPs have been cloned and sequenced. IGFBP-1 is the major IGFBP and has a low molecular weight. It has a twofold higher affinity for IGF-I than for IGF-II (Baxter et al., 1987). Studies using mutant IGF molecules indicate that the IGFBP-1 bind to the IGFs at the N-terminal

end of IGF molecules (Bayne et al., 1988). IGFBP-1 was first purified from human amniotic fluid (Povoa et al., 1984) and its complete structure was determined by complementary DNA (cDNA) sequence analysis (Brewer et al., 1988). Two isoforms of IGFBP-1 were isolated from amniotic fluid. One form can bind to the $\alpha_5\beta_1$ integrin receptor on cell surface and form disulfide-linked multimers when added to the media of culturing cells (Busby et al., 1989). IGFBP-1 was also purified from conditioned medium of human HepG2 cells (Povoa et al., 1985). Essentially identical N-terminal sequence were reported for the IGFBP isolated from human amniotic fluid and HepG2 human hepatoma cell-conditioned medium (Povoa et al., 1985). Koistinen et al., (1986) reported the IGFBP-1 derived from human decidua and purified from human placenta had identical N-terminal sequence to that of amniotic fluid and HepG2 cell derived IGFBP-1. Later, another protein reported and subsequently identified as IGFBP-1 is pregnancy-associated endometrial α_1-globulin which is secreted by decidualized endometrium during pregnancy (Waites et al., 1989). Identical cDNA sequence for IGFBP-1 derived from HepG2, human placenta and decidual were reported (Lee et al., 1988; Brinkman et al., 1988; Julkunen et al., 1988; Luthman et al., 1989; Brewer et al., 1988).

III.2.1 Molecular characterization of IGFBP-1

Complementary DNA clones have been isolated from human decidua, placenta, liver and HepG2 cells for IGFBP-1 (Rechler and Brown, 1992). A protein polymorphism has been identified at residue Ile/Met 228 in IGFBP-1 purified from amniotic fluid (Luthman et al., 1989). IGFBP-1 contains an N-terminal cluster of 12 cysteines and a C-

terminal cluster of six cysteines. This 18 cysteines are conserved in rat and bovine IGFBP-1. All 18 cysteines appear to be involved in intrachain disulfide linkages, but the function is not yet clearly delineated (Brinkman et al., 1991). An Arg-Gly-Asp (RGD) sequence is located near COOH terminal of human IGFBP-1 at residues 221-223 (Kiefer et al., 1991). RGD sequences are present in extracellular matrix proteins and might mediate adhering of this integrins to cell surfaces (Hynes 1987; Ruoslahti et al., 1987). There are no classic N linked glycosylation sites in human IGFBP-1. However, there are several regions rich in serine and threonine which might be glycosylated (Julkunen et al., 1988). The function of the IGFBP-1 glycosylation has not been defined. A ProGluSerThr (PEST) sequence is found in proteins which have rapid turn over. The presence of PEST regions in the IGFBP-1 protein and the ATTTA motifs in 3' untranslated region in IGFBP-1 mRNA may account for the rapid clearance rate of IGFBP-1 levels *in vivo* (Shaw et al., 1986). Human IGFBP-1 normally circulates in serum as a single, highly phosphorylated protein with phosphorylation on serine residues (Westwood et al., 1994). Serine phosphorylation results in a 6-fold increase in the affinity of IGFBP-1 for IGF-I and is important for maintenance of high affinity binding for this growth factor (Johns et al., 1991; Jones et al., 1993). Phosphorylation may be an important post-translational modification that regulates the capacity of IGFBP-1 to modulate IGF bioactivity. The stimulatory form of IGFBP-1 is mostly nonphosphorylated.

Rat IGFBP-1 cDNA clones were isolated by this laboratory from libraries established from rat decidua and rat regenerating liver (Murphy et al., 1990). The mature rat IGFBP-1 consists of a 247 residues and a signal peptide of 25 amino acids. Rat

IGFBP-1 is highly homologous to human IGFBP-1 precursor. It contains the same 18 cysteines, the RGD sequence, but there are fewer potential O-glycosylation sites compared with human IGFBP-1 (Murphy et al., 1990; Brinkman et al., 1991a).

The rat IGFBP-1 mRNA has been reported as a single 1.6kb transcript with four ATTTA motifs in the 3'- untranslated region (Murphy et al., 1990). The order of abundance of IGFBP-1 mRNA was deciduoma tissue $\geq$ liver >> kidney >> uterus > brain. The human IGFBP-1 gene is a single-copy gene. It is 5.7kb in length and contains four exons (Brinkman et al; 1988). The tentative genomic localization of IGFBP-1 has been reported by using a cDNA clone and *in situ* hybridization. It is located to the region 7p14-p12 site of chromosome 7 (Brinkman et al., 1988).

III.2.2 Regulation of IGFBP-1

Development regulation

Serum IGFBP-1 levels in human are elevated in fetal life and decline postnatally (Drop et al., 1984a; Drop et al., 1984b.; Pancal-Roessler et al., 1989). Fetal serum IGFBP-1 levels appear to rise in the time of midgestation and is about 10-20-fold higher than maternal serum levels (Fant et al., 1988). A elevated IGFBP-1 level in amniotic fluid in midgestation was also reported (Baxter et al., 1987). It is supposed that IGFBP-1 may important in the regulation of the biological effects of the IGF during fetal life when growth hormone has very limited function (Browne et al., 1989). Fetal and amniotic IGFBP-1 levels fall in late gestation. After birth serum IGFBP-1 levels decline progressively until puberty and remain at low levels constantly in adulthood (Giudice et

al., 1992). These data were consistent with the hepatic levels of IGFBP-1 mRNA. For example, in the Rhesus monkey, both liver IGFBP-1 mRNA and serum IGFBP-1 were higher in the fetus and first year of life than after puberty (Liu et al., 1991). Similar results for hepatic IGFBP-1 mRNA have been demonstrated in the human (Brinkman et al., 1988a) and the rat (Murphy et al., 1990; Ooi et al., 1990). These observations suggest that serum IGFBP-1 levels are regulated at the level of hepatic mRNA abundance.

Metabolic and hormonal regulation

It has been reported that IGFBP-1 mRNA increased 10-fold in rat liver after food deprivation (Murphy et al., 1990), and this increase was reversed after refeeding. Administration of insulin to fasted rats caused hypoglycemia and further decrease in GH, but it was ineffective in reducing IGFBP-1 mRNA (Murphy et al., 1991). Similar result has also been reported by Lewitt et al., (1992) who observed an increase in serum IGFBP-1 levels in the rats with insulin-induced hypoglycemia. These studies suggests that insulin might not directly regulate IGFBP-1 expression in fasted rats. GH administration to fasted rats reduces the elevated IGFBP-1 mRNA within 1 hour. GH deficiency results in the increased IGFBP-1 mRNA in fasted rat liver (Murphy et al., 1991).

Insulin has inhibitory effects on IGFBP-1 expression level *in vitro* and *in vivo*. *In vitro* studies have shown that insulin treatment inhibit IGFBP-1 levels in rat H35 hepatoma cells, rat primary hepatocytes, in human HepG$_2$ cells and human fetal liver explants. The inhibitory effect of insulin in H35, HIIE and HepG$_2$ cells is at the transcription level (Powell et al., 1991; Villafuerte et al., 1992; Orlowski et al., 1991 and Lewitt et al., 1989). Insulin also inhibits IGFBP-1 *in vivo*. Elevated IGFBP-1 levels and hepatic mRNA have

been demonstrated in STZ induced diabetic rats (Ooi et al., 1990; Unterman et al., 1990). The rise of IGFBP-1 mRNA levels in the diabetic rats were due to a remarkable increase in gene transcription. Insulin infusion to these rats decreased IGFBP-1 levels and hepatic and kidney IGFBP-1 gene transcription (Ooi et al., 1992; Luo and Murphy, 1991). It has been demonstrated that insulin suppresses rat IGFBP-1/ CAT-reporter constructs in primary hepatocyte cultures and a 41 bp region from -118 to -78 has been identified as the mediator of insulin suppression of CAT activity (Robertson et al., 1994). It has been observed that IGFBP-1 levels are elevated in insulin-dependent diabetes mellitus (IDDM) (Crosby et al., 1992). A study has been shown that more IGFBP-1 crosses intact endothelial barriers when insulin is co-perfused. This suggests that insulin stimulates the translocation of this binding protein out of the vascular compartment (Bar et al., 1990).

A study from this laboratory has demonstrated 6 and 18-fold increases in hepatic IGFBP-1 mRNA levels and circulating IGFBP-1 concentrations in hypophysectomized rats (Senevirante et al, 1990). The rate of hepatic IGFBP-1 gene transcription increased 4 fold. GH treatment on these hypophysectomized rats reduces IGFBP-1 concentrations and normalizes IGFBP-1 mRNA levels. The effect of GH was transient and at the transcription level. Further studies in this laboratory have shown that insulin treatment to hypophysectomized rats did not reduce hepatic IGFBP-1 expression and hypoglycemia (Murphy et al, 1991). This observation suggests that GH may effect on IGFBP-1 expression directly.

Glucocorticoids have been demonstrated to increase human plasma IGFBP-1 levels independent of insulin (Conover et al., 1993). Hepatic IGFBP-1 mRNA was

transiently increased 2-fold 1 hour after injection of dexamethasone (Luo et al., 1990). A greater increase in IGFBP-1 mRNA abundance (up to 10 fold) was seen after 6 days of dexamethasone administration with some increase in serum IGFBP-1 measured by immunoblotting. Increased IGFBP-1 levels have also been demonstrated in growth-retarded fetal rats after dexamethasone administration (Price et al., 1990). IGFBP-1 mRNA was increased 8.5 fold in fetal liver without significant change in mRNA for IGFs and type I receptors. This suggests that IGFBP-1 may be important in the development of dexamethasone-induced fetal growth retardation. A number of *in vitro* studies have also shown that dexamethasone significantly stimulates IGFBP-1 protein and IGFBP-1 transcription in H4IIE rat hepatoma cells and primary adult rat hepatocytes (Orlowski et al., 1990; Unterman et al., 1991; Villafuerte et al., 1992). The transcriptional effects of dexamethasone are mediated through the IGFBP-1 promoter *cis* elements located between 357 and 103 bp, 5' to the cap site (Powell et al., 1993). Both somatostatin and octreotide have stimulatory effect in IGFBP-1 expression in humans when administrated subcutaneously (Ezzat et al., 1991; Lieberman et al., 1992). A study suggests that only the highly phosphorylated species of IGFBP-1 is under hormonal control (Westwood et al., 1994).

Other positive regulators of IGFBP-1 include glucagon, IGF-I and sex steroids. Glucagon has been shown to have stimulatory effects on IGFBP-1 production by human fetal liver explants *in vitro* (Lewitt et al., 1989), but the result is in contrast with human HepG2 cells and primary rat hepatocytes (Villafuerte et al., 1992). Hilding et al., (1993) also observed elevated serum IGFBP-1 levels in response to glucagon treatment *in vivo*.

III.2.3 Actions of IGFBP-1

IGFs circulate in association with specific binding proteins (IGFBPs) which thought to modulate the availability and the biological effects of IGFs on target tissues. In serum about 75% of the IGFs are associated with IGFBP-3 and an acid labile subunit. IGFBP-1 is the only one of the six IGFBPs which has the unique characteristic of rapid *in vivo* regulation in plasma (Holly, 1991). IGFBP-1 has a half-life of approximately 90 min determined by infusing of pure IGFBP-1 into rats (Young et al., 1992). Like IGFBP-3, IGFBP-1 is also present in plasma in concentrations sufficient to alter IGF action. IGFBP-1 contains the unsaturated serum IGF-binding sites (Guler et al., 1989). Since both IGFs and IGFBP-1 are produced by multiple tissues, IGFBP-1 not only regulates IGFs in serum, but also acts as local modulators for IGFs. Most of the known actions of IGFBP-1 are associated with its ability to bind the IGFs.

In vitro

Inhibition of IGF actions

IGFBP-1 has been found to inhibit IGF actions when present in molar excess in a number of *in vitro* studies. IGFBP-1 inhibited IGF-I-stimulated aminoisobutyric acid uptake in the cultured JEG 3 choriocarcinoma cells (Ritvos et al., 1988) and IGF-I-stimulated ^{3}[H] thymidine incorporation in porcine smooth muscle cells and fibroblasts (Busby et al., 1988). The inhibition effects of IGFBP-1 on IGF-I mediated mitogenesis were also found in other cell types including FRTL5 rat thyroid cells, human osteosarcoma cells, chick embryo pelvic cartilage, chick embryo fibroblasts and human granulosa cells

(Frauman et al., 1989; Burch et al., 1990; Liu et al., 1991; Angervo et al., 1991). The mechanism of IGFBP-1 inhibitory effect on IGF-I is mediated by formation of IGF:IGFBP complexes which cannot bind to IGF receptor. In human secretory phase endometrium, IGFBP-1 directly inhibits the binding of IGF-I to its cell receptors (Rutanen et al., 1988). In human granulosa-luteal cells, IGF binding to its receptor was also inhibited at the presence of a molar excess of IGFBP-1 in serum-free medium. The predominant effect of IGFBP-1 is inhibition of IGF action and this inhibitory effect requires excess IGFBP-1 or phosphorylated form of IGFBP-1. However, the stimulation effect by destri-IGF-I which is an IGF-I analog in human osteosarcoma cells was not inhibited by IGFBP-1 (Campbell and Novak, 1991). IGFBP-1 has been demonstrated to inhibit glucose incorporation into BALB/C3T3-fibroblasts in the absence of IGF-I. This suggests that IGFBP-1 may directly inhibit glucose incorporation (Okajima et al., 1993). Termination of inhibition may require the release of IGFs from IGF:IGFBP1 complexes by mass action proteolysis or other mechanisms. In the conditioned media of human osteosarcoma cells, plasmin treatment results in the dissociation of IGFs from IGF:IGFBP-1 complex and the recovery of biologically activity IGF-I is 60%. The IGFBP-1 inhibitory effect of IGF-I-stimulated DNA synthesis was subsequently reversed (Campbell et al., 1992).

Enhancement of IGF actions

Several studies have demonstrated that IGFBP-1 can enhance IGF-1 mediated actions *in vitro*. The addition of a purified IGFBP-1 fraction from amniotic fluid increased IGF-I stimulated DNA synthesis in porcine vascular smooth muscle cells 4-8 fold in the

presence of platelet-poor plasma (PPP) (Elgin et al., 1987). Similar effect was found in chick embryo fibroblasts, mouse embryo fibroblasts, human fibroblasts and human keratinocytes. Koistinen et al (1990) have observed that coincubations of IGF-I with IGFBP-1 stimulate DNA synthesis on human fetal skin fibroblasts and inhibit IGF binding paradoxically. The authors postulated that a complex of IGFBP-1 and IGF-I might result in sustained release of IGF-1 to receptors which would result in mitogenic potentiation. The IGF-II induced mitogenic effect was unaffected (Kratz et al., 1992). The authors suggested that the interaction between IGFs and their binding proteins may induce a different response depending upon the ligand and the target cell. It is apparent that the enhancement may also depend on the presence of PPP (Elgin et al, 1987). The addition of IGFBP-1 inhibits IGF-I or II stimulated migration in porcine vascular smooth muscle cells after wounding. The migration response of IGFBP-1 requires an intact RGD sequence (Gockerman et al., 1995). Busby et al. (1988) found that the association of an IGFBP with the cell membrane or matrix might be necessary for its potentiative action of IGF-1.

In vivo

There are limited data regarding IGFBP-1 action *in vivo*. It has been observed that IGFBP-1 injection into rats results in a small increase in blood glucose (Lewitt et al., 1992). The authors suggest that fluctuating IGFBP-1 levels might modulate the hypoglycemic activity of unbound IGFs in the circulation because IGFBP-1 concentrations are depend on metabolic status. Pekonen et al. (1988) demonstrated that IGFBP-1 inhibit the hypoglycemic effect of equimolar infusions of human IGF-I by blocking IGF-I

association with its receptors on human endometrial membrane. This suggests IGFBP-1 directly inhibits receptor association. Mohn et al (1991) have found that one of the most highly expressed immediate-early genes in liver regeneration encodes the rat homologue of the low-molecular-weight IGFBP-1, but the physiological significance of this finding is unknown. Studies have shown that increased IGFBP-1 expression is involved in mediating the marked growth retardation (Price et al., 1992). As assessed by solution hybridization assays and Northern blot analysis, there was an 8.5 fold increase in IGFBP-1 mRNA expression in the livers of dexamethasone-induced fetal growth retardation rats compared to that in sham-injected controls.

To further examine the functions of IGFBP-1 *in vivo*, human IGFBP-1 transgenic mice have been generated. The transgene was expressed in a number of tissues. No significant alterations were found in fertility, reproductive productivity or somatic growth. An alteration in growth was only found in the brain which has a modest, 5-15% reduction in brain size among the tissues studied. No marked alteration in fasting serum glucose or in the tolerance of young adult transgenic mice to an ip glucose load was observed (Dai et al., 1994). In this laboratory, transgenic mice that overexpress rat IGFBP-1 have been generated. The transgene was highly expressed in the brain, uterus, lung, kidney and heart but little expression was detected in the liver (Rajknmar et al., 1995). The transgenic mice demonstrated a phenotype characterized by modest reduction in birth weight , reduced brain size and fasting hyperglycemia. The brain size of these transgenic mice was reduced remarkably, as much as 30-40%. These data suggest that IGFBP-1 may function to inhibit IGF action *in vivo* and that this inhibition selectively impairs development of organs.

III.3. Generation of transgenic mice

Transgenic mice are generated by direct microinjection of a purified DNA fragment into the pronuclei of fertilized one-cell mouse eggs. The injected eggs are then transferred into pseudopregnant foster mothers. The offspring from foster mothers were screened for transgene. Rat IGFBP-1 transgenic mice have been generated in this lab. Four transgenic mouse strains were generated with a fusion gene composed of the mouse phosphoglycerate kinase promoter (PGK) and a 5 kb BamH1-EcoR1 fragment containing the entire coding region of the rat IGFBP-1 gene. Microinjection of the fusion gene was performed by using fertilized C57BL/6JXCBA F1 zygotes. The micro-injected embryos were then transferred into CD-1 foster mice. Marked reduction in litter size , birth weight and brain size, fasting hyperglycemia and glucose intolerance were observed in offspring of founders 57C, 195C and 227A (Rajkumar et al., 1995). Furthermore, a reduction in estrogen induced uterine DNA synthesis in mice from the 57C and 277A strains has been reported (Rajkumar et al., 1996). Given that the reduction in brain weight is the most remarkable, these rat IGFBP-1 transgenic mice should prove useful in defining IGF actions during brain development.

Based on the data that IGFs stimulate cell proliferation and survival and that IGFBP-1 inhibits IGF actions, our hypothesis is that IGF-I and IGFBP-1 has a critical role in brain development.

The studies in this thesis were focused on approaches to determine whether overexpression of the transgene modulated the actions of IGFs during brain development.

The first study was to determine the abnormalities of the brain in rat IGFBP-1 transgenic mice. The second study was to investigate the role of IGFBP-1 underlying the impaired brain development. The third study was to explore the role of IGFBP-1 in brain wound healing.

IV.MATERIAL AND METHODS

IV.1. Materials

Restriction enzymes were obtained from Pharmacia (Canada) Inc. (Baie d'Urfe, Quebec), Radioisotopes were supplied from ICN Biochemical Canada Ltd. (Mississauga, Ontario) and Amersham Canada Ltd. (Oakville, Ontario). Nick translation kits were obtained from Amersham Canada Ltd. X-Ray film (XAR) was obtained from Kodak Laboratories. Calf thymus DNA was purchased from Sigma Chemical Co. (USA). The protein assay dye reagent was obtained from Bio-Rad laboratories (CA, USA). Terminal transferase, Biotin-16-2'-deoxyuridine-5'-triphosphate (biotin-16-dUTP) and Streptavidin-POD conjugate were obtained from Boehringer Mannheim (Canada). 5'-Bromo-2'-Deoxy-Uridine (BrdU) was supplied by Sigma Chemical Co. (USA). Biotinylated rabbit anti rat IgG (mouse adsorbed) was obtained from Vectors Lab, Inc. (CA, USA).

Rabbit anti-rat IGFBP-1 antiserum was kindly provided by Dr. M. Lewitt, University of Sydney, Australia). A polyclonal antibody against glial fibrillary acidic protein (GFAP) which was obtained from Dimension Laboratories Inc. (Ontario, Canada) was applied to identify astrocytes. Additional anti-GFAP antibodies and other antibodies tested are listed in Table 1 with the dilution employed for immunohistochemistry.

For those primary antisera raised in rabbit, the second antibody applied was goat anti-rabbit IgG and the third antibody was rabbit peroxidase anti-peroxidase (PAP) which were purchased from Sternberger Monoclonals Inc., Baltimore, USA for the PAP method. For those primary antisera raised in mouse, the second antibody applied was goat anti-mouse IgG and the third antibody was mouse PAP (Sternberger Monoclonals Inc.).

25

Secondary antibody for immunofluorescence was a fluorescein isothiocyanate (FITC)-conjugated goat anti rabbit antibody (Jackson ImmunoResearch Laboratories, Inc.).

IV.2. Tissue preparation

A total of 225 mice were used in the present study. Adult male CD-1 wild type or non-transgenic littermates and IGFBP-1 transgenic mice of the 57C strain weighting 25-35 g were deeply anesthetized with equithesin and perfused transcardially on ice. The thoracic cavity and diaphragm were opened to expose the heart. The left ventricle was pierced by a 23 gauge needle attached to the tube of a perfusion pump. The right atrium was then cut to allow blood and perfusion fluid outflow. Prefixative solution consisted of 15 ml of cold 50 mM sodium phosphate buffer (PB) containing 0.8% saline, 0.1% sodium nitrite and heparin (1 unit/ml). Following prefix, different fixative solutions were selected in order to optimize detection of the antibodies used. The fixative solutions used were either Lana's fix or 4% paraformaldehyde. The Lana's fixative solution consists of 40 ml of 0.16 M PB containing 4% paraformaldehyde and 0.2% picric acid adjusted to pH 6.9. The 4% paraformaldehyde fixative solution (pH7.4) consists of 40 ml of 0.1 M PB containing 4% paraformaldehyde adjusted to pH 7.4 per mouse. The brains were freed of the dura mater, removed and post-fixed for 2 h in 10 ml of the fixative solution. For cryoprotection, the brain was either transferred to 10 ml of 50 mM PB containing 15% sucrose (pH 7.4) for subsequent sectioning on the cryostat or transferred to 10 ml of 50 mM PB containing 25% sucrose and 10% glycerol (pH7.4) for sectioning on the sliding microtome. Neonatal mice were sacrificed by decapitation and brains were removed immediately and immersion

fixed in 4% paraformaldehyde for 6 hours. Following fixative solution, the brain was transferred to 15% sucrose for cryostat sectioning. For histological staining, BrdU labeling and TUNEL, transverse sections (10 μm) were cut on a cryostat at -20°C, mounted onto gelatin-coated slides and stored at -80°C until use. For immunohistochemistry, sections were cut on a sliding microtome and collected in 0.1 M phosphate buffer, 0.04% NaN₃ (pH 7.4) at 4°C.

IV.3. Screening of IGFBP-1 transgenic mouse

Isolation of mouse genomic DNA from tail

1-2 cm of mouse tails were cut and digested with proteinase K (0.05 mg/ml) in 50 mM Tris (pH8), 100 mM EDTA, 0.5% SDS at 55°C for overnight. DNA was extracted by phenol and phenol/chloroform(1:1). After extraction, DNA was precipitated in 3 M sodium acetate (pH6) and 100% ethanol. The DNA pellet was washed with 70% ethanol, dried briefly in vacuum and then dissolved in TE buffer (pH8) for use.

Labeling of probes

The probe DNA was taken to be nick translated by introducing radioactivity labeled nucleotides under the catalysis of Escherichia coli DNA polymerase I (Feinberg et al. , 1983). The nick translation reaction was carried out as described by the supplier of the kit. Briefly, 100-200 ng DNA and 7 μl of 3,000 Ci/mmol (alpha-32P) dCTP were used per nick translation reaction. Radioactively labeled DNA was separated from free (alpha-32P)dCTP by G50 Sephadex gel filtration chromatography. Before adding probe into the

hybridization solution, it was boiled for 5 min and cooled rapidly on ice to separate the two DNA strands.

Southern blot analyses of mouse genomic DNA

The absorbency of isolated DNA was measured at 260 nM and 280 nM to get the concentration of DNA. 10-15 μg mouse genomic DNA were digested with the restriction enzymes EcoR1 and Sst1 at 37°C overnight. The digested DNA and DNA fragment marker were separated by electrophoresis on 1% agarose gel. The gel was then washed in 1.5 M NaOH for 20 min to denature the DNA and neutralized in 0.5 M Tris and 3.0 M NaCl (pH7.0) for 40 min. DNA was then transferred to nitrocellulose filters. The filters were left at 80°C oven for 2-4 hours.

Hybridization

The filters were first prehybridized at 42°C in a solution containing 50% (v/v) formamide, 20 mM NaH_2PO4 (pH7.0), 4xSSC, 2 mM EDTA, 4x Denhard' solution, 0.1% SDS and 100 μg/ml sonicated, denatured salmon sperm DNA. After the prehybridization the filters were hybridized with the ^{32}P-labeled probe for 12-20 h at 42°C in the same solution . The probe was the same 0.5 kb EcoR1-Xho1 fragment of the PGK-1 promoter used to construct the fusion gene after it was labeled by nick translation labeling. The membranes were washed in 2x SSC and 0.1% SDS for 30 min at room temperature, then in 0.1x SSC and 0.1% SDS for 15 min at 65 °C.

Exposure

Nitrocellulose membrane were exposed to Kodak XAR film at -70°C with an intensifying screen for a period of several hours to 3 days (Southern et al. , 1975).

IV.4. Fluorometric quantification of brain DNA

Tissue homogenization

Fluorometric quantification of DNA was performed as previously described (Downs and Wilfinger et al. , 1983). Fresh brains were collected from IGFBP-1 transgenic mice of 57C stain and CD-1 wild type mice of different age groups and washed in phosphate-buffered saline (100 mM sodium phosphate, 150 mM NaCl, pH7.4), blotted, and weighed. The brains were then homogenized in 2.0-10.0 ml of AT solution (1 N NH_4OH, 0.2% Triton X-100) and incubated at 37°C for 10 min. A 100 μl aliquot of the solution was diluted to 1.0 ml with assay buffer (1xTNE) and centrifuged at 4000 RPM for 20 min. Then the supernatant was placed on ice.

Standard solutions

Calf thymus DNA was used for stock solution. A stock solution of DNA was adjusted spectrophotometrically to a concentration of 50 μg DNA/ml H_2O. Before each assay, 2 μl of the DNA stock solution was diluted with the same assay buffer described previously and placed on ice. The Hoechst 33258 was dissolved in water at a concentration of 200 μg/ml and stored in 4°C as stock solution. Before assay, the stock solution was diluted to a final concentration of 100 ng/ml with assay buffer and covered with foil paper.

DNA measurement

Total DNA was measured fluorometrically in a Hoefer DNA Fluorometer Model TKO100 and emission wavelengths set at 460 nm. Hoechst solution and blank solution

29

were pipetted into the cuvette, then the standard DNA was utilized for fluorescence measurements. After the DNA standards were assayed, the brain DNA levels were determined.

IV.5. Quantitation of brain protein

The amount of protein from all samples was measured in duplicate by the method of Bradford (Bradford et al., 1976). The protein determination method is a dye-binding assay which involves the binding of Coomassie Brilliant Blue to protein. A differential color change of a dye occurs in response to various concentrations of protein. The binding of the dye to protein causes a shift in the absorption maximum of the dye from 465-595 nm. Briefly, dye reagent was prepared by diluting 1 part Dye Reagent Concentrate with 4 parts distilled water and filtered through Whatman #1 filter. A protein standard of five dilutions was prepared with BSA, which is representative of the protein solution to be tested. Frozen brains were homogenized in 5 ml of 10 mM Tris-HCl, pH7.4, containing 1 mM EDTA and 1% SDS, using a Polytron at a setting of 6 for 10 s. 20 μl of a 1:20 diluted homogenate was pipetted into test tube, then 1 ml of protein reagent was added to each test tube of samples and the standards. The content of each tube was mixed by vortexing. The mixtures were incubated at room temperature for at least 5 minutes. Absorption at 595 nm was measured. The weight of protein was plotted against the corresponding absorbance resulting in a standard curve used to determine the protein in unknown samples.

IV.6. Identification of programmed cell death *in situ* via TUNEL

DNA breaks were detected *in situ* by nick end labeling. The methods used in this study is a modification of Gavrieli et al. (1992). The terminal deoxynucleotidyl transferase (TdT) -mediated dUTP-biotin nick end labeling (TUNEL) method is based on the specific binding of terminal deoxynucliotidyl transferase (TdT) to 3'-OH ends of DNA, ensuring the synthesis of a polydeoxynucleotide polymer. Briefly, brains were removed from neonatal mice and immerse fixed in 4% paraformaldehyde (pH7.4) for 6 hours at 4°C and transferred to 15% sucrose solution for cryoprotection. Cryostat sections air dried and rinsed in PBS. Subsequently, tissue were digested with 2 μg/ml proteinase K at room temperature (RT) for 15 minutes. The proteolytic pretreatment considerably enhanced the TdT reaction. After washed in PBS, sections were covered with 2% H_2O_2 and washed with PBS. TdT buffer solution containing 0.3U/μl TdT and 0.04 nmol/μl biotinylated dUTP were added to cover the sections and incubated in humidified environment for 60 minutes at 37°C. Reaction was terminated in TB buffer (300 mM sodium chloride and 30 mM sodium citrate) for 15 minutes, and then washed in PBS. Tissues were treated with 2% aqueous solution of BSA for 10 minutes at RT. After washing in PBS, they were incubated with peroxidase labeled streptavidin for 30 min at RT and finally stained with diaminobenzidine-H_2O_2 solution. If necessary, sections were counter-stained with hematoxylin. To confirm the staining specificity, a positive control was performed. Tissues were treated with 0.7 μg/ml DNase I in DNase buffer (pH 7.2) for 10 min before treatment with TdT. Negative controls included omission of TdT or biotinylated substrate

31

from the buffer solution. TUNEL positive cells were counted and statistical analysis was performed using Student's t test.

IV.7. Labeling with BrdU to determine cell proliferation.

BrdU is a thymidine analog that is incorporated into the DNA of dividing cells during S-phase and can be visualized by immunocytochemisty. In order to label the proliferating cells at different time course, neonatal mice at P2 were injected with BrdU (300 mg/kg, dissolved in 0.007N NaOH in 0.9% NaCl) every 12 hours for 2 times. Animals were killed 12 hours after the BrdU injections. The brains were removed and fixed in 4% paraformaldehyde for 6 hours at 4°C and then kept in 15% sucrose at 4°C for 48 hours. Sections were cut on a cryostat at -20°C and collected on gelatin coated slides. Subsequently sections were air dried and washed in PBS. The sections were treated with in 2.8N HCl for 15 min at room temperature to denature the DNA. This was followed by 3 x 10 min washes with the PBS and treated with 4% normal rabbit serum for 30 min to block nonspecific binding sites. The sections were subsequently incubated for 48 hours (4°C) in a primary Rat BrdU (1:100) directed against single-stranded DNA containing BrdU. After 3 washes, the sections were incubated in a biotinylated rabbit anti-rat IgG (1:1000) for 1 hour at room temperature. After three washes, the sections were treated with 0.1% H_2O_2 for 15 min in order to inhibit endogenous peroxidase activity. The sections were washed and incubated in Strepavidin-POD conjugate for one hour at room temperature. After washing in PBS for 3 x 10 min and 50 mM Tris for 10 min, the labeling was demonstrated with 0.2% diaminobenzidine (DAB) in 50 mM Tris (pH7.4) plus

32

0.0045% hydrogen peroxide. Reaction product from the above protocol stained BrdU-positive cell nuclei dark brown (Takahashi et al., 1992). Counts of BrdU labeled cells in dentate gyrus, ventricular zone and subventricular zone were averaged from 3 separate and non adjacent sections chosen from the same approximate rostrocaudal level of each animal aided by image analysis system (Neurolucida).

.

IV.8. Stab wound surgical procedures

To study the effect of lesions on GFAP immunoreactivity in astroglial cells in transgenic mice of 57C strain, male CD-1 wild type mice and transgenic mice aged 2 months were injured. Animals were anesthetized with equithesin and placed in a stereotaxic apparatus. The animal's head was held in the stereotaxic instrument with fixation of the head at three points. Two points were the bony external auditory meatuses on each side. Two bars are inserted into these meatuses until firmly fixed and the head is held rostrally by placing the incisors over a bar and firmly fixing the palate directly behind the incisors against the bar. The skull angle was kept constant by placing bregma and lambda on the same horizontal plane. A lesion was made in the right cerebral hemisphere by pushing a rotating dental drill to open the scalp and inserting a micro-dissecting knife through the burr hole in the skull and then withdrawing. The burr hole was made in the skull of animals without trespassing of the dura mater. The measurement of lesion was 2.0 mm lateral to midline, 4.0 mm anterior to lambda and 4.4 mm ventral in male CD-1 wild type; 1.6 mm lateral to midline, 2.8 mm anterior to lambda and 3.2 ventral in male strain 57C transgenic mice. A lesion crossing the parietal cerebral cortex, the CA1 field of the

dorsal hippocampus and the dentate gyrus was produced. The scalp was sutured with one or two stitches. After surgery all animals are individually housed at a 12 hour light /dark cycle and had free access to food and water. The animals were sacrificed seven days after the injury and immunocytochemical assays were performed as described in detail as bellow.

IV.9. Immunohistochemistry and histological staining

All brains were cut transverse at 20 μm on a sliding microtome and sections were washed overnight at 4°C in PBST (0.1 M PBS containing 0.3% triton X-100). Sections were processed by the PAP method and immunofluorescence (Staines et al., 1986).

IV.9.1 Peroxidaxe anti-peroxidase (PAP) method

Sections were preblocked for 30 min in PBS containing either 0.3% Triton , 5% normal goat serum. Followed by incubation with primary antibody (polyclonal) diluted in PBST containing 2% normal goat serum (NGS) for 48 hr at 4°C. The antibodies used are listed in Table 1. The peroxidase anti-peroxidase (PAP) method was used to detect bound antibodies. After primary incubation, free floating sections were washed in PBST for 1 hr and then incubated for 1.5 hr at room temperature with goat anti- rabbit IgG diluted 1:100 in PBST with 2% NGS. After a further 1 hr wash sections were incubated with rabbit PAP diluted 1:500 in PBST with 2% NGS. The sections were then washed in PBST for 30 min and subsequently in 50 mM Tris-HCl buffer (pH 7.4). Sections were incubated with 0.02% 3,3-diaminobenzidine (DAB) and 0.0045% hydrogen peroxide in Tris-HCl buffer and washed in Tris-HCl buffer. Sections were mounted onto slides from gelatin-

alcohol, dehydrated in serial concentrations of ethanol and coverslipped with Lipshaw mounting medium.

IV.9.2 Immunofluorescence.

Sections were incubated for 48 hr with anti-GFAP antibody diluted 1:400 in PBST and then incubated for 1.5 hr at room temperature in PBST with FITC conjugated goat anti rabbit antibody diluted at 1:50. Sections were washed in PBST for 10 min and in 50 mM Tris-HCl buffer (pH 7.4) for two 10 min. Sections were mounted onto slides from 50 mM Tris-HCl buffer, pH 7.4, air-dried and then coverslipped with anti-fade medium. Sections were viewed with a Leitz Dialux 20 microscope equipped with Ploempak filter cubes L3 (excitation 450-490 nm; band pass 500-550 nm).

IV.9.3 Nissl staining and myelin staining.

Every fifth serial coronal section through the whole brain was stained for Nissl substance with thionin and used to measure the area of the dentate gyrus and hippocampus. Cryostat sections were first rehydrated for 15 min in 50 mM PBS, counterstained in 0.025% thionin for 5 min, then dehydrated in ascending levels of alcohol and cleared in Histoclear. Finally sections were coverslipped with Lipshaw mounting medium. For histological staining of myelin, sections on slides were defatted and stained with 0.1% Luxol Fast Blue at 55°C-60°C for overnight and differentiated in 0.05% LiCO$_3$ and 70% EtOH, then dehydrated through ascending series of EtOH, clear with several changes of Histoclear and coverslip. Measurements of the transection area of the anterior commissure, dentate gyrus and hippocampus were done with Neurolucida, a computer-assisted image analysis system (MCID, Imaging Research). The thickness of corpus

callosum was measured under the light microscope. The quantification of brain structure was conducted on Morph computer-assisted quantification system..

IV.10. Systemic administration of Kainic acid (KA)

Male wild-type and IGFBP-1 mice of 2 months old were injected i.p with 30 mg/Kg kainic acid (Sigma) which was diluted in 0.9% saline. Animals were observed for abnormal behavior 2 hr after the injection of kainic acid and maintained for 7 days. For histological examinations of the brains, mice were perfused transcardially with 4% paraformadehyde (pH = 7.4), the brains were immediately removed and then postfixed in the above solution for 2 hours. 20 μm sections through the dorsal hippocampus were cut, stained either with thionin or incubated with GFAP antibodies and studied by light microscopy.

IV.11. Statistical analysis

Statistical analyses were performed by the Student's t test to determine the significance of the differences between wild type and transgenic mice. Values are means ± SEM. The definition of statistical significance was $p < 0.05$.

V. RESULTS

V.1. Sites of Rat IGFBP-1 transgene expression in the brain

To identify the sites of transgene expression in brain, immunocytochemistry were performed in transgenic and wild-type mice. Immunohistochemistry with an antiserum specific for rat IGFBP-1 was used to localize the transgene product in the brain. The major sites of rat IGFBP-1 expression are in the CA2 and CA3 region of hippocampus, hypothalamus and thalamus at hippocampus level and lateral septal nuclei and nuclei vertical limb diagonal band at striatum level. No immunostaining was found in the brain of wild type mice indicating that the endogenous mouse IGFBP-1 might be not expressed or expressed at very low levels in brains of normal mice.

V.2. Expression of rat IGFBP-1 in transgenic mice result in brain growth retardation

The brains of IGFBP-1 overexpress mice were smaller than those of age matched wild-type mice. Morphologically, the size of most brain structures also was decreased in IGFBP-1 transgenic mice, but no obvious abnormalities were observed compared to their normal littermates. All major parts of the brain were present and the sizes of all parts were reduced to similar extents as compared with wild type (Fig. 1). The brain weights of adult IGFBP-1 mice were 40.1% below weights of age matched wild-type mice, whereas the body weights were reduced by 11.84%. Furthermore, the brain weights of transgenic

mice from each age group were found to be reduced compared to wild-type mice (Table 2). IGFBP-1 transgenic mice exhibited 23% reduction of brain weight at the age of P0 and 32% reduction at the age of P12. To examine whether the reductions in brain weights were related to the reduction in overall somatic growth, brain weights were also calculated as a function of body weight. In adult group, brain to body weight ratios were significantly smaller in transgenic mice, 32%: 1.06 ± 0.126, means $\pm$ SE (N = 10) in transgenic mice vs. 1.55 ± 0.218 (N = 10) in non-transgenic mice.

To determine whether the brain weight reduction was due to a reduced total cell number, total brain DNA and protein content were measured in transgenic and wild-type. Total brain DNA and protein were significantly reduced. In each age group of transgenic mice the relative decreases in DNA and protein were not as much as the reductions in brain weight. For example while the brain weights of adult transgenic mice were decreased by 40.10%. Total DNA content was reduced by 15.5% in transgenic mice. This result suggests that a simple reduction in cell number does not account entirely for the reduction in brain weight and size, other components of the brain, such as myelin, may also be reduced. In order to find out at which developmental stage of the brain growth retardation occurs, brain weights, DNA and protein contents from transgenic mice and wild type mice were measured at p0 (The first 24 h after birth as postnatal day 0), p12 and adult (>56 days). Brain weights, DNA content and protein content were significantly less than those of their non-transgene littermates or CD-1 wild type at the age of P0 and thereafter (Table 2). The body weight difference was only significant in adult mice. Relatively large

38

variances in body weight among sucking mice accounted for the lack of significance in mice < 2 weeks age.

V.3. The thickness of corpus callosum is decreased in transgenic mice

The intensity of myelin staining was markedly reduced in the IGFBP-1 transgenic mice. In IGFBP-1 transgenic mice, the decreased intensity of myelin staining was obvious in corpus callosum, but not in brainstem and cerebellum. The fiber bundle forming the corpus callosum showed a marked reduction in thickness (Table 3). Histological staining for myelin with Luxol fast blue revealed a weakly stained fiber bundle in transgenic mice. The thickness of the medial part of the corpus callosum in transgenic mice was reduced by 62% (n=6, P< 0.001) compared with corresponding anterior-posterior at frontal horn level in non-transgenic mice. In contrast, the distance from the cortical to basal surface of the brain was only reduced by 22.34% (n =4, p< 0.0001). The cross sectional area of the anterior commissure was also reduced in transgenic mice. Histological staining for myelin with Luxol fast blue revealed a weakly stained fiber bundle that occupied a 20.30% smaller area than the same structure at a corresponding level in wild type mice (Table 3). However, when the anterior commissure area was normalized to whole section area, the area of the transgenic mouse brain was not smaller than that of the controls.

V.4. The area of dentate gyrus is reduced in transgenic mice

Visual inspection of the hippocampus showed that the cell number in the dentate gyrus and pyramidal layer of IGFBP-1 mice is dramatically reduced (Fig. 2). The

morphology of dentate granule neurons as visualized by Nissl staining in IGFBP1 mice was not different from wild-type. Cell bodies in the dentate gyrus were packed at higher density in Nissl-stained sections, the area occupied by dentate gyrus which consist of exclusively dentate granule neurons, was reduced by 72.37% (n=4, p< 0.0001, Table 4). Nissl staining positive granule neurons in dentate gyrus were counted from the corresponding levels in four each wild-type and transgenic mice at two months old. The packing densities of neurons in the dentate gyrus in wild-type and IGFBP-1 transgenic mice were: 16733 ± 287 and 18675 ± 329 cells/mm^2 (means $\pm$ SEM, n=4, P < 0.005) respectively, amounting a 10.4% increase in IGFBP-1 mice. The total area of hippocampus which consists mainly of pyramidal neurons, was reduced by 54.57% (n=4, p< 0.0001). So both the area of hippocampus and dentate gyrus are decreased notably in transgenic, which are more than the decrease seen in whole section area and overall brain weight.

V.5. The distribution and density of astrocytes immunopositive for GFAP

The distribution and staining intensity of glial fibrillary acidic protein (GFAP) immunopositive astrocytes was identical in the hippocampus of wild type and transgenic mice (Fig. 3 AB). The counting of cell number per area showed increased cell density by 11% in transgenic mice compared to wild type (475 ± 12.6 vs 427.7 ± 14.4, n = 4, p <

0.05, mean ± SEM). However, the size of astroglia body together with their processes in transgenic mice are not significantly different from that of wild-type mice .

V.6. The astrocytic reaction in the transgenic mice after a stab wound

In lesioned wild-type and transgenic mice, a dense network of strong positivity of immunoreactive for glial fibrillary acidic protein (GFAP) was observed surrounding the lesion tract. Reactive GFAP-positive astrocytes with very thick and long processes were found in the cortex around the lesion, in the white matter for a long distance, in the dorsal hippocampus of the same hemisphere and in the white matter of the contralateral hemisphere. A similar pattern of immunostaining was also observed in the sections near the wound (Fig. 3). As studied by light microscope, the morphology of GFAP positive astroglia did not show obvious difference between the test group and control group. However, GFAP-positive cell density in the lesioned hippocampus was increased by 7% in stab wound transgenic mice compared with that of intact transgenic mice. The cell density in lesioned wild-type mice was increased by 31% compared with intact wild-type mice. Therefore, the extent of increase in astrocyte density in transgenic mice was reduced remarkably compared to wild type mice (Fig. 6).

V.7. Kainic acid-induced neuron damage in the transgenic mice

The result showed here is from our preliminary experiment. Rats have been used for kainic acid induced brain lesion in a number of studies. The dosage treatment on mouse have not been reported. Schwob et al. (1980) reported system injections of 12

mg/kg kainic acid produce a seizure activity and neuronal damage on rats. We tried the same dosage on mice, but it had no effect. We increased the dosage to 18 mg/kg, 24 mg/kg, 30 mg/kg and 50 mg/kg. At 50 mg/kg, the toxicity of KA lead to death of all transgenic mice (n = 3) within 1 hr after the injection, while the wild-type survive. The dentate granule neurons are highly resistant to KA treatment, but pyramidal neurons in the CA1, CA2, part region of CA3 were preferentially lost in the surviving wild-type mouse brain. At the dosage below 30mg/kg, we did not find any abnormalities in both wild-type and transgenic mice. At the dosage of 30 mg/kg, 3 of the transgenic mice died. Brain lesion was found in the 2 surviving IGFBP-1 transgenic mice, whereas no obvious abnormalities were seen in wild-type mice (n = 2). In transgenic mice, some of the dentate granule neurons which have been damaged severely and apparently irreversibly, are dark and shrunken. However, most dentate granule neurons adjacent to the degenerating cells are apparently normal. The distribution and morphology of astroglia in wild-type and transgenic were unaffected.

V.8. The numbers of apoptotic cells are increased in the transgenic mice

The apoptotic cells were labeled by TUNEL. Apoptotic cells show intense dark brown staining, shrunken nuclei and nuclear fragments. No staining of the cytoplasm was observed. Corresponding anterior-posterior levels of coronal sections of hippocampus between wild-type and transgenic mice were chosen to represent the distribution of dying cells using the TUNEL assay on preparations at p0, p15 and adult. Apoptotic cells were found in the ependymal layer lining the lateral ventricle, cortex and the developing

hippocampus. Apoptotic cells were absent in the adult mouse both in IGFBP1 transgenic and CD1 wild type. Very few positive apoptotic cells were seen in both transgenic and wild type mice at p15. In p0 group, transgenic pups had increased number of apoptotic cells in hippocampus (7.33 ± 0.67 vs 4.67 ± 0.33, n = 3, p < 0.05) compared to wild type mice.

V.9. BrdU labeled proliferating cells are reduced in transgenic mice

At striatum level, the positive nuclei are sparsely distributed in cortex, corpus callosum, caudate putamen and heavily labeled nuclei are located in subventricular zone (SVZ), ventricular zone (VZ) and indusium griseum (IG). The counts of BrdU -labeled nuclei in SVZ and VZ in wild type and IGFBP-1 transgenic mice sacrificed on postnatal day 3 indicate a significant decrease by 18.51% of labeled cells in transgenic mice (Fig. 3, Table 5). At the level of the hippocampus, positive nuclei are recognizable in the cortex, corpus callosum and ventricle area and high density of labeled nuclei are concentrated in the developing dentate gyrus of hippocampus. The counts of positive nuclei showed a remarkably decrease of as much as 40.74% in the dentate gyrus area in IGFBP-1 transgenic mice in comparison with wild-type.

V.10. Immunohistochemistry for OX42, acetylcholinesterase (AChE), ED-1 and axons

43

OX42 immunostaining was performed on sections at striatum level from 4 wild type and 4 transgenic mice at 2 months of age. The distribution and morphological features of microglia cells in transgenic mice were not different from that of wild type under light microscope. AChE staining for cholinergic neurons was detected on sections from 3 wild type and 3 transgenic mice at the age of 2 months. The morphological features and distribution of AChE positive cells were identical in wild type and transgenic mice under light microscope. Monoclonal ED-1 antibody was used to detect macrophagic markers. Two wild type and two transgenic mice at age of two months were used. No immunoreactivities were found in all the animals. Immunoreactivities for neurofilament proteins were detected in two wild type and two transgenic mice. No morphological differences of panaxonal-immunoreactive fibers were found between wild type and transgenic mice.

VI. DISCUSSION

A number of studies have reported that IGFs expression in the brain during embryogenesis and adult life in rodents. The expression of IGF-I mRNA in the rat brain peaks around late embryonic to early postnatal development time points (Bartletl et al., 1991; Bartletl et al., 1992; Aguado et al., 1992). IGF receptors and IGFBPs are also expressed in the brain throughout development (Brown et al., 1989; Bondy and Lee., 1993a; Cerro et al., 1993; Lee et al., 1993). Furthermore, the IGFs have been shown to be growth factors for developing CNS neurons, astrocytes and oligodendrocytes (Han et al., 1987; Shemer et al., 1987; Nelson et al., 1991; McMorris et al., 1986; Barres et al., 1992; Drago et al., 1991). The IGFBP-1 expressed in transgenic mice is one of the family of IGF binding proteins that modulates the actions of the IGFs by controlling their serum and tissue concentrations and bioavalability. They also function to transport the IGFs from their sites of synthesis. IGFBP-1 can inhibit the actions of the IGFs in a concentration dependent manner. IGFBP-I is not expressed in brain normally. In our studies of IGFBP-1 transgenic mice, we have observed brain growth retardation. DNA and protein assays reported here consistently showed a significantly reduction in IGFBP-1 transgenic mice at P0, P12 and adult. This indicates total brain cell number is reduced and it is consistent with the transgene, rat IGFBP-1, expression inhibiting the actions of the IGFs on neural cell proliferation and/or survival and suggests that IGF-I plays an important role in the generation of brain cells *in vivo*. Since brain DNA and protein were reduced significantly in transgenic mice at P0, this indicate brain growth retardation may occur not only

45

postnatally but also prenatally or perinatally. Therefore, the expression of rat IGFBP-1 regulates IGF growth effect on brain during fetal life. Our observation is different from the report of D'Ercole et al (1994) which they found the brain growth retardation in hIGFBP-1 transgenic mice occurred at 2 weeks of age . In our study, the reduced cell number, however, is not as great as the reduction in brain weight. This result suggests that a simple reduction in cell number does not account entirely for the reduction in brain weight We also found that the intensity of myelin staining was less in transgenic mice and the corpus callosum thickness was significantly decreased in transgenic mice. Other component of the brain, such as myelin, might be also involved in the reduction in brain weight.

In the mouse CNS, myelination occurs during the first 4 postnatal weeks, with an initial period of oligodendrocyte proliferation and a partially overlapping consecutive period of myelin synthesis (Morell et al., 1972; Matthieu et al., 1973). A number of studies have demonstrate that IGF-I has important role in myelination. IGF-I can promotes oligodendrocyte survival and/or proliferation (Nogochi et al., 1982; King et al., 1988). *In vitro* studies have reported that IGF-I substantially increase oligodendrocyte number by increasing the proliferation and differentiation of progenitor cells as well as survival of oligodendrocytes (McMorris et al., 1988). IGF-I may act directly on oligodendrocytes or oligodendrocyte precursors since both cell types have cell surface type I IGF receptors and type II IGF receptors. While *in vivo* studies also showed that IGF-I promote myelination. Carson et al (1993) reported enhancement of CNS myelination in IGF-I overexpressing mice. Later, Ye et al. (1995) reported increased myelination in IGF-1 transgenic mice and decreased myelination in hIGFBP-1 transgenic mice. They found that cerebral cortical

proteolipid protein (PLP) and myelin basic protein (MBP) mRNAs which are myelin protein genes consistently exhibit 50% decreases in IGFBP-1 transgenic mice. The percentage of oligodendrocyte labeled with a PLP cRNA probe in the corpus callosum and cerebral cortex also reduced in hIGFBP-1 transgenic mice. In our study, measurement of white matter structures showed that the anterior commissure was decreased in size less than the decrease in total brain size, whereas corpus callosum was decreased 62% compared with wild-type littermates. Myelin in the cerebellum as visualized by Luxol Fast Blue myelin staining was not different from wild type. Myelin is generated earlier in cerebellum than other forebrain regions (Foran and Peterson 1992), we postulated that the transgene is expressed at relatively later period in the cerebellum. IGFBP-1 overexpression might affect the number of axons forming white matter tracts.

Previous studies have demonstrated that IGF-I has mitogenic effect on neuronal cell proliferation. IGF-I has neurotrophic actions on neuronal cells from the brain (Knusel & Hefti, 1991; Ishii et al., 1991). Its *in vitro* effects include regulation of mitotic cycle in sympathetic neuroblasts, stimulation of neurite outgrowth, neurotransmitter, synthesis and electrical activity in many types of neurons (DiCiccio and Black 1988; Recio et al., 1988; Caroni and Grandes 1990; Alzenman & Devellis 1987; Knusel et al., 1990). Masters and Raizada (1993) reported that neuronal cells express high-affinity IGF-I specific receptors and these receptors mediate IGF-I stimulated synthesis of proteins. By immunohistochemistry, we found the distribution of cholinergic neurons expressing AchE was unaffected. A similar result reported by Beck et al (1995), who found numbers and distribution of cholinergic neurons and dopaminergic neurons were not influenced in IGF-I

47

gene disruption mice. However, our further studies found remarkable loss of dentate granule cells, suggesting that IGF-I has selectively effects on different type of neurons.

The generation, migration and differentiation of neurons are generally complete soon after birth. However, neurogenesis continues in the dentate gyrus of hippocampus after birth (Rakic et al., 1985). IGF-I receptor mRNA is expressed in all neurons within the developing hippocampus. Postnatal neurogenesis in the hippocampus is concentrated in the hilus of the dentate gyrus. Granule cells begin to arise from the dentate germinal zone (DGZ) at postnatal day 4 hippocampal formation (Bondy and Lee, 1993b). Hippocampal pyramidal neurons are principal cells in CA1-4 that appear mostly from E17 to E20 (Altman and Bayer 1990a; 1990b). In this study, the gross morphology of dentate granule neurons as visualized by Nissl staining in IGFBP-1 mice was not different from wild type. However, the granule cells in transgenic are packed tightly together with an higher density which increased by 10.4% compared with wild-type. The decrease in total brain size and the area occupied by hippocampus was exceeded by the 72.37% reduction of the dentate gyrus area, indicating specific and remarkable loss or failure to generate of dentate granule neurons in transgenic mice. This finding suggests rat IGFBP-1 transgene modulates IGF-I action in the generation of dentate granule neurons. Further studies on cell proliferation with the immunohistochemical demonstration of BrdU uptake - a marker of cellular proliferation at P3 showed a remarkably greater number of positive nuclei in wild-type than that of transgenic mice. Since the age P3 is the period of neurogenesis in the dentate gyrus, whereas glial cells are generated later than this period, we postulate that the BrdU labeled cells in the dentate gyrus are mainly dentate granule neurons. Our

present observation confirmed that the decreased dentate granule neurons are due to reduced cell generation. This finding also demonstrate that IGFBP-1 modulate IGF-I mitogenic effect on neurons *in vivo*.

In rodent CNS, neurons and glial cells are derived from embryonic neuroepithelial cells of the neural plate. During CNS development, neuroepithelial cells divide and begin to migrate from the neuroepithelium, these are the first neurons. Most neurons are born in the ventricular and subventricular zones (VZ and SVZ) during brain development. It is supposed each region of the ventricular zone is intermixed with progenitor cells capable of generating all the types of neurons and glia. From these proliferative regions, cells migrate to reach their appropriate targets where they differentiate into neurons. Glial cells are generated via intermediate precursor cells in subventricular zone. The subventricular zone generates neurons, astrocytes, microglia and oligodendrocytes (Lois and Alvarez-Buylla, 1993; Skoff et al., 1995). Gliogenesis follows neurogenesis, the production of oligodendrocytes usually following that of astrocytes. Skoff et al (1995) have reported that the neurons are predominant cells in the cerebral cortex of neonatal rat and more glial cells were generated two weeks later. We have observed 18.51% reduction of labeled BrdU positive nuclei in the VZ and SVZ in IGFBP-1 transgenic mice at P3. Our finding indicates IGFBP-1 have inhibitory effect on the dividing precursor cells in the walls of lateral ventricles. We postulate this effect is via its interaction with IGF-I. Since VZ and SVZ are the proliferative regions for most neurons and glia in developing brain, we conclude the inhibited generation of brain cells are mainly account for the loss of total brain cells of IGFBP-1 transgenic mice rather than the increased apoptotic cells observed

in transgenic mice brain as we discuss later.

The insulin-like growth factor controls the cell proliferation also by protecting cells from apoptosis (Jung et al., 1996). *In vitro*, IGF-I not only has mitogenic activity for primary neurons and SH-SY5Y human neuroblastoma cell line, it can also rescues SH-SY5Y human neuroblastoma cells from hyperosmotic induced programmed cell death (Matthews and Feldman, 1996). In this study, the number of apoptotic cells in IGFBP-1 transgenic mice are increased compared with wild-type mice. Our finding strongly suggest the involvement of IGFBP-1 in certain pathways of programmed cell death, most likely, through modulation of the actions of IGF-I. Since the proportion of apoptotic cell is very small, we suggest the increased programmed cell death plays less important role in the decreased brain weight of IGFBP-1 transgenic mice compared with other growth parameters.

Astroglial cells provide physical support for the other cellular elements, their processes surround neurons and their processes often end on the walls of blood vessels, serve metabolic and nutritive function for the neuron. Astrocytes are highly enriched in IGF-I receptors, which unlike neurons, retain the ability to proliferate in the adult brains. IGF-I has stimulatory effects on astrocyte multiplication and glucose uptake (Shemer et al., 1987; Werner et al., 1989a; Masters et al., 1991). IGF-I binds to their respective receptors and stimulate the phosphorylation of the receptor β-subunits in neuronal and astroglia cells in primary culture. Tranque et al (1992) reported that IGF-I can rapidly activate protein kinase-C (PKC) in astrocytes and PKC activation is involved in the mitogenic effects of IGF-I on astroglia cells. From this study, we found that the

distribution of GFAP immunopositive astrocytes in IGFBP-1 mice is identical to that in wild type mice. Quantification of the cell density have showed that the astroglia cell density in hippocampus in transgenic is increased by 11% compared with wild type mice. When the cell density (cell number per mm^2) was normalized by the whole hippocampus area, the overall number of astroglia cells in transgenic mice is still less than that of wild type since the area of hippocampus decreased in transgenic mice compared with wild type mice (2.09 ± 0.19 vs 4.60 ± 0.27, n = 4, p < 0.0001). So the effect of IGFBP-1 transgene expression is to inhibit astroglia proliferation. Quantification of astroglia cell size using a computer assisted image analysis system found no significantly difference between transgenic and wild-type. Our finding demonstrate that the differentiation of astrocytes *in vivo* is not influenced by IGF-I or IGFBP-1 modulated IGF-I actions. The inhibition effect of astroglia proliferation in transgenic mice might be mediated by the IGFBP-1 inhibitory effects on IGF.

During brain development, neurons and astrocyte express IGF-I and its receptor. High levels of IGF-I and its receptor are detected in developing brain, while the adult brain contain low level of this growth factor and its receptor. (Garcia-Segura et al., 1991; Garcia-Estrada et al., 1992; Werner et al., 1989b). IGF-I is also recognized as trophic factor for wound healing (Mueller et al., 1994). Increased IGF-I, IGFBP-2 and IGFBP-3 were induced in astrocytes following hypophysectomizedic-ischemic injury and injection of IGF-1 into lateral ventricle improved outcome.(Williams et al., 1993). Faber-Elman et al. (1996) found IGF-I, in combination with HB-EGF, is a wound-related factor which stimulates astrocyte migration *in vitro*. IGF-I has also been shown to stimulate astrocyte

migration in response to axonal injury *in vitro* (Faber-Elman et al., 1996). Neuronal survival has also been found to be increased by IGF-I. *In vivo* studies have shown IGF-I treatment following brain hypophysectomizedic-ischemic injury can reduce neuronal loss (Williams et al., 1992; Johnston et al., 1996). Thus, the IGF system, might play an important role in response to brain injury. To study the effects of IGF system to neuronal damage and reactive astrocyte after brain damage, we applied kainic acid to induce neurodegeneration and stabwound to induce a mechanical lesion in the brain.

Following mechanical injury, reactive astrocyte expressing high levels of glial fibrillary acidic protein and IGF-I were found in a wilder distribution along the lesioned area and beyond (Garcia-Estrada et al., 1992). In rat, on the 2nd day after injury, significant astrocyte divisions were found by double staining reactive astrocytes expressing vimentin and GFAP and the autoradiographic recording of DNA synthesis. In this study, the percentage of astrocyte density increase after stab wound is decreased in transgenic mice compared with wild type (Fig. 5). So the overall reactive astrocyte are much more than the number seen in transgenic mice. Our finding suggests that IGF-1 promotes astrocyte proliferation near the lesion site. The inhibition effect of astroglia proliferation in transgenic mice might be mediated by the IGFBP-1 inhibitory effects on IGF. The expression of IGFBP-1 inhibits these actions of IGF-I. Our finding that reactive astrocytes were present in transgenic mice, indicate other trophic factors may response to brain injury or that IGFBP-1 only partly block the trophic action of IGF-I on astroglia.

Kanic acid is a potent neuroexcitatory and neurotoxic analogue of glutamate. Pollard et al. (1994) have observed that KA induced cell death in the rat brain has

apoptotic features. The commonly affected areas include the olfactory cortex, amygdaloid complex, hippocampus and related parts of the thalamus and cortex. We postulate IGF-I, like TGF-β1 (Morgan et al., 1993), also responds to KA induced neurodegeneration. The brains may produce neurotrophic factors (IGF-I) following brain damage induced by KA. IGF-I might serve either to restrict the extent of neuronal loss or to facilitate functional recovery. The observation from our preliminary study of KA induced brain injury in IGFBP-1 transgenic was compatible with previous reports that IGF-I has potential to rescue neuronal loss. In addition, the dentate granule neurons were preferentially damaged by KA in transgenic mice, suggest IGFBP-1 overexpression may have selective effect in cell type and brain region.

In summary, our findings demonstrate that IGF-I together with its binding protein-IGFBP-1 are important during brain development and have important role in brain injury.

VII. TABLES

Table 1. Summary of antibodies tested to probe for differences in protein expression and cell type in wild-type and IGFBP-1 transgenic mice

Antibody	Dilution	Producer	Specificity cell type
Monoclonal against OX42	1:100	Sternberger	microglia
Monoclonal against astrocyte*	1:500	Swant	astroglia
Monoclonal against GFAP*	1:100	Serotec	astroglia
Monoclonal against GFAP*	1:50	Boehringer	astroglia
Monoclonal against GFAP*	1:1000	Sternberger	astroglia
Polyclonal against GFAP	1:800	DAKO	astroglia
Polyclonal against 5-HT*	1:1000	Dr. Kimura	serotonergic neuron
Polyclonal against AchE	1:1000	Sternberger	cholinergic neuron
Monoclonal against DβH*	1:500	Chemison	nonadrenergic and adrenergic neuron
Monoclonal against tyrosine hydroxylase*	1:1000		catecholaminergic, dopaminergic, noradrenergic and adrenergic neuron
Monoclonal against ED-1	1:1000	Serotec	macrophage
Monoclonal against panaxonal	1:1000		neurofiliment
Polyclonal against Cx43	1:1000	Elliot Herzberg	Cx43 (gap junction protein)
Polyclonal against IGF-I*	1:2000	Dr. Murphy	IGF-I
Polyclonal against IGFBP-1	1:500	Dr. M. Lewitt	IGFBP-1

* indicates the antibody did not work well or not work at all.

Table 2. Comparison of Body Weight, Brain weight and Total Content of DNA protein in IGFBP-1 transgenic mice and wild-type mice

Measurement	Wild-Type	Tg	Difference (%)
P0 (n = 4)			
Body Weight, g	1.633 ±0.038	1.453 ± 0.054	11%*
Brain Weight, g	0.1027 ± 0.0049	0.07928 ± 0.0012	23%**
Brain DNA, µg	362.7 ± 14.1	313.7 ± 8.4	13.7%***
Brain Protein, mg	5.73 ± 0.18	5.03 ± 0.05	12.2%**
P12 (n = 4)			
Body Weight, g	8.25 ± 0.49	7.49 ± 0.15	9.20%*
Brain Weight, g	0.40 ± 0.010	0.27 ± 0.004	32%*****
Brain DNA, µg	752 ± 9.24	669.3 ± 6.42	11%*****
Brain Protein, mg	27.2 ± 0.23	20.9 ± 0.71	23%**
Adult (> 56 days, n ≥5 or more)			
Body Weight, g	32.1 ± 0.62	28.3 ± 0.85	11.84%****
Brain Weight, g	0.4990 ± 0.011	0.2989 ± 0.017	40.1%****
Brain DNA, µg	809.6 ± 31.7	684 ± 7.7	15.5%***
Brain Protein, mg	47.69 ± 1.82	36.61 ± 0.87	23%****

Transgenic and wild-type mice killed at the ages indicated. CD-1 wild type mice or non-transgenic littermates of IGFBP-1 transgenic mice are used as control group. The brains were removed and homogenized. Aliquots of each homogenate were used for determinate of protein. and DNA. Values are means ± SEM. The significance of differences was evaluated by student's t test. NS indicates not significant at $p > 0.05$. * indicates not significance, ** $p < 0.01$, *** $p < 0.05$, **** $p < 0.005$. ***** $p < 0.0001$.

Table 3. Measurement of White Matter Tracts

	Wild-Type	Tg	N	Difference(%)
Anterior Commissure				
Area (mm^2)	0.113 ± 0.007	0.090 ± 0.02	3	20.3%*
Corpus Callosum				
Thickness (μm)	281.67 ± 9.1	106.67 ± 6.15	6	62%**
Section Cortical to				
Basal (mm)	4.93 ± 0.04787	3.825 ± 0.04787	4	22.34%***

Areas of each wild-type and transgenic mice were quantified in brain sections at corresponding anterior-posterior levels of coronal sections. Values are means ± SEM. The significance of differences was evaluated by Student's t test.

* indicate p< 0.05, ** p < 0.001 and *** p<0.0001 between transgenic and wild-type.

Table 4. Area (mm^2) of Section, Hippocampus and Dentate Granule cell layer

	Wild-Type	Tg	N	Difference(%)
Section	45.74 ± 1.56	30.46 ± 2.57	5	33.40%*
Hippocampus	4.60 ± 0.27	2.09 ± 0.19	4	54.57%**
Dentate Gyrus	0.286 ± 0.01	0.079 ± 0.006	4	72.37%**

Serial transverse sections (20 μm) through the entire extent of the hippocampus in wild type and transgenic mice were stained with thionin and areas were determined as described in Materials and Methods. Areas of each wild-type and transgenic mice were quantified in brain sections at corresponding anterior-posterior levels of coronal sections. Values are means ± SEM and statistical significance was evaluated by Student's t test.

* indicate p< 0.001 and ** p< 0.0001 between transgenic and wild-type.

Table 5. Number of BrDU positive proliferating cells at P3

	Wild Type	Transgenic mice	Difference(%)
Hippocampus			
Dentate gyrus	189 ± 7.92	112 ± 3.34	40.74%[*]
VZ and SVZ	104 ± 3.52	84.75 ± 5.94	18.51%[**]

Immunopositive cells were counted in brain sections at corresponding anterior-posterior levels six each of wild-type and IGFBP-1 transgenic mice for the level of dentate gyrus, seven wild-type and 4 transgenic mice for the level of lateral ventricle were used. Values are means $\pm$ SEM. The significance of differences was evaluated by Student's t test.

* Significantly different from wild type ($p < 0.0001$)

** Significantly different from wild type ($p < 0.05$)

VIII. FIGURES

Fig. 1 Comparison of brains (midline sagittal sections) from age matched IGFBP-1 transgenic mouse (B) and wild-type mouse (A) at P56. Note all major structures including cerebral cortex(Cx), striatum(St), hippocampus(H), thalamus(Thal), colliculus(Col), cerebellum(Cb), brain stem(BS) are present in the brain from IGFBP-1 transgenic mouse and the sizes of all parts were decreased to similar extents as compared with control mice. Magnifications: A,B x40
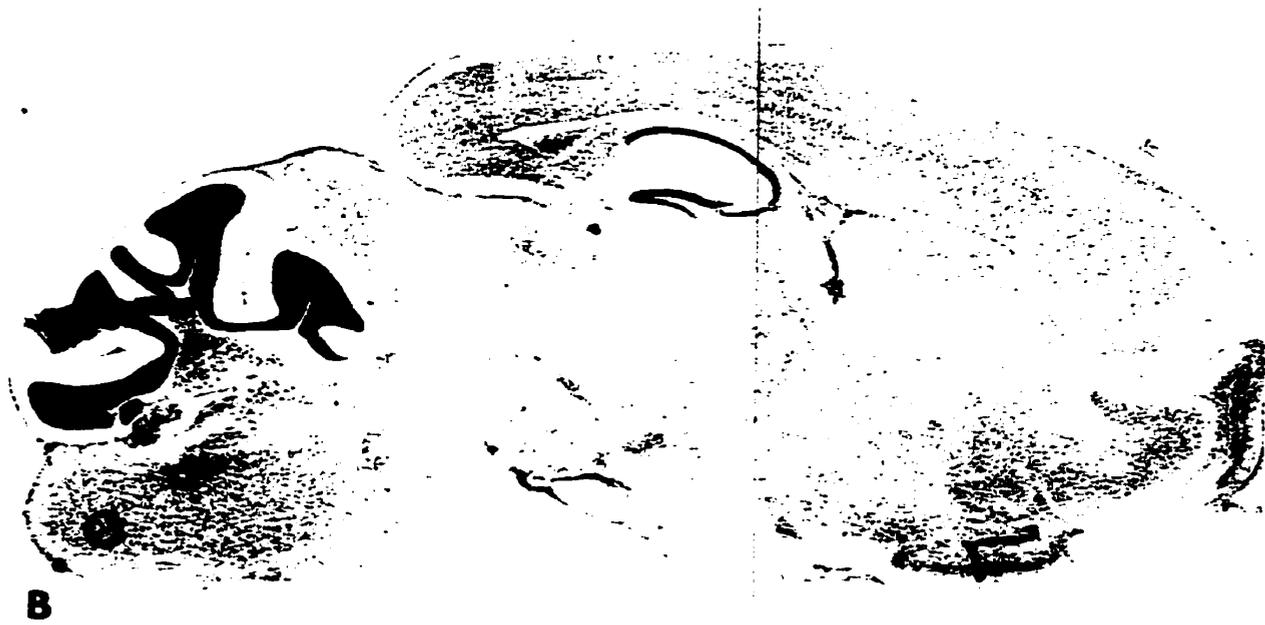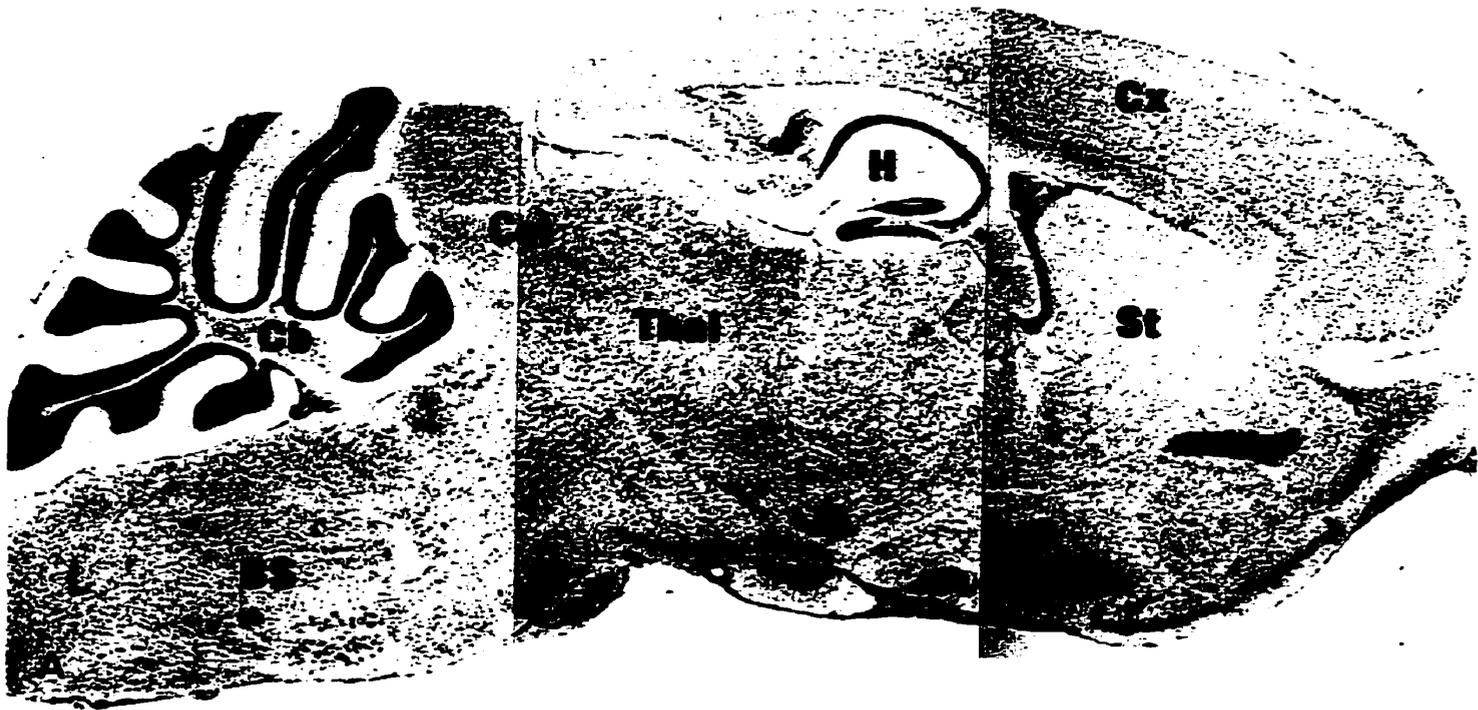
Fig.1

Fig. 2 Photomicrographs of hippocampus (cross section) from IGFBP-1 transgenic and control mice.

A,B: Nissl staining of the dorsal hippocampus illustrate remarkable reduction in size of the dentate gyrus (arrow) and hippocampus in transverse sections from IGFBP-1 transgenic mice (B) compared with wild type control (A).

Magnifications: A,B x70

Fig.2

Fig. 3 Photomicrographs of glial fibrillary acidic protein (GFAP) immunopositivity in the hippocampus of intact and stab wound mice

A,B: GFAP labeled astrocytes in hippocampus of an intact wild type mouse (A) and an intact transgenic mouse (B). C,D: GFAP labeled astrocytes in hippocampus of wild type control mouse at 7 days post-injury in non-lesion side (C) and lesion side (D). Note the high density of reactive, GFAP-positive astrocytes around the lesion site (arrow) and in the non-lesion, contra lateral hippocampus (C). E,F: Same level as C and D at 7 days post-injury in non-lesion side (E) and lesion side (F)of hippocampus from a IGFBP-1 transgenic mouse. Note significantly fewer reactive, GFAP-positive astrocytes were detected around the wound (arrow) and in the non-lesion, contra lateral hippocampus compared with the corresponding area from wild type mouse. G,H: Reactive, GFAP labeled astrocytes were also distributed in adjacent sections next to the lesion. Note the astrocytes (arrow head) are found with very thick and long processes in the hippocampus of a wild type mouse (G) compared with the astrocytes (arrow head) in the corresponding level of a transgenic mouse (H).

Magnifications: A,B x320; C,D,E,F x260; G,H x560
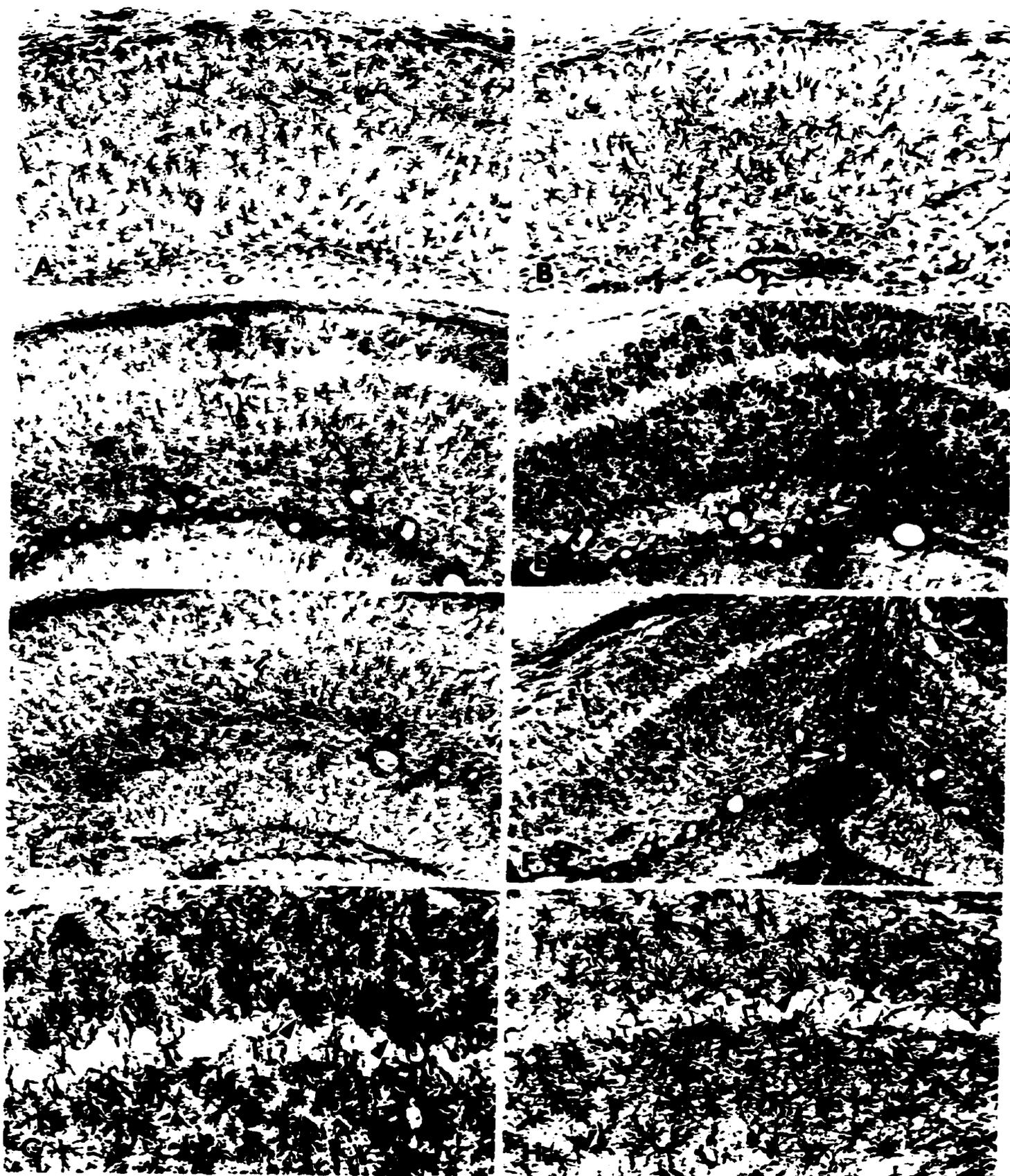
Fig. 3

Fig. 4 Photomicrographs of Brdu-labeled cells at frontal horn at P3 from control wild-type (B) and IGFBP-1 transgenic (D) mice. Note the remarkably decreased number of positive nuclei (arrow) in a transgenic mouse(D) compared with a control mouse(B). A, C: Sections at the same levels of B and D respectively were counter stained with thionin. Note high density of Brdu-labeled proliferating cells in ventricular zone and sub ventricular zone near lateral ventricle (LV).

Magnifications: A,C x560; B,D x320

Fig.4

Fig. 5 Percentage increase of astrocyte density in hippocampus of wild type and transgenic mice after stab wound. Mean of astrocyte density percentage from intact mice is 100%. Values represent means ± SEM from four to six mice. p < 0.0001.
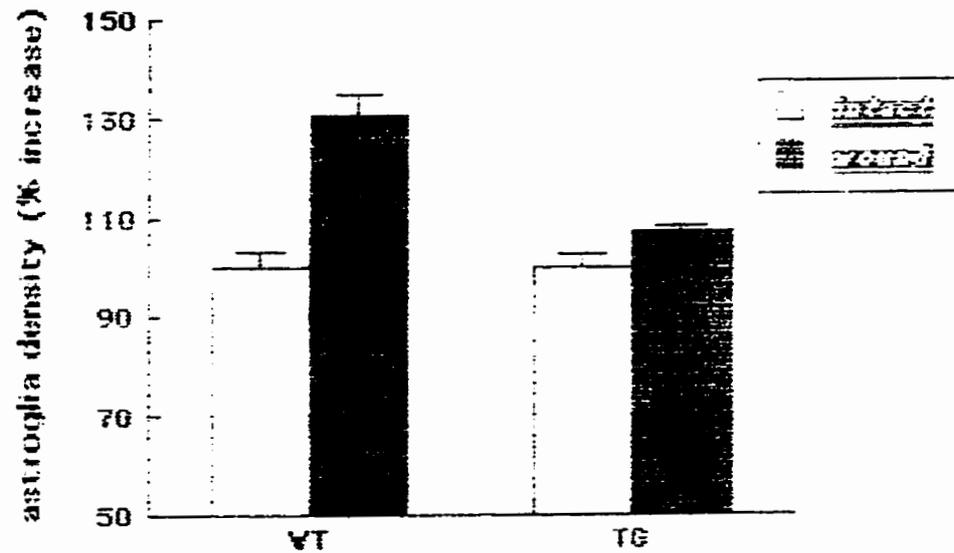
IX. REFERENCES

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

Aguado, F., Fernandez, T., Martinez Murillo, R., Rodrigo, J., Cacicedo, L., and Sanchez Franco, F. (1992) Immunocytochemical localization of insulin-like growth factor I in the hypothalamo-hypophyseal system of the adult rat. *Neuroendocrinology* 56: 856-863

Alzenman, Y., and Devellis, J. (1987) Brain neurons develop in a serum and glial free environment: effects of transferrin, insulin, insulin-like growth factor-I and thyroid hormone on neuronal survival, growth and differentiation. *Brain Res* 406: 32-42

Altman, J., and Bayer, S.A. (1990a) Migration and distribution of two populations of hippocampal granule cell precursors during the perinatal and postnatal periods. *J Comp Neurol* 301: 365-381

Altman, J., and Bayer, S.A. (1990b) Prolonged sojourn of developing pyramidal cells in the intermediate zone of the hippocampus and their settling in the stratum pyramidale. *J Comp Neurol* 301: 365-381

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

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

Barres, B.A., Hart, I.K., Coles, H. S. R., Burne, J. F., Voyvodic, J. T., Richardson, W. D., and Raff, M. C. (1992) Cell death and control of cell survival in the oligodendrocyte lineage. *Cell* 70:31-46

Barres, B.A., and Raff, M.C. (1994) Control of oligodendrocyte number in the developing rat optic nerve. *Neuron* 12: 935-942

Bartlett, W.P., Li, X.S., and Williams, M. (1992) Expression of IGF-I mRNA in the murine subventricular zone during postnatal development *Mol Brain Res* 12: 285-291

Bartlett, W.P., Li, X.S., Williams, M., and Benkovic, S., (1991) Localization of insulin-like growth factor-I mRNA in murine central nervous system during postnatal development. *Dev Biol* 147: 239-250

Baxter, R.C., Martin, J.L., and Wood, M.H. (1987) Two immunoreactive binding proteins for insulin-like growth factors in human amniotic fluid: relationship to fetal maturity *J. Clin Endocrinol Metab* 65, 423-431

Bayne, M.L, Applebaum, J., and Chicchi, G.G. (1988) Structural analogs of human insulin like growth factor I with reduced affinity for serum binding proteins and the type 2 insulin-like growth factor receptor *J Biol Chem* 263: 6233-6239

Beck, F, Samani, N.J, Byrne, S., Morgan, K., Gebhard, R., and Brammar, W.J. (1988) Histochemical localization of IGF-I and IGF-II mRNA in the rat between birth and adulthood. *Development* 104: 29-39

Beck, K.D., Powell-Braxton, L, Widmer, H.R., Valverde, J., and Hefti, F (1995) Igf1 gene disruption results in reduced brain size, CNS hypomyelination, and loss of hippocampal granule and striatal parvalbumin-containing neurons. *Neuron* 14: 717-730

Behringer, R.R., T.M. Lewin, C.J. Quaife, R.D. Palmiter, R.L. Brinster and A.J. D'Ercole. (1990) Expression of insulin-like growth factor I stimulates normal somatic growth in growth hormone-deficient transgenic mice. *Endocrinology* 127: 1033-1040.

Bhrissenden, J.E., Ullrich, A., and Francke U. (1984) Human chromosomal mapping of genes for insulin-like growth factors I and II and epidermal growth factor. *Nature* 310: 781-784

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

Bondy, C.A., Werner, H., Roberts, C.T., and LeRoith, D. (1990) Cellular pattern of insulin-like growth factor-1 and type 1 IGF receptor gene expression in early organogenesis: comparison with IGF-II gene expression. *Mol. Endocrinol* 4: 1386-1398

Bondy, C.A., and Lee,W.H. (1993a) Correction between insulin-like growth factor (IGF)-binding protein 5 and IGF-I gene expression during brain development. *J Neurosci* 13: 5092-5104

Bondy, C.A., and Lee,W.H. (1993b) Patterns of insulin-like growth factor and IGF receptor gene expression in the brain. *Annals New York Academy of Sciences* 692: 33-43

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein binding. *Analytical Biochemistry.* 72, 248-254

Brewer, M.T., Stetler, G.L., Squires, C.H., Thompson, R.C., Busby, W.H., Clemmons, D.R. (1988) Cloning, characterization and expression of a human insulin-like growth factor binding protein. *Biochem Biophus Res Commun* 152: 1289-1297

Brinkman, A., Groffen, C., Lortleve 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* 7:2417-2433

Brinkman, A., Kortleve, D.J., Schuller, A.G.P., Zwarthoff, E.C., and Drop, S.L.S. (1991a) Site-directed mutagenesis of the N-terminal region of IGF binding protein 1; Analysis of IGF binding capability. *FEBS Lett.* 291:264-268

Brinkman, A., Kortleve, D.J., Zwarthoff, E.C., and Drop, S.L.S. (1991b) Mutations in the C-terminal part of insulin-like growth factor (IGF)-binding protein-1 result in the dimer formation and loss of IGF binding capacity. *Mol Endocrinol* 5:987-994

Brown, A.L., Chiarotti, L., Orlowski, C.C., Mehlman, T., Burgess, W.H., Ackerman, E.J., Bruni, C.B., 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

Burch, W.W., Correa, J., Shaveley, J.E., and Powell, D.R. (1990) The 25k Dalton insulin-like growth factor (IGF) Binding protein inhibits both basal and IGF mediated growth in chick embryonic pelvic cartilage in vitro. *J Clin Endocrinol Metab* 70:173-180

Burrin, J.M., Paterson, J.L., and Sharp, P.S., and Yeo, T.H. (1987) Monoclonal and polyclonal antibodies compared for radioimmunoassay of somatomedin-C in patients with acromegaly or hypopituitarism. *Clin Chem* 33:1593-1596

Busby, W.H., Hossenlopp, P., Binoux, M., and Clemmons, D.R. (1989) Purified preparations of the amniotic fluid IGF binding protein contain multimeric forms that are biologically active. *Endocrinology* 125:773-777

Busby, W.H., Klapper, D.G., and Clemmons D.R. (1988) Purification of a 31000 dalton insulin like growth factor binding protein from human amniotic fluid. *J Biol Chem* 263:2289-2296

Campbell, P.G., and Novack, J.F. (1991) Insulin like growth factor binding protein inhibits IGF action in human osteosarcoma cells. *J Cell Physicol* 149:293-300

Campbell, P.G., Novak, J.F., Yanosick, T.B., and McMaster, J.H. (1992) Involvement of the plasmin system in dissociation of the insulin-like growth factor-binding protein complex. *Endocrinology (Baltimore)* 130:1401-1412

Canalis, E., Centrella, M, Burch W, and McCarthy, T.L. (1989) Insulin-like growth factor I mediates selective anabolic effects of parathyroid hormone in bone cultures. *J. Clin Invest* 83:60-65

Caroni, P., and Grandes, P. (1990) Nerve sprouting in innovated adult skeletal muscle induced by exposure to elevated levels of insulin-like growth factors. *J Cell Biol* 110: 1307-1313

Carson, M.J., Behringer, R.R., Brihster, R.C., Mcmorris, F.A. (1993) Insulin-like growth factor I increased brain growth and central nervous system myelination in transgenic mice. *Neuron* 10: 729-740

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. *Regul Pept* 48: 189-198

Conover, C.A., Divertie, D., and Lee P.D.K. (1993) Cortisol increased plasma insulin-like growth factor binding protein-1 in humans. *Acta Endocrinol* 128: 140-143

Cotter, T.G., Lennon, S.V., Glynn, J.G., and Martin, S.J. (1990) Cell death via apoptosis and its relationship to growth , development and differentiation of both tumor and normal cells. *Anticancer Res* 10:1153-1159

Crosby, S.R., Tsigos, C. Anderton, C.D., Gordon, C, Young, R.J., and White, A. (1992) Elevated plasma insulin-like growth factor binding protein-1 levels in type 1 (insulin-dependent) diabetic patients with peripheral neuropathy. *Diabetologia* 35: 868-872

Czech, M.P., (1989) Signal transmission by the insulin-like growth factor *Cell* 59: 235-238

Dai, Z., Xing, Y., Boney, C., Clemmons, D.R., and D'Ercole, A.J. (1994) Human insulin-like growth factor binding protein-1 transgenic mice: characterization and insights into the regulation of IGFBP-1 expression. *Endocrinology* 135: 1316-1327

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

Daughaday, W.H., K. Hall, W.D. Salmon, J.R., J.L. Van Den Brande and J.J. Van Wyk (1987) On the nomenclature of the somatomedins and insulin-like growth factors. *Endocrinology* 121: 1911-1912

D'Ercole, A.J., Dai, Z., Xing, Y., Boney, C., Wilkie, M.B., Lauder, J. M. and Han, V.K. (1994) Brain growth retardation due to the expression of human insulin like growth factor binding protein -1 in transgenic mice: an in vivo model for the analysis of IGF function in the brain. *Dev Brain Res* 82: 213-222

DiCiccio-Bloom, E. and Black, I.B. (1988) Insulin growth factors regulate the mitotic cycle in cultured rat sympathetic neuroblasts. *Proc Natl Acad Sci USA* 85:4066-4070

Downs, T.R. and Wilfinger, W.W. (1983) Fluorometric quantification of DNA in cells and tissues. *Anal Biochem* 131: 538-547

Drago, J., Murphy, M. Carroll, S.M., Harvey R.P., and Bartleth, P.F. (1991) Fibroblast growth factor-mediated proliferation of central nervous system precursors depends on endogenous production of insulin-like growth factor I. *Proc Natl Acad Sci USA* 88: 2199-2203

Drop, S.L.S., Kortleve, D.J. and Guyda, H.J. (1984a) Isolation of a somatomedin binding protein from preterm amniotic fluid. development of a radioimmunoassay. *J Clin Endocrinol Metab* 59: 899-907

Drop, S.L.S., Kortleve, D.J. and Guyda, H.J., and Posner B.I. (1984b) Immunoassay of a somatomedin-binding protein from human amniotic fluid: levels in fetal neonatal, and adult sera. *J Clin Endocrinol Metab* 59:908-915

Elgin, R.G., Busby, W.H., Jr., and Clemmons D.R. (1987) An insulin-like growth factor (IGF) binding protein enhances the biologic response to IGF-I *Proc Natl Acad Sci USA* 84:3254-3258

Ernst M. Heath J.K., and Rodan G. (1989) Estrodiol effects on proliferation, messenger ribonucleic acid for collagen and insulin-like growth factor-I, and parathyroid hormone-stimulated adenylate cyclase activity in osteoblastic cells from calvariae and long bones. *Endocrinology* 125: 825-833

Ezzat, S., Ren, S-G, Braunstein, G. D. and Melmed, S. (1991) Octreotide stimulates insulin-like growth factor binding protein-1: a potential pituitary independent mechanism for drug action. *J. Clin Endocrinol Metab.* 75: 1459-1463

Faber, E.A., Solomon, A., Abraham J. A., and Marikovsky, M., and Schwarta, M. (1996) Involvement of wound-associated factors in rat brain astrocyte migratory response to axonal injury: *in vitro* simulation. *J Clin Invest* 97: 162-171

Fant, M., Munro, H., and Moses, A.C. (1988) Production of insulin-like growth factor binding protein(s) (IGF-BPs) by human placenta: variation with gestational age. *Placenta* 9: 397-407

Feinberg A., and Vogelstein B. (1983) A technique for radio-labeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem*. 132:6-13.

Finch, C.E., Laping, N.J., Morgan, T.E., Nichols, N.R., Pasinetti, G.M. (1993) TGF-beta 1 is an organizer of responses to neurodegeneration. *J Cell Biochem* 53: 314-322

Florini J.R, and Magri K.A. (1989) Effects of growth factors on myogenic differentiation. *Am J Physiol* 256:C701-C711

Florini J.R., and Ewton D.Z. (1990) Highly specific inhibition of IGF-I-stimulated differentiation by an antisence oligodeoxyribonucleotide to myogenin mRNA. No effects on other actions of IGF-I. *J Biol Chem* 265:13435-13437

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

Foran, D.R. and Peterson, A.C. (1992) Myelin acquisition in the central nervous system of the mouse revealed by an MBP Lac Z transgene. *J Neurosci* 12: 4890-4897

Franuman, A.G., Tsuzaki, S., and Moses, A.C. (1989) The binding characteristics and biological effects in FRTL5 cells of placental protein-12, and insulin-like growth factor-binding protein purified from human amniotic fluid. *Endocrinology* 124:2289-2296

.

Garcia-Segura, L.M., Perez, J., Pons, S., Rejas, M.T., and Torres-Aleman, I (1991) Localization of Insulin-like growth factor (IGF-I)-like immunoreactivity in the developing and adult rat brain. *Brain Res* 560: 167-174

Garcia-Estrada, J., Garcia-Segura, L.M., and Torres-Aleman, I (1992) Expression of insulin-like growth factor I by astrocytes in response to injury. *Brain Res* 592: 343-347

Gavrieli, Y., Sherman , Y. Ben, and Osasson, S.A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Bil.* 119, 493-501.

Geduspan, J.S., and Solursh M. (1993) Effects of the mesonephros and insulin-like growth factor I on chondrogenesis of limb explants. *Dev Biol* 156:500-508

Geier A., Haimshon M., Beery R., Hemi R., and Lunenfeld B. (1993) Insulin-like growth factor-I inhibits cell death induced by cycloheximide in MCF-7 cells: a model system for analyzing control of cell death. *In Vitro Cell Dev Biol* 28A:725-729

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 281: 413-417

Giudice, L.C., Dsupin, B.A., Iruin, J.C. (1992) Steroid and peptide regulation of insulin-like growth factor-binding proteins secreted by human endomatrial stromal cells is dependent on stromal differentiation. *J Clin Endocrinol Metab* 75:1235-1241

Gockerman, A., Prevette, T., Jones, J.I., and Clemmons, D.R. (1995) Insulin-like growth factor (IGF) -binding proteins inhibit the smooth muscle cell migration responses to IGF-I and IGF-II. *Endocrenology* 136(10): 4168-73

Goldstein, S., Unterman, T.G., and Phillips, L.S. (1987) Nutrition and somatomedin. XV. Growth plate, growth factor and biologically active somatomedins in tats with streptozotocin-induced diabetes. *Ann Nutr Metab* 31: 367-377

Guler, H.P., Zapf, J., and Froesch, E.R. (1987) Short-term metabolic effects of recombinant human insulin-like growth factor-I in healthy adults. *N Engl J Med* 317:137-140

Guler, H.P., Zapf, J., Schmid, C., and Froesch, E.R., (1989) Insulin-like growth factors I and II in healthy man. Estimations of half-lives and production rates. *Acta Endocrinol* 121:461-468

Hall, K., and Sara, V.R. Growth and somatomedins. (1983) *Vitam Horm* 40: 175-233

Han, V.K.M., Lunder, J.M., D'Ercole, A.J.. (1987) Characterization of somatomedin/insulin-like growth factor receptors and correlation with biological action in cultured neonatal rat astroglial cells. *J Neurosci* 7: 501-511

Han, V.K.M., Lund, P.K., Lee, D.C. and D'Ercole, A.J. (1988) Expression of somatomedin insulin-like growth factor messenger ribonucleic acids in the human fetus: identification, characterization, and tissue distribution. *J Clin Endocrinol Metab*. 66: 22-429

Hansson, H.A., Brandsten, C., Lossing, C., and Petruson, K. (1989) Transient expression of insulin-like growth factor I immunoreactivity by vascular cells during angiogenesis. *Exp. Mol. Pathol*. 50: 125-138

Harris, D.A. , Van Vliet, G., Egli, C.A., Grumbach, M. M., Kaplan, S. L., Styne, D.M. and Vainsel, M. (1985) Somatomedin-C in normal puberty and in true precocious puberty before and after treatment with a potent luteinizing hormone-releasing hormone agonist. *J. Clin. Endocrinol. Metab.* 61: 152-159

Hilding, A., Brismar, K., Thoren, M., and Hall, K., (1993) Glucagon stimulates insulin-like growth factor binding protein-1 secretion in healthy subjects, patients with pituitary insufficiency, and patients with insulin-dependent diabetes mellitus. *J Clin Endocrinol Metab.* 77: 1142-1147

Hizuka, N., Takano, K., Shizume, K., Asakawa, K., Miyakawa, M., and Tanaka, I. (1986) Insulin-like growth factor I stimulate growth in normal growing rats. *Eur J Pharmacol* 125: 143-146

Holly, J.M.P. (1991) The physiological role of IGFBP-1. *Acta Endocrinol* 124(suppl 2):55-62

Hynes, R.O. (1987) Intergrins: A family of cell surface receptors. *Cell* 48: 549-554

Humbel, R.E. (1984) Insulin-like growth factors, somatomedins, and multiplication stimulating activity: chemistry, in *Hormonal Proteins and Peptides, Vol. 12* (Li C. H., ed), pp. 57-79. Academic Press, New York.

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

Ishii, D.N., Wang, C., and Li, Y. (1991) Second messengers mediating gene expression essential to neurite formation directed by insulin and insulin like growth factors. *Adv. Exp. Med. Biol.* 293:361-378

Johnes J. I., and Clemmons, D.R. (1995) Insulin-like growth factors and their binding proteins: Biologicla actions *Endocrine Reviews* 16: 3-34

Johnson, T.R., Blossey, B.K., Denko, C.W., Ilan, J. (1989) Expression of insulin-like growth factor I in cultured rat hepatocytes: effects of insulin and growth hormone. *Mol Endocrinol* 3: 580-587

Johnston, B.M., Mallard, E.C., Williams, C.E., and Gluckman, P.D. (1996) Insulin-like growth factor-1 is a potent neuronal rescue agent after hypoxic-ischemic injury in fetal lambs. *J Clin Invest* 97: 300-308

Jones, J.I., Gockerman, A., Busby, W.H., Wright, G., and Clemmons D.R. (1993) Insulin-like growth factor binding protein 1 stimulates cell migration and binds to the $\alpha5\beta1$ integrin by means of its Arg-Gly-Asp sequence. *Proc Natl Acad Sci USA* 90:10553-10557

Julkunen, M., Koistinen, R., Aalto-Setala, K., Seppala, M., Janne, O.A., and Kontula, K. (1988) Primary structure of human insulin-like growth factor-binding protein/placental protein 12 and tissue-specific expression of its mRNA. *FEBS Lett.* 236:295-302

Jung, Y., Miura, M., and Yuan, J. (1996) Suppression of interleukin-1 beta-converting enzyme-mediated cell death by insulin-like growth factor. *J Biol Chem* 271: 5112-5117

Kiefer, M.C., Ioh, R.S., Bauer, D.M. and Zapf, J. (1991) Molecular cloning of a new human insulin-like growth factor binding protein. *Biochem Biophys Res Commun* 176:219-225

Knusel, B., Michel, P.P., Scheaber J.S., and Hefti, F. (1990) Selective and nonselective stimulation of central cholinergic and dopaminergic development *in vitro* by NGF, FGF, EGF, IGFs-I and II. *J Neurosci* 10: 556-570

Knusel, B. and Hefti, F. (1991) Trophic actions of IGF-I, IGF-II , and insulin on cholinergic and dopaminergic brain neuron. *Adv. Exp. Med. Biol.* 293: 351-360

Kozak, L.P., McLean, G.K., and Eicher, E.M. (1974) Linkage of phosphoglycerate kinase in the mouse *Biochem Genetics* 11: 41-47

Koistinen, R., Itkonen, O, Selenius, P, and Seppala, M. (1990) Insulin-like growth factor-binding protein-1 inhibits binding of IGF-I on fetal skin fibroblasts but stimulates their DNA synthesis. *Biochem Biophys Res Commun* 173: 408-415

Kratz, G., Lake, M., Ljungstrom, K., Forsberg, G., Haegerstrand, A., and Gidlund, M. (1992) Effect of recombinant IGF binding protein-1 on primary cultures of human keratinocytes and fibroblasts: selective enhancement of IGF-1 but not IGF-2-induced cell proliferation. *Exp Cell Res* 202:381-385

Kuzuya, H., Matsuura, N., Sakamoto, M., Makino, H., Sakamoto, Y., Kadowaki, T., Suzuki, Y., Kobayashi, M., Akazawa, Y., Nomura, M., Yoshimasa, Y., Kasuga, M., Goji, K., Nagataki, S., Oyasu, H., and Imura, H., (1993) Trial of insulin-like growth factor I therapy for patients with extreme insulin resistance syndromes. *Diabetes* 42:696-70

Lee, W.H., Michels, K.M. and Bondy, C.A. (1993) Localization of insulin-like growth factor binding protein-2 messenger RNA during postnatal brain development: correlation with insulin-like growth factors I and II. *Neuroscience* 53: 251-265

Lee, Y.L., Hintz, R.L., James, P.M., Lee, P.K.D. Shively, J.E., and Powell D.R. (1988) Insulin-like growth factor (IGF) binding protein complementary deoxyribonucleic acid from human HEPG2 hepatoma cells: Predicted sequence suggests an IGF binding domain different from those of the IGF-I and IGF-II receptors. *Mol. Endocrinol* 2:404-411

Lewitt, M.S., and Baxter, R.C. (1989) Regulation of growth hormone-independent insulin-like growth factor binding protein (BP-28) in cultured human fetal liver explants. *J Clin Endocrinol Metab* 69: 246-252

Lewitt, M.S., Saunders, H., and Baxter, R.C. (1992) Regulation of insulin-like growth factor binding protein-1: the effects of insulin-induced hypoglycemia. *Endocrinology* 131: 2357-2364

Li, S., Resnicoff, M. and Baserga, R. (1996) Effect of mutations at serines 1280-1283 on the mitogenic and transforming activities of the insulin-like growth factor I receptor. *J Biol Chem* 271: 12254-12260

Lieberman, S.A., Bukar, J., and Chen, S.A.(1992) Effects of recombinant human insulin-like growth factor-I (rhIGF-I) in total and free IGF-I concentrations, IGFBPs and glycemic response in humans. *J Clin Endocrinol Metab* 75: 30-36

Liu, F., Powell, D.R., Styne, D.M., and Hintz, R.L. (1991) Insulin-like growth factors (IGFs) and IGF-binding proteins in the developing rhesus monkey. *J. Clin. Endocrinol. Metab.* 72:905-911

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

Liu, L., Brikman, A., Blat, C., and Harel, L. (1991) IGFBP-1, an insulin like growth factor binding protein is a cell inhibitor. *Biochem Biophys Res Commun* 174:673-679

Lois, C., and Alvarez-Buylla, A. (1993) Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. *Proc. Natl. Acad. Sci. U. S. A.* 90: 2074-2077

Lowe, J.R., W.L., Adao, M., Werner, H., Roberts, C.T., and LeRoith, D. (1989) Regulation by fasting of rat insulin-like growth factor I and its receptor. Effects on gene expression and binding. *J. Clin Inbest* 84:619-26

Luo, J.M., Reid, R.E., and Murphy, L.J. (1990) Dexamethasome increase hepatic insulin-like growth factor binding protein-1 (IGFBP-1) mRNA and serum IGFBP-1 concentrations in the rat. *Endocrinology* 127: 1456-1462

Lu, K., and Campisi, J. (1992) Ras proteins are essential and selective for the action of insulin-like growth factor 1 late in the G1 phase of the cell cycle in BALB/c murine fibroblasts. *Proc Natl Acad Sci USA* 89:3889-3893

Luna, A.M., Wilson, D.M., Wibbelsman, D.M., Brown, R.C., Nagashima, R.J. Hintz, R.L. and Rosenfeld, R.G. (1983) Somatomedins in adolescence: a cross-sectional study of the effect of puberty on plasma insulin-like growth factor I and II levels. *J. Clin. Endocrinol. Metab.* 57: 268-271

Lund, P.K., B.M. Moats-Staats, M.A. Hynes, J.G. Simmons, M. Jansen, A.J. D'Ercole and J.J. Van Wyk. (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.

Luo, J.M., and Murphy, L.J. (1991) Differential expression of insulin-like growth factor-I and insulin-like growth factor binding protein-1 in the diabetic rat. *Mol Cell Biochem* 103: 41-51

Luthman, H., Soderling-Barros, J., Persson, B., Engberg, C., Stern, I., Lake, M., Franzen, S-A, Israelsson, M., Raden, B., Lindgren, B., Hjelmqvist, L., Enerback, S., Carlsson, P., Bjursell, G., Povoa, G., Hall, K., and Jornvall, H. (1989) Human insulin-like growth factor

binding protein. Low molecular mass form: Protein sequence and cDNA cloning. *Eur J Biochem* 180: 269-265

Masters, B.A., Werner, H., Roberts, C.T., Leroith, D., and Raizada, M.K. (1991) Developmental regulation of insulin like growth factor-1 stimulated glucose transporter in rat brain astrocytes. *Endocrenology* 128: 2548-2557

Masters, B.A., Werner, H., Roberts, C.T., Leroith, D., and Raizada, M.K. (1991) Insulin like growth factor-1 (IGF-I) receptors and IGF-I action in oligodendrocytes from rat brains. *Regul Pept* 33:117-131

Masters, B.A., and Raizada, M.K. (1993) Insulin like growth factor-1 (IGF-I) receptors and IGF-I action in neuronal cultures from the brains. *Ann N Y Acad Sci* 692:89-101

Mathews, L.S., Norstedt, G., and Palmiter, R.D. (1986) Regulation of insulin-like growth factor I gene expression by growth hormone. *Proc Natl Acad Sci USA* 83: 9343-9347

Mathews, L.S., Hammer, R.E., Behringer, R.R., D'Ercole, A.J., Bell, G.I., Brinster, R.L., and Palmiter, R.D. (1988a) Growth enhancement of transgenic mice expressing human insulin-like growth factor-I. *Endocrinology* 123:2827-2833

Mathews, L.S., Hammer, R.E., Brinster, R.L., Palmiter, R.D. (1988b) Expression of insulin-like growth factor I in transgenic mice with elevated levels of growth hormone is correlated with growth. *Endocrinology* 123:433-437

Matthews, C.C., and Feldman, E.L. (1996) Insulin-like growth factor I rescues SH-SY5Y human neuroblastoma cells from hyperosmotic induced programmed cell death. *J Cell Physiol* 166(2): 323-331

Matthieu, J.M. Widmer, S., and Hershkowitz, N. (1973) Biochemical changes in mouse brain composition during myelination. *Brain Res* 55: 391-402

Mauras, N., Horber, F.F., and Haymond, M.W. (1992) Low dose recombinant human insulin-like growth factor-I fails to affect protein anabolism but inhibits islet cell secretion in humans. *J Clin Endocrinol Metab* 75:1192-1197

McCarthy, T.L., Centrella, M., and Canalis, E. (1990) Cortisol inhibits the synthesis of insulin-like growth factor-I in skeletal cells. *Endocrinology* 126: 1569-1565

McConkey, D.J., Hartzell, P., Nicotera, P., Wyllie, A.H., and Orrenius, S. (1988) Stimulation of endogenous endonuclease activity in hepatocytes exposed to oxidative stress. *Toxicol Lett.* 42: 123-130

McMorris, F.A., Smith, T.M., Desalvo, S., and Furlanetto, R.W. (1986) Insulin-like growth actor I/somatomedin C: a potent inducer of oligodendrocyte development. *Proc Natl Acad Sci USA* 83: 822-826

McMorris, F.A., and Dubois-Dalcq, M. (1988) Insulin-like growth factor I promotes cell proliferation and oligodendroglial commitment in rat glial progenitor cells developing in vitro. *J Neurosci Res* 21: 199-209

Mochizuki, H., Hakeda, Y., Wakatsuki, N., Usui N., Akashi, Ssato, T., Tanaka, K., and Kumegawa, M. (1992) Insulin-like growth factor-I supports formation and activation of osteoclasts. *Endocrinology* 131:1075-1080

Mohn, K.L., Melby, A.E., Tewari, D.S., Laz, T.M., and Taub, R. (1991) The gene encoding rat insulin-like growth factor-binding protein 1 is rapidly and highly induced in regenerating liver. *Mol. Cell. Biol.* 11:1393-1401

Morell, P., Greenfield, S., Constantino-Ceccarini, E., and Wisniewski, H. (1972) Changes in protein composition of mouse brain myelin during development. *J Neurochem* 19: 2545-2554

Mozell, R.L., and McMorris, F.A. (1991) Insulin-like growth factor I stimulates oligodendrocyte development and myelination in rat brain aggregate cultures *J Neurosci Res* 30:382-390

Morgan, D.O., Edman, J.C., and Standring, D.N. (1987) Insulin-like growth factor II receptor as a multifunctioal binding protein. *Nature* 329: 301-307

Mueller, R.V., Hunt, T.K., Tokunaga, A., and Spencer, E.M. (1994) The effect of insulin-like growth factor I on wound healing variables and macrophages in rats. *Arch Surg* 129: 262-265

Murphy, L.J., Bell, G.I., Duckworth, M. and Friesen, H.G. (1987a) Identification, characterization, and regulation of a rat complementary deoxyribonucleic acid which encodes insulin-like growth factor-I. *Endocrinology* 121:684-691

Murphy, L.J., Murphy, L.C., and Friesen, H.G.. (1987b) Estrogen induces insulin-like growth factor-1 expression in the rat uterus. *Mol Endocrinol* 1: 445-450

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

Murphy, L.J., Seneviratne, C., Moreira, P., and Reid, R.. (1991) Enhanced expression insulin-like growth factor binding protein-1 in the fasted rat: The effects of insulin and growth hormone administration. *Endocrinology* 128: 689-696

Muta, K., and Krantz, S.B. (1993) Apoptosis of human erythroid colony-forming cells is decreased by stem cell factor and insulin-like growth factor I as well as erythroprotein. *J Cell Physiol* 156:264-271

Nielsen, F.C., Wang, E., and Gammeltoft, S. (1991) Receptor binding, endocytosis, and mitogenesis of insulin-like growth factors I and II in fetal rat brain neurons. *J Neurochem* 56:12-21

Okajima, T., Iwashita, M., Takeda, Y., Sakamoto, S., Tanabe, T., Yasuda, T., and Rosenfeld, R.G. (1993) Inhibitory effects of insulin-like growth factor (IGF)-binding proteins-1 and -3 on IGF-activated glucose consumption in mouse BALB/c 3T3 fibroblasts. *J Endocrinol* 136:457-470

Olchovsky, D., Bruno, J.F., Gelato, M.C., Song, J., and Berelowitz, M. (1991) Pituitary insulin-like growth factor-I content and gene expression in the streptozotocin-diabetic rat: evidence for tissue-specific regulation. *Endocrinology* 128: 923-928

Ooi, G.T., and Herington, A.C. (1990) Recognition of insulin-like-growth-factor-binding proteins in serum and amniotic fluid by an antiserum against a low-molecular-mass insulin-like-growth-factor-inhibitor/binding protein. *Biochem J.* 267(3): 615-620

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 mRNAs encoding rat insulin-like growth factor binding proteins rIGFBP-1 and rIGFBP-2. *Mol. Endocrinol* 4:321-328

Ooi, G.T., Tseng, LY-H, Tran, M.Q., Rechler, M.M. (1992) Insulin rapidly decreased insulin-like growth factor-binding protein-1 gene transcription in streptozotocin-diabetic rats. *Mol Endocrinol* 6:2219-2228

Orlowski,C.C., Ooi, G.T., and Rechler, M.M. (1990) Dexamethasone stimulates transcription of the insulin-like growth factor binding protein 1 gene in H4-II-E rat hepatoma cells. *Mol Endocrinol* 4: 1592-1599

Orlowsli,C.C., Ooi, G.T., Brown D. R., Yang YW-H, Tseng LY-H and Rechler, M.M. (1991) Insulin rapidly inhibits insulin-like growth factor binding protein 1 gene expression in rat H4-II-E hepatoma cells: The dominant role of insulin. *Mol Endocrinology* 5: 1180-1187

Pahlman, S., Meyerson, G., Lindgren, E., Schalling, M., and Johansson, I. (1990) Insulin-like growth factor I shifts from promoting cell division to potentiating maturation during neuronal differentiation. *Proc Natl Acad Sci USA* 88:9994-9998

Pancal-Roessler, M.K., and Lee P.D.K. (1989) 25kDa insulin-like growth factor binding protein in fetal sera. *Clin Res* 37:458A .

Pekonen, F., Suikkari, A.M., Makinen, T., and Rutanen, E.M. (1988) different insulin-like growth factor binding species in human placenta and decidua. *J Clin Endocrinol Metab* 67: 1250-1257

Pledger, W.J., Stiles, C.D., Antoniades, H.N. and Scher, C.D. (1978) An ordered sequence of events is required before BALB/C3T3 cells become committed to DNA synthesis *Proc. Natl Sci. USA* 75, 2839-2843

Pollard, H., Charriant-Marlangne, C., Cantagrel, S., Represa, A., Roboin, O., Morean, J., and Ben-Ari, Y. (1994) Kainate-induced apoptotic cell death in hippocampal neurons. *Neuroscience* 63:7-18

Povoa, G., Roovete, A., Hall, R., (1984) Cross reaction of serum somatomedin-binding protein in a radioimmunoassay developed for somatomedin-binding protein isolated from human amniotic fluid. *Acta Endocrinol Copenh* 107:563-570

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

Powell D.R., Lee, P.D.K., DePaolis, L.A., Morris, S., Suwnaichkul, A. (1993) Dexamethasone stimulates expression of insulin-like growth factor binding protein-1 in HEP G2 human hapatoma cells. *Growth Regul* 3: 11-13

Powell, D.R., Suwanichkul., Cubbage, M.L., Depaolis, L.A, Snuggs, M. B., and Lee, P.D.K. (1991) Insulin inhibits transcription of the human gene for insulin-like growth factor-binding protein-1 *J Biol Chem* 266: 18868-18876

Price, W.A. Stiles, A.D., Moats-Staats, B.M., D'Ercole, A.J. (1990) Gene expression of insulin-like growth factors (IGFs), the type 1 IGF receptor, and IGF-binding proteins in dexamethasone-induced fetal growth retardation. *Endocrinology* 130:1424-1432

Price, W.A. Stiles, A.D., Moats-Staats, B.M., D'Ercole, A.J. (1992) Changes in insulin-like growth factors (IGF-I and II), IGF-binding protein and IGF receptor transcript abundance after uterine artery ligation. *Pediatr Res* 32:291-295

Rakic, P. (1985) Limits of neurogenesis in primates. *Science* 227: 1054-1056

Rajkumar, K., Barron, D., Lewitt, M.S., and Murphy, L.J. (1995) Growth retardation and hyperglycemia in insulin-like growth factor binding protein-1 transgenic mice. *Endocrinology* 136: 4029-4034

Rajkumar, K., Dheen, T., Krsck., M. and Murphy, L.J. (1995) Impaired estrogen action in the uterus of insulin-like growth factor binding protein-1 transgenic mice. *Endocrinology* 137: 1258-1264

Recio-Pinto, E., Rechler, M.M., and Ishii, D.N. (1988) Insulin and insulin-like growth factor receptors regulating neurite formation in cultured human neuroblastoma cells. *J Neurosci Res* 6: 1211-1219

Rechler, M.M. (1993) Insulin-like growth factor binding proteins *Vitam Horm* 47:1-114

Ritvos, O., Ranta, T., Jalkanen, J., Suikkari, A.M., Voutilainen,R., Bohn, H., and Rutanen, E.M., (1988) Insulin-like growth factor (IGF) binding protein from human decidua inhibits the binding and biological action of IGF-I in cultured choriocarcinoma cells. *Endocrinology* 122:2150-2157

Roberts, C.T. J.R., Lasky, S.R.., Lowe, WL J.R., Seaman, W.T., and Leroith, D. (1987) Molecular cloning of rat insulin-like growth factor I complementary deoxyribonucleic acids: differential messenger ribonucleic acid processing and regulation by growth hormone in extra hepatic tissues. *Mol Endocrinol* 1:243-248

Robertson, D.G., Murino, E.M., Thule, P.M., Seneviratne, C.K., and Murphy, L.J. (1994) Insulin and glucocorticoids regulate IGFBP-1 expression via a common promoter region. *Biochem Biophys Res Commun* 200:226-232

Rodriguze, Tarduchy, G., Collins, M.K., Garcia, L., and Lopez Rivas, A. (1992) Insulin-like growth factor-I inhibits apoptosis in IL-3-dependent hemopoietic cells. *J Immunol* 149:535-540

Rosenfeld, R.G., Wilson, D.M., Lee, P.D.K and Hintz, R.L. (1986) Insulin-like growth factors I and II in evaluation of growth retardation *J Pediatr* 109: 428-433

Rossetti, L., Frontoni, S., Dimarchi, R., DeFronzo, R.A., and Giaccari, A. (1991) Metabolic effects of IGF-I in diabetic rats. *Diabetes* 40:444-448

Rutanen, E-M., Pekonen, F., and Makinen, T. (1988) Soluble 34K binding protein inhibits the binding of insulin-like growth factor I to its own cell receptors in human secretory phase endometrium: evidence for autocrine/paracrine regulation of growth factor action. *J Clin Endocrinol Metab* 66:173-180

Ruoslahti, E., and Piersbacher, M.D. (1987) new perspectives in cell adhesion: RGD and integrins. *Science* 238:491-497

Salmon, W.D., J.R. and W.H. Daughaday. (1957) A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. *J. Lab. Clin. Med.* 116: 408-419

Sara, V.R., and Hall, K. (1990) Insulin-like growth factors and their binding proteins. *Physiol Rev* 70:591-614

Scheiwiller, E., Guler, H.P., Merryweather, J., Scandella, C., and Maerki, W.(1986) Growth restoration of insulin-deficient diabetic rats by recombinant human insulin-like growth factor-I. *Nature* 323:169-171

Schoenle, E.J., Zapf, J., Humbel, R.E., and Froesch, E.R. (1982) Insulin-like growth factor I stimulate growth in hypophysectomized rats. *Nature* 296:252-253

Schoenle, E.J., Zenobi, P.D., Torresani, T., Werder, E.A., Zachmann, M., and Froesch, E.R. (1991) Recombinant human insulin-like growth factor I (rhIGF I) reduces hyperglycemia in patients with extreme insulin resistance. *Diabetologia* 34:675-679

Schwob, J. E., Fuller, T., Price, J.L., and Olney, J.W. (1980) Widespread patterns of neuronal damage following systemic or intracerebral injections of Kainic acid: a histological study. *Neuroscience* 5: 991-1014

Senevirante, C., Luo, J., and Murphy, L.J. (1990) Transcriptional regulation of rat insulin-like growth factor binding protein-1 expression by growth hormone. *Mol Endocrinol* 4: 1199-1204

Shaw, G., and Kamen, R. (1986) A conserved AU Sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* 46; 659-667

Shemer, J., Raizada, M. K., Masters, B., Ota, A., and Leroith, D. (1987) Insulin like growth factor I receptors in neuronal and glial cells: characterization and biological effects in primary culture. *J Bilo Chem* 262:7693-7699

Silbergeld, A., Litwin, A., Bruchis, S., Varsano, I., and Laron, Z. (1986) Insulin-like growth factor I (IGF-I) in healthy children, adolescents and adults as determined by a radioimmunoassay specific for the synthetic 53-70 peptide region. *Clin. Endocrinol* 25: 67-74

Skoff, R.P., and Kanapp, P.E.. (1995) The origins and lineage's of microglial cells. In: *Neuroglia* Edited by Kettenmann, H and Kanson, B. R. Oxford University Press

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol.* 98:503-517

Spinas, G.A., Zapf, J., Landolt, A.M., Stuckmann, G., and Froesch, E.R. (1987) Pre-operative treatment of 5 acromegalics with a somatostatin analogue endocrine and clinical observations. *Acta Endocrinol Copenh* 114: 249-256

Staines, W.A., Yamamoto, T., Daddona, P.E., and Nagy, J.I. (1986) Adenosine deaminase containing hypothalamic neurons accumulate 5-HTP: A dual color immunofluorescence procedure using a new fluorescence marker. *Neurosci Lett* 70:1-5

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

Takahashi, T., Nowakowski, R.S., and Caviness, V.S. (1992) BUDR as an S-phase marker for quantitative studies of cytokinetic behavior in the murine cerebral ventricular zone. *J. Neurocytol.* 21, 185-197

Tollefsen, S.E., Lajara, R., McCusker, R.H., Clemmons, DR, and Rotwein, P (1989) Insulin-like growth factors (IGF) in muscle development. *J Biol Chem* 264: 13810-13817

Tranque, P.A., Calle, R., Naftolin, F., and Robbins, R. (1992) Involvement of protein kinase-c in the mitogenic effect of insulin-like growth factor-I on rat astrocytes. *Endocrinology* 131(4): 1948-1954

Turkalj, I., Keller, U., Ninnis, R., Vosmeer, S., and Stauffacher, W. (1992) Effect of increasing doses of recombinant human insulin-like growth factor-I on glucose, lipid, and leucine metabolism in man. *J Clin Endocrinol Metab* 75:1186-1191

Ullrich, A. Gray, A. and Tam, A.W. (1986) Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. *EMBO J* 5: 2503-2512

Unterman, T.G., Oehler, D.T., Murphy, L.J., and Lacson, R.G., (1991) Multihormonal regulation of insulin-like growth factor binding protein-1 in rat H4IIE hepatoma cells: the dominant role of insulin. *Endocrinology* 128: 2693-2701

Unterman, T.G., Patel, K., Mahathre, V.K., Rajamohan, G., Oehler, D.T., and Becker, R.E. (1990) Regulation of low molecular weight insulin-like growth factor binding proteins in experimental diabetes mellitus. *Endocrinology* 126: 2614-2624

Van Buul-Offers, S., Ueda, I. and Vand den Brande, J.L. (1986) Biosynthetic somatomedin C (SMC/IGF-I) increased the length and weight of snell Dwarf mice *Pediatr Res* 20:825-827

Van Wyk, J.J. (1984) In *Hormonal proteins and peptides*, Vol. XII (Li, C. H., ed) pp81-125, Academic Press, Orlando FL. USA

Villafuerte, B.C., Goldstein, S., Robertson, D.G., and Phillips, L.S. (1991) Regulation of insulin-like growth factor binding protein in primary cultures of normal rat hepatocytes. *Diabetes* 40: 837-841

Waites, G.T., James, R.F.L., and Bell, S.C. (1989) Human "pregnancy-associated endometrial α-globulin", an insulin-like growth factor-binding protein: Immunohistological localization in the decidua and placenta during pregnancy employing monoclonal antibodies. *J Endocrinol* 120:351-357

Westwood, M., Gibson, J.M., Daries, A.J., Young, R.J., White, A. (1994) The phosphorylation pattern of insulin like growth factor-binding protein-1 in normal plasma is different from that in amniotic fluid and changes during pregnancy. *J Clin Endocrinol Metab* 79: 1735-1741

Werner, H., Raizada. M. K., Mudd, L.M., Foyt, L., Simpson, A., Roberts, C.T., and Leroith, D. (1989a) Regulation of rat brain/HepG2 glucose transporter gene expression by insulin and IGF-I in primary culture of neuronal and glial cells. *Endocrinology* 125: 314-320

Werner, H., Woloschak, M., Adamo, M., Shen-Orr, Z, Roberts, C.T., and Leroith, D. (1989b) Developmental regulation of rat insulin-like growth factor I receptor gene. *Proc Natl Acad Sci USA* 86: 7451-7455

Williams, C., Guan, J., Miller, O., Beilharz, E., McNeill, H., Sirimanne, E., and Gluckman, P. (1993) The role of the growth factors IGF-I and TGF-β_1 after hypoxic-ischemic brain injury. *Annals New York Academy of Sciences* 692: 306-307

Yamasaki, H., Prager, Da, and Melmed, S. (1993) Structure-function of the human insulin-like growth factor-I receptor: a discordance of somatotroph internalization and signaling. *Mol Endocrinol* 7:681-685

Ye, P., Carson, J., and D'Ercole, A.J. (1995) In vivo actions of insulin-like growth factors (IGF-I) on brain myelination: studies of IGF-I and IGF binding protein-1 (IGFBP-1) transgenic mice. J Neurosci 15: 7344-7356

Young, S.C., Miles, M.V., and Clemmons, D.R., (1992) Determination of the pharmacokinetic profiles of insulin-like growth factor binding proteins-1 and -2 in rats. *Endocrinoloy* 131:1867-1873

Zapf, J., Hauri, C., Waldvogel, M., and Froesch, E.R. (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