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Insulin-Like Growth Factor Binding Protein-2 Inhibits Proliferation in MAC-T Cell Cultures

By

Maria E. Zavala

AN ABSTRACT OF A THESIS

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ABSTRACT

Insulin-Like Growth Factor Binding Protein-2 Inhibits Proliferation in MAC-T Cell Cultures

By

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Insulin-like growth factor-I (IGF-I) is a potent mitogen for mammary epithelial cells *in vitro*. Insulin-like growth factor binding protein-2 (IGFBP-2) is the predominant IGFBP synthesized by mammary epithelial cells *in vitro* and is thought to regulate IGF-I action. To investigate the direct effects of IGFBP-2 and IGF-I on proliferation in mammary epithelial cells, IGFBP-2 was added to cultures of MAC-T cells on collagen I with and without IGF-I. Addition of IGFBP-2 inhibited the proliferation of MAC-T cells in the presence and absence of IGF-I. This inhibition acted in a dose dependant manner where 100 ng/ml IGFBP-2 inhibited proliferation to a greater extent than 10 ng/ml IGFBP-2 ($P < 0.0001$). However, the magnitude of the effect was greater in the presence of IGF-I ($P < 0.001$). For example, 100 ng/ml IGFBP-2 decreased proliferation by 40%. in the absence of IGF-I, while 100 ng/ml IGFBP-2 decreased IGF-I-stimulated cell proliferation by 51% in the presence of IGF-I. In conclusion, IGFBP-2 inhibits the proliferation of MAC-T cells *in vitro*. This suggests IGFBP-2 may play a role in regulating mammary epithelial cell proliferation *in vivo*.

For My Beloved Husband James

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LIST OF ABBREVIATIONS

DMSO ..... Dimethy sulfoxide
DNA ..... Deoxyribonucleic acid
df ..... degrees of freedom
GH ..... Growth hormone
IGF ..... Insulin-like growth factor
IGFBP ..... Insulin-like growth factor binding protein
MAC-T ..... Mammary alveolar cells transfected
mRNA ..... messenger Ribonucleic acid
MW ..... Molecular weight
SV 40 ..... Simian virus 40
RGD ..... Arganine-Glycine-Aspartate

INTRODUCTION

In ruminants, the number of the secretory cells in the mammary gland is a major factor that determines milk yield (Tucker, 1987). Efforts to increase this number have the potential to increase milk yield and efficiency of production. Selective breeding of dairy cows has led to great increases in secretory cell number and therefore milk yield (Forsyth, 1996). A greater understanding of the physiological factors responsible for this increase in cell number may lead to further improvements in milk production. One such factor is growth hormone (GH). Studies of GH have revealed the existence of a potent mitogen for the secretory cells of the bovine mammary gland. This mitogen is insulin-like growth factor-I (IGF-I). Increasing our knowledge of these regulatory influences in the IGF-I system may enable further increases in mammary secretory cell number and thereby improve milk production.

Administration of exogenous GH to dairy cows has mitogenic and mammogenic effects on the bovine mammary gland (Sejrsen et al., 1986; Peel and Bauman, 1987). Since GH receptors have not been detected in bovine mammary tissue *in vitro* and GH has no effect on proliferation of this tissue *in vitro*, GH is not believed to act directly on mammary tissue *in vivo* (Akers, 1985; Baumrucker and Stemburger, 1989). However, its actions may be carried out indirectly through IGF-I. Insulin-like growth factor-I is a protein that is predominantly produced by the liver of animals and humans (Daughaday and Rotwein, 1989). However, because IGF-I is produced by many tissues in addition to the liver (D'Ercole et al., 1984), GH is considered to act by stimulating IGF-I in target tissues,

which then acts in an autocrine or paracrine manner on local cells (Holly and Wass, 1989).

When IGF-I binds to membrane receptors on secretory cells in the mammary gland it stimulates cell proliferation of these secretory or epithelial cells *in vitro* (Jones and Clemmons, 1995; McGrath et al., 1991). Though the majority of IGF-I is produced in the liver, it is also secreted locally by the stromal cells of the mammary gland (Campbell et al., 1991). The localized production of IGF-I and its mitogenic effects in mammary cultures suggest that IGF-I is a factor of great importance in stimulating mammary epithelial cell proliferation *in vivo*.

Insulin-like growth factor binding proteins (IGFBPs) are a family of proteins that regulate IGF-I activity. Insulin-like growth factor binding proteins have a higher affinity for IGF-I than does the membrane receptor for IGF-I and are thought to play a regulatory role in IGF action (Jones and Clemmons, 1995). Insulin-like growth factor binding proteins are synthesized in a wide variety of tissues in the body and may bind to IGF-I either in the tissues or in the blood. In the mammary gland, epithelial cells secrete IGFBPs, particularly IGFBP-2 (Campbell et al., 1991; Hodgkinson et al., 1991; and Collier et al., 1993).

Serum IGFBP-2 concentration increases when ruminants enter a catabolic state as during lactation, fasting, and mild insulinopenia (Sharma et al., 1994; Cohick et al., 1989; Ooi, 1990). In addition, serum concentrations of IGFBP-2 decrease while IGF-I mRNA and

serum IGF-I protein concentration both increase during treatment with GH (Sharma et al., 1994; Cohick et al., 1989; Ooi, 1990). To investigate the direct effects of IGFBP-2 on IGF-I on mammary epithelial cells, an *in vitro* model was developed.

Validation of several *in vitro* models of bovine mammary epithelial cells has already been achieved (McGrath, 1987; Baumrucker and Stemburger, 1989; Peri et al., 1992; and Huynh et al, 1991). One such model employs the MAC-T cell line. The MAC-T cells are a nontransformed cell line derived from bovine mammary alveolar cells. These cells proliferate in response to IGF-I treatment (Woodward et al., 1994) and have been shown to bind IGF-I via cell surface receptors for the IGF-I ligand (Zhao et al., 1992). Primary bovine mammary cells and tissue also bind and proliferate in response to IGF-I treatment (Shamay et al., 1988; Baumrucker and Stemburger, 1989; and McGrath et al., 1991). Thus, the MAC-T cells provide a useful model for studying the role of the IGF system in mammary growth and development.

The objective of this thesis was to determine the effects of IGFBP-2 addition on IGF-I-stimulated mitogenic activity in MAC-T cell cultures. A series of three experiments was designed to determine the effects of IGF-I and to determine the effects of IGFBP-2 addition on MAC-T cell proliferation in my system. The specific objectives of experiments were:

1. To determine the effects of exogenous IGF-I on MAC-T cell proliferation for concentrations of IGF-I ranging from 0.001 ng/ml to 100ng/ml.

2. To determine the effects of exogenous des (1-3) IGF-I on MAC-T cell proliferation for concentrations of des (1-3) IGF-I ranging from 0.01 ng/ml to 100 ng/ml.
3. To determine the effect of IGFBP-2 addition on IGF-I stimulated mitogenic activity in MAC-T cell cultures.

Though the addition of IGFBPs to mammary cell cultures has been accomplished in several laboratories (McGuire et al., 1992, Oh et al., 1993a, 1993c; Yee et al., 1994; and Chen et al., 1994), the addition of IGFBP-2 to the MAC-T cells has never been reported. Efforts to establish the role of the IGFBPs in mediating IGF-I mitogenic activity or in actions independent of IGF-I may provide insight into the regulation of mammary growth and development. Increasing our understanding of this regulation has potential to increase the efficiency of milk production.

Review of Literature

Insulin-like growth factor-I

Insulin-like growth factor-I (IGF-I) is a 7.6 kDa protein composed of approximately 70 amino acids (Daughaday and Rotwein, 1989). The IGF-I molecule is composed of four domains on a single peptide chain. These domains are designated A, B, C, and D; A and B domains are homologous with A and B chains of the insulin molecule (Thissen et al., 1994). The IGF-I molecule also shares structural homology across several species. The IGF-I molecule has an identical amino acid sequence in the bovine, human, and pig, differing from ovine IGF-I by one amino acid and from murine IGF-I by five amino acids (Wong et al., 1989).

Insulin-like growth factor-I is found solubilized in serum, embedded in extracellular matrix, and distributed throughout the extracellular spaces surrounding cells. The highest concentration of IGF-I is found in blood (Furlanetto et al., 1977). The source of serum IGF-I is believed to be the liver (D'Ercole et al., 1984). However, IGF-I is produced in most organs (D'Ercole et al., 1984); such as the kidney, liver, heart and testes; and by many cell types such as fibroblasts, smooth muscle, skeletal muscle, liver, granulosa, Sertoli, and decidua cells (Sara and Hall, 1990). Production of IGF-I at multiple sites in the body leads to the concept that IGF-I acts by endocrine, autocrine, and paracrine mechanisms (Figure 1) (Schlecter et al., 1986; and Holly and Wass, 1989).

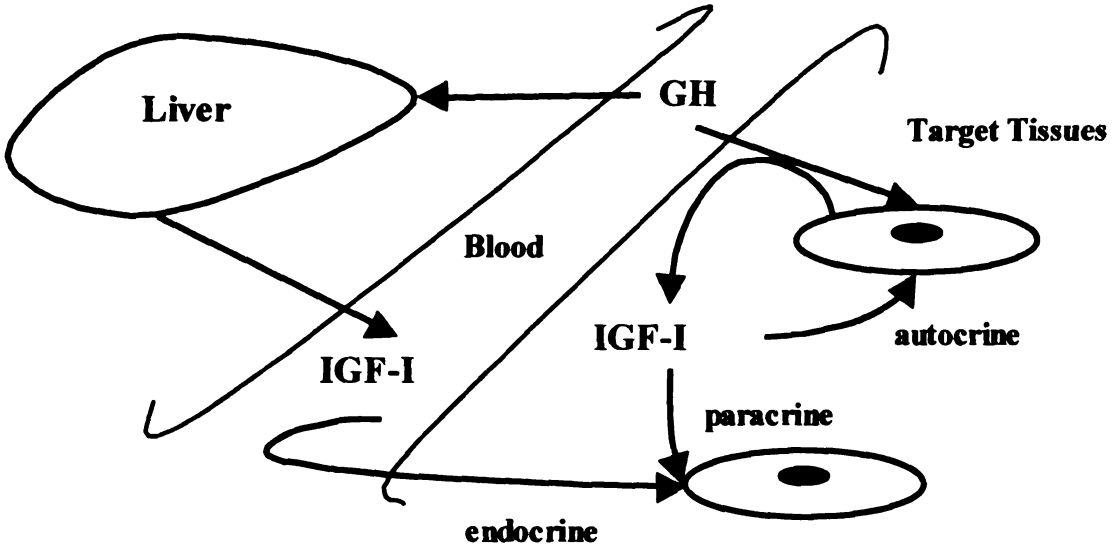

Figure 1. IGF-I may act in an endocrine, autocrine, and/or paracrine fashion. GH can act upon the liver and/or the tissues stimulating IGF-I secretion into either the blood or the extracellular space. The IGF-I secreted can act locally in an autocrine or paracrine fashion, or can be carried by the blood to target tissues and act in an endocrine fashion.

Regulation of IGF-I production

One of the principle hormonal stimuli for IGF-I production is growth hormone (GH) (Copeland et al., 1980). Growth hormone binding to the GH receptor increases serum IGF-I by inducing IGF-I synthesis in the liver (Bichell et al., 1992). Hypothalamic GH-releasing hormone (GHRH) regulates the inductive effect of GH on IGF-I (Shibaski et al., 1986), such that high levels of circulating IGF-I provide a negative feedback to the

hypothalamus that decreases the secretion of GHRH and GH (Ceda et al., 1987). This negative feedback loop provides a mechanism for acute regulation of IGF-I production by liver (Hill, 1989), as well as other tissues where IGF-I production is under control of GH (Figure 2).

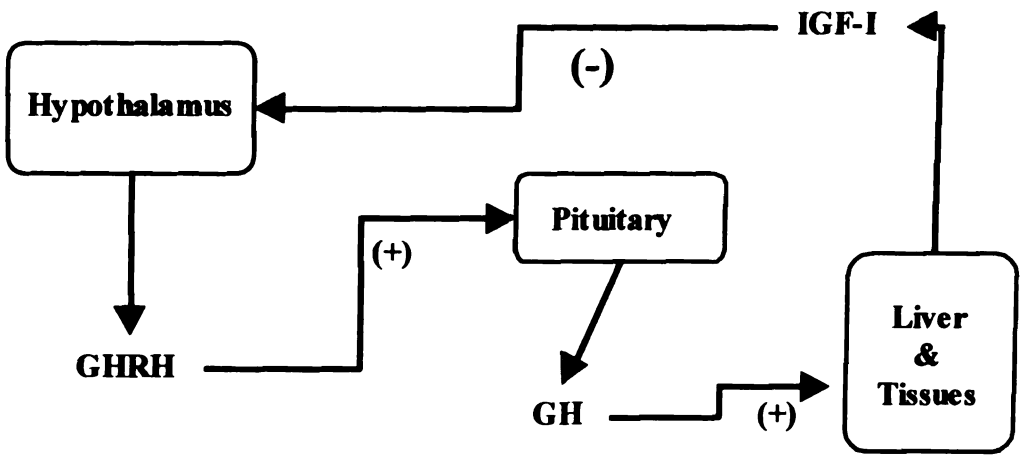

Figure 2. GH binding to the liver and tissues stimulates the production of IGF-I. High levels of circulating IGF-I provide a negative feedback to the hypothalamus that results in a decrease in the secretion of GHRH, a decrease in pituitary secretion of GH, and thereby a decrease in the production of IGF-I.

In addition to hormonal stimulation, nutrition plays a primary role in regulation of IGF-I production (Thissen et al., 1994). Suppression of serum IGF-I levels by energy/protein deprivation has been well documented in many different species (Clemmons and Underwood, 1991; McGuire et al., 1992; and Bauman and Vernon, 1995). Furthermore, the decline of serum IGF-I during dietary restriction is independent of the increase in pituitary GH secretion that occurs at the same time. This decline in serum IGF-I

concentration results from a decrease in hepatic IGF-I production and an increase in the clearance of IGF-I from the blood (Clemmons and Underwood, 1991). Multi-level regulation of growth factors like this link diet to growth in the body. Interestingly, nutrient excess does little to raise the concentration of IGF-I in serum, meanwhile nutritional deficiency reduces the concentration of IGF-I in serum (Thissen et al., 1994). In addition, serum IGF-I in obese subjects are reported to be either low or high (Thissen et al., 1994). Further discussion of the nutritional regulation of IGFs can be obtained in the review by Thissen et al. (1994).

IGF-I action

Insulin-like growth factor-I action is mediated through the type-I IGF receptor. The type-I IGF receptor is a transmembrane receptor that shares structural homology with the insulin receptor (Hintz et al., 1972). The IGF-I ligand initiates signal transduction by binding to the cystine-rich regions of the alpha subunit's extracellular domain. Tyrosine kinase activity resides in the cytoplasmic beta domain, which causes auto-phosphorylation of insulin receptor substrate-I and propagation of the cytoplasmic signal by intracellular machinery (Jones and Clemmons, 1995). Insulin-like growth factor type-I receptors are present in a wide variety of cell types, and are critical in regulating the effects of IGF-I at the cellular level (Sara and Hall, 1990).

The *in vitro* action of IGF-I has been established in a wide variety of cells. In general, the effects of IGF-I can be separated into two categories: acute and chronic. The acute effects of IGF-I include anabolic effects on protein and carbohydrate metabolism. The

chronic effects of IGF-I include effects on cell replication and differentiation. Jones and Clemmons (1995) have reviewed the effects of the IGFs in multiple cell types and determined that the end result of IGF action is dependent on cell type, cell environment, and stage of cell differentiation. Other factors known to modulate the effect of IGF-I on the target cells are the insulin-like growth factor binding proteins (IGFBPs).

IGFBPs

Insulin-like growth factor-I is present in circulation and extracellular space almost entirely bound to IGFBPs (Jones and Clemmons, 1995). The high affinity of these proteins for IGF-I suggests that the IGFBPs are involved in the mediation of IGF-I action (Oh et al., 1993b; Rosenfeld et al., 1994). Furthermore, reversibility of this binding of IGF-I with any one of the IGFBPs provides modulation of the interactions between IGF-I and its receptor.

Of all the serum protein fractions with high affinity for IGF-I, more than ten have been identified (Rechler, 1997, Baumrucker and Erondu, 2000), and seven have been cloned (Binoux, 1995; Oh et al., 1996). Of these binding proteins, six have been shown to bind IGF-I with high affinity (IGFBPs 1-6), and nine have been shown to bind IGF-I with much lower affinity (IGFBP related proteins 1-9) (Baumrucker and Erondu, 2000). These binding proteins are commonly referred to as IGFBP-1, -2, -3, -4, -5, and –6. Similar to IGF-I, the IGFBPs are synthesized ubiquitously, although their expression varies with tissue origin, developmental stage, and environment (Rechler et al., 1997). The IGFBPs-1 through -6 are structurally related proteins, but are encoded by different

genes (Rechler et al., 1993). The six molecular species of IGFBPs have molecular masses that range between 25 and 45 kDa and can be detected by western ligand and immunoblotting techniques (Hossenlopp et al., 1986). The structural differences between the six IGFBPs also confer their difference in affinities for IGF-I (Table 1) (Oh et al., 1993b).

Table 1. A summary of the structural characteristics of human IGFBPs –1 through -6. Adapted from Rosenfeld et al. (1994); and Jones and Clemmons (1995).

Structural Characteristic	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6
MW	25,271	31,355	28,717	25,957	28,553	22,847
Number of amino acids	234	289	264	237	252	216
RGD Sequence	+	+	-	-	-	-
Glycosyla-tion sites	-	-	+	+	+	+
Phos-phorylation	+	-	+	-	+	NA**
Proteolysis	-	+	+	+	+	NA**

****Data for some characteristics are not yet available (NA).**

Further analysis of the structures of the IGFBPs reveals the presence of an RGD (Arginine-Glycine-Aspartate amino acid) sequence. This sequence is involved in ligand recognition by integrin receptors and suggests that IGFBPs may associate with the cell surface and carry out actions independent of IGF-I binding. The RGD sequences have been identified on IGFBP-1 and -2 (Brewer et al., 1988). However, only IGFBP-3 appears capable of specific binding to cell membrane receptors (Oh et al., 1993 a, c), even though this IGFBP lacks an RGD sequence.

Another theory regarding the ability of IGFBPs to act independently of IGF-I suggests the involvement of postranslational processing. IGFBP-3 undergoes limited proteolysis in serum (Hossenlop et al., 1990). The IGFBP-3 products of this proteolysis are two different types of fragments with different activities. One has a weak affinity for the IGFs and is only a weak antagonist of IGF action. The other lacks affinity for the IGFs but nevertheless inhibits IGF-stimulated mitogenesis, thus acting by a mechanism that is independent of the IGFs (Lalou et al., 1996). Thus, not only are there at least six distinct IGFBP genes producing distinct translational products, but *in vivo*, some IGFBPs undergo postranslational modifications that may alter their biological activity.

Other modifications to IGFBP structure include phosphorylation, glycosylation, and changes in location such as cell surface association vs. embedding in the extracellular matrix (Jones and Clemmons, 1995). These alterations add another dimension to the regulation of IGF-I action by the IGFBPs. However, the effects of these alterations on

the affinity of the IGFBPs for IGF-I, and the physiological significance of these alterations have not been determined.

IGFBP production

Most cells tested appear to secrete at least one form of IGFBP though the level of secretion and profile of IGFBP produced can vary dependant on cell type, tissue origin, developmental stage, hormonal and nutritional environment. Discussions of these factors and details of their impact on IGFBP production can be found in reviews by Baxter and Martin (1989), Rechler et al. (1993), and Ferry et al. (1999).

Though the relative amounts of each of the IGFBPs vary, factors associated with fluctuations of IGFBPs-1 through -3 have been evaluated in serum. The major factors affecting serum IGFBP levels are diet and serum GH. In general, dietary restriction decreases IGFBP-3 concentrations while it increases serum IGFBP-1 and -2 (Thissen et al., 1994). These changes in serum levels of each of the IGFBPs are parallel to changes observed in abundance of their respective mRNA levels in the liver (Thissen et al., 1994). Because IGF-I and insulin-like growth factor binding protein-3 concentrations in serum increase during anabolic states and decrease during catabolic states, IGFBP-3 is thought to enhance IGF-I action in target tissues (Thissen et al., 1994). In addition, because IGFBP-2 increases and IGF-I decreases during catabolic states, IGFBP-2 is thought to inhibit IGF-I action in target tissues (Thissen et al., 1994).

Aside from the influence of the diet on serum concentrations of IGFBPs, GH also regulates IGFBP concentrations in serum. In humans, GH dependency of IGFBP-3 is evident from the reduced serum concentrations of IGFBP-3 measured during states of GH deficiency and insensitivity (Rosenfeld et al., 1994). In cattle, elevated concentrations of IGFBP-3 in serum are observed during administration of GH (Vicini et al., 1991; Cohick et al., 1992; and VanderKooi et al., 1995). Similarly to IGFBP-3, IGFBP-2 also displays a relationship with GH. When exogenous GH is administered to cattle, serum concentrations of IGFBP-2 decrease (Vicini et al., 1991; Cohick et al., 1992; McGuire et al., 1992). The relationship between GH and IGFBP-2 and -3 are of interest because IGFBP-2 and -3 are the predominant IGFBPs in serum; thus their concentrations are relevant to the availability and action of serum IGF-I (Rosenfeld et al., 1994).

The effects of GH on the serum concentrations of the IGFBPs described above become uncoupled by undernutrition (Clemmons and Underwood, 1991). This phenomenon is evident as circulating concentrations of IGFBP-3 decrease rapidly upon fasting even though GH levels are increased (Clemmons and Underwood, 1991). Insulin-like growth factor binding protein-2 also becomes uncoupled during undernutrition as serum concentrations of IGFBP-2 are highest when GH is high even though GH is known to decrease serum levels of IGFBP-2 when applied exogenously (Vicini et al., 1991). These results demonstrate the complexity of nutritional status on the GH/IGF axis.

IGFBP action

There are several biological properties that are shared among the IGFBPs. Shared properties have led to development of several theories describing the relationship between the IGFBPs and IGF-I. Some of these are: 1) IGFBPs regulate the transport of IGF-I from the blood to the extravascular space; 2) IGFBPs prolong the half-life of IGF-I and decrease its rate of clearance from blood; 3) IGFBPs target IGF-I to specific tissues or cells; 4) IGFBPs directly modulate the interaction between IGF-I and the type-I IGF receptor; and 5) IGFBPs have direct effects on cellular functions that are mediated by cell surface IGFBP receptors (Jones and Clemmons, 1995). Though evidence in the literature suggests the IGFBPs may possess many of these roles, the biological role of each specific IGFBP is not well defined.

The IGFBPs have multiple modes of action: endocrine, paracrine and autocrine. Soluble IGFBPs are believed to attenuate IGF-I action (Elgin et al., 1987). Cell-associated IGFBPs may either attenuate or enhance IGF-I action (Jones and Clemmons, 1995). While the physical properties of the IGFBPs have been the target of extensive investigations during the last decade, the diversity among the IGFBPs and their effects on different target cell systems prevents the formation of a common mechanism of action that would explain all of their functions.

The role of the GH-IGF-I axis in bovine mammary gland growth and lactation

Mammary Growth

During puberty, bovine mammary tissue enters a phase of allometric growth. The number of mammary secretory cells that develops during this period may play an important role in determining future milk yield (Tucker, 1987). Several studies have examined the relationship between GH and pubertal bovine mammary growth. One such study has shown that circulating GH is positively correlated with mammary growth in peri-pubertal heifers (Sejrsen et al., 1983). In addition, several studies have shown that GH administration to pubertal heifers increases mammary parenchyma (Sejrsen et al, 1986; Sejrsen et al., 1999). In contrast, overfeeding heifers results in a decrease in endogenous GH and decreased mammary development (Sejrsen et al., 1983). Thus GH is believed to play an important role in promoting pubertal mammary growth.

During the dry period, the bovine mammary gland undergoes substantial growth and development in preparation for lactation. Due to a dearth of data on the effect of GH on mammogenesis during the dry period, much of the support for a stimulatory effect of GH on mammogenesis during this period stems from data using other ruminant models. For example, studies conducted in pregnant ewes and goats suggest that GH-stimulates an increase in mammary parenchyma (Stelwagen et al., 1993; Knight et al., 1994). The stimulatory effect of GH on mammogenesis in these ruminants during the dry period suggests that GH may act as a bovine mammary mitogen. However, since GH does not directly bind bovine mammary tissue; the effect of GH on mammogenesis is likely mediated by other factors. One potential factor believed to mediate the mammogenic

effects of GH on the bovine mammary gland is IGF-I. Further evidence of the role of IGF-I in the regulation of mammogenesis comes from studies employing the use of tissue and cell culture systems as described below.

The ability of IGF-I to stimulate proliferation of bovine mammary tissue *in vitro* is well established. Culture systems using bovine mammary tissue slices, mammary organoids, mammary epithelial cells, and mammary epithelial cell lines have all shown that IGF-I is a potent mitogen of bovine mammary tissue *in vitro* (Shamay and Gertler, 1986; Shamay et al., 1988; Baumrucker and Stemburger, 1989; McGrath et al., 1991; and Woodward et al., 1994; Woodward et al., 1996). This information is consistent with the idea that IGF-I mediates the mammogenic effects of GH on the mammary gland.

Lactation

Treatment with exogenous GH stimulates milk production in the dairy cow (Peel and Bauman, 1987). Two potential mechanisms for GH-stimulated increases in milk production are an increase in secretory cell number or in the synthetic capacity of each cell of the bovine mammary gland. During lactation, administration of GH stimulates an increase in milk production that takes place without significant changes in the ratio of milk yield to feed intake (Tyrrell et al.1982). These data suggest that GH can coordinate more efficient use of nutrient stores and protein synthesis. Several studies have lent support to this hypothesis by demonstrating that GH treatment increases total RNA per gland, as well as key lactogenic enzymes such as acetyl CoA carboxylase, acetyl CoA synthetase, and fatty acid synthase (Baldwin, 1990; Bauman and Vernon, 1995). This

GH-stimulated increase in the synthetic capacity of the gland is coupled to increases in nutrient availability and mammary blood flow (Davis et al., 1988; Fullerton et al., 1989; Peel and Bauman, 1989). Thus during lactation, GH has been shown to play a lactogenic role in the bovine mammary gland. However, because GH does not bind to bovine mammary tissues (Akers, 1985; Baumrucker and Stemburger, 1989), the mechanisms whereby GH mediates these increases in the synthetic capacity of the bovine mammary gland are unknown.

One of the possible mechanisms by which GH may exert its effects on milk production by the bovine mammary gland is through the actions of IGF-I. As discussed in a previous section, GH stimulates the production of IGF-I (Copeland et al., 1980). Furthermore, administration of exogenous GH to lactating cows causes an increase in IGF-I concentrations in the blood and milk (Cohick et al., 1989; Prosser et al., 1989). These data show that GH stimulates an increase in IGF-I in the blood and may also stimulate the production of IGF-I locally by mammary stromal tissue (Campbell et al., 1991). Thus GH-stimulated IGF-I may affect mammary tissue in an endocrine and paracrine fashion. Regardless of its source, IGF-I can act directly on mammary tissue because type-I IGF receptors that have been detected in the bovine mammary gland (Dehoff et al., 1988).

The effect of IGF-I on milk production by the bovine mammary gland has been examined using several approaches. For example, Prosser and coworkers (1990) used close-arterial infusion of IGF-I into the mammary gland of goats to show that the infusion of IGF-I

increased both milk yield and mammary blood flow. However, because the relationship between milk yield and mammary blood flow was not determined, the mechanism for the effect of IGF-I on milk yield in this study cannot be resolved. Another study conducted by Shamay and coworkers used an *in vitro* system to examine the effects of IGF-I on fatty acid synthesis and alpha lactalbumin synthesis in bovine mammary tissues (Shamay et al., 1988). The results of this study suggest that IGF-I is not lactogenic in the bovine (Shamay et al., 1988), therefore the lactogenic effects of GH administration may be mediated by factors other than IGF-I.

A second mechanism that could be involved in the effect of GH on milk production is an increase in the number of secretory cells in the gland. However, in contrast to the mammogenic effects of GH/IGF-I during puberty and the dry period, treatment of cows in mid-lactation with GH has no effect on total mammary DNA (Baldwin, 1990). Thus the effect of GH on the bovine mammary gland during lactation may largely involve a sustainable increase in the synthetic capacity of the gland in contrast to an increase in secretory cell number. However, due to the limited data available it is difficult to determine whether there is truly a change in secretory cell number during this physiological stage. It must be noted that data on the effect of GH on the amount of secretory cells is limited and often employs the use of rudimentary techniques. In addition, studies supporting a role for the GH/IGF-I axis as a mechanism mediating bovine mammogenesis draw their support from loosely based assumptions regarding the mechanisms mediating the effects of the GH/IGF-I axis. Thus to better evaluate the role of GH on mammogenesis during lactation, future work is needed to characterize the

population and regulatory mechanisms controlling secretory cell numbers in the bovine mammary gland.

In conclusion, these data show that the mechanism involved in the GH-stimulated increase in milk yield involves an increase in the synthetic capacity of the bovine mammary gland; whether or not an increase in secretory cell number occurs is not clearly known. Furthermore, the effects of GH-administration on milk yield and/or secretory cell number appear to be dependent on the physiological stage of GH administration. Future studies employing the use of more precise techniques and/or that target the mechanism that mediates the effects of GH/IGF-I axis will greatly enhance our understanding of growth and lactation in the bovine mammary gland.

IGFBPs in bovine mammary tissue

Insulin-like growth factor binding proteins are synthesized in bovine mammary tissue. Immuno-histochemical staining has determined that IGFBP-2 is localized to the luminal surface of epithelial cells of bovine mammary tissue slices (Collier et al., 1993). Insulin-like growth factor binding protein-2 is also the predominant form of IGFBP secreted by primary bovine mammary epithelial cells when cultured in serum-free media (McGrath et al., 1991; and Woodward et al., 1996). These cultures also produce IGFBP-3 especially when IGF-I is added to the media (McGrath et al., 1991). Insulin-like growth factor binding protein-1 and -4 were also detected in the mRNA analysis of bovine mammary tissue (Sharma et al., 1994), but the presence of these proteins in mammary tissue or primary cell cultures has not been reported.

Determining the role of the IGFBPs in mammary cell growth and development is complicated by difficulties in maintaining bovine mammary cell cultures. Methods for culturing bovine mammary epithelial cells were developed several years ago (Shamay and Gertler, 1986). However, use of these techniques has not been wide spread. Underdevelopment of primary bovine mammary epithelial cell culture techniques is likely due to the problems associated with biological variability and cell recoveries that are associated with its use (Forsyth, 1989; and Akers, 1990). The development of bovine mammary epithelial cell lines provides an alternative to using primary cells thereby easing some of the difficulties involved with studying growth and development of the mammary gland at the cellular level.

The MAC-T cell line

The MAC-T cell line is a well-characterized mammary epithelial cell line that was derived from primary bovine mammary alveolar cells (Huynh et al., 1991). The MAC-T cells were immortalized by stabile transfection with the SV-40 large-T antigen but not transformed. The MAC-T cells exhibit epithelial morphology and stain positively for cytokeratin and negatively for vimentin, which indicates that these cells are of epithelial origin. The MAC-T cells are also able to differentiate when induced by addition of extracellular matrix and prolactin. The differentiated phenotype was characterized to include 1) the ability to form secretory domes with a lumen from a pavement of columnar cells; 2) increased casein mRNA abundance; 3) increased alpha S and beta casein secretion; 4) increased number and size of casein secretory vesicles; and 5) increased

lactose synthesis and secretion (Huynh et al., 1991). Thus, the MAC-T cell line provides a useful model for the study of bovine mammary epithelial cells *in vitro*.

As discussed above, studying the role of IGF system at the cellular level is complicated by endogenous production of IGFBPs by many cell types. The MAC-T cells secrete IGFBP-1, -2, -3, -4, and -6 in serum-free media (Cohick and Turner, 1998). The predominant IGFBP produced by MAC-Ts in serum-free media is IGFBP-2, which is also the predominant IGFBP produced by primary bovine mammary epithelial cells in serum-free culture (McGrath et al., 1991; and Woodward et al., 1996).

The MAC-T cells also secrete low levels of IGF-I (Romagnolo et al., 1992). Approximately 22 ng IGF-I /10^6 cells was measured in conditioned media from MAC-T cells after 72 h of culture (Romagnolo et al., 1992). Conditioned media containing endogenous IGF-I also stimulates proliferation in MAC-T cell cultures (Romagnolo et al., 1992). Because MAC-T cells both secrete and respond to IGF-I in culture, IGF-I is thought to act in an autocrine and/or paracrine fashion.

Proliferation stimulated by IGF-I is likely mediated by the IGF type I receptor in MAC-T cell cultures. Since IGF type I receptors are expressed by most if not all tissues in the body including the bovine mammary gland (Nissley et al., 1985; Dehoff et al., 1988), they are likely expressed by MAC-T cells. Using Scatchard analysis, the binding of IGF-I to MAC-T cell surfaces was shown to be specific, saturable, and time dependent (Zhao et al., 1992). Though these data support the existence of type-I IGF receptors on MAC-T

cell surfaces, additional experiments verifying the presence of the type-I receptor and its mediation of IGF-I action in MAC-T cell cultures have not been reported.

Summary

The effect of nutrition and GH on milk production and the mammary gland are well documented. The effects of these influences on mammary growth and development are likely mediated at least in part by the IGF system. Insulin-like growth factor-I is a potent mitogen in the bovine mammary gland. Systemic and local production and local binding indicate that IGF-I regulates mammary cell growth in an endocrine, autocrine, and/or paracrine fashion.

A family of high affinity IGF binding proteins further regulates IGF-I action. These proteins are also produced in the bovine mammary gland. Insulin-like growth factor binding protein-2 is the predominant form of IGFBP secreted by bovine mammary tissue *in vitro* and has been localized to the luminal surfaces of mammary epithelial cells in bovine mammary tissue slices (McGrath et al., 1991; Collier et al., 1993). The mRNA for this IGFBP is also detected in bovine mammary tissue (Sharma et al., 1994). These findings suggest IGFBP-2 plays a role in mediating the effects of IGF-I on mammary growth and development. However, due to the complexity of the IGF system and the difficulty in studying its local effects on the mammary gland *in vivo*, the precise role of IGFBP-2 in the mammary gland is not known.

The use of *in vitro* systems has aided in the study of the effects of the IGF system at the cellular level. Using *in vitro* systems has enabled mammary physiologists to examine the

direct effects of the production and action of the IGF system on bovine mammary epithelial cells. My study focuses on the effect of IGFBP-2 on IGF-I stimulated mitogenic activity in mammary epithelial cell cultures. This model employs the MAC-T mammary epithelial cell line, which is a well-characterized cell line, derived from bovine mammary alveoli. Information gathered in these experiments may increase our understanding of the effects of the IGF system on mammary epithelial cell growth. Efforts to enhance understanding of the IGF system and mammary growth and development have potential for economic impact in the dairy industry. Manipulation of such factors could increase the number of secretory epithelial cells and thereby milk production by the bovine mammary gland. Increasing our knowledge of factors that regulate mammary growth can foster increases in milk production and productive efficiency.

Materials & Methods

Materials

Receptor grade recombinant human insulin-like growth factor-I (IGF-I) (MW = 7649), receptor grade recombinant analog of human insulin-like growth factor-I (des (1-3) IGF-I) (MW = 7366), and recombinant bovine insulin-like growth factor binding protein-2 (IGFBP-2) (MW = 30776) were purchased from GroPep (Adelaide, Australia). Dulbeco's modified eagle medium (DMEM), HEPES buffer, Dulbecco's phosphate buffered saline (DPBS) (with and without calcium and magnesium), and antibiotic-antimycotic solution (100X) containing pennicillin (10,000 IU/ml), streptomycin (10 mg/ml), and amphotericin B (25 μg/ml), were obtained from GibcoBRL (Grand Island, NY). Fetal Bovine Serum (FBS), trypsin-EDTA solution (10X), Hank's Buffered Saline Solution (HBSS), gentamycin solution, bovine insulin, glutathione, soybean trypsin inhibitor, sodium selenite, and apo-transferrin were obtained from Sigma Chemical Company (St. Louis, MO). [Methyl-^{3}H] thymidine (specific activity 50 Ci/mmol) was purchased from ICN Pharmaceuticals, Inc. (Irvine, CA). Rat tail collagen I and Matrigel were purchased from Collaborative Biomedical Products (Bedford, MA). Twenty-four well cell culture plates and 75 cm^2 cell culture flasks were purchased from Corning (Corning, N.Y.). All other reagents used were of analytical grade.

Model development

Growing stock cultures

The MAC-T cell line is an established bovine mammary epithelial cell line (Huynh et al, 1992). The MAC-T line was routinely cultured as described by Huynh et al. (1992). Briefly, MAC-T cells were cultured in 75 cm tissue culture flasks with DMEM F-12 medium supplemented with 10% FBS, antibiotic-antimycotic solution (10%), and gentamycin (1%). Growing cultures were fed every 48 h and kept in a humidified, 37^0C, 5% CO_2 incubator. Stock cultures were harvested with a trypsin-EDTA solution (0.10% w/v) and cryogenically preserved.

Cryogenic preservation

Cells were preserved by methods described by Weber et al. (1999) where preserved cells were kept in freezing medium composed of serum-free medium supplemented with FBS (44%), and DMSO (6%) and stored in liquid nitrogen until use.

Plating cells

In preparation for proliferation experiments, cells were processed and plated by methods originally described by Woodward et al. (1994) where aliquots of MAC-T cells were thawed and washed in DMEM with antibiotic-antimycotic solution (10%) and gentamycin (1%). The MAC-Ts were counted using a hemocytometer and plated at a density of 2×10^4 cells/ml.

Selection of culture substratum

To fulfill the requirement of MAC-T cells for a substratum (Hyunh et al., 1992), growth of MAC-T cells was tested on various substrata. Matrigel was the first extracellular matrix (ECM) attempted. Matrigel is composed of laminin, collagen IV, fibronectin, enactin, and various proteoglycans (Becton Dickenson Labware, 1998). Matrigel is a reconstituted basement membrane isolated from the Engelbreth-Holm-Swarm tumor cell line. Matrigel was chosen because it more closely resembles the composition of the basement membrane in the mammary gland than other ECMs available. However, because my thymidine incorporation assay requires adherence of cells to the plate that could not be achieved with Matrigel, the cultures grown on the Matrigel could not be assayed, and use of the Matrigel substratum was discontinued.

The next substratum attempted was collagen I. Collagen I was chosen because studies comparing attachment of murine mammary epithelial cells on various ECMs demonstrated epithelial cells grown on collagen I had the strongest attachment efficiency compared to the other ECMs tested (Xie and Haslam, 1997). MAC-T cells cultured on collagen I were adherent during the thymidine incorporation assay and thus collagen I was employed as the substratum for this model system.

Serum-free medium

Serum may contain factors that influence cell growth such as IGF-I. Serum may also contain factors that interact with exogenous growth factors such as IGFBPs. Therefore, serum-free medium was chosen for these experiments because including serum in media

may not only confound our studies of the IGF system on MAC-T cell proliferation, but serum has been shown to cause variations in growth rates and chemosensitivities in human mammary cells (Emerman et al., 1987).

Serum-free medium was prepared essentially according to Shamay and Gertler (1986). The recipe was modified as follows. The media DMEM F-12 was used instead of Media 199 because it is more nutrient dense. Albumin was deleted from the media because albumin may contain fatty acids that can modulate growth (Nilausen, 1996). Furthermore, albumin may contain other proteins such as IGFBPs. Insulin was added at 10 ng/ml rather than at 100ng/ml because insulin concentrations greater than 10 ng/ml cause stimulation of the type I IGF receptor (Winder et al., 1989; Bauman and Vernon, 1993). The contents of our culture medium were DMEM F-12 (GibcoBRL, Grand Island, NY), HEPES buffer (7.4 μg/ml), antibiotic-antimycotic solution (10%), gentamicin (50 mg/ml), glutathione (1 μg/ml), soybean trypsin inhibitor (1 μg/ml), sodium selenite (1 ng/ml), insulin (10 ng/ml), and apo-transferrin (5 μg/ml).

Thymidine incorporation assay

Incorporation of [methyl- ^{3}H] thymidine into DNA was assayed to evaluate the proliferative responses of MAC-T cells to growth factor treatment. The MAC-T cells were originally plated and incubated for 48 or 72 h prior to assay. This incubation time allowed cells to attach to the substratum and synchronize in the G0/G1 phase of the cell cycle (Zavizion et al., 1993).

After the 1 or 2 d incubation, the cultures were washed with DPBS, and fresh media containing growth factor treatments was applied. The thymidine incorporation assay was performed according to Xie and Haslam (1997). After treatment for 16 h, 5 μCi of [methyl-^{3}H] thymidine was added to each well. The cultures were incubated for 2 h more, and then assayed for [methyl-^{3}H] thymidine incorporation into DNA that occurs only during the S phase of the cell cycle. To assay for incorporated [methyl-^{3}H] thymidine, cells were washed with ice cold HBSS, 10% trichloroaceitic acid, and 90% ethanol. After the last wash, lysis buffer containing 0.5 *M* NaOH and 0.1% Triton X-100 (v/v) was added and the solution containing the cell lysates was neutralized with 5 m*M* hydrochloric acid. Radioactivity was measured using a liquid scintillation counter.

Determination of plating efficiency

To determine whether the method used for plating cells was distributing equal amounts of cells per well, the amount of DNA per well was measured in MAC-T cell cultures. The method from West et al. (1985) was used to determine total DNA per well for MAC-T cell cultures. Briefly, MAC-T cells were cultured as above, except that the media used in this experiment were serum-free medium (SFM), SFM + 1% fetal bovine serum (FBS), and SFM + 2% FBS. MAC-T cell cultures were maintained for 66 h before they were assayed for total DNA per well. This 66 h time point was chosen because it is at this point and afterwards that the mitogenic activity of IGF-I was measured by the thymidine incorporation assay.

After 66 h of culture, the medium was aspirated and cells were washed with ice cold DPBS. Cultures were then incubated with 10 *mM* EDTA (pH 12.3) until all cells were lysed. The EDTA solution was then neutralized with 1 *M* KH_2PO_4 and sonicated. Aliquots of each well were added to a microtiter plate and 33258 Hoescht dye dissolved in 100 *mM* NaCl and 10 *mM* Tris (pH 7.0) was added to each aliquot. Aliquots were read on a fluorescent plate reader. Total DNA per well was determined using a standard curve made from calf thymus DNA.

Preparation of growth factor treatments

Treatments containing growth factors or binding proteins were prepared 30 min prior to use. Lypohilized growth factors were solubilized in 10 m*M* hydrochloric acid and diluted with serum-free medium to the desired concentration. Medium containing growth factor treatments was applied to cells directly after dilution.

Experimental design

Experiment 1: The objective of this experiment was to measure the response of MAC-T cell cultures to different levels of IGF-I. The levels of IGF-I chosen were 0 ng/ml, 0.001 ng/ml, 0.01 ng/ml, 0.1 ng/ml, 1 ng/ml, 10 ng/ml, and 100 ng/ml. The MAC-T cell's response to these levels of IGF-I was measured in cpm [methyl-^{3}H] thymidine incorporated/well. The responses from nine wells per treatment were assayed. This experiment was repeated three times.

Experiment 2: The objective of this experiment was to assess the impact of endogenous IGFBPs on the mitogenic response of MAC-T cell cultures to IGF-I-stimulation. Des (1-3) IGF-I molecule is a truncated IGF analog with decreased affinity for the IGFBPs (Ross et al., 1989). The use of des (1-3) IGF-I provided an indirect assessment of the effect of IGFBP on the mitogenic activity of IGF-I.

To measure the effects of IGF-I vs. des (1-3) IGF-I on MAC-T cell proliferation, IGF-I and des (1-3) IGF-I were added to MAC-T cell cultures. Each of the IGFs was added at the following concentrations: 0.1ng/ml, 1 ng/ml, 10 ng/ml, and 100 ng/ml. The MAC-T cell's response to these levels of IGF-I was measured in cpm [methyl-^{3}H] thymidine incorporated/well. The responses from nine wells per treatment were assayed. This experiment was repeated five times.

Experiment 3: The objective of this experiment was to measure the effect of IGFBP-2 on MAC-T cell cultures with and without a stimulatory concentration of IGF-I. The concentrations of IGF-I were 1 ng/ml and 10 ng/ml. These concentrations were chosen because the response to 1 ng/ml was half-maximal, and 10 ng/ml was maximal in this system. The concentrations of IGFBP-2 that were chosen were 10 ng/ml and 100 ng/ml. These concentrations were chosen because the addition of 100 ng/ml IGFBP-2 (3.2 n*M* IGFBP-2) was in molar excess of the concentrations of IGF-I tested, and 10 ng/ml IGFBP-2 (0.32 n*M* IGFBP-2) was in molar excess of 1 ng/ml IGF-I (0.13 n*M* IGF-I) but not in excess of 10 ng/ml of IGF-1 (1.3 n*M* IGF-I). Thus the two concentrations of IGFBP-2 were chosen to enable us to determine the effects of IGFBP-2 in excess of concentrations of IGF-I at the concentrations of half-maximal and maximal response.

Insulin-like growth factor-I and IGFBP-2 treatments were added to MAC-T cell cultures in combination and alone. The MAC-T cell's response to these levels of IGF-I and IGFBP-2 was measured in cpm [methyl-^{3}H] thymidine incorporated/well. The responses from nine wells per treatment were assayed. This experiment was repeated three times.

Statistical analysis

The general linear model procedure (PROC GLM) of SAS (SAS Institute Inc., 1998) was used in all statistical analyses performed. Data from experiments 1 and 2 were analyzed using Dunnett's T-test, comparing IGF treatments to serum-free medium as a control. Comparisons among treatments in experiment 3 were analyzed as non-orthogonal contrasts. All data are presented as least square means (LSMEANS) plus or minus the standard error of the mean (SEM).

Results

Plating efficiency

MAC-T cell cultures were plated in SFM, SFM supplemented with 1% FBS (v/v), and SFM supplemented with 2% FBS. Plating efficiency for MAC- T cells in these media was tested by measuring total DNA in μg DNA/well for nine wells per media treatment. The average, standard error of the mean, and coefficient of variation for each medium are reported in Table 2. The coefficient of variation between wells (n=9) was less than 10% for each type of media tested. This demonstrates that the plating techniques used were distributing cells evenly across culture wells in my experiments.

Table 2. Comparison of plating efficiency in MAC-T cell cultures in serum free media (SFM), and SFM supplemented with 1% and 2% fetal bovine serum (FBS). After 66 h of culture in each media tested, the DNA/well was measured. The average DNA/well, standard error of the mean (SEM), and coefficient of variation (C.V.) are reported for culture wells (n=9) grown in the presence of each media.

Media	Average μg DNA /well	SEM	C.V.
SFM	2.65	0.01	8%
1% FBS	2.76	0.01	7%
2% FBS	2.76	0.01	7%

Experiment 1: IGF-I Mitogenic Activity

The effect of adding IGF-I to MAC-T cell cultures in concentrations is shown in Figure 3. Concentrations of IGF-I within the range of 0.01 ng/ml to 100 ng/ml stimulated proliferation in a dose dependent manner when compared with serum-free media alone ($P < 0.05$). Maximal stimulation of proliferation occurred at 10 ng/ml in these experiments. Concentrations of 1 ng/ml stimulated proliferation at approximately half the maximal rate.

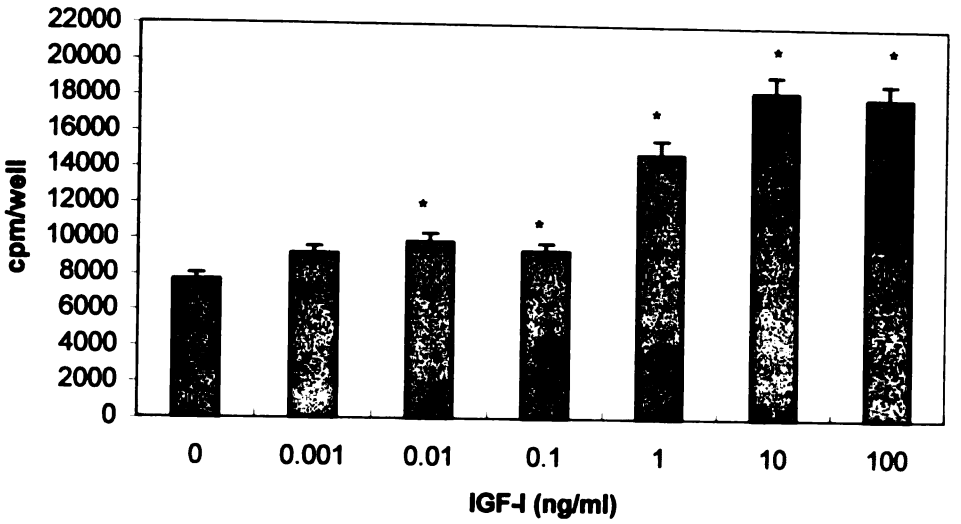

Figure 3. The mitogenic effect of IGF-I in MAC-T cell cultures. Concentrations of IGF-I within the range of 0 to 100 ng/ml were applied to MAC-T cell cultures (n=9) for 18 h. The mitogenic effect of IGF-I was determined by [methyl-^{3}H] thymidine incorporation into DNA during the last 2 h of treatment. The (*) indicates concentrations of IGF-I that stimulated proliferation significantly when compared with SFM ($P < 0.05$).

Experiment 2: Des (1-3) IGF-I Mitogenic Activity

The mitogenic activity of des (1-3) IGF-I, a recombinant analog of IGF-I with a reduced affinity for IGFBPs (Ballard et al., 1987), was also measured in MAC-T cell cultures (Figure 4). Des (1-3) IGF-I in concentrations ranging from 0.1 ng/ml to 100 ng/ml stimulated proliferation in a dose dependent manner compared with serum-free media alone ($P < 0.05$). Maximal stimulation of proliferation was observed with 10 ng/ml of

des (1-3) IGF-I. The mitogenic activity of des (1-3) IGF-I was not different from that of IGF-I at any concentration tested ($P > 0.05$).

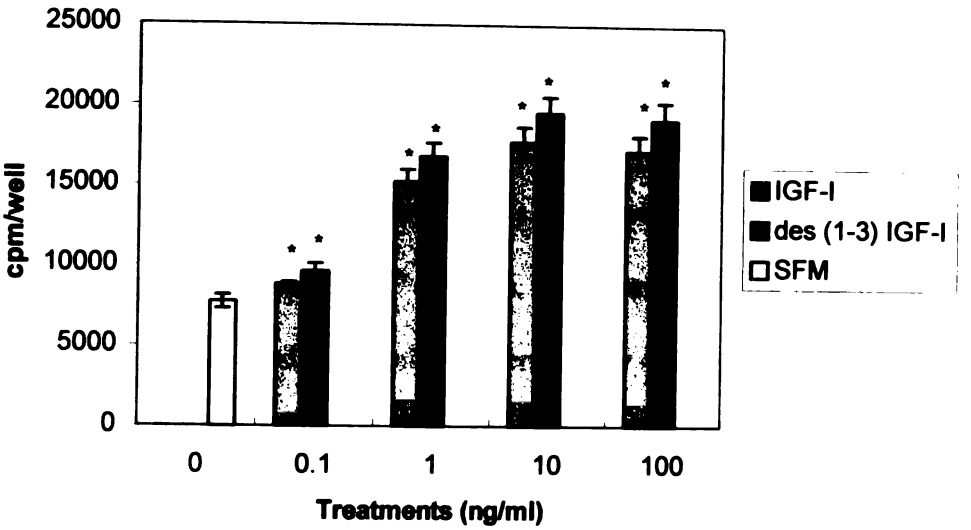

Figure 4. The mitogenic effects of IGF-I vs. des (1-3) IGF-I in MAC-T cell cultures. Concentrations of insulin-like growth factor-I (IGF-I) and des (1-3) IGF-I within the range of 0 to 100 ng/ml were applied to MAC-T cell cultures (n=9) for 18 h. The mitogenic effects of IGF-I and des (1-3) IGF-I were determined by measuring [methyl- ^{3}H] thymidine incorporation into DNA during the last 2 h of treatment. The (*) indicates IGF-I and des (1-3) IGF-I concentrations that stimulated proliferation significantly when compared with serum free media (SFM) ($P < 0.05$). The mitogenic activity of des (1-3) IGF-I was not different from that of IGF-I at any concentration tested ($P > 0.05$).

Experiment 3: Inhibition of Mitogenic Activity by IGFBP-2

Effects of IGFBP-2 addition on IGF-I stimulated mitogenic activity in MAC-T cell cultures are shown in Figure 5. Addition of 10 and 100 ng/ml IGFBP-2 inhibited MAC-T cell proliferation in the presence and absence of IGF-I ($P < 0.0001$). This inhibition acted in a dose dependent manner where 100 ng/ml IGFBP-2 inhibited proliferation to a greater extent than 10 ng/ml IGFBP-2 ($P < 0.0001$). However, the magnitude of the effect was greater in the presence of IGF-I ($P < 0.001$). For example, in the absence of IGF-I, 100 ng/ml IGFBP-2 decreased basal cell proliferation by 40% or 6600 cpm/well. Meanwhile, 100 ng/ml IGFBP-2 decreased proliferation of cells treated with 10 ng/ml IGF-I by 51% or by 17300 cpm/well.

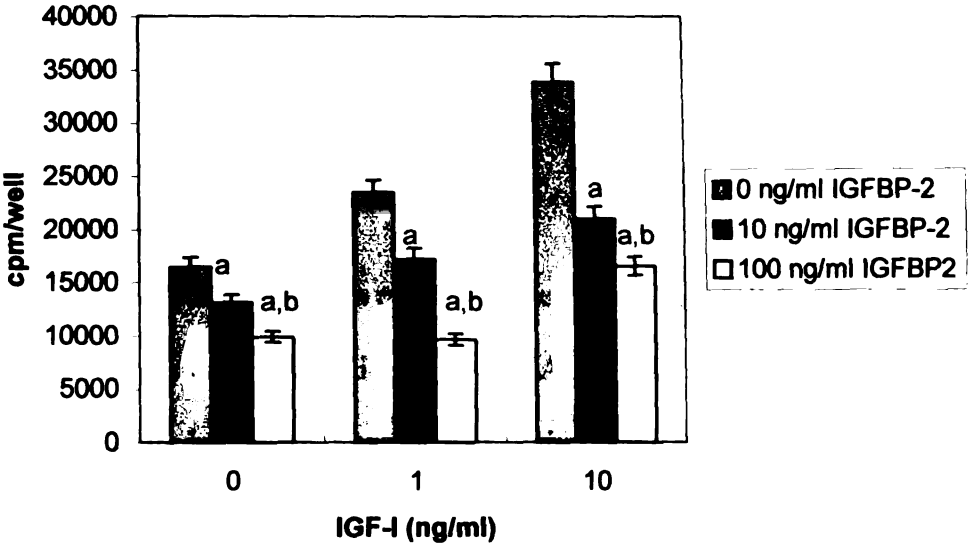

Figure 5. The inhibition of MAC-T cell proliferation by IGFBP-2. Insulin-like growth factor binding protein –2 (IGFBP-2) was added to MAC-T cell cultures (n=9) for 18 h with and without stimulatory concentrations of IGF-I. The effect of IGFBP-2 on MAC-T cell proliferation was determined by [methyl- ^{3}H] thymidine incorporation into DNA during the last 2 h of treatment. The (a) indicates addition of IGFBP-2 inhibited cell proliferation ($P < 0.0001$). The (b) indicates 100 ng/ml IGFBP-2 inhibited proliferation to a greater extent than 10 ng/ml IGFBP-2 ($P < 0.001$).

Discussion

IGF-I mitogenic activity

Insulin-like growth factor-I is a potent mitogen in MAC-T cell cultures. Insulin-like growth factor-I stimulates proliferation of MAC-T cells in a dose-dependent fashion. These results are in agreement with prior studies using MAC-T cells (Woodward et al., 1994, 1996; Zhao et al., 1992), primary bovine mammary epithelial cells, and mammary tissue explants (Shamay et al., 1988; Baumrucker and Stemburger, 1989; McGrath et al., 1991; and Peri et al., 1992). A comparison of these *in vitro* studies to that of my own demonstrates my MAC-T model parallels other bovine mammary *in vitro* models with respect to the IGF system. Furthermore, use of the MAC-T cell model may aid in increasing our understanding of the role of the IGF system in bovine mammary growth and development.

Des (1-3) IGF-I mitogenic activity

Des (1-3) IGF-I is a truncated IGF-I analog characterized by its increased mitogenic activity when compared to intact IGF-I. Des (1-3) IGF-I's increased mitogenic activity is attributed a decreased affinity for IGFBPs (Ross et al., 1989). Evidence in the literature shows truncated forms of IGF-I stimulate mammary epithelial cell proliferation to either a similar or greater extent when compared with intact IGF-I (Peri et al., 1992; McGrath et al., 1991; and Romagnolo et al., 1994). My data show no difference between des (1-3)

IGF-I and IGF-I mitogenic activity in MAC-T cell cultures at the concentrations of IGF tested.

One possible explanation for the similarity between IGF-I and des (1-3) IGF-I mitogenic activities is that these IGFs were added at concentrations that were significantly greater than the concentrations of endogenous IGFBPs. If the IGFs were added at molar concentrations that were higher than the molar concentrations of the endogenous IGFBPs then the IGFBPs would likely have a negligible impact on IGF-stimulated proliferation. This idea is supported by data from Ross et al. (1989) where higher concentrations of exogenous IGFs were required to exert biological effects in cultures that secreted IGFBPs. Therefore, if the endogenous IGFBPs had no effect on IGF action in my culture system, then addition of IGF-I or des (1-3) IGF-I would produce similar mitogenic responses as is shown by my data.

Another explanation for the similarities between IGF-I and des (1-3) IGF-I mitogenic activities is that the IGFBPs produced by the MAC-T cells in this study are not inhibitory to IGF-stimulated mitogenic activity. The MAC-T cells secrete IGFBP-1, -2, -3, -4, and -6 in serum-free media (Cohick and Turner, 1998). However, the effect of the majority of these IGFBPs on IGF action in MAC-T cell cultures is not known.

IGFBP-2 inhibition

My data show for the first time that IGFBP-2 inhibits proliferation of MAC-T cell cultures. These findings are consistent with other cell culture systems in which exogenous IGFBPs inhibit IGF-I action (Gopinath et al., 1988; Rivitos et al., 1988; and McCusker et al., 1991). The inhibitory effect of IGFBP-2 is of interest because IGFBP-2 is synthesized and secreted by bovine mammary tissue and thus may play a role in the regulation of mammary growth and development (McGrath et al, 1991; Collier et al., 1993).

My data show that IGFBP-2 inhibits MAC-T cell proliferation both in the presence and absence of exogenous IGF-I. Though these data might suggest that IGFBP-2 inhibits MAC-T cell proliferation in a manner that is independent of the presence of IGF-I, the mechanism involved in IGFBP-2 inhibition, as well as the role of IGF-I in IGFBP-2 inhibition, remain unclear because MAC-T cells secrete low levels of IGF-I (Romagnolo, 1994). Although IGF-I accumulation in conditioned medium was not measured in my MAC-T cell culture system, Romagnolo measured 21.7 ng IGF-I secreted per 10^6 cells after 72 h of culture in a similar MAC-T cell culture system (1994). These data suggest that the 2×10^4 MAC-T cells/ml in my cultures would have secreted 0.4 ng IGF-I/ml of media after 72 h of culture. Because my cultures were assayed after only 17 h of culture, they could have accumulated up to 0.1 ng IGF-I/ml of media. This concentration of IGF-I has been shown to stimulate cell proliferation in this assay system (see Results of Experiment 1). Therefore it is possible that in the absence of exogenous IGF-I, addition of IGFBP-2 inhibited proliferation stimulated by endogenous IGF-I in these MAC-T cell

cultures. To determine the mechanism behind IGFBP-2 inhibition of MAC-T cell cultures, mechanistic studies are required. Furthermore, studies that address the mechanisms behind IGFBP-2 inhibition of MAC-T cell proliferation will increase our understanding of the role of IGFBP-2 in the regulation of growth, development, and the IGF system in the bovine mammary gland.

In our studies, the inhibitory effect of IGFBP-2 on IGF-I-stimulated cell proliferation in the MAC-T cell assay system may be mediated directly or indirectly (Figure 6). For example, the binding of IGFBP-2 to IGF-I would inhibit proliferation of MAC-T cells indirectly because it involves the binding and sequestering of IGF-I (Figure 6a-b). Meanwhile the binding of IGFBP-2 to a cell surface receptor may directly inhibit proliferation (Figure 6c). Although our studies did not test the mechanisms behind the inhibitory effect of IGFBP-2 on IGF-I-stimulated mitogenic activity, several other studies have investigated the inhibitory effects of exogenous IGFBPs on IGF-I action in vitro. The results of these studies are discussed below.

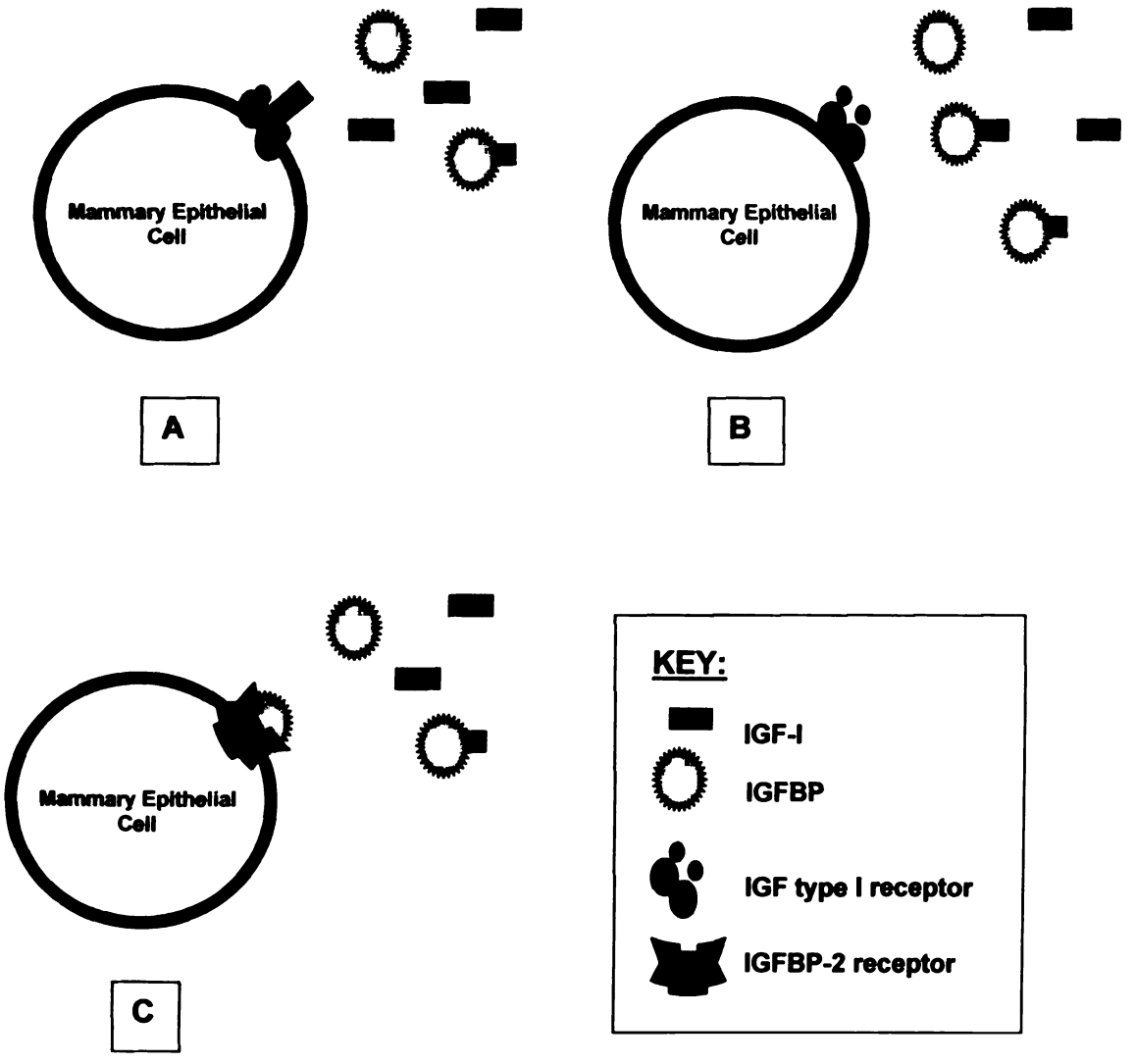

Figure 6. The modulation of IGF-I action by IGFBPs. In (A) free IGF-I binds to the type I IGF receptor on the mammary epithelial cell. In (B) IGFBP binds IGF-I and decreases the amount of IGF-I binding to the Type I receptor on the mammary epithelial cell. In (C) IGFBP binds an IGFBP receptor thereby inhibiting IGF action. Thus, the addition of IGFBPs inhibits IGF-I-stimulated cell proliferation.

Several studies have demonstrated that the addition of IGFBPs and IGF-I to cultured cells results in decreased IGF-I action due the sequestering of IGF-I by IGFBPs (Gopinath et al., 1988; Rivitos et al., 1988; and McCusker et al., 1991). In these studies, exogenous IGFBPs were shown to limit the availability of labeled IGF-I for binding to its cell-surface receptor. Thus the inhibition of IGF-I action in these cultures was attributed to binding of IGF-I to IGFBPs in the culture medium which indirectly inhibited IGF-I action in the target cells.

Another possible mechanism for the inhibitory effects of IGFBPs in cell cultures is the direct effect of IGFBP-2 on target cells. Oh and coworkers (1993a) demonstrated that IGFBP-3 inhibited proliferation in cultures of human breast cancer cells by binding to cell-surface proteins. Furthermore, the addition of IGF-I to these cultures attenuated the inhibitory effect of IGFBP-3 by forming IGF-I-IGFBP-3 complexes, thereby preventing cell surface binding of IGFBP-3 (Oh et al., 1993b). This finding implies that in some cases, IGFBPs act independently of IGF-IGFBP binding and can trigger their own effects independent of IGF-I on target cells.

Though the ability of IGFBP-2 to bind to cell surfaces is unknown, the genes for IGFBP-2 does encode an RGD sequence which is suggestive of cell surface binding (Brewer et al., 1988). Therefore the ability of IGFBP-2 to bind to MAC-T cell surfaces and act independently of IGF-I binding to inhibit cell proliferation is a potential mechanism for IGFBP-2 inhibitory action in MAC-T cell cultures.

Summary

My hypothesis was that IGFBP-2 would inhibit IGF-I-stimulated proliferation of MAC-T cells in culture. I found that IGFBP-2 inhibited IGF-I-stimulated MAC-T cell proliferation as measured by thymidine incorporation. Addition of IGFBP-2 also inhibited basal proliferation of MAC-T cells in serum-free media. The mechanism by which IGFBP-2 inhibits MAC-T cell proliferation in this target cell system remains unknown however it may involve IGF-I-dependant or independent mechanisms as described above.

Future research to elucidate the mechanism behind IGFBP-2's inhibition of mammary epithelial cell proliferation may provide insight on the role of IGFBP-2 in the regulation of mammary growth in the bovine. Increasing our understanding of factors such as IGFBP-2 that limit mammary epithelial cell proliferation will enable researchers to develop safe methods to increase proliferation and thereby increase the efficiency and production of milk.

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