THE ROLE OF INSULIN-LIKE GROWTH FACTOR-I (IGF-I) AND IGF-BINDING PROTEINS IN MAMMARY GLAND DEVELOPMENT

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(ABSTRACT)

Development of the mammary gland is likely mediated by locally produced growth factors acting in concert with circulating mitogens. To investigate the importance of mammary synthesis of insulin-like growth factor-I (IGF-I) and IGF-binding proteins (IGFBP), the initial objective was to evaluate the physiological effects of recombinant IGF-I synthesis in the mouse mammary gland. Expression of recombinant IGF-I was targeted by the mouse mammary tumor virus - long terminal repeat (MMTV-LTR) to the mammary glands of two lines (15 and 29) of transgenic mice. Mammary synthesis of recombinant IGF-I increased the frequency of appearance of mammary alveolar buds (71% vs. 21%) in transgenic compared with non-transgenic CD-1 mice. During lactation, mammary synthesis of recombinant IGF-I reduced the amount of endogenous native IGF-I secreted into milk of transgenic mice. Regardless of transgenesis, a shift in the milk IGFBP profile from predominantly IGFBP-3 to a lower molecular weight IGFBP occurred between d 8 and d 12 of lactation. The altered composition of milk from transgenic line 29 dams reduced by 27% the average daily gain of suckling litters,

compared with CD-1 dams. Moreover, mammary glands of transgenic mice were less regressed after weaning than controls and were characterized by the presence of more organized secretory lobules.

The second overall objective was to evaluate the regulation and physiological effects of mammary IGF-I and IGFBP synthesis in prepubertal heifers. Serum and extracts of mammary tissue at 5% concentration in media stimulated DNA synthesis 545% and 28%, respectively, in primary mammary epithelial organoids in collagen gel culture. Addition of IGFBP-3 strongly inhibited this growth response. High feeding level tended to increase IGFBP-3 levels in mammary tissue and reduced by 30% the growth response to mammary tissue extracts. Somatotropin increased the mitogenic response to mammary extracts at high feeding level and increased the tissue content of IGF-I by 46%.

In summary, local synthesis of IGF-I and IGFBP is influenced by feeding level and exogenous somatotropin and contributes substantially to effects on mammary cell proliferation. Interactions of locally produced IGFBP-3 with IGF-I and other growth factors appear to be especially important when mammary growth is modulated by feeding level.

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BM	basal medium
bST	bovine somatotropin
cDNA	complementary deoxyribonucleic acid
Da	dalton
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
FL	feeding level
i.p.	intraperitoneally
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding proteins
kD	kilodaltons
μCi	microcurie
MG	mammary gland
mIGF-I	murine insulin-like growth factor-I
mRNA	messenger ribonucleic acid
MMTV-LTR	mouse mammary tumor virus - long terminal repeat
PCR	polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
ST	somatotropin
SV	simian virus
WAP	whey acidic protein

CHAPTER 1

REVIEW OF LITERATURE

I. Introduction

The primary purpose of the mammary gland is to produce milk for the nourishment of the developing offspring. During early pre- and post-natal life, the milk-synthesizing or parenchymal portion of the gland extends into the relatively expansive mammary fat pad at a growth rate isometric with the rest of the body. Additional growth and differentiation in response to pregnancy and lactation yields a gland capable of milk synthesis and secretion (Tucker, 1987). Hormonal regulation of development of the gland is a fascinating subject for many, including animal scientists trying to improve profitability. In particular, the future milk yield potential of ruminants depends on optimum development of the tissue foundation that is established around the time of puberty (Akers, 1990).

The ontogeny of development proceeds in discontinuous phases in relation to physiological state. The bulk of mammary epithelial growth takes place during allometric growth phases in the peripubertal period and during pregnancy. Control of mammary development is highly species-dependent, in that 48 to 94% of total mammary growth is achieved during pregnancy depending upon species (Tucker, 1969; Knight and Peaker, 1982). Although the development associated with pregnancy is relatively greater than that prior to puberty, evidence indicates that manipulation of mammary growth

during the peripubertal period can markedly affect the future milk yield potential (Sejrsen et al., 1990). Thus, for scientists seeking to optimize milk production in dairy animals, the peripubertal period has been of increasing interest.

In the prepubertal heifer, exogenous somatotropin can stimulate mammary growth, although the underlying mechanisms are unclear (Sejrsen et al., 1986). Circulating levels of IGF-I have been implicated along with potential changes in local mammary synthesis of IGF-I. The rate of growth of heifers provides an additional opportunity for modulation of mammary growth. Specifically, a high rate of gain reduces prepubertal mammary growth (Sejrsen and Purup, 1997). Because circulating somatotropin levels are positively related to mammary growth (Sejrsen et al., 1983), the observed decrease in somatotropin levels in heifers on a high feeding level has been implicated in the negative effects of a high feeding level on mammary development. The issue is complicated by the positive relationship between circulating IGF-I levels and growth rate, indicating that serum IGF-I levels do not consistently explain effects on mammary growth. In the rat, somatotropin directly stimulates mammary synthesis of IGF-I (Kleinberg et al, 1990). Taken together with the positive effects of IGF-I synthesis on growth of the rat mammary gland (Ruan et al., 1992), local IGF-I levels may be relatively more important than circulating IGF-I to mammary growth in ruminants.

Insulin-like growth factor-I is a potent mitogen for a variety of cell types. The mammary gland synthesizes IGF-I in the stromal portion (Yee et al., 1989), supporting a paracrine role for IGF-I to influence development of the epithelium. Ultimately, the biological effects of IGF-I are regulated by IGFBP that control the availability of IGF-I to

its target tissues (Conover, 1996). Synthesis of IGFBP in mammary cells is influenced by IGF-I (McGrath et al., 1991), suggesting that IGFBP regulate local as well as systemic IGF-I actions. However, the effects of mammary IGF-I synthesis on peripubertal mammary development have not been described. To test the hypothesis that mammary IGF-I synthesis plays a role in early mammary growth, the initial objective was to evaluate the effects of recombinant IGF-I (rIGF-I) synthesis in the mammary glands of transgenic mice on peripubertal gland growth. The demonstration of a role for mammary IGF-I synthesis in gland development, and its putative role as a survival factor for mammary epithelial cells, supported the study of effects of rIGF-I expression on lactation and involution of the mammary gland. These studies illustrated the importance of mammary IGF-I synthesis in mammary growth and provided the basis for evaluation of gene expression of IGF-I, the type 1 IGF receptor, and IGFBP in the mammary glands of prepubertal heifers. The underlying objective was to test the hypothesis that the effects of feeding level and exogenous somatotropin on heifer mammary development are mediated in part by locally produced factors. The final objective was to determine if changes in mammary expression of IGF-I and IGFBP induced by feeding level and exogenous somatotropin mediate a portion of the associated effects on mammary development.

II. Morphogenesis of the Mammary Gland

From birth until puberty

At birth, the mammary parenchyma consists of a limited duct system embraced by a surrounding stroma. In mice, these ducts are decorated by a single layer of luminal epithelial cells and will eventually serve as channels for milk secretion in the mature gland (Imagawa et al., 1994). The epithelial compartment is separated from stroma by a basement membrane composed of extracellular matrix elaborated from both cell types (Pitelka, 1978). In rodents, mammary ducts exhibit end buds which subsequently disappear within a few days following cessation of stimulation by maternal hormones (Imagawa et al., 1994). Epithelial tissue in the heifer mammary gland at birth consists of a primary sprout and multiple secondary sprouts (Anderson, 1978) that give rise to the gland cistern and to major ducts lined by a double layer of epithelium.

Prior to puberty, the duct system extends in concert with growth of adipose and connective tissue at a rate approximately isometric with body growth. Terminal end buds reappear between 3 and 4 weeks in the mouse, signaling the onset of ductal arborization and directing the extension of ducts to the perimeter of the fat pad (Daniel and Silberstein, 1987). The terminal end bud comprises body cells, which give rise to luminal and ductal mammary epithelial cells, and the cap cells, which differentiate into myoepithelial cells (Humphreys et al., 1996). Ductal spacing is maintained while terminal end bud cells proliferate into the surrounding fatty stroma (Faulkin and DeOme, 1960). Differentiation of end bud cells to form myoepithelial, luminal, and ductal cells is

followed by a decreased rate of cell division (Bresciani, 1968). Development of the ruminant mammary gland is considerably less well delineated (Akers, 1990) but contrasts markedly with that of rodents. Unlike the rodent, the prepubertal ovine mammary gland does not exhibit terminal end buds (Ellis and Akers, 1995). Further, the relatively closely-packed mammary ducts in the ruminant are surrounded by connective tissue and do not directly abut adipocytes as in the rodent mammary gland (Akers and Sejrsen, 1998).

Peri- and postpubertal development

Around 21 days of age in the rat, mammary area commences an allometric rate of growth 3.5 times that of body surface area (Sinha and Tucker, 1966). In heifers, mammary DNA increases 5.5 times faster than body surface area between three and nine months of age (Sinha and Tucker, 1969b). These increases in mammary growth rates begin well in advance of the first estrus. Strain differences appear to contribute variation to this allometric growth in mice and rats (Imagawa et al., 1994).

The rate of mammary growth in rodents slows around 50 to 60 days of age (Tucker, 1969). Further DNA synthesis occurs primarily in alveolar cells, which are present in virgin mouse glands depending on the strain (Bresciani, 1968). In heifers, mammary growth ceases to be allometric after about 9 months of age; in fact, mammary DNA per unit body weight did not increase from 9 to 16 months of age (Sinha and Tucker, 1969b). At puberty, the mammary parenchyma comprises about 10-12% duct

cells, 2-3% ductular lumen, 50% connective tissue and 35% fat cells (Sejrsen et al., 1982).

During the early postpubertal period, mammary cell proliferation varies in a manner that coincides with stage of the estrous cycle (Dulbecco et al., 1982; Vonderhaar, 1988). During proestrus in mice, buds form at the ductular tips; in early estrus, ducts dilate and elongation of the buds occurs. Regression of buds occurs late in estrus, followed by a return to a similar appearance of the gland during the previous diestrus. Sinha and Tucker (1969a) showed that the greatest net accumulation of mammary DNA occurs primarily during the first three estrous cycles of the rat, then plateaus until conception. In general, morphological development peaks during estrus, while the mitotic index is highest during diestrus when the gland is least developed (Imagawa et al., 1994). Stages of estrus and metestrus are characterized by low mitotic indices.

In heifers, DNA content in the mammary gland is highest at estrus compared to other stages of the cycle (Sinha and Tucker, 1969b). Mammary DNA increases 118% between late luteal phase of the cycle and estrus, followed by regression during the early luteal phase of the cycle.

Gestation

Development of mammary epithelium is dramatic during gestation, constituting 48-94% of total mammary growth depending on the species (Tucker, 1969; Knight and Peaker, 1982). In early pregnancy in rodents, epithelial cells proliferate to fill interductal mammary fat pad spaces, by increased ductal branching and formation of lobuloalveolar

structures. Ultimately, epithelial development is limited to the area of the mammary fat pad (Hoshino, 1964). Myoepithelial cells encircle the alveoli and terminal ducts. In later pregnancy, little formation of new acinar structures occurs but individual cells undergo marked structural and biochemical differentiation to gain a polarized appearance with clearly evident organelles, secretory vesicles, and lipid droplets. Estimates of mammary DNA indicate that mammary growth occurs at a relatively constant rate. Autoradiographic studies showed that peaks in DNA synthesis occur on day 4, just prior to implantation and likely stimulated by progesterone and prolactin, and to a lesser degree on day 12, in association with the onset of placental contribution to gestational development (Traurig, 1967). The murine placenta synthesizes progesterone in quantities related to litter size, and there is a high correlation between mammary growth and the number of placentae in litter-bearing species.

Gestational growth of mammary parenchyma in ruminants involves a gradual displacement of adipose tissue among the ducts with closely spaced alveoli. Mammary parenchymal growth in ruminants during pregnancy is exponential and, in contrast to the situation in rodents, the ruminant mammary gland is almost fully developed at term.

Lactation

During early lactation, mammary DNA increases further in most species, although mammary growth in sheep is mostly complete by parturition (Anderson, 1975). Akers and co-workers reported that there is a 65% increase in mammary DNA in the bovine between 10 d prepartum and 10 d postpartum (Akers et al., 1981). Up to 40% of

mammary growth takes place during lactation in rats; in both mice and rats maximum mammary DNA content is reached by approximately day 10 of lactation. Additional development of the mammary gland can be induced by increasing milking frequency or suckling intensity (Tucker, 1966). If pregnancy is concurrent with lactation, the late stages of pregnancy are associated with reduced cell numbers in rats. Rats may resemble dairy cows in this respect as milk yield in cattle is reduced after the 5^{th} month of concurrent pregnancy (Tucker, 1969).

III. Regulation of Mammary Development

The basic structure of the mammary gland develops during the embryonic and fetal stages of life. It is intriguing, but so far unknown to what extent alterations in maternal or fetal endocrine environments might modulate the eventual secretory capacity of the mammary gland. Both prenatal and postnatal growth of the mammary gland occurs in response to specific hormones and growth factors that stimulate growth, morphogenesis, and differentiation. There is, however, a notable species variation in the hormonal requirements for gland development (Imagawa et al., 1994).

The ovary

The importance of secretions from the ovary in mammary development has been demonstrated in several species. Early work in rodents (Cowie, 1949; Lyons et al., 1958) showed that estrogen, progesterone, and somatotropin were critical to full lobuloalveolar

development in rodents. The ovary is critical during prepubertal allometric growth of the mammary gland in rodents (Silver, 1953; Cowie, 1949; Flux, 1954). In dairy heifers, ovariectomy at 2.5 months of age nearly abolishes further parenchymal growth (Purup et al., 1993), indicating that ovarian secretions are involved. Interestingly, somatotropin stimulation of mammary growth in heifers in this study was relatively ineffective in the absence of the ovary. In contrast to the situation in mice and heifers, mammary growth in sheep is not dependent on the ovary (Ellis et al., 1998; Wallace, 1953).

Estradiol and progesterone both stimulate DNA synthesis at the terminal end bud tips; whereas progesterone is required to increase DNA synthesis in ductal epithelium (Bresciani, 1968). Estrogen is the primary stimulator of duct growth in young mice but may induce progesterone receptors in cells of the end bud, as progesterone alone can substitute for estrogen (Imagawa et al., 1994). The mechanism of estrogen action is still undefined but evidence suggests that both direct and indirect local effects on the mammary epithelium are involved.

Somatotropin

It is well documented that exogenous somatotropin (ST) stimulates mammary growth in dairy heifers (Sejrsen et al., 1986; Purup et al., 1993), lambs (Johnsson et al., 1986; McFadden et al., 1990), prepartum ewes and heifers (Stelwagen et al., 1992; Stelwagen et al., 1993) and rodents (Flux, 1957) as well as milk production in lactating dairy cattle (Bauman and Vernon, 1993). In spite of the imposing body of literature on

ST effects on the mammary gland, the mechanism by which ST exerts its actions on the mammary gland remains a topic of considerable debate.

Several lines of evidence suggest a direct action of ST on the mammary gland. Somatotropin receptor gene expression was detected in poly(A)+ RNA from the rat mammary gland (Rosato et al., 1994). Further, ST receptor mRNA or protein has been identified in bovine (Chapter 5; Glimm et al., 1990; Hauser et al., 1990), porcine and ovine (Jammes et al., 1991) mammary tissue. Expression of the putative ST receptor mRNA is common to a number of tissues other than the mammary gland (Lucy et al., 1998).

The ability of ST to enhance mammary development has been demonstrated. In rats, a direct action of ST on mammogenesis was implied by stimulation of unilateral mammary development by local ST implants. This was in contrast to implants of rat prolactin or other prolactin variants, suggesting that the effects occurred through the ST receptor and not the prolactin receptor (Feldman et al., 1993). Further, ST enhancement of milk synthesis was noted in treated but not contralateral glands using local ST implants (Flint and Gardner, 1994). It is generally accepted that ST does not interact directly with bovine mammary epithelial cells to yield an effect (Bauman and Vernon, 1993); however, results of these studies do not discount the possibility of a paracrine effect of ST possibly mediated by local production of IGF-I. Mammary fibroblasts and adipocytes display ST binding (Kelly et al., 1991). Moreover, Sheffield and Anderson (1986) demonstrated that ST increases proliferation of mammary fibroblasts in culture. Growth hormone may

stimulate synthesis of IGF-I in target tissues which then acts locally on cells of the target tissue (Holly and Wass, 1989).

Dietary regulation

Many studies have investigated the idea that an increased growth rate could reduce the age at first calving, with earlier milk production offsetting the higher feed costs. Multiple reports demonstrated that rapid growth rates in heifers led to reduced milk production (Gardner et al., 1977; Little and Kay, 1979). Using twin heifers to reduce genetic variability between treatment groups, Swanson (1960) suggested that a reduction in secretory tissue development in the udder contributed to the negative effects of a high rate of gain on milk yield. Pritchard et al. (1972) also showed that a high feeding level reduced mammary development at first estrus in untreated heifers, whereas feeding level did not affect mammary development in heifers fed melengestrol acetate. Results from later Danish experiments indicated that this negative effect on mammary development was a result of a high feeding level prior to or around the time of puberty (Sejrsen, 1978) but not after puberty (Sejrsen et al., 1982). Foldager and Sejrsen (1987) suggested that the critical period begins by 90 kg of body weight and ends at approximately 300 kg body weight in larger dairy breeds.

Nutrition is a key regulator of the somatotropin-IGF-I axis (Thissen et al., 1994). Energy level can modulate mammary growth, but the mechanisms involved are not well understood. Because circulating somatotropin concentrations are positively related to mammary growth (Sejrsen et al. 1983), decreased serum somatotropin levels have been

implicated in the negative effects of a high rate of gain on mammary growth (Sejrsen and Purup 1997). However, the bovine mammary gland lacks specific receptors for bST (Akers, 1985), indicating an indirect mechanism of action which may involve IGF-I. Circulating IGF-I levels are increased by bST but are reduced by restriction of feed intake (McGuire et al., 1992). Regulation of mammary development is complex; thus, it is not surprising that changes in circulating IGF-I concentrations do not consistently explain the accompanying changes in mammary growth. Alternatively, IGF-I and IGFBP are synthesized in the bovine mammary gland, supporting the idea that changes in local production of IGF-I may be important (Glimm et al., 1988; Sharma et al., 1994).

Insulin-like Growth Factor-I (IGF-I)

Insulin-like growth factor-I (IGF-I) is a single-chain 7646 Da peptide. It displays structural similarity to IGF-II and to insulin, but the IGF display distinct receptor binding and biological effects (Sara and Hall, 1990). The IGF-I molecule has an identical 70-amino acid sequence in the bovine, human and pig, differing from ovine IGF-I at position 66 (alanine is substituted for proline) and from murine IGF-I by 5 amino acids (Wong et al., 1989). Insulin-like growth factor-I functions to mediate normal ST-dependent postnatal growth, particularly in skeletal and cartilage tissue, whereas IGF-II is considered to be important in fetal development and is not apparently regulated by ST (Tripathy and Benz, 1994).

According to the original somatomedin hypothesis proposed by Salmon and Daughaday (1957), ST stimulates liver synthesis of IGF-I, which is transported via the

circulation to target tissues where it mediates the actions of ST. Increased concentrations of circulating IGF-I in response to exogenous ST may effect such an endocrine action in stimulating milk yield in lactating dairy cows (Cohick et al., 1989; Davis et al., 1987) and in lactating goats (Davis et al., 1989). The primary source of IGF-I for the mammary gland and its regulation are not defined, and the issue is complicated by the likelihood that mammary development is directed by both autocrine and systemic endocrine actions (Wilde et al., 1990). Administration of exogenous ST to heifers (Purup et al., 1993) and lactating cows induces an increase in circulating IGF-I concentrations, and the temporal pattern of the IGF-I response corresponds with its proposed role in mediating ST effects on milk yield (Bauman and Vernon, 1993). However, attempts to demonstrate an effect of IGF-I on milk production in vivo have met with limited success. Increasing systemic IGF-I concentrations by jugular infusion of IGF-I did not stimulate milk yield, despite serum IGF-I concentrations being increased to similar levels as in ST-treated animals (Davis et al., 1989). On the other hand, close-arterial infusion of free IGF-I into the pudic artery of lactating goats for 6 h stimulated milk production in the infused gland, but not in the noninfused gland possibly as a result of association of free IGF-I with plasma IGF-I binding proteins upon recirculation (Prosser et al., 1990). Furthermore, comparison of lactating goats differing in responsiveness to ST treatment showed that increased milk yield corresponded with increased concentrations of IGF-I in milk and mammary tissue, while one animal in which ST treatment did not increase milk yield had unaltered IGF-I concentrations in milk and mammary tissue (Prosser et al., 1991). Thus mammary IGF-I may play a key role in the galactopoietic effects of bST. Hodgkinson et

al. (1991) studied the tissue distribution of a recombinant IGF-I variant following intravenous administration to lactating sheep and concluded that most of the IGF-I in lactating mammary epithelium is derived from blood, whereas mammary connective tissue contained significantly less of the IGF-I variant found in the mammary gland.

The mammary gland also may be a target tissue for ST-induced IGF-I production, as exogenous ST increases IGF-I mRNA synthesis within the rat mammary gland (Kleinberg et al., 1990), and IGF-I synthesis in prepubertal heifer mammary tissue depending on the rate of body weight gain (Weber et al., 1996). In addition, ST administration increased the presence of immunoreactive IGF-I in the cytoplasm of mammary epithelial cells from lactating cows (Glimm et al., 1988) though the source of the IGF-I was not determined. Most evidence indicates that mammary tissue synthesis of IGF-I occurs in stromal cells, potentially for a paracrine effect on nearby epithelium (Cullen et al., 1992). Receptors for IGF-I have been located on mammary epithelial cells from heifers (Purup et al., 1995), cows (Dehoff et al., 1988; Hadsell et al., 1990), sheep (Winder et al., 1993), and rats (Lavandero et al., 1991). The mitogenic activity in vitro of IGF-I on mammary epithelial cells from a number of species has been well established. Insulin-like growth factor-I enhances DNA synthesis in cultures of normal mammary epithelial cells of the mouse (Imagawa et al., 1986); rat (Ethier et al., 1987); sheep (Winder et al., 1989); and the cow (Shamay et al., 1988; Baumrucker and Stemberger, 1989; McGrath et al., 1991). In vivo, implants of native IGF-I as well as des-(1-3)-IGF-I, an IGF-I variant lacking 3 amino-terminal residues, were shown to substitute for ST in mammary development in hypophysectomized castrated male rats, though estrogen is

required for a full effect (Ruan et al., 1992, 1995). In pregnant cows, intramammary infusion of IGF-I stimulates mammary development (Collier et al., 1993). On the other hand, the inability to detect specific receptors for ST in mammary tissue from cattle (Akers, 1985; Keys and Djiane, 1988; Purup et al., 1995) or sheep (McFadden et al., 1990) by standard ligand binding assays suggests that ST does not act directly on the mammary gland. Moreover, addition of ST to explants from bovine lactating mammary tissue in culture had no stimulatory effect on synthesis of milk components; and exogenous ST did not stimulate proliferation in clonal bovine mammary epithelial cells (Woodward et al., 1994). Alternatively, regulation of ST receptor expression may occur at a post-transcriptional level, or the receptor number may be too low to allow detection by conventional assays.

The IGFBP

It has long been recognized that the insulin-like growth factors are present in the circulation as high molecular-weight forms (Bürgi et al., 1966; Jakob et al., 1968). However, not until nearly ten years following the identification of the amino acid sequences for the IGF were the first two human IGFBP described in similar detail (Povoa et al., 1984; Martin and Baxter, 1986). The IGF-I in most body fluids is bound to one of six specific, high-affinity IGFBP that modulate their biological effects (Thissen et al., 1994). Similar to the IGF, the IGFBP are synthesized by a variety of body tissues. Availability of the IGF-I to its receptor is further regulated by certain IGFBP-specific proteases that are produced by multiple body tissues and are also found in the circulation

(Zapf, 1995). More recently, a closely related gene family that includes connective tissue growth factor, immediate early gene products, *cyr* 61, and *nov* (postulated to be an avian proto-oncogene) has been designated as the IGFBP-related proteins until further analyses of their physiological roles as low-affinity binding proteins (Baxter et al., 1998). It has been proposed that the specificity of IGFBP gene expression may modulate their local effects, whereas alterations in serum IGFBP levels may regulate the bioavailability of IGF-I (Lemozy et al., 1994).

IGFBP-1. IGFBP-1 is a 25-kD protein that contains the RGD recognition sequence for membrane integrin receptors, suggesting the possibility for cell effects independent of IGF-I. Sites of production include the liver, decidua, and kidneys. Serum levels are mainly controlled by insulin and corticosteroids, by transcriptional regulation of hepatic IGFBP-1 synthesis. Insulin appears to inhibit IGFBP-I production, leading to low serum levels of IGFBP-1 in cases such as fasting or poorly controlled type I diabetes.

Reported effects of IGFBP-1 include both inhibition and stimulation of IGF-I activity. Phosphorylated forms demonstrate a higher affinity for IGF-I and generally inhibit IGF-I action; the opposite is true for dephosphorylated IGFBP-1. In vivo, IGFBP-1 levels are regulated by nutritional status. During fasting states, increased IGFBP-1 curbs the hypoglycemic effects by binding IGF and decreasing the levels of free IGF-I (Collett-Solberg and Cohen, 1996). Similar to the in vivo situation, in vitro studies indicate that addition of IGFBP-1 to cells in culture inhibits growth in an IGF-dependent fashion (Jones and Clemmons, 1995). It is possible that IGFBP-1 causes a controlled release of IGF-I, because concentrations that can inhibit IGF-I binding to its receptor

have been shown to enhance IGF-I-stimulated DNA synthesis (Collett-Solberg and Cohen, 1996). Movement of IGF-I across the endothelium may be partly controlled by IGFBP-1 and appears to be insulin-dependent in certain tissues (Bar et al., 1990; Lewitt et al., 1993).

IGFBP-2. IGFBP-2 is a 31-kD protein that does not undergo phosphorylation or glycosylation. It also contains an RGD sequence but does not appear to bind membrane integrin receptors, although cell surface association has been demonstrated. IGFBP-2 appears to mainly inhibit IGF-dependent effects on cell growth, as shown in multiple cell lines by addition of IGFBP-2 to cultures (Jones and Clemmons, 1995). Interestingly, however, IGFBP-2 knockout mice do not exhibit an altered phenotype. Age is a major regulator of IGFBP-2 in serum, with levels being high in early and late life and low in young adults. In addition, serum IGFBP-2 levels are elevated in response to somatotropin deficiency or in fasting situations, particularly in the case of protein restriction (Collett-Solberg and Cohen, 1996).

IGFBP-3. The IGFBP-3 protein has a molecular weight of 29 kD in the non-glycosylated form. In serum, IGFBP-3 is found in the glycosylated form with a molecular weight of 40 to 44 kD. Body reserves of IGF-I are primarily complexed with IGFBP-3 (Jones and Clemmons, 1995), either as a binary complex or as a ternary complex with the acid-labile subunit (Baxter and Martin, 1989). The major tissue sources of IGFBP-3 and its regulation of synthesis have not been determined. Its concentrations in serum are under complex regulation by age and somatotropin secretion. Serum levels increase during childhood to a peak around puberty and then decline. In

dwarf rats, Lemmey et al. (1997) showed IGF-I administration induced an increase in serum IGFBP-3 without a corresponding increase in liver IGFBP-3 mRNA. In contrast, somatotropin consistently increased IGFBP-3 mRNA levels in multiple tissues, implying that somatotropin might have a direct role in regulation of IGFBP-3 synthesis.

IGFBP-3 has both inhibitory and stimulatory actions in vitro, depending on the cell type and culture conditions. Addition of exogenous IGFBP-3 generally inhibits IGF-I-stimulated growth, presumably by preventing association of IGF-I with its cell surface receptor. Further, cells transfected with the IGFBP-3 gene display a reduced growth rate (Cohen et al., 1993). On the other hand, DeMellow and Baxter (1988) showed that IGFBP-3 addition to culture media inhibited growth of skin fibroblasts, whereas a pre-incubation of IGF-I with IGFBP-3 followed by removal of IGFBP-3 potentiated the effects of IGF-I. More recently, Conover and Powell (1991) showed that low molecular weight forms of IGFBP-3 were bound to the cell surface after a pre-incubation with IGFBP-3 and washing. It was concluded that IGFBP-3 may function to slowly release bound IGF-I to the cells and thus protect the IGF-I receptor from downregulation.

IGFBP-4. The molecular weight of IGFBP-4 is 24 kD, or 28 kD in its glycosylated state. Multiple cell types produce IGFBP-4 locally in tissues, and IGFBP-4 is present in all body fluids. Little information exists regarding its regulation of expression. Binding of IGFBP-4 to cell membranes has been demonstrated, but IGFBP-4 is mainly present in an extracellular soluble state (Collett-Solberg and Cohen, 1996). In vitro studies have shown quite conclusively that IGFBP-4 inhibits IGF-I activity. This

effect is accomplished by competitive binding of IGFBP-4 to free IGF-I, decreasing the availability of free IGF-I in the extracellular environment (Mohan et al., 1995).

IGFBP-5. IGFBP-5 has a molecular weight of 29 kD, or between 29 and 32 kD in its glycosylated forms. It is present in high amounts in bone cells, cerebrospinal fluid, connective tissues, and is the major IGFBP produced by the kidneys. Similar to the pattern for IGFBP-3, levels of IGFBP-5 decline after puberty. Growth of a number of cell types is inhibited by IGFBP-5, including granulosa cells, renal cells, and osteosarcoma cells. More recently, Tonner et al. (1997) suggested a role in the induction of apoptosis because IGFBP-5 is upregulated in the involuting mammary gland, similarly to the involuting prostate, thyroid, and in atretic ovarian follicles.

IGFBP-6. IGFBP-6 has a molecular weight of 34 kD and differs from other IGFBP in demonstrating O-glycosylation. Interestingly, it is the only IGFBP that exhibits a significant binding preference for IGF-II compared with IGF-I. Multiple cells express IGFBP-6, including ovarian cells, prostate cells, breast cancer cells, and fibroblasts (Bach et al., 1994).

In addition to the above functions that have been ascribed to the IGFBPs, a variety of IGF-independent functions have been demonstrated (Collett-Solberg and Cohen, 1996). To date, only IGFBP-1 and IGFBP-3 have been shown to effect independent actions in cultures. In an estrogen receptor-negative human breast cancer cell line that is not growth-responsive to the IGF, exogenous IGFBP-3 alone reduces DNA synthesis in a dose-dependent manner (Oh et al., 1993). In estrogen-responsive cells, IGFBP-3 also inhibits estradiol-stimulated cell proliferation (Pratt and Pollak,

1994), supporting the concept of cross-talk between the steroids and the IGF axis in control of growth (Newton et al., 1994; Westley and May, 1994). Further interactions between IGFBP-3 and locally produced growth factors in growth modulation were shown by TGF-β-induced stimulation of IGFBP-3 synthesis in breast cancer cells (Huynh et al., 1996; Oh et al., 1995) and the demonstration that IGFBP-3 antisense oligonucleotides suppressed the inhibitory effect of TGF-β on cell proliferation (Oh et al., 1995). The IGFBP-3 binds directly to a cell surface receptor to cause inhibition of growth (Oh et al., 1993).

Another aspect of IGFBP regulation of cell growth involves the IGFBP proteases, which are known to regulate IGFBP-3 proteolysis in pregnancy serum (Rutishauser et al., 1993). To date, at least three classes of IGFBP proteases have been identified, including the kallikreins, matrix metalloproteinases, and cathepsins (Collett-Solberg and Cohen, 1996). Action of these proteases reduces the affinity of IGFBP for IGF, stimulating the release of IGF for transport to extravascular space or for receptor binding. Additionally, the IGFBP fragments may bind specifically to the cell membrane and modulate IGF actions.

IV. SUMMARY

Insulin-like growth factor-I is a strong mitogen for mammary epithelium from prepubertal heifers and has been credited with mediating at least a portion of the action of somatotropin on the mammary gland. Circulating IGF-I likely influences mammary growth in an endocrine fashion, but also its role as a local stimulator of cell proliferation is evident in a number of tissues. Appropriate modulation of mammary-specific synthesis of IGF-I, particularly through understanding of physiological regulation of mammary IGF-I synthesis and activity, might offer insight into new strategies to enhance mammary development and future milk yield potential in dairy cattle. Therefore, the objective of this study was to investigate the physiological regulation of mammary IGF-I and IGFBP synthesis, and to describe the effects of local recombinant IGF-I expression on mammary development and lactation.

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CHAPTER 2

EXPRESSION OF OVINE INSULIN-LIKE GROWTH FACTOR-I (IGF-I) STIMULATES ALVEOLAR BUD DEVELOPMENT IN MAMMARY GLANDS OF TRANSGENIC MICE

ABSTRACT

To determine whether murine mammary growth is modulated by local IGF-I production, expression of recombinant IGF-I was directed to the mammary glands of transgenic mice using an ovine prepro IGF-I cDNA under control of the mouse mammary tumor virus-long terminal repeat (MMTV-LTR) promoter. Bioactivity of recombinant IGF-I in transgenic mouse milk extracts was demonstrated by a concentration-dependent increase in [^{3}H]thymidine incorporation in clonal bovine MAC-T mammary cells compared with control mouse milk extracts; moreover, addition of excess rhIGFBP-3 abolished the increase in [^{3}H]thymidine incorporation attributed to recombinant IGF-I in transgenic mouse milk. Recombinant IGF-I was produced in mammary tissue of virgin and pregnant transgenic mice and secreted into milk of lactating mice. However, recombinant IGF-I was not detected in serum from transgenic mice; and ligand blot analysis of serum IGFBP indicated no differences due to transgene presence. In peripubertal virgin mice at 49 d of age, the frequency of appearance of mammary alveolar buds was significantly higher in MMTV-IGF-I than in CD-1 mice and was

unaffected by ovariectomy or estradiol treatment. In conclusion, mammary synthesis of recombinant IGF-I enhances the rate of development of alveolar buds in mammary glands of virgin transgenic mice.

INTRODUCTION

Insulin-like growth factor-I (IGF-I) has been implicated in many biological processes including prenatal and postnatal development of a number of tissues (Jones and Clemmons, 1995). In vitro observations on the mitogenicity of IGF-I suggest an important role in growth stimulation of mammary epithelial cells, but not necessarily a regulatory one (Imagawa et al., 1990). Insulin-like growth factor-I is synthesized in mammary stroma (Cullen et al., 1992) and is generally considered to act in a paracrine fashion via epithelial cell IGF-I receptors. Clonal bovine mammary epithelial cells expressing recombinant IGF-I exhibited an accelerated growth rate (Romagnolo et al., 1992).

Significant physiological effects of hIGF-I overexpression directed by a metallothionein promoter in transgenic mice have been described, although its impact on mammary growth was not reported (Mathews et al., 1988). Using transgenic mouse models to examine the effects of local mammary IGF-I synthesis, researchers showed that expression of recombinant IGF-I under control of the whey acidic protein promoter inhibited normal involution (Hadsell et al., 1996; Neuenschwander et al., 1996), while high levels of α_{s1}-casein promoter-directed IGF-I expression in the mammary glands of

transgenic rabbits was without significant effect on mammary function (Wolf et al., 1997). However, the effects of mammary-specific synthesis of IGF-I in the developing mammary gland have not been described. When IGF-I-containing implants were placed into the mammary glands of hypophysectomized, gonadectomized, estradiol-treated immature rats for five days, Ruan et al. (1992) showed that IGF-I mimicked the action of ST in mammary development by stimulating epithelial cell differentiation into terminal end buds and alveolar structures. Similarly, using whole mouse mammary gland organ culture, Richert and Wood (1997) suggested that activation of the IGF receptor may mediate significant ductal and alveolar growth. Taken together with evidence that mammary IGF-I gene expression displays developmental regulation during fetal and neonatal stages (Morgan et al., 1996; Lee et al., 1993), these findings indicate that IGF-I may play an important role in early mammary development.

Our objective was to examine the effects of increased mammary IGF-I synthesis on development of the peripubertal mouse mammary gland, using a transgenic mouse model expressing recombinant ovine IGF-I under control of the mouse mammary tumor virus - long terminal repeat (MMTV-LTR) promoter. In this study, we report the transgene expression of biologically active IGF-I in virgin, pregnant and lactating mice and describe its effects on peripubertal mammary gland development.

MATERIALS AND METHODS

Transgene

Details on the construction of the MMTV-IGF-I transgene (Fig. 2) have been described elsewhere (Romagnolo et al., 1992). The 3.7 kb construct contains a 0.7 kb cDNA encoding an ovine exon-2 prepro IGF-I (Ohlsen et al., 1993) under control of the MMTV-LTR promoter. The construct was isolated from the expression vector pMMTV-IGF-I (Romagnolo et al., 1992) following digestion with the restriction endonucleases BglII and NdeI (USB, Cleveland, OH), excised from a 1% agarose gel, purified using GeneClean (BIO 101 Inc., La Jolla, CA), and filtered through a 0.45 µm spin filter.

Generation of transgenic mice

The linearized MMTV-IGF-I fragments were microinjected into the pronuclei of embryos from CD-1 mice (Charles River Laboratories, Wilmington, MA) housed four per cage in a temperature (21 ± 1 °C) and light (12 h light/day)-controlled environment. Rodent chow and tap water was available to mice ad libitum. Tail DNA was isolated from the resulting offspring and screened for presence of the transgene by PCR. Tail DNA served as the template in a 25-µl reaction including each of the following: 1X Taq polymerase buffer, 0.2 mM dNTP's, 0.4 µM oligonucleotide primers, 1.5 mM $MgCl_2$, and 0.625 U Taq polymerase (Promega Corp., Madison, WI). The oligonucleotide primers 3c (5'-GATGCCAGTCACATCCTCCTCGC-3'; exon 3, nucleotides 190-212) and 4a (5'-GAGCCTTGGGCATGTCGGTG-3'; exon 4, nucleotides 383-403) amplified

a 329-bp sequence in the ovine IGF-I cDNA (Ohlsen et al., 1993). Initial denaturation of the samples was performed at 98 °C for 2 min followed by 40 cycles at 56 °C for 45 sec, 72 °C for 45 sec, and 94.5 °C for 45 sec. Non-transgenic mouse DNA was used as a negative control, and a dilution series of MMTV-IGF-I plasmid standards served as a positive control.

Transgenic founders were subsequently mated with nontransgenic CD-1 mice to generate transgenic lines. All pregnant or lactating mice used in this study were mated at six to seven weeks of age and were primigravid. Transgene copy number for individuals within each line was determined by Southern blotting using 10 µg BamHI-digested tail DNA. Digested samples were electrophoresed through a 0.8% agarose gel and transferred to nitrocellulose membranes (NitroPlus, MSI, Westborough, MA). Membranes were hybridized overnight at 42 °C with the 0.7 kb ovine IGF-I cDNA [^{32}P]-labeled by random priming, washed according to manufacturer's instructions and exposed to Kodak XAR-5 film at -80 °C with intensifying screens.

To evaluate the activity of the glucocorticoid-responsive promoter, a test group of virgin mice were treated with dexamethasone following steroid priming of mammary gland development. Beginning at 28 to 30 d of age, control and transgenic F_1 females (n = 2/line) were injected subcutaneously with estrogen (1 µg/ml) and progesterone (1 mg/ml) for 9 days (Sheffield et al., 1985). Twelve hours after the final steroid injection, mice were injected i.p. with dexamethasone (Sigma, St. Louis, MO; 50 µg in saline with 1% ethanol and 1 mg/ml gum arabic) every 12 h for a total of 5 injections. Mice were

sacrificed by cervical dislocation 12 h after the final injection and tissue was removed for analysis of recombinant IGF-I concentration.

RNA analysis

Total RNA was extracted from tissues using TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH). Twenty micrograms of total RNA was electrophoresed on a 1% agarose-0.66M formaldehyde gel and transferred to nylon membranes (ZetaProbe, BioRad, Hercules, CA) by vacuum blotting (BioRad, Hercules, CA). Northern blots were processed as described above for Southern blots.

Total RNA prepared from the fourth inguinal mammary gland was used in confirming mammary transgene expression by RT-PCR. The oligonucleotide primer 6a (5'-CTGCACTCCCTCTGCTTGTG-3'; exon 6, nucleotides 426-445) was hybridized to 1 µg of total RNA in 10 µl at 95 °C for 5 min followed by cooling for 15 min at 37 °C (Ohlsen et al., 1993). Reverse transcription was performed in a 25 µl reaction volume of 1X first strand buffer (50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$), 200 U of M-MLV reverse transcriptase (Life Technologies, Gaithersburg, MD), 2.0 mM dNTP's, 10 mM DTT, and 40 U rRNAsin (Promega Corp., Madison, WI) at 37 °C for 1 h. To remove RNA remaining at the end of the incubation period, samples were digested with 1 µg of RNase A for 15 min at 37 °C, followed by incubation at 37 °C in 0.1N NaOH and 0.03M EDTA. Samples were neutralized and fractionated on Sephadex G-50 spin columns before precipitation of cDNA with ethanol in the presence of ammonium

acetate. The cDNA product was analyzed by PCR as described above for the detection of the MMTV-IGF-I transgene.

[^{3}H]Thymidine incorporation

Mammary tissue was obtained from female virgin mice (n = 6/stage) at 60 and 90 d and at 12 d for pregnant mice. For pregnant animals (n = 6), tissues for explants and for IGF-I assay were obtained from the fourth inguinal glands. Approximately 150 mg of explants (1-3 mg each) were incubated in 2 ml DMEM (Life Technologies, Gaithersburg, MD) containing 2 µCi [^{3}H]thymidine (specific activity 67 Ci/mole; ICN Biomedicals, Irvine, CA) in a shaking water bath at 37 °C for 1 h.

Because we anticipated that preparation of homogeneous explants from virgin mouse mammary glands would be difficult, 60 and 90 d mice were injected with [^{3}H]thymidine (2 µCi/g BW) for measurement of DNA synthesis in the mammary gland. After 1 h the right fourth inguinal gland was removed for analysis. Incorporation of [^{3}H]thymidine into mammary DNA from both virgin and pregnant mice was used as a measure of DNA synthesis. The validity of both methods of measuring [^{3}H]thymidine incorporation described above has been previously discussed (Woodward et al., 1993).

Mitogenic activity in transgenic mouse milk

The mitogenic activity in transgenic and nontransgenic mouse milk from primiparous 80 d transgenic mice was compared for the ability to stimulate [^{3}H]thymidine incorporation in the MAC-T bovine mammary epithelial cell line (Huynh

et al., 1991). Skim milk samples from d 12 lactation were extracted to remove IGFBP (Breier et al., 1991), lyophilized, and resuspended in serum-free DMEM. The MAC-T cells were cultured in 24-well plates and serum-starved for 72 h before addition of milk extracts with or without 320 ng/ml rhIGFBP-3 (Celtrix Pharmaceuticals, Santa Clara, CA) for 16 h at 37 °C. Cells were then pulsed for 2 h with 1 μCi of [^{3}H]thymidine per well. At the end of the incubation, cells were harvested for measurement of [^{3}H]thymidine incorporation (Zhao et al., 1992).

Radioimmunoassay for IGF-I and ligand blotting

Extraction of IGF-I from plasma, milk, and mammary tissue homogenates was performed using an acid-ethanol cryoprecipitation method according to Breier et al. (1991). Two hundred microliters of skim milk or plasma was mixed with acid-ethanol extraction buffer at a ratio of 1:4. For mammary tissue, one hundred milligrams of frozen crushed tissue was homogenized in 1 ml of extraction buffer. Following extraction, the supernatant was frozen at -20 °C until radioimmunoassay for IGF-I as previously described (Shimamoto et al., 1992). Recombinant IGF-I and total IGF-I in extracts were measured using a mouse anti-human IGF-I monoclonal primary antibody (Kerr, 1989) and with a rabbit anti-human IGF-I polyclonal primary antibody (antiserum UB3-189; Hormone Distribution Program of NIDDK and the National Hormone and Pituitary Program), respectively. The lower limit of detection for assays using the monoclonal antibody was approximately 10 ng/ml. Approximately 85% of IGF-I was recovered when added to milk and plasma in this assay (data not shown).

Plasma profiles of IGFBP were analyzed by Western ligand blotting (Hossenlopp et al., 1986). Blood samples were centrifuged for 15 min at 12,000 × g, and the resulting plasma fraction was recovered. Samples (4 µl) were dissolved in reducing SDS-polyacrylamide gel sample buffer and separated overnight by 12.5% SDS-PAGE at constant current. Proteins were blotted to a nitrocellulose membrane (MSI, Westborough, MA). Blots were incubated with [^{125}I]IGF-I and exposed to Kodak XAR-5 film at -70 °C for 5 to 7 days with intensifying screens.

Effects of oIGF-I expression on mammary development

Effects of transgene expression on mammary development were examined using mammary gland whole mounts. Mice were randomly selected from CD-1 and transgenic mouse populations housed similarly to avoid bias due to stage of the estrous cycle on mammary development. Prepubertal female mice in the F_3 and F_4 generations were ovariectomized or sham-operated at 35 d of age, as early as possible following PCR analysis of transgenesis before puberty, and allowed to recover until hormone injections commenced at 42 d of age. Animals within each group (n = 6 per treatment) were designated to receive 1 of 3 hormone injections i.m. for 7 d: dexamethasone (5 µg/d); estradiol (1 µg/d); or placebo. Mice were sacrificed by cervical dislocation at 49 d of age. Fourth inguinal mammary glands were removed, spread onto microscopy slides, fixed overnight in Histochoice (Amresco, Solon, OH), and subsequently processed as described by Strum (1979).

Statistical analysis

Statistical analysis was performed using the GLM procedure in SAS (SAS Institute, Inc., 1985) (Appendix A). For analysis of bioactivity of recombinant IGF-I, the statistical model included transgene presence, milk extract concentration, and transgene presence × milk extract concentration. Linear contrasts were used to determine effects of the addition of milk extracts from control and transgenic mice on [^{3}H]thymidine incorporation into MAC-T cells. The first contrast compared the means of [3H]thymidine incorporation into DNA of MAC-T cells for transgenic and control mouse milk extract. The second contrast compared the difference in [3H]thymidine incorporation stimulated by transgenic and control mouse milk extracts within each concentration. Chi-square analysis was used to test for transgene and treatment effects on the number of mice exhibiting alveolar buds. Contrasts were performed to test for significance in measurements of [^{3}H]thymidine incorporation. Values are presented as least squares means ± SEM.

RESULTS

Characterization of MMTV-IGF-I transgenic mice

Six transgenic founder mice were generated by pronuclear microinjection and identified by PCR analysis. Transgenic mouse lines were established from each of the six founder animals. Southern blot analysis of F_1, F_2, and F_3 offspring from two founders are shown in Fig. 1. After Bam HI digestion of tail DNA, all mice showed an

endogenous 14 kb band. Transgenic Line 15 and Line 29 mice showed a faint band at 1.1 kb corresponding to the middle Bam HI-digestion product of the transgene (Figure 2). Line 29 mice showed an additional band at 3.2 kb. Line 15 mice showed four additional bands at 6.7, 7.5, 8.5 and 17 kb corresponding to the variable-length 5' segment of the integrated transgene copies following Bam HI digestion. These results indicate the presence of four copies of the transgene in Line 15 and one copy in Line 29; further, these bands were present in F_1, F_2 and F_3 animals indicating that the transgene copies were stably inherited.

To determine initially whether each transgenic line was expressing the transgene in mammary tissue, extramammary tissues and the fourth inguinal mammary glands from 80 d mice and mice on d 14 of lactation were screened for presence of recombinant IGF-I mRNA by Northern blotting and RT-PCR. Mammary tissue from transgenic mice produced a transgene IGF-I mRNA distinguishable from the endogenous IGF-I mRNA found in liver tissue (Figure 3). Mammary RNA from control mice was not loaded equally on this blot compared to transgenic mice, possibly contributing to the apparent absence of endogenous IGF-I expression in non-transgenic mammary tissue; also, this may reflect a limitation of detection by Northern analysis. The spleen, salivary gland, kidney, and brain expressed mRNA for the IGF-I transgene (depending on the line) at lower levels in comparison with lactating mammary tissue (Table 1). Transgene expression in brain tissue was detected in one out of two Line 15 mice. Mammary expression of transgene IGF-I ranged from 47 to 140% of endogenous liver IGF-I mRNA

for both transgenic lines. Transgene expression was not detected in liver or heart tissue in either line.

To determine whether the transgene mRNA was translated in mammary tissue, tissue homogenates were analyzed by radioimmunoassay using a mouse anti-human IGF-I monoclonal antibody that detected ovine and bovine but not mouse IGF-I. Recombinant IGF-I was detected at low levels of approximately 2 ng/100 mg in the fourth inguinal mammary glands of virgin mice of transgenic lines 15 and 29. On d 12 of pregnancy, transgene expression was increased to approximately 4 ng/100 mg. To induce greater activity of the glucocorticoid-responsive MMTV promoter, a test group of virgin mice were treated with dexamethasone. Dexamethasone-treated transgenic virgin mice from lines 15 and 29 responded with mammary recombinant IGF-I production of 11.8 ± 0.6 and 21.9 ± 0.4 ng/100 mg tissue (n = 2/line). This acute dosage of dexamethasone was chosen based on its reported ability to induce expression of MMTV-LTR fusion genes over a short term (Stewart, 1988).

Lactating mice from transgenic lines 15 and 29 secreted 2859 ± 374 ng/ml and 1008 ± 109 ng/ml recombinant IGF-I into milk on d 16 of lactation, respectively (n = 2 to 4). Recombinant IGF-I was not detected in plasma from transgenic virgin, pregnant, or lactating females. Plasma IGF-I concentrations averaged 362 ± 33 and 218 ± 20 ng/ml in 60 d virgin and d 12 pregnant CD-1 mice, respectively (n = 6) and did not differ in transgenic mice ($P > .10$). Native milk IGF-I concentrations in CD-1 mice (n = 4) averaged 552 ± 69 ng/ml on d 16 of lactation.

Profiles of IGFBP in serum from control and transgenic animals were evaluated using Western ligand blotting. Four bands corresponding to (I) 39-43-kD IGFBP-3, (II) 32-kD IGFBP-2, (III) 28-kD IGFBP-1, and (IV) 24-kD IGFBP-4 were detected in pooled plasma from CD-1 and MMTV-IGF-I mice (Figure 4) (Donovan et al., 1994). Differences in abundance of IGFBP were not detected between control and transgenic animals in individual or pooled samples; however, pregnancy dramatically reduced serum levels of IGFBP-3 and increased IGFBP-4 levels compared with virgin mice.

Bioactivity of recombinant IGF-I

To compare the mitogenic activity in milk from transgenic mice and control mice, milk extracts from a mouse of line 29 and from non-transgenic mice were cultured with MAC-T cells. The enhanced mitogenic activity of transgenic mouse milk relative to control mouse milk was demonstrated by a greater stimulation of [^{3}H]thymidine incorporation in MAC-T cells ($P < .05$) (Figure 5). Using a pool of milk extracts obtained from different mice, a subsequent study confirmed the ability of extracts to stimulate cell proliferation as well as the ability of excess rhIGFBP3 to inhibit the mitogenic activity in milk extracts (Table 2).

[^{3}H]Thymidine incorporation

The relative number of cells actively synthesizing DNA in explants of mammary tissue from non-transgenic and transgenic pregnant mice was compared by measurement of [^{3}H]thymidine incorporation over a 1 h period. Virgin mice were injected with

[^{3}H]thymidine and mammary tissue was recovered 1 h later. Subsequent analysis indicated no differences in [^{3}H]thymidine incorporation among CD-1 and MMTV-IGF-I virgin mice or pregnant mice (Table 3). Not surprisingly, [^{3}H]thymidine incorporation was markedly increased in tissue from pregnant mice ($P < .001$).

Effects on mammary development

Comparison of mammary whole mounts from MMTV-IGF-I mice with those from CD-1 mice was used to evaluate the effects of IGF-I expression on peripubertal gland development. The single-copy Line 29 was selected for this study because transgene recombination events influencing expression were detected by Southern analysis in several Line 15 individuals at the time of the experiment. Mammary whole mounts were prepared from non-transgenic and transgenic mice that had been ovariectomized or sham-operated and subsequently treated with placebo, estradiol, or dexamethasone. Because mammary DNA synthesis at 60 d of age did not differ between control and transgenic mice, mammary development was analyzed in younger, 50 d mice following ovariectomy and hormone treatment. Histological observation of the methylene blue-stained glands showed that a greater percentage of transgenic mice (24/34, 71%) displayed alveolar buds in mammary parenchyma compared to CD-1 mice (7/34, 21%) ($P < .01$) (Table 4). In those mice showing alveolar buds, the buds were distinguishable throughout the ductular network (Figure 6). The proportion of mice showing alveolar buds was not different ($p>0.10$) between intact and ovariectomized CD-1 (3/17 versus 4/17) or MMTV-IGF-I mice (14/18 versus 10/16), respectively. Similarly, there was no difference between

placebo- and estrogen-treated CD-1 (4/12 versus 3/10) or MMTV-IGF-I mice (9/12 versus 9/11), respectively. However, dexamethasone reduced the number of animals exhibiting alveolar buds regardless of transgene presence ($P = .05$). Body weight of treatment groups was not different ($P > .10$).

DISCUSSION

Production of IGF-I within the mammary gland was achieved in this study using the model system of transgenic mice producing recombinant ovine IGF-I under control of the MMTV-LTR promoter. Our study investigated the influence of locally produced IGF-I on growth and development of the virgin mouse mammary gland. Ovine IGF-I, which differs from murine IGF-I by five amino acids (Wong et al., 1989), was used to evaluate the effects of IGF-I synthesis in vivo based on our earlier studies of the role of recombinant IGF-I produced by clonal mammary epithelial cells in vitro (Romagnolo et al., 1992). We report the characterization of transgene expression in virgin and pregnant animals, evaluation of biological activity of the recombinant IGF-I, and analysis of its effects on mammary development.

Tissue-specific expression of biologically active recombinant IGF-I protein was observed in two lines of MMTV-IGF-I mice at virgin, pregnant, and lactating stages. Previous work using the WAP-IGF-I transgenic mouse (Hadsell et al., 1996; Neuenschwander et al., 1996) or transgenic rabbits carrying the bovine α_{s1}-casein promoter to direct IGF-I expression (Wolf et al., 1997) focused on transgene effects

during the stages of pregnancy and lactation, when promoter activity is maximal. In contrast, the MMTV promoter utilized in our model becomes active in the virgin animal and is more strongly induced during pregnancy and lactation. Northern analysis of the tissue specificity of the transgene revealed low-level expression in salivary gland, kidney, spleen and brain, similar to the pattern of MMTV expression noted in other studies (Mok et al., 1992). However, recombinant protein was not detected in serum of transgenic mice of any stage, using a monoclonal IGF-I antibody that does not recognize mouse IGF-I. Further, western blot analysis of serum IGFBP showed no differences due to transgene expression. Absence of the 43-kD IGFBP-3 in serum is characteristic of pregnant mice regardless of transgenesis; this degradation of IGFBP-3 was originally observed during human and rat pregnancy and is attributed to proteolysis by a specific serum protease (Rutishauser et al., 1993). Thus, the MMTV-IGF-I mice exhibited tissue-specific transgene expression which allowed us to evaluate the effects of locally produced IGF-I on the virgin mammary gland. Bioactivity of recombinant IGF-I was demonstrated by stimulation of DNA synthesis in MAC-T cells above that derived from mitogenic activity inherent in mouse milk. Proliferative effects of recombinant IGF-I were blocked in vitro by addition of IGFBP-3, one of the IGF-I binding proteins secreted by mouse mammary epithelial cells which may serve to regulate IGF-I actions (Fielder et al., 1992).

The role of mammary-synthesized IGF-I remains undetermined. Much evidence exists, however, to suggest that IGF-I may be important in early mammary development (Akers, 1990). Clearly IGF-I is a mitogen for mammary tissue in vitro, as demonstrated using collagen gel culture of normal undifferentiated mammary epithelial cells of the

mouse (Imagawa et al., 1986), rat (Ethier et al., 1987), sheep (Winder et al., 1989), and the cow (Shamay et al., 1988). The effects of IGF-I in the immature mammary gland have been reviewed by Kleinberg (1997). Ruan et al. (1992) demonstrated that implants of both intact and des(1-3)IGF-I induced development of alveolar structures and terminal end buds in the mammary glands of hypophysectomized, castrated, and estradiol-treated sexually immature male rats. Likewise, mammary IGF-I expression in our transgenic virgin mice induced formation of similar alveolar buds. In contrast, IGF-I did not affect lobulo-alveolar development of mouse mammary glands in whole organ culture (Plaut et al., 1993). This discrepancy may possibly be related to the different methods of in vivo transgenesis and in vitro organ culture used in the two studies.

The increased incidence of alveolar budding in non-transgenic mice in our study compared with that of Ruan et al. (1995) may be related to the later age at which ovariectomy was performed in our animals, the shorter time period of recovery before treatments were applied, or species differences. Such alveolar buds develop around the time of puberty in response to ovarian secretions, and mature into alveolar structures during pregnancy (Russo and Russo, 1987). Treatment with dexamethasone depressed alveolar bud development despite its ability to induce IGF-I transgene expression in virgin mice. Such a result may be attributed to a general catabolic effect of acute dexamethasone treatment on body tissues. Alternatively, dexamethasone has been shown to inhibit growth hormone-induced IGF-I mRNA synthesis in the liver (Luo and Murphy, 1989) and may have exerted its effect on alveolar bud development by reducing endogenous liver IGF-I synthesis.

Evaluation of the rate of mammary growth may be an important indicator of the effects of IGF-I on an absolute level. DNA synthesis, as measured by [^{3}H]thymidine incorporation into mammary explants, was not different between MMTV-IGF-I and CD-1 virgin mice. The lack of difference in mammary DNA synthesis, despite the observed difference in presence of alveolar budding, suggests that IGF-I may enhance the rate of early virgin mammary development occurring prior to 60 d of age.

During pregnancy, IGF-I stimulated DNA synthesis or cell proliferation in mammary tissue slices from pregnant cows (Baumrucker and Stemberger, 1989), collagen gel culture of cells from pregnant heifers (McGrath et al., 1991), and in primary cells as well as in response to in vivo infusion of IGF-I (Collier et al., 1993). Our results did not demonstrate an effect of local IGF-I synthesis on whole mount appearance of transgenic mouse mammary glands during pregnancy, although additional evaluations are justified as the density of ductular and alveolar structures may render potential differences visually undetectable.

Similar to more defined mechanisms of action in other tissues, mammary production of IGF-I under control of circulating ST may contribute to the growth and differentiation of mammary epithelium (Walden et al., 1997). The relative importance of systemic versus local production of IGF-I for the development of the mammary gland remains controversial, as mammary IGF-I expression is considerably lower than that in liver and other reproductive tissues (Forsyth, 1996). However, mammary IGF-I expression can be influenced by exogenous ST (Kleinberg et al., 1990), plane of nutrition (Weber et al., 1996), and is developmentally regulated (Simmen, 1991), suggesting a

potential role in mammary growth. In our study, epithelial cell synthesis of IGF-I induced alveolar bud development in peripubertal mice, potentially at a faster rate or at an earlier age than in non-transgenic mice. Additional studies are in progress to evaluate the hormonal factors contributing to this effect as well as the consequences of mammary IGF-I expression in lactating MMTV-IGF-I mice.

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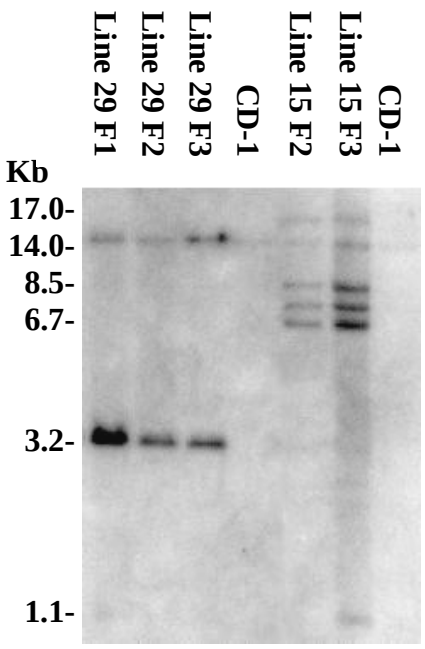

Figure 1. Southern analysis of transgene copy number in transgenic MMTV-IGF-I and nontransgenic CD-1 mice. Blots were hybridized with a [^{32}P]-IGF-I cDNA as described in Materials and Methods and exposed for 10 d. Each lane contains 10 μg DNA digested with Bam HI. Sizes are shown in kb.

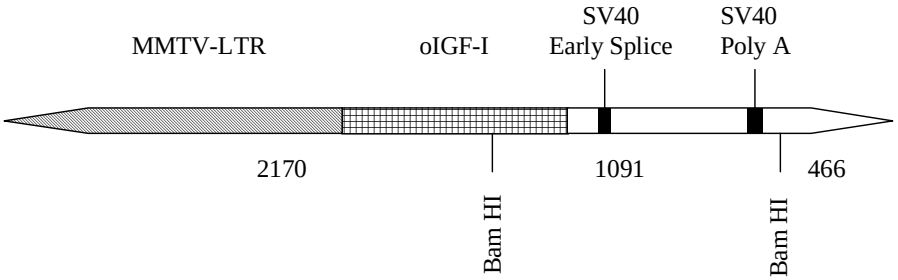

Figure 2. Map of the MMTV-IGF-I transgene (3.7 kb). Segments are the MMTV-LTR promoter and oIGF-I cDNA that were assembled in the expression vector pMSG as described previously (Romagnolo et al., 1992). SV40 early splice and poly A sites are indicated. The sizes (kb) of the fragments generated by Bam HI digestion of the construct are shown.

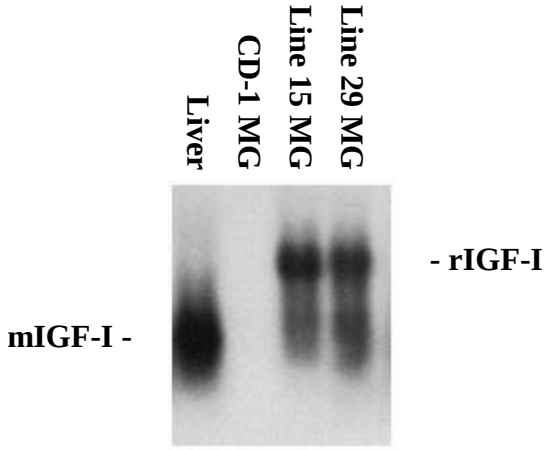

Figure 3. Northern analysis of tissue-specific IGF-I expression in tissues of mice at d 14 lactation. Total RNA was prepared from CD-1 liver and mammary glands (MG) of one non-transgenic CD-1 and one transgenic mouse from Lines 15 and 29 and loaded at 20 μg/lane. Blots were hybridized overnight with a [^{32}P]-IGF-I cDNA and exposed to film for 12 d. The lower band corresponds to endogenous mouse IGF-I (mIGF-I) and the upper band derives from recombinant IGF-I (rIGF-I) expression.

Table 1. Tissue-specific mRNA expression of recombinant IGF-I (rIGF-I) in tissues from transgenic mice at d 14 lactation (n = 1 or 2/line).

	rIGF-I expression (% of mammary rIGF-I expression within line, densitometric units)[1]	
Tissue	**Line 15**	**Line 29**
Mammary (lactating)	100	100
Spleen	0	45
Brain	13	0
Kidney	0	10
Salivary gland	0	6
Heart	0	0
Liver	0	0

[1]Relative abundance of mRNA for rIGF-I in mammary tissue was 2874 and 2640 densitometric units for lines 15 and 29, respectively. Endogenous IGF-I mRNA abundance in liver was 2477, 2595, and 3063 densitometric units for CD-1, line 15 and line 29, respectively.

Table 2. Effect of rhIGFBP-3 (320 ng/ml) on mean [^{3}H]thymidine incorporation into MAC-T cells in response to basal medium (DMEM; Dulbecco's Modified Eagle Medium), rhIGF-I, or milk extracts (20 µl/well) (means ± SEM, n=2/pool)

Treatment	**[^{3}H]thymidine incorporation (cpm×10^4)**	
	-BP3	**+BP3**
DMEM	1.6 ± .09	1.5 ± .07
rhIGF-I (1 ng/well)	5.3 ± .46	1.6 ± .04[a]
rhIGF-I (20 ng/well)	14.3 ± .70	2.2 ± .12[a]
Line 29 pool	11.1 ± .39	1.7 ± .23[a]
Line 15 pool	10.0 ± .41	1.7 ± .13[a]

[a]Different from treatments without added IGFBP-3 ($P < .001$).

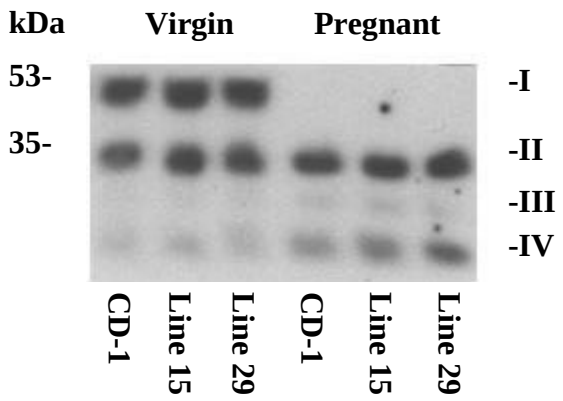

Figure 4. Western ligand blot analysis of plasma pools (n = 6/lane) from nontransgenic or MMTV-IGF-I mice at 60 d of age or d 12 of pregnancy. Blood samples were obtained via cardiac puncture. Plasma was collected and electrophoresed by SDS-PAGE, blotted to nitrocellulose, and probed with [^{125}I]-IGF-I. Four bands corresponding to (I) 39-43 kD IGFBP-3, (II) 32-kD IGFBP-2, (III) 28-kD IGFBP-1, and (IV) 24-kD IGFBP-4 were detected in pooled plasma from CD-1 and MMTV-IGF-I mice. Blots were exposed for 7 days.

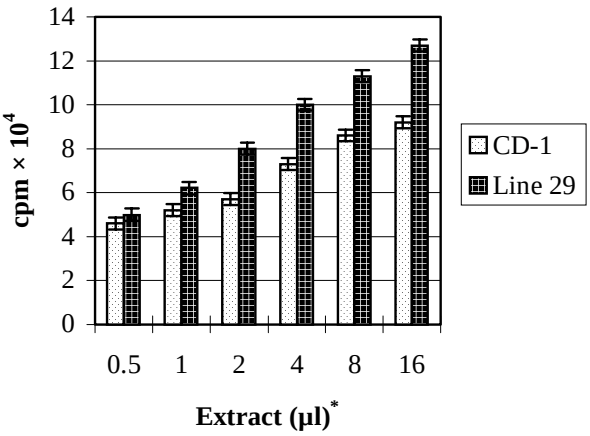

Figure 5. Mean [^{3}H]thymidine incorporation into MAC-T cells in response to pooled milk extracts from CD-1 and transgenic MMTV-IGF-I mice (n = 2/line). MAC-T cells were cultured in 24-well plates for 16 h followed by a pulse with 1 µCi [^{3}H]thymidine/well for 2 h. Bars represent means ± SEM from 4 wells at each concentration. [a]MMTV-IGF-I differs from CD-1 controls at 2 µl extract and greater ($P < .05$).

Table 3. Mean [^{3}H]thymidine incorporation (cpm × 10^2/µg DNA) into mammary explants from 60 d or 90 d virgin or d 12 pregnant CD-1 and transgenic MMTV-IGF-I lines 15 and 29 mice (means ± SEM, n = 6/line).

Mouse Line	**[^{3}H]thymidine incorporation (cpm × 10^2/µg DNA)**		
	60 d	**90 d**	**Pregnant**
CD-1	50 ± 3.5	46 ± 11.2	296 ± 36.0
Line 15	34 ± 5.4	31 ± 5.4	215 ± 28.9
Line 29	45 ± 8.9	48 ± 8.8	279 ± 24.6

Table 4. Effect of ovariectomy and hormone treatment (NH = placebo, E2 = estrogen, Dex = dexamethasone) on presence of alveolar buds in mammary glands of CD-1 and MMTV-IGF-I mice

Mouse Line	Total[a]	Treatment[a]		
		NH	E2	Dex[b]
CD-1	7/34	4/12	3/10	0/12
MMTV-IGF-I	24/34[c]	9/12	9/11	6/11

[a]Results are expressed as the number of animals exhibiting alveolar structures/total number of animals.
[b]Reduced overall number of mice exhibiting alveolar buds compared with NH ($P = .05$).
[c]Different from CD-1 ($P < .01$).

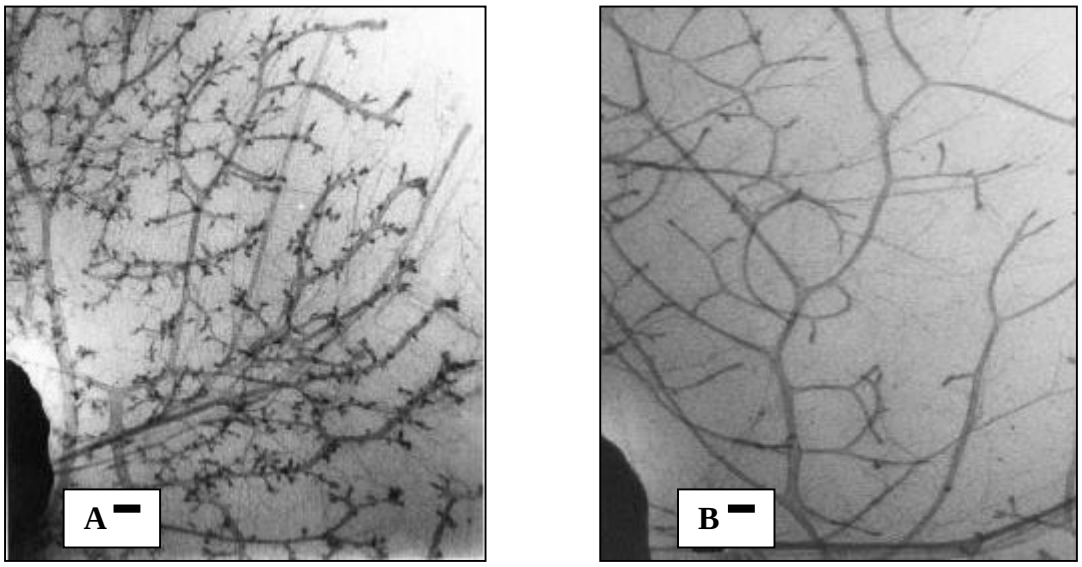

Figure 6. Histological comparison of methylene blue-stained mammary gland whole mounts in the peripubertal period from (A) MMTV-IGF-I transgenic and (B) CD-1 mice. Tissue was taken from 50 d virgin mice. Whole mounts were processed as described in Materials and Methods. Each bar equals 500 µm. Photos represent 24/34 transgenic Line 29 and 7/34 non-transgenic CD-1 mice from a study investigating the effects of ovariectomy and hormone treatments (estradiol, dexamethasone, or placebo). Histological appearance of alveolar buds was not affected by ovariectomy or hormone treatment.

CHAPTER 3

OVEREXPRESSION OF INSULIN-LIKE GROWTH FACTOR-I MODULATES MAMMARY INVOLUTION AND BINDING PROTEIN SYNTHESIS IN TRANSGENIC MICE

ABSTRACT

To investigate the role of IGF-I and IGFBP in the lactating mammary gland, transgenic mice were generated using an ovine prepro IGF-I cDNA under control of the mouse mammary tumor virus-long terminal repeat (MMTV-LTR) promoter. Mammary synthesis of recombinant IGF-I (rIGF-I) significantly reduced the contribution of endogenous IGF-I to total IGF-I found in milk of transgenic mice throughout lactation. Analysis by SDS-PAGE of milk from MMTV-IGF-I and CD-1 mice showed no apparent differences in milk protein composition. However, protein content was reduced in milk from both transgenic lines compared with CD-1 mice. A shift in the milk IGFBP profile from predominantly IGFBP-3 to a lower molecular weight IGFBP was evident between d 8 and d 12 of lactation. Overall, there was a greater concentration of IGFBP in milk from transgenic mice. The altered milk composition led to reduced growth rates in litters suckling transgenic compared with CD-1 dams from d 2 to d 14 of lactation. In mammary glands at 7 d involution, rIGF-I synthesis impaired mammary involution, as

indicated by presence of more organized secretory lobules and ducts in transgenic mice. Recombinant IGF-I synthesis in mammary tissue modulates endogenous mammary IGF-I and IGFBP synthesis and mammary function, likely via autocrine or paracrine effects on stromal and epithelial cells.

INTRODUCTION

Milk contains multiple growth factors that may contribute to regulation of mammary gland function during lactation and to development of the suckling neonate. Among these is insulin-like growth factor-I (IGF-I), a 7.6-kD peptide that plays a role in mouse mammary development (Chapter 2; Weber et al., 1998) and involution (Chapter 3; Hadsell et al., 1996; Neuenschwander et al., 1996). Modulation of lactation by IGF-I expression has not been reported despite high-level expression of IGF-I transgenes directed to the lactating mammary gland (Hadsell et al., 1996; Neuenschwander et al., 1997; Wolf et al., 1996). Expression of IGF-I in milk has been described for rats (Donovan et al., 1991), humans (Baxter et al., 1984), cows (Francis et al., 1986), and pigs (Simmen et al., 1988; Donovan et al., 1994).

The first objective of this study was to evaluate the effects of rIGF-I synthesis on mammary IGFBP expression and gland function during lactation. The second objective was to study the effects of rIGF-I synthesis on involution of the mammary gland.

MATERIALS AND METHODS

Generation of transgenic mice

Details on transgene construction and generation of the MMTV-IGF-I transgenic founder mice have been described elsewhere (Chapter 2; Weber et al., 1998). Transgenic founders were mated with nontransgenic CD-1 mice (Charles River Laboratories, Wilmington, MA) to generate the two transgenic lines used in this study.

Milk composition

Milk protein expression in pools of milk from control and transgenic mice at d 4, 8, 12 and 16 of lactation (1 to 4 mice per stage) was evaluated by SDS-PAGE using 10 µl skim milk mixed with 10 µl 200 mM EDTA, pH 7.0. Samples (160 µg milk whey protein) were loaded on a non-reducing 9 - 18% SDS-polyacrylamide gel and separated overnight at constant current. Protein bands were visualized by silver staining.

Concentrations of total and recombinant IGF-I were evaluated by radioimmunoassay in milk samples. Total IGF-I in milk was not measured in these Line 15 animals because of low sample volume and a priority on measurement of recombinant IGF-I. Extraction of IGF-I from milk was performed using an acid-ethanol cryoprecipitation method according to Weber et al. (1998). Radioimmunoassays were conducted according to Shimamoto et al. (1992) using a mouse anti-human IGF-I monoclonal primary antibody (Kerr et al., 1989) and a rabbit anti-human IGF-I

polyclonal primary antibody (antiserum UB3-189; Hormone Distribution Program of NIDDK and the National Hormone and Pituitary Program).

Profiles of IGFBP in the same milk samples on d 4, 8, 12, and 16 of lactation were analyzed by Western ligand blotting (Hossenlopp et al., 1986). Samples were centrifuged at 12,000 × *g* for 30 min. Ten microliters of the supernatant was dissolved in 10 µl non-reducing SDS-polyacrylamide gel sample buffer and separated overnight by 12.5% SDS-PAGE at constant current. Proteins were blotted to a nitrocellulose membrane (MSI, Westborough, MA). Blots were incubated with [^{125}I]IGF-I and exposed to Kodak XAR-5 film at -70 °C with intensifying screens.

Milk protein content was evaluated in milk samples obtained from primiparous CD-1 and transgenic line 15 and line 29 mice (n = 10 to 12 mice/line) on d 12 of lactation. Measurements were performed using the bicinchoninic acid assay (Pierce Life Technologies, Rockford, IL). Bovine serum albumin was used as a standard. Milk fat percentage was determined using a hematocrit tube sample of fresh milk.

Plasma IGF-I and IGFBP

Blood was obtained by cardiac puncture at sacrifice from primiparous lactating nontransgenic CD-1 and transgenic line 15 and 29 mice (n=6/line) on days 4 and 14 of lactation. Samples were centrifuged for 15 min at 12,000 × g, and the plasma fraction was recovered and stored at -20 °C. Concentrations of total and recombinant IGF-I were determined in acid-ethanol extracted samples by radioimmunoassay as described above.

Abundance of IGFBP in plasma samples was evaluated by Western ligand blotting analysis as described above.

[^{3}H]Thymidine incorporation

Incorporation of [^{3}H]thymidine into mammary DNA from lactating mice was used as a measure of DNA synthesis. Mammary tissue was obtained from d 4 and d 14 lactating primiparous mice (n = 6/stage). Tissues for explants and for IGF-I assay were obtained from the fourth inguinal glands. Approximately 150 mg of explants (1-3 mg each) were incubated in 2 ml DMEM (Life Technologies, Gaithersburg, MD) containing 2 µCi [^{3}H]thymidine (specific activity 67 Ci/mole; ICN Biomedicals, Irvine, CA) in a shaking water bath at 37 °C for 1 h. DNA concentration was determined according to Woodward et al. (1993).

RNA analysis

Total RNA was extracted from mammary tissue from primiparous mice at d 14 of lactation using TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH). Twenty micrograms of total RNA was electrophoresed on a 1% agarose-0.66M formaldehyde gel and transferred to nylon membranes (ZetaProbe, BioRad, Hercules, CA) by vacuum blotting (BioRad, Hercules, CA). Membranes were hybridized for 2 h at 68 °C in QuikHyb hybridization buffer (Stratagene, La Jolla, CA) with a 0.7 kb ovine IGF-I or a 440 bp rat IGFBP-3 cDNA [^{32}P]-labeled by random priming. Northern blots were washed 2 × 15 min in 1X SSC, 5% SDS at room temperature and 1 × 30 min in

0.1X SSC, 0.5% SDS at 65 °C and exposed to Kodak XAR-5 film at -80 °C with intensifying screens for 2 d.

Litter growth rates

Growth rates of litters fostered on non-transgenic and transgenic lines 15 and 29 primiparous dams (n = 6/line) were examined from d 2 to d 14 of lactation. Two-day old pups from separate non-transgenic dams were fostered on dams at d 2 of lactation; and litters were standardized to 14 pups in an initial study using transgenic lines 15 and 29. Weights of dams and litters were taken at 0900 h each day. In one or two litters per line, one pup that appeared unhealthy was replaced with a non-transgenic pup at a similar developmental stage before taking litter weights.

In a follow-up weigh-suckle-weigh study milk yield was measured in non-transgenic and transgenic line 15 mice (n = 6/line) with litters standardized to 12 pups. Transgenic line 29 was not included due to lack of available animals at the time of the study. Weights of dams and litters were taken at 0900 h each day. On d 8, 10, 12 and 14 of lactation, litters were separated from dams at 0900 h for 4.5 h. Litters were weighed prior to being returned to dams, and the daily litter weight was taken after a 1.5 h suckling period. Milk yield of dams was calculated by the difference in litter weights prior to and after suckling. Dam weights were also taken at the end of the suckling period.

Effects of oIGF-I expression on involution

The effects of transgene expression on mammary involution in CD-1 and transgenic lines 15 and 29 (n = 6 mice/line) were examined on histological sections of mammary tissue taken at d 7 of involution. Dams in their fifth or sixth lactations were separated from pups at d 12 of lactation. After 7 d, mammary tissue pieces were excised from the fourth inguinal glands, fixed in 10% neutral buffered formalin, and processed. In addition, fourth inguinal mammary glands were removed, spread onto microscopy slides, fixed overnight in Histochoice (Amresco, Solon, OH), and subsequently processed as described by Strum (1979).

Statistical analysis

Statistical analysis was performed using the GLM procedure in SAS (SAS Institute, Inc., 1985) (Appendix B). Values are presented as least squares means ± SE. The statistical model included block and transgene presence. Data for comparison of litter growth rates in CD-1 and line 15 mice did not differ between the two studies and were combined for purposes of statistical analysis. Litter growth rates were analyzed using a model with block and transgene. Native IGF-I concentrations were compared for 4, 8, 12, and 16 d using the repeated measures analysis procedure in GLM with a model that included transgene.

RESULTS

Milk protein analysis

Milk protein composition was analyzed by SDS-PAGE of pooled samples on d 4, 8, 12, and 16 of lactation. Stage of lactation effects were evident for multiple bands (Figure 1). However, milk protein composition did not appear to grossly differ between CD-1 and transgenic lines 15 and 29 (Figure 1).

Concentrations of IGF-I in milk and plasma were evaluated by radioimmunoassay using polyclonal and monoclonal antibodies to IGF-I. Lactating mice from the CD-1 strain and from transgenic line 29 (n = 4/line) secreted 600 ± 283 and 822 ± 245 ng/ml of total IGF-I into milk on d 16 of lactation, respectively. Recombinant IGF-I in the same pool of milk samples from transgenic line 29 averaged 701 ± 47 ng/ml. Native IGF-I concentrations in milk were calculated from the difference of total and recombinant IGF-I measurements. On d 4, 8, and 16 of lactation, native IGF-I secretion into milk was reduced in transgenic mice ($P < .05$; Figure 2A), although total IGF-I levels in milk of transgenic mice were consistently higher than CD-1 mice during lactation (Figure 2B).

Abundance of IGFBP in milk samples was analyzed by ligand blotting. Multiple bands were detected, corresponding to 39-43 kD (IGFBP-3), approximately 32 kD, and approximately 29 kD (Figure 3). Whereas IGFBP-3 was the predominant IGFBP in milk on d 4 and 8 of lactation, there was a shift between d 8 and d 12 to a greater relative abundance of a lower molecular weight IGFBP on d 12 and 16. Abundance of all IGFBP appeared to be greater in transgenic mouse milk compared with that of CD-1 controls.

On overexposed blots (not shown), a band of approximately 29 kD became evident on d 12 and 16 of lactation in samples from CD-1 mice and by d 8 in samples from transgenic line 15.

Milk protein concentration was evaluated in milk samples obtained at d 14 of lactation from primiparous mice. At d 14 of lactation, milk protein concentrations tended to be lower in transgenic line 15 (131 ± 6 mg/ml; $P < .07$) and in transgenic line 29 (133 ± 8 mg/ml; $P < .09$) than CD-1 controls (151 ± 6 mg/ml). Mean values for milk fat percentage in samples from CD-1 mice were 34.5 ± 4.6, 28 ± 5.4, 32.7 ± 5.2, and 25.3 ± 3.3 on d 4, 8, 12, and 16 of lactation, and did not differ for transgenic line 15 and 29 mice ($P > .10$).

Plasma IGF-I and IGFBP

Plasma IGF-I concentrations averaged 421 and 304 ± 38 ng/ml in d 4 and d 14 lactating CD-1 mice (n = 6/stage), respectively, and decreased during this period ($P < .05$). Plasma IGF-I concentrations in line 15 did not differ significantly from controls on d 4 (333 ± 38 ng/ml; $P < .12$); neither was there a change in concentration by d 14 (319 ± 38 ng/ml; $P < .79$). Line 29 exhibited lower plasma IGF-I levels on d 4 compared with controls (306 ± 38 ng/ml; $P < .05$) but unlike controls was unaffected by stage of lactation (391 ± 38 ng/ml; $P > .12$). Recombinant IGF-I was not detected in plasma from transgenic lactating females. Analysis of plasma IGFBP revealed four bands corresponding to 39-43 kD IGFBP-3, 32 kD IGFBP-2, 28 kD (putatively IGFBP-1), and

24 kD (putatively IGFBP-4) (data not shown). Abundance of these IGFBP did not appear to be affected by transgenesis.

[^{3}H]Thymidine incorporation

The relative number of mammary cells undergoing DNA synthesis in explants of mammary tissue from lactating CD-1 and transgenic lines 15 and 29 mice was compared by measurement of [^{3}H]thymidine incorporation over a 1-h period. [^{3}H]thymidine incorporation into mammary explants from line 29 on d 4 of lactation was higher than that of CD-1 mice (Table 1; $P < .05$). Line 15 did not differ from CD-1 on d 4, and neither transgenic line differed from CD-1 on d 14 ($P > 0.10$). Concentration of DNA in mammary explants did not differ as a result of transgenesis ($P > 0.10$).

RNA analysis

To determine if IGF-I transgene expression in mammary tissue modulated local production of IGFBP-3, mammary tissue from mice (n = 3/line) on d 14 of lactation was analyzed for presence of recombinant IGFBP-3 mRNA by northern blotting. Mammary tissue from both non-transgenic and transgenic mice produced an IGFBP-3 mRNA (Figure 4). Levels of IGFBP-3 mRNA consistently appeared more abundant in mammary tissue of CD-1 mice compared with tissue from both transgenic lines. Liver tissue from a CD-1 mouse served as a positive control and exhibited an mRNA transcript similar in size to that detected in mammary tissue. Equal loading of lanes was confirmed by visual evaluation of ethidium bromide staining.

Litter growth rates

Litter weights of fostered litters suckling CD-1 compared with transgenic line 15 or 29 were compared over d 2 to d 14 of lactation. Litter weights of litters suckling transgenic line 29 dams were reduced compared with CD-1 dams (3.78 vs. 5.21 ± .11 g/d, respectively; $P < .001$) (Figure 5). Growth rates of pups suckling transgenic line 15 dams were not significantly lower than controls (4.24 vs. 4.94 ± .31 g/d, respectively; $P > .13$) (Figure 6). Milk yield, as measured by weigh-suckle-weigh, did not differ as a result of transgenesis ($P > .10$); average gains of pups after the suckling periods on d 8, 10, 12, and 14 of lactation were 2.15 and 2.34 ± .20 g/d for pups suckling CD-1 and line 15 dams, respectively.

Effects of oIGF-I expression on involution

Comparison of mammary whole mounts from transgenic lines 15 and 29 with those from CD-1 mice did not indicate apparent effects of transgenesis on mammary involution (data not shown). Histological comparison of hematoxylin-stained sections of mammary tissue revealed a pattern of reduced involution in both transgenic line 15 and 29 mice compared with CD-1 mice, characterized by retention of more organized secretory lobules and ducts (Figure 7).

DISCUSSION

Synthesis of ovine IGF-I targeted to the mammary glands of transgenic mice has previously been shown to stimulate mammary development in prepubertal mice (Chapter 2; Weber et al., 1998). This study presents our subsequent investigation into the effects of IGF-I transgene expression on lactation and involution of the mouse mammary gland. We report the expression of native IGF-I and IGFBP in mammary tissue and milk and its modulation by transgene IGF-I synthesis. The effects of mammary IGF-I overexpression on milk composition, growth of suckling litters, and on involution of the mammary gland were also characterized.

In an earlier study describing the effects of transgene expression on peripubertal mammary development, we reported the tissue-specific expression of ovine IGF-I in mammary tissue during lactation (Chapter 2; Weber et al., 1998). Transgene IGF-I was not detected in blood from virgin, pregnant, or lactating mice, suggesting that observed effects on mammary tissue were locally mediated. In addition, ligand blot analysis of serum from lactating mice did not show apparent differences in IGFBP profiles (data not shown), further supporting the tissue specific localization of the transgene product. Plasma IGF-I concentrations decreased from early to late lactation in CD-1 mice; however, transgenic mice did not display a similar change in plasma IGF-I. The reason for this discrepancy is unknown. It is possible that tissues other than the liver can contribute to circulating IGF-I levels; alternatively, an unknown mediator may provide feedback from IGF-I expressing tissues to modulate endogenous IGF-I secretion.

The physiological source of IGF-I in milk is unknown; however, the observed reduction in endogenous IGF-I secretion into milk of transgenic mice suggests the existence of a local regulatory mechanism. Synthesis of IGF-I in normal mammary tissue of CD-1 mice was not detectable by northern analysis, whereas mRNA for the transgene IGF-I was clearly evident. Secretion of IGFBP-3 and -2 into milk was increased in transgenic mice, whereas mRNA for IGFBP-3 was lower in mammary tissue from transgenic mice (Figure 4). These results support the suggestion of Donovan et al. (1995) for rats that milk IGFBP-3 may arise from the maternal circulation. However, similar to the ovarian IGF-I system (Adashi et al., 1997), species differences exist between rats and mice in that rat mammary tissue expressed only IGFBP-2 and -4 mRNA.

Despite the visual similarity in milk protein composition from transgenic and CD-1 mice (Figure 1), milk protein concentrations tended to be lower in MMTV-IGF-I than CD-1 mice. Milk fat percentage was not affected, nor was milk yield different as measured by the weigh-suckle-weigh procedure. Altered milk composition of transgenic dams likely contributed to the reduction in average daily gain of litters suckling transgenic compared with control dams. Specifically, IGF-I in milk is a potent contributor to growth and development of the gastrointestinal tract in newborn rat pups (Staley et al., 1998). In calves, colostrum intake or oral administration of IGF-I can modulate plasma IGF-I levels (Hammon and Blum, 1997; Baumrucker and Blum, 1994). It is possible that rIGF-I levels in milk modulated intestinal growth or endogenous IGF-I levels in plasma to reduce litter growth rates particularly at 10 d of age and older. Alternatively, the lower endogenous IGF-I levels in milk from transgenic dams may have

been responsible for the slower growth rates of suckling litters. Interestingly, mammary DNA synthesis at d 4 of lactation was increased in one transgenic line, indicating that locally synthesized IGF-I may promote growth of mammary cells rather than synthesis of milk in early lactation when the mouse mammary gland is approaching its peak DNA content.

Similar to earlier studies in which IGF-I expression was directed to the mammary glands of transgenic mice using whey acidic protein gene regulatory sequences (Hadsell et al., 1996; Neuenschwander et al., 1996), we observed inhibited involution of the mammary gland in multiparous MMTV-IGF-I mice. The mechanism by which epithelial cell synthesis of IGF-I modulates involution of the epithelium is not clear but may relate to the local IGFBP profile. Specifically, a key role has been proposed for IGFBP-5 in the induction of apoptosis in the rat mammary gland (Tonner et al., 1997). A similar mechanism may exist for the mouse mammary gland. It is intriguing that the presence of an IGFBP similar in molecular weight to IGFBP-5 is detectable in mouse milk by d 8 of lactation in transgenic mice, and by d 12 in CD-1 mice (Figure 3).

The ability of the mammary gland to modulate local synthesis and secretion of components of the IGF system, as well as the process of involution, emphasizes the importance of locally produced growth factors in gland development and function. An increased understanding of the local regulation of growth factor synthesis and activity may provide insight on potential mechanisms to enhance mammary epithelial cell productivity during lactation.

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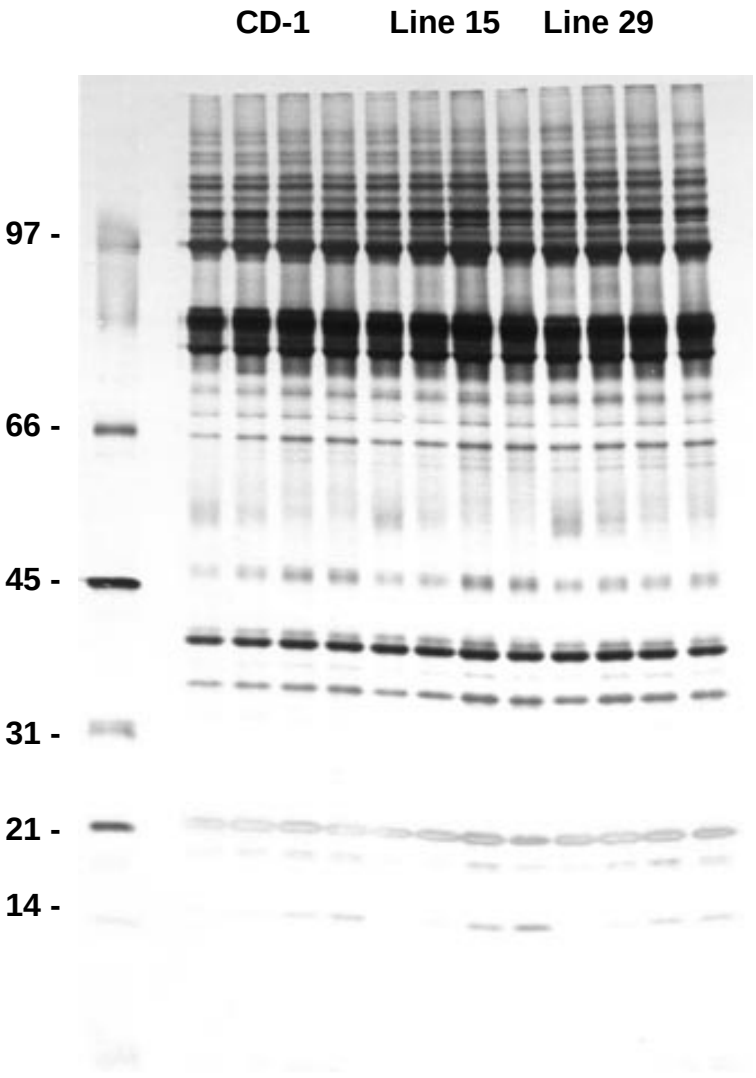


Figure 1. Milk proteins in pooled mouse milk from non-transgenic CD-1 and transgenic Line 15 and Line 29 mice on d 4, 8, 12, and 16 of lactation. Samples were separated by 9 - 18% SDS-PAGE and visualized by silver staining. Migration of molecular mass markers is shown on the left.

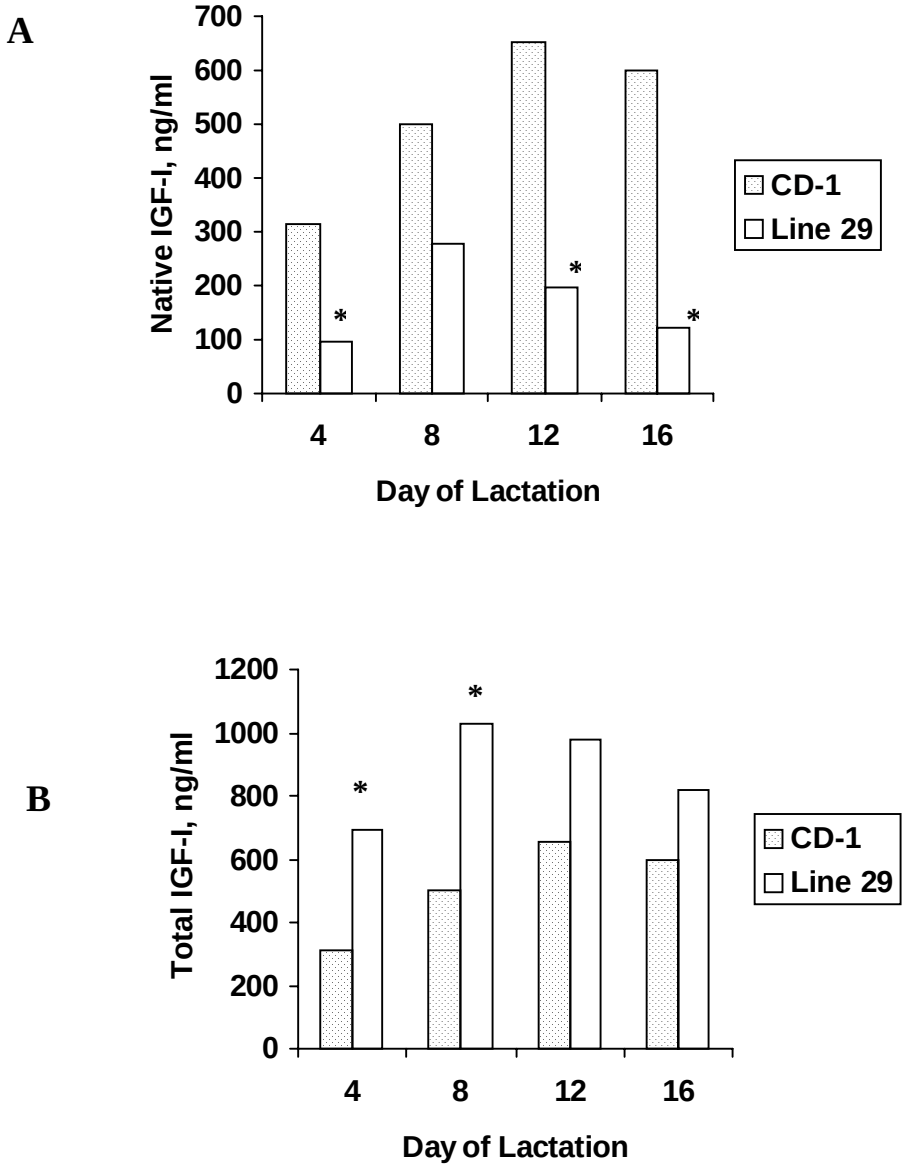


Figure 2. Mean concentrations (ng/ml) of native IGF-I (A) and total IGF-I (B) in skim milk from CD-1 or transgenic line 29 mice (n = 3 or 4) on d 4, 8, 12 and 16 lactation. *CD-1 is different from Line 29 ($P < .05$).

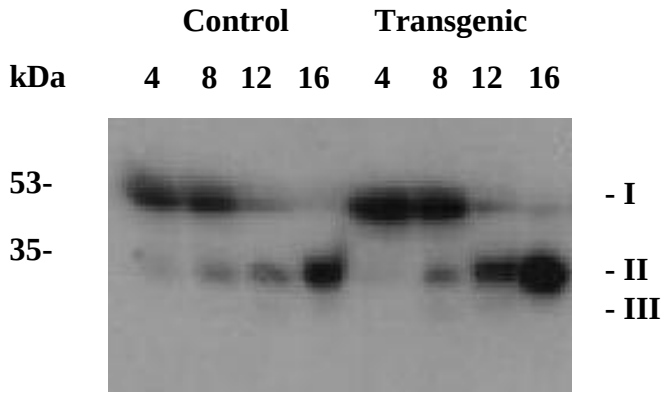


Figure 3. Western ligand blot analysis of whey samples (n = 6/lane) from nontransgenic CD-1 or transgenic line 15 mice on d 4, 8, 12, and 16 of lactation. Milk samples were centrifuged at 12,000 × g for 30 min. Ten microliters per lane of the supernatant was separated by SDS-PAGE, blotted to nitrocellulose and probed with [^{125}I]-IGF-I. Three bands corresponding to (I) 39-43 kD IGFBP-3, (II) ~ 32 kD (IGFBP-2), and (III) ~ 29 kD were detected in pooled samples from CD-1 and transgenic mice. Blots were exposed for 4 d.

Table 1. Mean [^{3}H]thymidine incorporation (cpm × 10^2/µg DNA) and DNA concentration (µg/mg) in mammary explants from d 4 or d 14 lactating non-transgenic CD-1 and transgenic lines 15 and 29 mice (means ± SEM, n = 6/line).

Mouse Line	**d 4**		**d 14**	
	[^{3}H]thymidine incorporation	**DNA (µg/mg)**	**[^{3}H]thymidine incorporation**	**DNA (µg/mg)**
CD-1	44.1 ± 5.8	3.74 ± .38	29.7 ± 2.8	2.80 ± .24
Line 15	49.0 ± 11.1	3.47 ± .35	28.7 ± 5.9	2.93 ± .28
Line 29	89.0 ± 10.2	3.54 ± .37	30.7 ± 2.6	2.76 ± .22

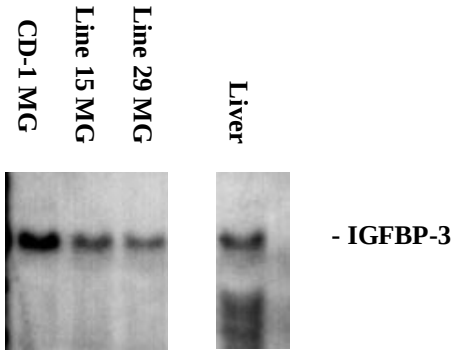


Figure 4. Representative northern analyses of IGFBP-3 mRNA in mammary tissue of non-transgenic CD-1 and transgenic line 15 and line 29 mice at d 14 of lactation. Total RNA was prepared from CD-1 liver and mammary glands (MG) of CD-1 and transgenic line 15 and 29 mice (n = 3/line) and loaded at 20 μg/lane. Blots were hybridized for 2 h with a [^{32}P]-IGFBP-3 cDNA and exposed to film for 2 d.

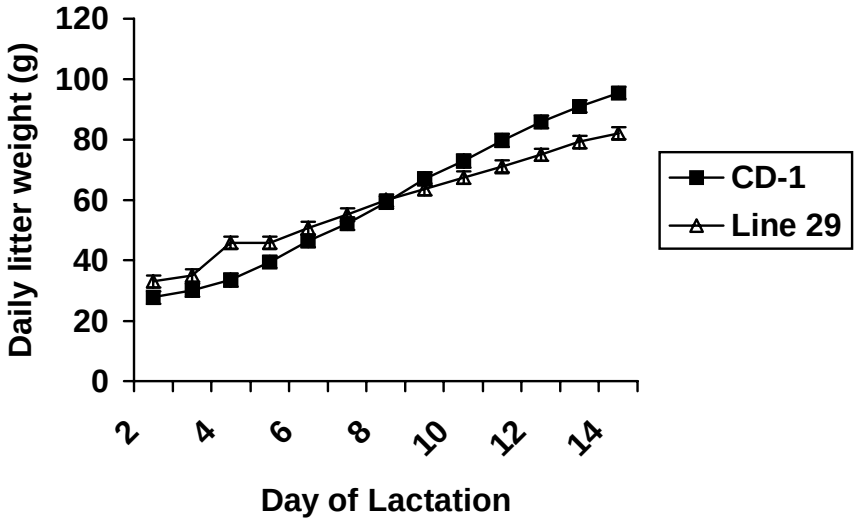


Figure 5. Daily litter weights (g/d) of fostered nontransgenic CD-1 litters (n = 14 pups) suckling CD-1 or transgenic line 29 dams (n = 6/line) from d 2 to d 14 of lactation. Data are means ± SEM.

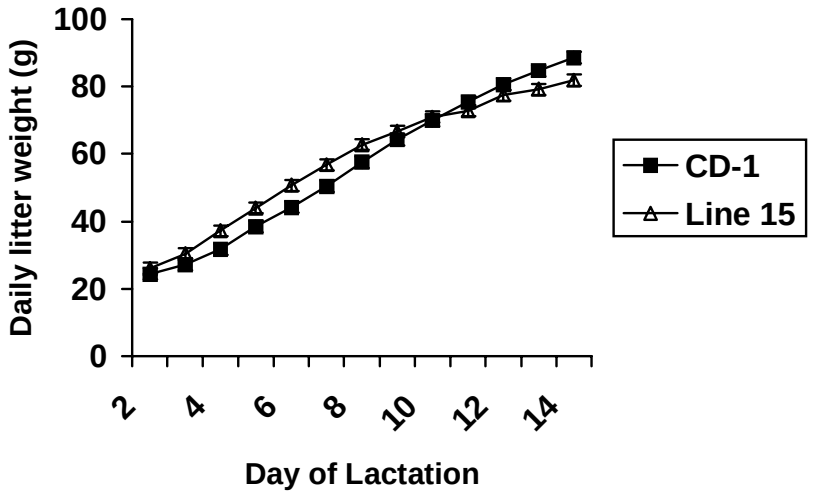
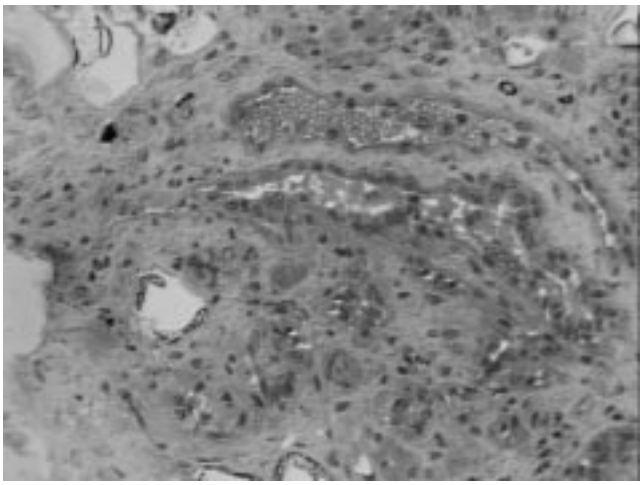


Figure 6. Daily litter weights (g/d) of fostered non-transgenic CD-1 litters (n=12 or 14 pups) suckling CD-1 or transgenic Line 15 dams (n=12/line) from d 2 to d 14 of lactation. Data are means ± SEM.

A



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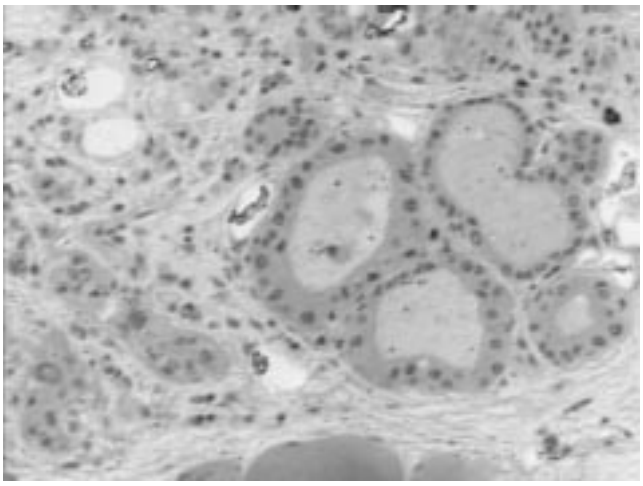

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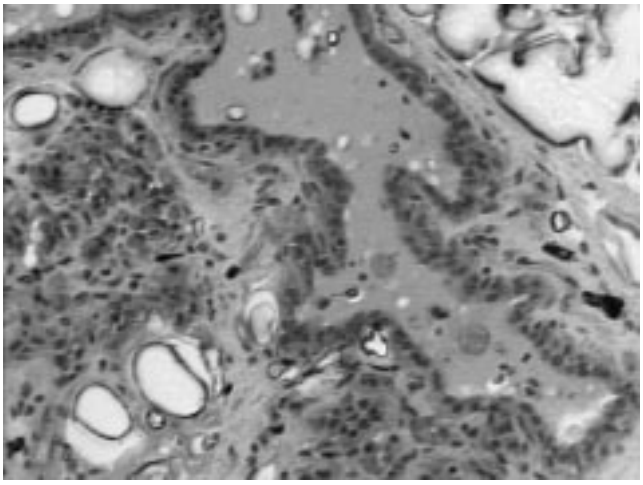

Figure 7. Histological comparison of representative sections of mammary tissue collected at 7 d involution from CD-1 (A) or MMTV-IGF-I transgenic mice from Line 15 (B) or Line 29 (C).

CHAPTER 4

NUTRITIONAL AND SOMATOTROPIN REGULATION OF THE MITOGENIC RESPONSE TO BOVINE MAMMARY TISSUE EXTRACTS

ABSTRACT

Our objective was to investigate the influence of feeding level and exogenous bovine somatotropin (bST) on the mitogenic response to extracts of mammary tissue from prepubertal heifers. Twenty-four prepubertal Friesian heifers were blocked according to body weight. Within each block, heifers were randomly assigned to placebo or bST (15 mg/d) treatment at low or high feeding level (0.55 or 1.1 kg/d average daily gain, respectively) in a 5-week experiment. Following slaughter, mammary extracts were prepared from parenchymal tissue. The mitogenic response to mammary extracts was tested using primary undifferentiated mammary epithelial organoids cultured for 4 to 5 d in collagen gels in serum-free medium supplemented with 5% of the mammary extracts. Cell proliferation was determined using [methyl-^{3}H]thymidine incorporation as a measure of DNA synthesis. High feeding level reduced DNA synthesis in response to mammary extracts. At low feeding level, somatotropin treatment decreased DNA synthesis in response to mammary extracts; whereas, at high feeding level, somatotropin increased DNA synthesis in response to mammary extracts. These results suggest that

locally produced growth factors are involved in regulation of mammary development when mammary growth is modulated by feeding level and bST treatment.

INTRODUCTION

Mammogenesis is modulated by circulating protein and steroid hormones that elicit their effects in part by subsequent modulation of paracrine or autocrine growth factors (Cunha and Hom, 1996). As these locally produced growth factors are defined in terms of temporal and physiological regulation of expression, knowledge of their interactive effects are increasingly important to understanding of mammary growth. Presently, regulation of growth factor gene expression in the prepubertal bovine mammary gland is poorly understood. Moreover, complex interactions between hormones and metabolic regulators make it difficult to predict the growth response of the mammary gland using in vivo experiments.

Peripubertal mammary development in heifers can be modulated by feeding level and exogenous bST. Circulating levels of somatotropin have been postulated to mediate at least a portion of the effects of both feeding and level and exogenous somatotropin (Sejrsen and Purup, 1997). In addition, recent studies have begun to elucidate the roles of systemic and locally produced growth factors in mammary growth. Specific factors that serve to mediate somatotropin actions or that exert effects in the mammary gland independent of somatotropin control include IGF-I and IGF-II, transforming growth factors α and β (TGFα and TGFβ), the fibroblast growth factors, platelet derived growth

factor (PDGF), and mammary-derived growth factor-1 (MDGF-1) (Plaut, 1993; Forsyth, 1991). Production of at least some of these locally produced factors appears to be influenced by different stages of gland development (Talhouk et al., 1996).

The focus of this study was to determine whether the mitogenic response to extracts of mammary tissue of prepubertal heifers was influenced by exogenous somatotropin or feeding level.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade unless otherwise stated. Human recombinant insulin-like growth factor-I (MW=7,646 Da) was purchased from Bachem AG (Bubendorf, Switzerland). Fetal bovine serum (FBS) was purchased from Life Technologies (Gaithersburg, MD). Basal medium was prepared from M199 with Earle's salts, L-glutamine, and Hepes buffer (Sigma, St. Louis, MO), sodium bicarbonate (24 mM), bovine serum albumin (2.6 mg/ml), transferrin (5 μg/ml), reduced glutathione (1 μg/ml), soybean trypsin inhibitor (1 μg/ml), bovine insulin (10 ng/ml), selenium (1 ng/ml) and antibiotic solution (0.2%) containing penicillin (50,000 IU/l) and streptomycin (50 μg/ml) were added to the medium. [Methyl-^{3}H]thymidine (specific activity 3.03 TBq/mmol) was obtained from Amersham International (Birkerød, Denmark).

Experimental design

Twenty-four Holstein Friesian heifers (233 ± 7 kg BW, 271 ± 4 d at slaughter) were blocked by body weight in an earlier experiment described by Vestergaard et al. (1995). Within blocks of 4, animals were randomly assigned to bST treatment at low or high feeding level in an experiment with a 2×2 factorial arrangement of treatments. Treatments continued for 5 weeks and consisted of daily bST injections (0 or 100 μg/kg body weight) at either low or high feeding level. The low feeding level utilized a roughage-based mixed ration available ad libitum and supported an average daily gain (ADG) of 0.55 kg/d. The high feeding level diet was a concentrate mixture available ad libitum along with 2 kg/d of the mixed ration and yielded an ADG of 1.1 kg/d.

One block of four heifers was killed each week depending on body weight. Heifers were stunned by captive bolt and sacrificed by exsanguination at a final body weight of 233 ± 7 kg. At slaughter, mammary glands were removed; mammary parenchyma was excised from the left half of the udder and frozen at -80 °C. Aqueous mammary extracts were prepared from thawed tissue essentially according to Waksman et al. (1991). Frozen slices (5 g total) were cut from larger tissue samples and homogenized in physiological saline (12 ml) for 1 min using a Polytron tissue homogenizer (Kinematica, Buch & Holm, Herlev, Denmark). A saline rinse (3 ml) of the Polytron tip was combined with the initial homogenate, and the total homogenate (3:1 saline to tissue) was shaken at 4 °C for 90 min. Homogenates were then filtered through a double layer of surgical gauze, and the filtrate was centrifuged for 45 min at 10,000 × *g* at 4 °C. The supernatant was recovered from below the uppermost fat layer and subsequently centrifuged at

105,000 × *g* for 1 h at 4 °C. The supernatant was filtered through sterile 0.2 µm filters and stored at -80 °C. Protein content of the mammary extracts was determined using the bicinchoninic acid assay (Pierce Life Technologies, Roskilde, Denmark).

Ligand blotting and IGF-I assay

Concentrations of IGF-I in mammary extracts were measured by ELISA (OCTEIA IGF-I; IDS Ltd., UK) (Norup & Vestergaard, 1996). For calculations, it was assumed that 1 g of mammary tissue was equivalent to a volume of 1 ml. Concentrations of IGFBP in mammary extracts were evaluated by Western ligand blotting (Hossenlopp et al. 1986). Samples (200 µg protein; 17 to 31 µl extract) were dissolved in non-reducing SDS-polyacrylamide (SDS-PAGE) gel buffer, heated to 60 °C for 15 min, and separated overnight on an SDS-polyacrylamide gel at constant current. Following transfer of proteins to a nitrocellulose membrane by electroblotting, the blots were incubated with [^{125}I]IGF-I and washed. Autoradiographs from the blots were exposed at -70 °C for 2 days.

Primary cell cultures

Mammary epithelial cells for testing of mitogenic response to extracts were prepared from mammary parenchymal tissue from two prepubertal Friesian heifers (198 and 223 kg body weight) essentially according to Shamay et al. (1988). Within 10 min of slaughter, mammary parenchyma was excised aseptically from the outer margin of parenchyma (outer 1/3) and minced finely with scissors. Tissue pieces were transferred

to a petri dish and weighed. Tissue was incubated in basal medium supplemented with DNase (277 µg/g tissue), insulin (67 µg/g tissue) and an antibiotic-antimycotic mixture, at a ratio of 10 ml medium per gram of tissue, for 10 min in a 37 °C shaking water bath. Tissue pieces were allowed to sediment for 5 min and excess medium was aspirated. Tissue was then digested in basal medium supplemented with collagenase (10 mg/g tissue), hyaluronidase (10 mg/g tissue), DNase (277 µg/g of tissue) and insulin (67 µg/g of tissue), at a ratio of 10 ml medium per gram of tissue, for 5 h in a 37 °C shaking water bath. Organoids were isolated by filtering the suspension through 200 µm Nitex and centrifuging the filtrate for 10 min at 300 × *g*. Organoids were suspended in basal medium and allowed to sediment for 8 min; then excess medium was aspirated and replaced with fresh medium for a total of 10 washes. Following suspension in basal medium supplemented with 44% FBS and 6% dimethyl sulfoxide (DMSO), organoids were frozen overnight at -80 °C and transferred the next day to liquid N_2.

Collagen gels were prepared essentially as described by Shamay et al. (1988). A collagen solution prepared from rat tail tendons was neutralized using a neutralizing solution (10× M-199 without bicarbonate and 0.33 M NaOH at a 2:1 ratio) at a ratio of about 6:1. Each well of 24-well plates (Nunc A/S, Roskilde, Denmark) was coated with 0.5 ml of the neutralized collagen. Before use, organoids were quickly thawed at 37 °C and washed in basal medium, then suspended in the cold neutralized collagen solution. The cell-collagen suspension (0.5 ml) was pipetted onto each collagen gel and allowed to gel in a 37 °C incubator for 30 min before 1 ml of basal medium was added. Cells were cultured for 5 d at 37 °C in air:CO_2 (95:5).

Treatment media were added 24 h after plating and were changed every 48 h. Results for [methyl-^{3}H]thymidine incorporation were determined on a liquid scintillation counter. Because variation was observed in the mitogenic response to basal medium in different experiments, results were normalized by dividing values for [^{3}H]thymidine incorporation per well by the average value for basal [^{3}H]thymidine incorporation in each assay. Results from five independent cell culture assays testing the effects of mammary extracts on two different cell preparations were then averaged and analyzed as described.

Statistical analysis

Statistical analyses were performed using the GLM procedure of SAS (SAS Institute, Inc. 1989) (Appendix C). Results were analyzed as a randomized complete block design using block and treatments (somatotropin, feeding level) as main effects, with cell preparation (heifer) and its interactions as the error term. Comparisons among treatments used non-orthogonal contrasts to generate F values. The F values obtained from contrasts were adjusted using the Bonferroni F test. Comparisons with $P < .05$ were considered to be significantly different. Values are presented as least squares means ± SEM of values obtained from triplicate samples.

RESULTS

This study assessed the overall effects of exogenous somatotropin and feeding level on the mitogenic response to extracts of mammary tissue from prepubertal heifers. Similar to previous experiments, maximal stimulation of DNA synthesis was induced by approximately 25 ng/ml of IGF-I. Addition of 10% FBS stimulated DNA synthesis approximately three times that of basal medium across five cell culture assays.

Addition of extracts of mammary tissue from prepubertal heifers stimulated [^{3}H]thymidine incorporation to a greater degree than 10% FBS or the greatest concentration of IGF-I used (100 ng/ml). There was a dose-dependent response to mammary extracts over the range of 2 to 8%, with maximal stimulation between 6 and 8% extract concentration in media (Chapter 6, Figure 2). A concentration near the middle of this range (5%) was used in cell cultures. Insulin concentrations in individual mammary extracts were undetectable. Concentrations of IGF-I in mammary extracts averaged 33.3 ± 1.8 ng/ml.

High feeding level inhibited [^{3}H]thymidine incorporation in response to mammary extracts (Figure 1; $P < .01$). At the low feeding level, bST treatment decreased [^{3}H]thymidine incorporation (Figure 2A; $P < .05$); whereas, bST at high feeding level increased [^{3}H]thymidine incorporation in response to mammary extracts (Figure 2B; $P < .05$). Extract protein concentrations were highly correlated with the mitogenic response ($r = .53$; $P < .007$) but were not significantly affected by heifer treatment ($P > .10$).

DISCUSSION

Prepubertal growth and development of the mammary gland is dependent on circulating peptide and steroid hormones, including somatotropin and estrogens (Topper and Freeman, 1980). It is evident from studies on normal ruminant mammary tissue that these hormones work in concert with local tissue mediators to affect growth of the mammary epithelium (Purup et al., 1998; Woodward et al., 1994). Mammary tissue synthesizes multiple growth factors that may influence mammary growth in a positive or negative fashion (Forsyth, 1991). In the case of IGF-I, the growth response is modulated by IGFBP which may also exert IGF-independent effects on growth (Jones and Clemmons, 1995).

The negative effect of a rapid growth rate on mammary development in prepubertal heifers has been well established (Sejrsen and Purup, 1997), although the underlying mechanisms have not been elucidated. The mitogenic response to extracts of mammary tissue from prepubertal heifers is reduced by a high feeding level, suggesting that local changes in growth factor concentrations are important for growth of the mammary gland. Our recent work (Purup et al., 1998; Chapter 5) demonstrated that a high feeding level increased the abundance of IGFBP-3 protein in mammary tissue from prepubertal heifers. Interestingly, IGFBP-3 in mammary tissue extracts was negatively correlated with the mitogenic response to those extracts, indicating a role for IGFBP-3 in the negative effect of a rapid growth rate. Interactions between IGFBP-3 and locally produced growth factors in growth modulation was also supported by evidence for TGF-β-induced

stimulation of IGFBP-3 synthesis in breast cancer cells (Huynh et al., 1996; Oh et al., 1995) and the demonstration that IGFBP-3 antisense oligonucleotides suppressed the inhibitory effect of TGF-β on cell proliferation (Oh et al., 1995). The presence of TGF-β1 in serum and mammary extracts from prepubertal heifers has been demonstrated (Purup et al., 1998). Also, IGFBP-4 which consistently inhibits the mitogenic activity of IGF-I in cultures, was increased by feeding level (Purup et al., 1998) and was negatively correlated with the mitogenic response.

Somatotropin administration to prepubertal heifers stimulates mammary development (Sejrsen et al., 1986; Purup et al., 1993). Specific receptors for ST as measured by ligand binding assays have not been detected in bovine mammary tissue (Akers, 1985), prompting studies to identify the mediators responsible for its effects on the mammary gland. In this study, somatotropin treatment at low feeding level decreased the mitogenic response to mammary extracts. In contrast, at high feeding level, somatotropin stimulated the mitogenic response to mammary extracts. It is likely that multiple growth factors synthesized in mammary tissue contribute to the mitogenic response. In conditioned medium from primary bovine mammary epithelial cells, the mitogenic activity was attributed to the effects of several growth factors including proteins of 6 kD and less, and a small fraction related to the fibroblast growth factors (Sandowski et al., 1993). Interactions of these and other potentially important factors in mammary gland development have not been described.

In conclusion, this study provides additional evidence for the endocrine control of mammary synthesis of positive and negative-acting factors. Feeding level and exogenous

somatotropin can modulate prepubertal mammary development in dairy heifers, potentially through mediation by locally produced factors. Elucidation of the interactive effects of these local factors will increase our understanding of the role of imbalanced expression of growth regulatory proteins in mammary gland development.

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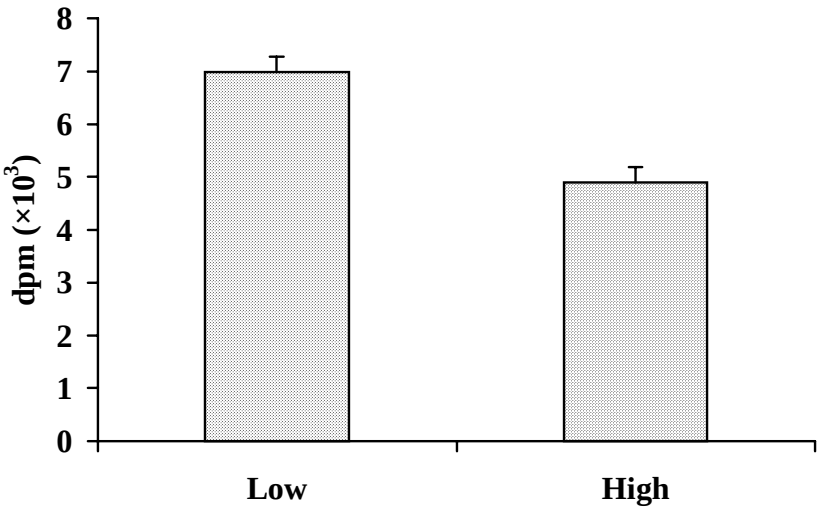

Figure 1. Effect of feeding level on [^{3}H]thymidine incorporation (dpm) into primary cultures of bovine mammary epithelial organoids in response to media containing 5% extracts of mammary tissue from prepubertal heifers. Results were normalized by dividing values for [^{3}H]thymidine incorporation per well by the average value for basal [^{3}H]thymidine incorporation in each experiment. Values are LS means ± SEM for cultures performed in triplicate using two different cell preparations.

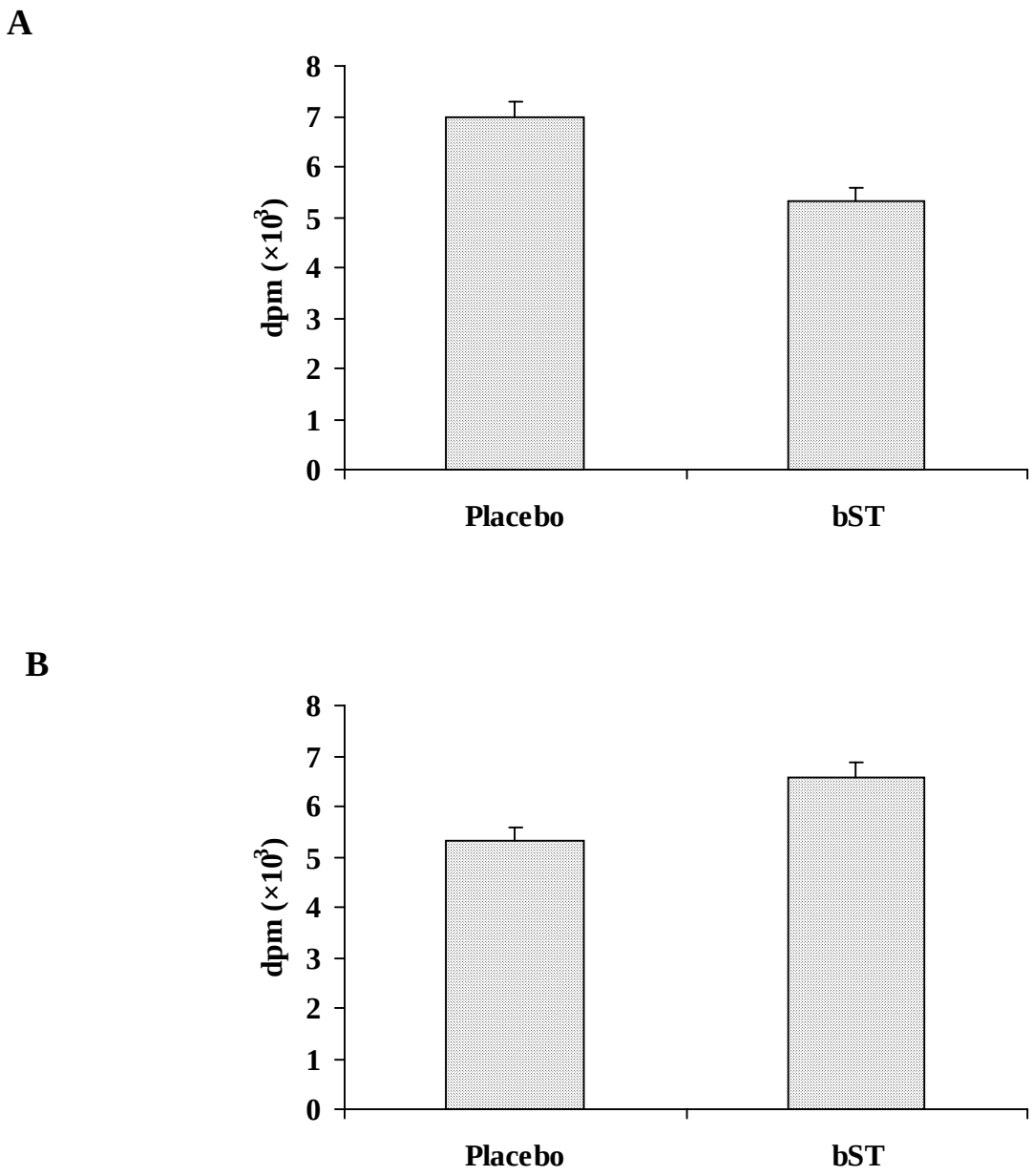

Figure 2. Effect of bST treatment at low feeding level (A) and high feeding level (B) on [^{3}H]thymidine incorporation (dpm) into primary cultures of bovine mammary epithelial organoids in response to media containing 5% extracts of mammary tissue from prepubertal heifers. Results were normalized by dividing values for [^{3}H]thymidine incorporation per well by the average value for basal [^{3}H]thymidine incorporation in each experiment. Values are LS means ± SEM for cultures performed in triplicate using two different cell preparations.

CHAPTER 5

REGULATION OF LOCAL SYNTHESIS OF BOVINE MAMMARY INSULIN-LIKE GROWTH FACTOR-I AND BINDING PROTEINS

ABSTRACT

Our objective was to investigate the mammary expression of insulin-like growth factor-I (IGF-I) and IGF-binding proteins in prepubertal heifers, and its regulation by bovine somatotropin (bST) and feeding level. Twenty-four prepubertal Friesian heifers were blocked according to body weight (BW). Within blocks, heifers were randomly assigned to daily bST treatment (0 or 15 mg/d) at low or high feeding level (.55 kg/d or 1.1 kg/d average daily gain (ADG), respectively) for 5 weeks. High feeding level, and somatotropin treatment at the low feeding level, tended to decrease abundance of IGF-I mRNA in mammary tissue. High feeding level reduced abundance of IGF-binding protein-1 mRNA in mammary tissue, but there was no significant effect of feeding level or somatotropin on other IGF-binding protein mRNAs. Content of IGF-I protein in tissue extracts was increased 46% by somatotropin compared with placebo at high feeding level. Somatotropin and high feeding level tended to increase IGF-binding protein-3 (40-43 kDa) abundance. High feeding level increased a 24-kD binding protein (IGF-binding protein-4) but reduced IGF-binding protein-2 (32 kDa) abundance in mammary extracts. These results indicate that effects of somatotropin treatment and feeding level on the

prepubertal mammary gland are mediated in part by alterations in local synthesis of IGF-I and IGF-binding proteins.

INTRODUCTION

Optimum development of the prepubertal bovine mammary gland is important to the future lactation potential of cows. Because the number of alveolar cells determines milk yield (Tucker, 1981), potential means to enhance development of the mammary epithelium have been extensively investigated. In the prepubertal heifer, exogenous somatotropin and feeding level can modulate mammary growth, although the pathways involved are not well understood. Administration of somatotropin stimulates prepubertal mammary growth (Sejrsen et al., 1986). Further, because circulating ST concentrations are positively related to mammary growth, decreased serum somatotropin levels have been implicated in the negative effects of a high rate of gain on mammary growth (Sejrsen et al., 1983; Sejrsen and Purup, 1997). However, as judged by direct ligand binding assays, the bovine mammary gland lacks specific receptors for bST (Akers, 1985), indicating an indirect mechanism of action which likely involves IGF-I. Mammary epithelial cells express the IGF-I receptor, and IGF-I stimulates mammary cell proliferation in culture (Purup et al., 1995). Circulating IGF-I levels are increased by bST but are reduced by restriction of feed intake (McGuire et al., 1992). Regulation of mammary development is complex; thus, it is not surprising that changes in circulating IGF-I concentrations do not consistently explain the accompanying changes in mammary

growth. Also, changes in serum IGF-binding proteins (IGFBP) do not appear to account for the reduction in mammary growth with a high rate of gain (Vestergaard et al., 1995). Alternatively, IGF-I mRNA is synthesized in the bovine mammary gland; thus, changes in local production of IGF-I may be important (Glimm et al., 1988, Sharma et al., 1994). The mammary gland appears to produce IGF-I exclusively in the stromal portion, supporting a paracrine role for IGF-I to influence development of the epithelium (Hauser et al., 1990, Yee et al., 1989). Synthesis of IGF-I mRNA has been demonstrated in mammary stroma from a pregnant heifer (Hauser et al., 1990) and in mammary tissue from lactating cows (Glimm et al., 1992). Ultimately, the biological activities of IGF-I are regulated by IGFBP that control the availability of IGF-I to its target tissues or cells (Thissen et al., 1994). The potent effects of IGF-I and IGFBP-3 on DNA synthesis in cultured mammary epithelial organoids from prepubertal heifers suggests that local mammary synthesis of IGF-I and IGFBP can modulate mammary growth in vivo (Purup et al., 1998, Chapter 6). Primary mammary organoids from both prepubertal (Chapter 6) and pregnant heifers (McGrath et al., 1991) secrete IGFBP-3 and -2 into cell culture medium. Synthesis of IGFBP is stimulated by IGF-I added to cell cultures, suggesting that the IGFBP regulate local as well as systemic IGF-I actions in the bovine. However, gene expression of IGF-I and IGFBP and its regulation in the mammary glands of prepubertal heifers have not been described. The objectives of this study were to 1) evaluate prepubertal mammary tissue for mRNA expression of IGF-I and IGFBP, and 2) determine the effects of exogenous somatotropin at two different feeding levels on mammary IGF-I and IGFBP synthesis.

MATERIALS AND METHODS

Experimental design

Twenty-four Holstein Friesian heifers (233 ± 7 kg BW; 271 ± 4 d at slaughter) were blocked according to BW in an earlier experiment described by Vestergaard et al. (1995). Within blocks of four, animals were randomly assigned to bST treatment at low or high feeding level in an experiment with a 2×2 factorial arrangement of treatments. Treatments continued for 5 weeks and consisted of daily bST injections (0 or 100 μg/kg BW) at either low or high feeding level. The low feeding level diet utilized a forage-based mixed ration available ad libitum and supported an average daily gain of 0.55 kg/d. The high feeding level diet was a concentrate mixture available ad libitum along with 2 kg/d of the mixed ration and yielded an average daily gain of 1.1 kg/d.

Tissue collection and RNA analysis

One block of four heifers was killed each week depending on BW. Heifers were stunned by captive bolt and sacrificed by exsanguination at a final BW of 233 ± 7 kg (271 ± 4 d). At slaughter, mammary glands were removed; mammary parenchyma was excised from the left half of the udder and frozen for later analyses. Total RNA was extracted from mammary parenchyma using TRIZOL reagent (Life Technologies, Grand Island, NY). Thirty micrograms of total RNA were electrophoresed on a 1% agarose-0.66M formaldehyde gel and transferred to nylon membranes (Hybond-N; Amersham

International, Buckinghamshire, England) by vacuum blotting (BioRad, Hercules, CA). Mammary RNA from each heifer in a block was prepared at the same time and evaluated on the same blot. Blots were hybridized overnight at 42 °C with a cDNA [^{32}P]-labeled by random priming, washed according to manufacturer's instructions, and exposed to Kodak XAR-5 film at –80 °C with intensifying screens. Blots were stripped in 0.1x SSC and 0.5% SDS at 95 °C twice for 20 minutes and were re-probed with an 18S cDNA labeled by nick translation. Values for mRNA abundance were normalized for loading differences using 18S rRNA abundance data.

Expression of IGF-I and IGFBP was evaluated by use of a 0.7 kb ovine IGF-I cDNA (Ohlsen et al., 1993), a 0.44 kb rat IGFBP-3 cDNA isolated from plasmid rIGFBP-3 (Smith et al., 1990), an EcoRI-HindIII fragment of the rat IGF-I-R cDNA (Werner et al., 1989), a 0.69 kb ovine IGFBP-2 cDNA isolated from plasmid 30KS+ (Delhanty and Han, 1992), and a 0.50 kb ovine IGFBP-1 cDNA isolated from plasmid sIGFBP-1.1 (Phillips et al., 1991).

Ligand blotting and IGF-I assay

Aqueous mammary extracts were prepared from thawed tissue essentially according to Waksman et al. (1991). Frozen slices (5 g total) were cut from larger tissue samples and homogenized in physiological saline (12 ml) for 1 min using a Polytron tissue homogenizer (Kinematica, Buch & Holm, Herlev, Denmark). A saline rinse (3 ml) of the Polytron tip was combined with the initial homogenate, and the total homogenate (3:1 saline to tissue) was shaken at 4 °C for 90 min. Homogenates were then filtered

through a double layer of surgical gauze, and the filtrate was centrifuged for 45 min at 10,000 × *g* at 4 °C. The supernatant was recovered from below the uppermost fat layer and subsequently centrifuged at 105,000 × *g* for 1 h at 4 °C. The supernatant was filtered through sterile 0.2 µm filters and stored at -80 °C. Concentrations of IGF-I in mammary extracts were measured by ELISA (OCTEIA IGF-I; IDS Ltd., UK) (Norup & Vestergaard, 1996). For calculations, it was assumed that 1 g of mammary tissue was equivalent to a volume of 1 ml.

Concentrations of IGFBP in mammary extracts were evaluated by Western ligand blotting (Hossenlopp et al., 1986). Samples (200 µg protein; 17 to 31 µl extract) from each of 20 heifers were dissolved in non-reducing SDS-polyacrylamide (SDS-PAGE) gel buffer, heated to 60 °C for 15 min, and separated overnight on an SDS-polyacrylamide gel at constant current. Following transfer of proteins to a nitrocellulose membrane by electroblotting, the blots were incubated with [^{125}I]IGF-I and washed. Autoradiographs from the blots were exposed at -70 °C for 2 days.

Statistical analysis

The general linear models procedure of SAS (SAS Institute, Inc. 1985) was used for analysis of dependent variables (Appendix D). Block, somatotropin treatment, feeding level, and the somatotropin × feeding level interaction were included in the model. When the interaction was significant ($P < 0.10$), *t*-tests were used to identify between-group differences. Pearson correlations were performed on the residuals after

adjusting for the block effect. Data are presented as least squares means ± SEM with corresponding P-values.

RESULTS

Northern analysis

Northern blot analysis of mammary parenchymal tissue from prepubertal heifers showed a major 7.5 kb transcript. Less abundant transcripts were detected around 9.5, 4.7, 1.2 and 0.8 kb (not shown). There was a tendency for an interaction of somatotropin and growth rate on mammary IGF-I expression (Table 1, $P < .08$). High feeding level reduced IGFBP-1 mRNA levels (Table 1, $P < .04$) and increased IGF-I receptor mRNA levels ($P < .02$). Somatotropin at low feeding level tended to decrease IGF-I (Table 1, $P < .08$) mRNA abundance. In contrast, expression of IGFBP-3 and -2 mRNA was unchanged in mammary tissue by somatotropin or feeding level (Table 1, $P > .10$).

Ligand blotting and IGF-I assay

Concentrations of IGF-I in aqueous extracts of mammary tissue were evaluated by ELISA. Tissue concentrations ranged from 110 to 182 pg IGF-I/mg tissue, with an interaction of feeding level and somatotropin treatment ($P < .01$; Table 2). Somatotropin at high feeding level increased IGF-I protein in mammary tissue extracts ($P < .001$).

The relative abundance of IGFBP in mammary extracts was evaluated by Western ligand blotting (Table 2). High feeding level increased IGFBP-2 ($P < .005$) and IGFBP-4 ($P < .02$), while IGFBP-1 was unchanged ($P > .10$). Somatotropin tended to increase IGFBP-3 content in mammary tissue extracts ($P < .10$).

Correlations between IGF-I and IGFBP in serum and mammary tissue

The significant positive correlation between IGF-I concentrations in serum and mammary tissue extracts from these animals ($r = .84$; $P < .001$) was previously reported (Purup et al., 1998). Tissue levels of IGFBP-3 were not strongly correlated with IGF-I concentrations in either serum ($r = .37$, $P < .08$) or tissue ($r = .17$, $P < .42$) . Tissue levels of IGFBP-2 were negatively correlated with IGF-I in serum ($r = - .68$, $P < .0002$) and in tissue ($r = - 0.51$; $P < .02$) but positively correlated with serum IGFBP-2 ($r = .89$; $P = .001$). Tissue IGFBP-4 levels were positively correlated with IGF-I in tissue ($r = .41$, $P < .05$) and in serum ($r = .61$; $P < .002$). On the other hand, tissue IGFBP-1 levels were not significantly correlated with serum or tissue IGF-I ($P > .10$).

DISCUSSION

Insulin-like growth factor-I is a strong mitogen for mammary epithelium from prepubertal heifers and has been implicated as a mediator of somatotropin action on the mammary gland. Circulating IGF-I likely influences mammary growth in an endocrine fashion, but also its role as a local stimulator of cell proliferation is becoming apparent in

a number of tissues. This study quantifies expression of IGF-I, the receptor for IGF-I and ST, and IGFBP-3, -2, -1, and -4 in mammary tissue from heifers and demonstrates their regulation by exogenous somatotropin and feeding level.

Production of IGF-I in most body organs, and its biological actions on multiple cell types, indicate that IGF-I exerts its mitogenic effects by paracrine or autocrine as well as endocrine modes of action (Jones and Clemmons, 1995). Data from this study show that the developing glands of prepubertal heifers synthesize IGF-I mRNA. Total RNA was isolated from mammary parenchyma, which on an area basis is comprised of 10 to 20% epithelial cells, 40 to 50% connective tissue, and 30 to 40% fat cells in heifers around this stage of development (Sejrsen and Purup, 1997). A major band at 7.5 kb was readily visible in mammary RNA from all heifers, with additional, less abundant transcripts evident at 9.5, 4.7, 1.2 and 0.8 kb. Except for the 9.5 kb transcript, this pattern of expression is similar to that reported in mammary RNA from lactating cows (Glimm et al., 1992, Sharma et al., 1994). The 9.5-kb band likely represents variability in either stability or processing of the mRNA transcripts and may be a developmental stage-specific phenomenon. The IGF-I transcripts appear to originate from the stromal portion of the gland (Hauser et al. 1990). Nonetheless, Collier et al. (1993) showed that direct infusion of IGF-I into the mammary glands of pregnant beef cows stimulated mammary growth. In vitro, IGF-I is also a potent mitogen for bovine mammary explants, mammary organoids (Purup et al., 1995), and isolated cells (Woodward et al., 1994). Taken together, this evidence indicates that mammary stroma can synthesize IGF-I for a paracrine effect on mammary epithelial cells.

The IGF-I in most body fluids is bound to specific, high-affinity IGFBP that modulates its biological effects (Thissen et al., 1994). The IGFBP are synthesized by a variety of body tissues. It has been proposed that the tissue specificity of IGFBP gene expression may modulate their local effects, whereas alterations in serum IGFBP levels may regulate the bioavailability of serum IGF-I to tissues (Lemozy et al., 1994). Results from northern analyses in this study revealed that mammary tissue from prepubertal heifers synthesizes mRNA for IGFBP-3, IGFBP-2, and IGFBP-1; expression of other IGFBP was not determined. Ligand blotting of aqueous mammary tissue extracts displayed IGFBP protein with relative molecular masses of 40-43, 32, 28, and 24 kD likely corresponding to IGFBP-3, -2, -1, and -4. Of the total IGFBP activity in mammary extracts, IGFBP-3 represented the majority (75%) as noted for serum from prepubertal heifers of a similar age (71%); although IGFBP-2 levels were substantially higher in serum (22%) than in extracts (7%) (Chapter 6).

Exogenous somatotropin stimulates mammary growth in prepubertal heifers (Sejrsen et al., 1986, Purup et al., 1993). The lack of somatotropin binding sites as measured by direct ligand binding assays in bovine mammary tissue microsomes, coupled with the potent mitogenic activity of IGF-I in vitro, led to the suggestion that IGF-I mediates somatotropin effects on the mammary gland. Circulating somatotropin concentrations are positively related to mammary growth, and serum IGF-I concentrations are increased by exogenous somatotropin (Sejrsen et al., 1983). However, the contribution of systemic versus local IGF-I and IGFBP to growth regulation in mammary tissue is a controversial topic. Interestingly, somatotropin receptor mRNA has

been identified in mammary tissue from prepubertal heifers (Table 1; Purup et al., 1998). This transcript potentially represents a common mRNA for the somatotropin receptor found in a variety of tissues (Lucy et al., 1998) and may exhibit translational regulation of expression. In the bovine, exogenous somatotropin caused a slight nonsignificant reduction in late lactating cows (Sharma et al., 1994) or decreased the abundance of IGF-I mRNA in mammary tissue from mid-lactating cows (Glimm et al., 1992). In prepubertal heifers on a low feeding level, our results also show that exogenous somatotropin tends to decrease IGF-I mRNA abundance although corresponding IGF-I protein concentrations were not affected. Concentrations of IGF-I were increased by somatotropin at a high feeding level, whereas IGF-I mRNA abundance displayed a numerical but insignificant increase. Somatotropin did not affect IGFBP mRNA abundance. Exogenous somatotropin tended to increase IGFBP-3 protein content in mammary tissue but did not affect concentrations of other IGFBP, indicating a possible differential requirement for IGFBP synthesis in the mammary gland.

The poor correlation between mRNA and protein abundance suggests that somatotropin regulation of mammary IGF-I and IGFBP synthesis in heifers is dependent on nutritional status. Such a discrepancy has been noted between serum IGF-I concentrations and liver IGF-I mRNA levels in rats and may derive from impaired translation of mRNA in animals on restricted feeding (Thissen et al., 1994) although the mechanisms involved are not understood. Similarly, a strong correlation between liver IGF-I mRNA and protein levels was noted in fed, but not fasted, sheep (Hua et al., 1993). It has been suggested that energy level may influence IGF-I gene transcription, whereas

mRNA translation is influenced by amino acid availability (McGuire et al., 1992). Undernutrition may limit nutrients essential to cells for IGF-I production (Clemmons and Van Wyk, 1981) and decrease availability of insulin which is important to IGF-I synthesis (Thissen et al., 1994).

Nutrition is a major regulator of the somatotropin-IGF-I axis and influences prepubertal development of the mammary gland. A high rate of gain leads to impaired development of the mammary epithelium and subsequently reduced milk yield (Sejrsen and Purup, 1997). The mechanism underlying this effect remains unclear. Circulating concentrations of somatotropin, but not IGF-I, are positively related to mammary growth. Nutrient restriction increases serum somatotropin but decreases serum IGF-I (Ketelslegers et al., 1996). In this study, a high feeding level tended to decrease mammary IGF-I mRNA abundance and increased IGF-I receptor mRNA abundance but did not affect IGF-I protein concentrations in extracts. Feeding level did not influence mRNA abundance for IGFBP. In contrast, a high feeding level tended to increase IGFBP-3 levels and significantly increased abundance of the 24-kD species. Amounts of IGFBP-2 and the 28-kD species were reduced. Thus, growth rate does not appear to influence IGF-I concentrations in mammary tissue but is capable of modulating local IGFBP levels.

Interestingly, IGFBP-3 concentrations, which in the circulation largely regulate the circulating body reserve of IGF-I (Lewitt et al., 1993), did not fluctuate similarly to IGF-I concentrations in mammary tissue. In prepubertal heifers in this study, high feeding level or somatotropin at low feeding level did not affect IGF-I concentrations but increased

IGFBP-3 levels in mammary extracts. Both bovine mammary epithelial cells (Romagnolo et al., 1994) and fibroblast cell lines show IGFBP-3 synthesis (Conover, 1990). Insulin-like growth factor-I is constitutively secreted following synthesis and does not accumulate in tissue storage sites. Body reserves of IGF-I are primarily complexed with IGFBP-3 (Jones and Clemmons, 1995), either as a binary complex or as a ternary complex with the acid-labile subunit (Baxter and Martin, 1989). The primary tissue sources of this serum IGFBP-3 and its regulation of synthesis have not been determined. In dwarf rats, Lemmey et al. (1997) showed IGF-I administration induced an increase in serum IGFBP-3 without a corresponding increase in liver IGFBP-3 mRNA. In contrast, somatotropin consistently increased IGFBP-3 mRNA levels in multiple tissues, implying that somatotropin might have a direct role in regulation of IGFBP-3 synthesis. The somatotropin-IGF-I axis is uncoupled when animals are on a restricted feeding level (McGuire et al. 1992), adding difficulty to interpretation of the combined effects of these factors on the mammary gland. However, the significant positive correlation between IGF-I concentrations in serum and mammary tissue extracts from these animals and the lack of a similar relationship between IGFBP-3 in serum and mammary tissue suggests differential regulation.

The roles of IGFBP in mammary epithelial cell proliferation have been most widely studied in relation to IGFBP-3. However, the importance of other IGFBP in serum and mammary tissue should not be discounted. Both IGFBP-1 and IGFBP-2 form low-molecular weight complexes with IGF-I that are capable of transporting IGF-I across capillary endothelium into target tissues (Jones and Clemmons, 1995). In this study,

protein levels of IGFBP-1 were not changed by heifer treatments or significantly correlated with tissue or serum IGF-I levels, indicating an undetermined source of regulation. On the other hand, IGFBP-2 tissue levels were decreased at a high feeding level; further, tissue levels were strongly correlated with serum IGFBP-2 but negatively correlated with serum IGF-I. Serum IGFBP-2 levels are typically elevated in response to somatotropin deficiency or in fasting situations (Collett-Solberg and Cohen, 1996). In respect to the strong correlations with serum IGF-I, mammary tissue levels of IGF-I, IGFBP-2 and IGFBP-4 levels appear to be related to factors in the circulation. A more complete understanding of the regulation of local mammary IGF and IGFBP synthesis and its significance to mammary development will require further investigation.

In conclusion, mammary tissue from prepubertal heifers expresses IGF-I, the IGF-I and the ST receptor, and IGFBP-3, -2, -1, and -4, supporting the idea of local regulation of mammary growth by the insulin-like growth factors. Exogenous somatotropin and feeding level clearly modulate mammary synthesis of IGF-I, the IGF-I receptor, and its binding proteins. In summary, changes in local availability of IGF-I and IGFBP likely mediate a portion of the effects of somatotropin and feeding level on mammary development in heifers.

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Table 1. Relative abundance of mRNA for IGF-I, IGF-binding proteins (IGFBP), IGF-I receptor (IGF-I-R), and ST receptor (ST-R) in mammary tissue from heifers (n = 24; n = 20 for IGF-I mRNA) receiving bST or placebo at low or high feeding level (FL).[1]

Feeding level	Low		High			Probability		
Treatment	Placebo	bST	Placebo	bST		FL	bST	FL × bST
					SEM			
IGF-I	866	328	295	517	196	.35	.44	.08
IGFBP-3	239	167	168	204	36	.64	.62	.16
IGFBP-2	422	375	321	404	71	.63	.80	.38
IGFBP-1	192	183	119	109	33	**.04**	.78	.99
IGF-I-R	657	901	1703	1147	212	**.02**	.48	.10
ST-R	93	96	78	110	9	.99	.08	.13

[1]Densitometric units for each mRNA expressed relative to 18S rRNA in that sample.

Table 2. Concentrations of IGF-I and relative protein abundance of IGF-binding proteins (IGFBP) in extracts of mammary tissue from heifers (n = 24) receiving bST or placebo at a low or high feeding level (FL).

Feeding level	Low		High			Probability		
Treatment	Placebo	bST	Placebo	bST		FL	bST	FL × bST
					SEM			
IGF-I[a]	110	116	125	182	8	**.0001**	**.002**	**.007**
IGFBP-3	460	1175	1174	1311	243	.11	.10	.26
IGFBP-2	156	167	105	52	25	**.005**	.42	.22
IGFBP-1	146	164	137	179	32	.93	.37	.73
IGFBP-4	52	124	172	189	33	**.02**	.20	.43

[a]Values for pg IGF-I/mg tissue were determined from IGF-I concentrations in an aqueous mammary extract containing 5 g tissue in 15 ml saline. It was assumed that 1 g tissue is equivalent to 1 ml volume.

CHAPTER 6

CONTRIBUTION OF INSULIN-LIKE GROWTH FACTOR-I (IGF-I) AND IGF-BINDING PROTEIN-3 (IGFBP-3) TO MITOGENIC ACTIVITY IN BOVINE MAMMARY EXTRACTS AND SERUM

ABSTRACT

Peripubertal development of the mammary gland is likely mediated by locally produced growth factors acting in concert with circulating mitogens. Our objective was to investigate the effect of recombinant human insulin-like growth factor-binding protein-3 (rhIGFBP-3) or insulin-like growth factor-I (IGF-I) antibodies on the IGF-I-related mitogenic activity of bovine serum and of mammary tissue extracts in primary mammary epithelial cell cultures. Cells were obtained from prepubertal female calf mammary tissue and cultured in three-dimensional collagen gels. A mammary parenchymal tissue extract (pooled from 20 prepubertal heifers) or serum (pooled from 3 heifers) at a concentration of 5% was added to the medium containing either rhIGFBP-3 or monoclonal or polyclonal antibodies to human IGF-I. Cell proliferation was evaluated using [methyl-^{3}H]thymidine incorporation as a measure of DNA synthesis. Addition of mammary extracts stimulated DNA synthesis 545% compared to basal medium. Addition of serum stimulated DNA synthesis by 28%. Mitogenic activity of serum and added IGF-I was abolished by addition of rhIGFBP-3 in equimolar concentrations with

IGF-I. For mammary extracts, mitogenic activity was inhibited by 35%, 50%, and 82% by addition of rhIGFBP-3 at 1, 2 and 4 times the molar IGF-I concentration in the extract. Addition of rhIGFBP-3 to basal medium reduced DNA synthesis by 26%, whereas IGF-I antibodies had no consistent effect. These results indicate that circulating and mammary-synthesized IGF-I and IGFBPs likely play a critical role in prepubertal development of the bovine mammary gland.

INTRODUCTION

Regulation of mammary growth and development is accomplished by a complex interplay of circulating hormones with local-acting growth factors. Classic studies in laboratory and domestic species established the involvement of hormones from the ovary and pituitary gland in hormonal control of mammary development (Forsyth, 1991). Subsequent investigations into the mechanisms underlying the effects of these hormones on mammary cell growth revealed the existence of potent systemic as well as mammary-derived growth factors that may mediate much of their activity (Oka et al., 1991). Moreover, Waksman et al. (1991) demonstrated that tissue extracts from bovine pituitary, kidney, uterus and mammary gland stimulated mammary cell DNA synthesis in a dose-dependent manner and concluded that normal mammary epithelial cell proliferation is likely influenced by a multitude of tissue-derived growth factors.

Exogenous bovine somatotropin (bST) stimulates peripubertal mammary growth in dairy heifers (Sejrsen et al., 1986) and sheep (McFadden et al., 1990). However, much

evidence indicates that bST functions by an indirect mechanism of action, mediated at least in part by insulin-like growth factor-I (IGF-I) (Akers, 1985; Kleinberg, 1997). The mitogenic effect of IGF-I on mammary explants (Baumrucker and Stemberger, 1989; Purup et al., 1995), or mammary organoids of bovine (Shamay et al., 1988, McGrath et al., 1991, Purup et al., 1995) and ovine (Winder et al., 1989) origin in collagen gel culture, and in mammary tissue of pregnant heifers (Collier et al., 1993) has been documented. In the mammary gland, IGF-I is synthesized locally by stromal tissue and is considered to act on adjoining epithelial cells via a paracrine mechanism of action (Cullen et al., 1992). It is likely that IGF-I of both systemic and local origins influences mammary cell proliferation, together with additional serum- and mammary-derived growth factors.

The mitogenic activity of IGF-I is modulated in vivo by a family of IGF-binding proteins (IGFBP), which are secreted from mammary epithelial cells in culture (McGrath et al., 1991, Campbell et al., 1991). In the circulation, IGF-I associates predominantly with IGFBP-3. Production of IGFBP-3 by mammary epithelial cells is stimulated by IGF-I, indicating a potential mechanism for regulation of IGF-I activity (Romagnolo et al., 1994, McGrath et al., 1991). Thus, IGFBP-3 modulation of IGF-I mitogenic activity in mammary tissue may be important in normal growth and development of the mammary gland.

Our objective was to evaluate the potential importance of IGF-I and IGFBP-3 in serum and mammary tissue extract in stimulating mammary epithelial cell proliferation. This paper describes the modulatory effect of IGFBP-3 and IGF-I antibody addition to

cultures of primary undifferentiated mammary epithelial cells when supplemented with IGF-I, serum or mammary gland extracts from prepubertal heifers.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade unless otherwise stated. Recombinant human insulin-like growth factor-I (MW=7,646 Da) was purchased from Bachem AG (Bubendorf, Switzerland). Glycosylated recombinant human IGFBP-3 (MW=47,000 Da) was obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-human IGF-I polyclonal antibody (IgG fraction) was purchased from Austral Biologicals (San Ramon, CA). The mouse anti-human IGF-I monoclonal antibody was generously provided by Novo Nordisk A/S, Gentofte, Denmark. Mouse anti-human IgG, deoxyribonuclease I (DNase), and type I-S hyaluronidase was obtained from Sigma (St. Louis, MO). Collagenase (type II; 273 U/mg) was purchased from Worthington Biochemical Corp. (Freehold, NJ). Fetal bovine serum (FBS) was purchased from Life Technologies (Gaithersburg, MD). Basal medium (BM) was prepared from M-199 with Earle's salts, L-glutamine, and Hepes buffer (Sigma, St. Louis, MO), sodium bicarbonate (24 mM), bovine serum albumin (2.6 mg/ml), transferrin (5 μg/ml), reduced glutathione (1 μg/ml), soybean trypsin inhibitor (1 μg/ml), bovine insulin (10 ng/ml), selenium (1 ng/ml) and antibiotic solution (0.2%) containing penicillin (50,000 IU/l) and streptomycin (50 ng/ml)

were added to the medium. [Methyl-^{3}H]thymidine (specific activity 3.03 TBq/mmol) was obtained from Amersham International (Birkerød, Denmark).

Source of mammary extracts and serum

Extracts were prepared from the mammary parenchyma of 20 prepubertal Friesian heifers (body weight and age at slaughter 237 ± 7 kg and 272 ± 4 d, respectively) that were part of a previous experiment (Vestergaard et al., 1995). At slaughter, mammary glands were removed and mammary parenchyma was excised from the left half of the udder and frozen at -80 °C. Aqueous mammary extracts were prepared from thawed tissue essentially according to Waksman et al. (1991). Frozen slices (5 g total) were cut from larger tissue samples and homogenized in physiological saline (12 ml) for 1 min using a Polytron tissue homogenizer (Kinematica, Buch & Holm, Herlev, Denmark). A saline rinse (3 ml) of the Polytron tip was combined with the initial homogenate, and the total homogenate (3:1 saline to tissue) was shaken at 4 °C for 90 min. Homogenates were then filtered through a double layer of surgical gauze, and the filtrate was centrifuged for 45 min at 10,000 × *g* at 4 °C. The supernatant was recovered from below the uppermost fat layer and subsequently centrifuged at 105,000 × *g* for 1 h at 4 °C. The supernatant was filtered through sterile 0.2 µm filters and stored at - 80 °C. Protein content of the mammary extracts was determined using the bicinchoninic acid assay (Pierce Life Technologies, Roskilde, Denmark). The mammary extracts (n = 20 heifers) were pooled and used for the in vitro experiments. Serum for in vitro experimentation

was obtained from 3 prepubertal Friesian heifers averaging 257 ± 7 days of age and 192 ± 14 kg body weight.

Primary Cell Preparation and Culture

Mammary epithelial cells were prepared from mammary parenchymal tissue from a prepubertal Friesian heifer (200 kg body weight; 242 d of age) essentially according to Shamay et al. (1988). Within 10 min of slaughter, mammary parenchyma was excised aseptically from the outer margin of parenchyma and minced finely with scissors. Tissue pieces were transferred to a petri dish and weighed. Tissue was incubated in basal medium supplemented with DNase (277 µg/g tissue), insulin (67 µg/g tissue) and an antibiotic-antimycotic mixture, at a ratio of 10 ml medium per gram of tissue, for 10 min in a 37 °C shaking water bath. Tissue pieces were allowed to sediment for 5 min and excess medium was aspirated. Tissue was then digested in basal medium supplemented with collagenase (10 mg/g tissue), hyaluronidase (10 mg/g tissue), DNase (277 µg/g of tissue) and insulin (67 µg/g of tissue), at a ratio of 10 ml medium per gram of tissue, for 5 h in a 37 °C shaking water bath. Organoids were isolated by filtering the suspension through 200 µm Nitex and centrifuging the filtrate for 10 min at 300 × *g*. Organoids were suspended in basal medium and allowed to sediment for 8 min; then excess medium was aspirated and replaced with fresh medium for a total of 10 washes. Following suspension in basal medium supplemented with 44% FBS and 6% dimethyl sulfoxide (DMSO), organoids were frozen overnight at - 80 °C and transferred the next day to liquid N_2.

Collagen gels were prepared essentially as described by Shamay et al. (1988). A collagen solution prepared from rat tail tendons was neutralized using a neutralizing solution (10× M-199 without bicarbonate and 0.33 M NaOH at a 2:1 ratio) at a ratio of about 6:1. Each well of 24-well plates (Nunc A/S, Roskilde, Denmark) was coated with 0.5 ml of the neutralized collagen. Before use, organoids were quickly thawed at 37 °C and washed in basal medium, then suspended in the cold neutralized collagen solution. The cell-collagen suspension (0.5 ml) was pipetted onto each collagen gel and allowed to gel in a 37 °C incubator for 30 min before 1 ml of basal medium was added. Cells were cultured for 5 d at 37 °C in air:CO_2 (95:5). Because treatment media were distributed across multiple culture plates, the basal medium and 10% FBS treatments were repeated four times in each experiment, to evaluate consistency of treatment effects across different culture plates. Treatment media were added 24 h after plating and were changed every 48 h.

Thymidine Incorporation

Incorporation of [methyl-^{3}H]thymidine incorporation was determined essentially according to Shamay et al. (1988). On d 3 of culture, 50 µl of basal medium containing 20 µCi/ml [methyl-^{3}H]thymidine] was added to each well. After 24 h of incubation, media were aspirated from the wells and collagen gels were transferred to microcentrifuge tubes. Gels were dissolved by the addition of 150 µl 25% acetic acid and subsequent incubation for 20 to 30 min at 37 °C. Cells were collected by centrifugation for 10 min at 500 × *g*. Following aspiration of the supernatant, 750 µl of

4% perchloric acid (PCA) were added and tubes were centrifuged as above. Subsequent washes were performed similarly with 750 µl of 80% ethanol, 100% ethanol and 4% PCA. The final supernatant was aspirated and cells were hydrolysed with 500 µl 6% PCA at 80 °C for 1 h. The suspension was transferred to scintillation vials, mixed with 5 ml of scintillation fluid (Picofluor 40; Packard Instruments, Greve, Denmark) and counted in a liquid scintillation counter.

Inhibition studies with IGFBP-3 and IGF-I antibodies

For the purpose of IGFBP inhibition studies, it was assumed that binding reactions between IGF-I and rhIGFBP-3 are monovalent; that is, one molecule of IGF-I binds one molecule of rhIGFBP-3. Consequently, equimolar ratios of IGF-I and rhIGFBP-3 require that the weight concentration (ng/ml) of rhIGFBP-3 be slightly more than 6-fold greater than that of IGF-I (47:7.6). For cell culture assays utilizing addition of recombinant IGF-I, a concentration of 50 ng/ml was utilized because this consistently corresponds with a maximal response in prior reports (Purup et al., 1995). Thus cultures containing 50 ng/ml IGF-I were supplemented with 400 ng/ml rhIGFBP-3, yielding an rhIGFBP-3:IGF-I ratio of approximately 1.3 (see Results section for other concentrations used).

For antibody inhibition studies, purity estimates supplied by the vendor were used to calculate molar ratio estimates. Assumptions were that each IgG molecule would bind one molecule of IGF-I, and that the average IgG molecule had a molecular weight of 120 kDa. Potential divalent binding of two IGF-I molecules, possible differences in classes

of antibody molecules (polyclonal) present or possible differences in binding affinities were not considered.

For experiments with either rhIGFBP-3 or antibody addition, these components were combined with IGF-I, serum, or mammary extracts 2 h prior to addition to cultured cells as indicated. Concentrations of IGF-I in individual mammary extracts, and serum obtained from three prepubertal heifers, were determined by an ELISA (OCTEIA IGF-I; IDS Ltd., UK) that was validated previously (Norup and Vestergaard, 1996).

Ligand blotting

Concentrations of IGFBPs in mammary extracts, and in conditioned media collected from cell cultures following 2 d in treatment media, were evaluated by Western ligand blotting (Hossenlopp et al., 1986). Samples (200 µg protein; 17 to 31 µl extract) from each of 20 heifers or 250 µl media were dissolved in non-reducing SDS-polyacrylamide (SDS-PAGE) gel buffer, heated to 60 °C for 15 min, and separated overnight on an SDS-polyacrylamide gel at constant current. Following transfer of proteins to a nitrocellulose membrane by electroblotting, the blots were incubated with [^{125}I]IGF-I and washed. Autoradiographs from the blots were exposed at - 70 °C for 2 days.

[^{125}I]IGF-I binding studies

The amount of free IGF-I in media following a 2 h preincubation with a dilution series of rhIGFBP-3 or the polyclonal IGF-I antibody was evaluated by affinity labeling

according to D'Ercole and Wilkins (1984). [^{125}I]IGF-I (3.9×10^6 cpm; specific activity 77 μCi/μg) was incubated in 0.1 M Dulbecco's phosphate-buffered saline, pH 7.5 (400 μl total) (Life Technologies, Grand Island, NY) at 37 °C for 2 h with rhIGFBP-3 or the polyclonal IGF-I antibody, at the same molar ratios used in cell culture. Investigation of tracer binding to rhIGFBP-3 or IGF-I antibody was performed by use of the cross-linking agent disuccinimidyl suberate (DSS; Pierce Chemical Co., Rockford, IL) followed by SDS-PAGE. Following a 2 h preincubation period, samples (.08 to 6.25 μg protein/ml) were treated with 4 μl 25 mM DSS in dimethylsulfoxide (final concentration .25 mM) for 30 min at room temperature. Samples (25 μl) were prepared for SDS-PAGE by addition of 25 μl sample buffer (5% SDS; 10% glycerol; 0.06 M Tris-HCl, pH 6.8; 5% 2-mercaptoethanol; and .02% bromophenol blue; final concentrations) and heated at 60 °C for 15 min (Hardouin et al., 1987). Free [^{125}I]IGF-I was separated from bound [^{125}I]IGF-I on a 10% polyacrylamide gel overnight at constant current. Autoradiographs from the gels were exposed at - 70 °C for 2 h. The percent of the total available [^{125}I]IGF-I bound to rhIGFBP-3 or IGF-I antibody was calculated by gamma counting of the radioactivity in individual gel sections.

Statistical analysis

Statistical analyses were performed using the GLM procedure of SAS (SAS Institute, Inc. 1989) (Appendix E). Significance of effects of factor addition to media (in comparison with basal medium) was tested by analysis of variance with a model that included treatment, followed by Dunnett's multiple comparison test. Comparisons with P

< .05 were considered to be significantly different. Values are presented as least squares means (LS means) ± SEM of values obtained from triplicate samples.

RESULTS

Mitogenic activity

To evaluate the contribution of IGF-I to mitogenic activity of mammary extracts and serum, primary bovine mammary organoids were embedded in attached collagen gels and grown for 4 days in treatment media (Figure 1). Organoids cultured with heifer serum consistently displayed a stellate appearance with broad outgrowths. In contrast, organoids cultured with mammary extracts, rIGF-I, or basal medium alone showed distinct needle-like projections. Figure 2a illustrates the dose-dependent effects on DNA synthesis of 10% FBS and rhIGF-I in concentrations ranging from 3.125 to 100 ng/ml during the final 24 h of a 4 d treatment period ($P < .05$ at all concentrations compared with basal medium (0)). Similar to previous experiments, maximal stimulation of DNA synthesis was provided by approximately 25 ng/ml of IGF-I. After 2 d in culture, organoids secreted IGFBP-3 and IGFBP-2 into media (Figure 3). Secretion of IGFBP-3 in particular was increased by addition of IGF-I to treatment media. Addition of 10% FBS stimulated DNA synthesis by 30% ($P < .05$). The response to basal medium and 10% FBS treatments did not differ according to position within the experiment ($P > .05$), indicating that cell number per well and DNA synthesis were not affected by time of plating or harvest.

Addition of serum from prepubertal heifers to the culture medium stimulated cell proliferation at concentrations of 4% and greater ($P < .05$) (Fig. 2b), with a dose-dependent response apparent over a range from 2 to 10% serum. As serum IGF-I concentration was 107 ng/ml, the increased DNA synthesis was apparent at an IGF-I concentration of 4.3 ng/ml in the medium (4% serum).

Mammary extracts, on the other hand, stimulated cell proliferation much more potently than serum or IGF-I alone, yielding a mitogenic response approximately twice that induced by the highest IGF-I concentration tested (Figure 2c). The dose-dependent response was different from basal medium at all levels ($P < .05$). Maximal stimulation of DNA synthesis occurred at a concentration between 6 and 8% of mammary extract in the culture medium. The IGF-I concentration within the mammary extract pool was 34.0 ng/ml; thus, maximal cell proliferation was achieved at an IGF-I concentration of less than 3 ng/ml in the medium. Of the IGFBP detected in mammary extracts, IGFBP-3 comprised 75% of the total, with lesser amounts of IGFBP-2 (7%) and a 28-kD species (9%) and 24-kD species (9%) putatively corresponding to IGFBP-1 and IGFBP-4 (data not shown).

Inhibition of mitogenic activity

To investigate the contribution of IGF-I to mitogenic activity in serum and mammary extracts, rhIGFBP-3 and antibodies to hIGF-I were added to media containing either 50 ng/ml of added rhIGF-I, 5% serum or 5% mammary extracts. These concentrations of serum and extracts were selected based on previous dose-response

experiments resulting in approximate half-maximal responses. Addition of a low concentration of rhIGFBP-3 (40 ng/ml) inhibited DNA synthesis in basal medium by 26% ($P < .05$); and addition of a higher concentration of rhIGFBP-3 (800 ng/ml) reduced DNA synthesis in basal medium by 21% ($P < .10$) (Figure 4). Addition of polyclonal or monoclonal IGF-I antibodies to cultures in basal medium did not show a consistent effect on DNA synthesis. Addition of a high concentration (2000 ng/ml) of a non-immune IgG fraction inhibited DNA synthesis in response to basal medium ($P < .05$) whereas a low concentration (12.5 ng/ml) had no effect ($P > .05$).

With IGF-I supplemented media, antibodies and rhIGFBP-3 were added at an antibody or IGFBP-3:IGF-I molar ratio of 1/16, 1/8, 1/4, 1/2, 1, and 2. Corresponding concentrations of rhIGFBP-3 in media were 25, 50, 100, 200, 400 and 800 ng/ml. Similarly, antibody concentrations were 62.5, 125, 250, 500, 1000 and 2000 ng/ml. When added to medium supplemented with 50 ng/ml of IGF-I, equimolar and greater concentrations of rhIGFBP-3 reduced DNA synthesis to the level of basal medium ($P < .05$) (Figure 5). Monoclonal and polyclonal antibodies negated approximately 25% of the mitogenic activity of IGF-I when added at equimolar and greater concentrations ($P > .05$).

To evaluate the effect of inhibition of IGF-I mitogenic activity on serum-induced cell proliferation, antibodies and rhIGFBP-3 were added at an antibody or rhIGFBP-3:IGF-I molar ratio of 1/2, 1, 2 and 4 (Figure 6) in medium with 5% serum. Corresponding concentrations of rhIGFBP-3 in media were 20, 40, 80 and 160 ng/ml. Antibody concentrations were 50, 100, 200 and 400 ng/ml. Addition of rhIGFBP-3

elicited a dose-related complete inhibition of the mitogenic activity at half the molar concentration of IGF-I and above ($P < .05$). Addition of IGF-I antibodies or a non-immune IgG fraction did not significantly inhibit the mitogenic activity of serum ($P > .05$).

Similarly, antibodies and rhIGFBP-3 were added with 5% mammary extracts at similar calculated antibody or rhIGFBP-3:IGF-I molar ratios as given above (Figure 7). Corresponding concentrations of rhIGFPB-3 in media were 5, 10, 20 and 40 ng/ml. Antibody concentrations were 12.5, 25, 50 and 100 ng/ml. Addition of rhIGFBP-3 inhibited cell proliferation at all levels ($P < .05$). An equimolar concentration of rhIGFBP-3 abrogated 35% of the mitogenic activity in extracts, but the dose-dependent inhibition of rhIGFBP-3 was proportionally greater at 2 (50% inhibition) and 4 times (82% inhibition) the molar concentration of IGF-I. Addition of IGF-I antibodies inhibited up to one half of the mitogenic activity in mammary extracts.

[^{125}I]IGF-I binding studies

The relative binding activity of rhIGFBP-3 and the IGF-I antibody (pAb)for [^{125}I]IGF-I was determined following a 2 h incubation, corresponding to time of treatment media addition to cell cultures. Complexes of rhIGFBP-3 and [^{125}I]IGF-I or antibody and [^{125}I]IGF-I were covalently bound using the cross-linking agent disuccinimidyl suberate and separated from free [^{125}I]IGF-I by SDS-PAGE. Samples containing rhIGFBP-3 and [^{125}I]IGF-I exhibited one band corresponding to the protein complex, that was not evident in a sample containing only [^{125}I]IGF-I, and one lower

molecular weight band corresponding to free [^{125}I]IGF-I (Figure 8A). This protein complex band increased in intensity in samples containing increasing concentrations of rhIGFBP-3, and there was a corresponding decrease in intensity of the free [^{125}I]IGF-I band. At an IGFBP-3:IGF-I molar ratio of 1/8, 1/2, 1, and 2, the amount of bound [^{125}I]IGF-I was 4.4, 18.0, 37.9, and 70.4%, respectively, of the total [^{125}I]IGF-I available. Samples containing the IGF-I antibody and [^{125}I]IGF-I did not show any bands corresponding to complexes of the antibody and [^{125}I]IGF-I; only free [^{125}I]IGF-I was detected (Figure 8B). However, the presence of low amounts of an [^{125}I]IGF-I and IGF-I antibody complex in the samples was detected by size exclusion chromatography and gamma counting of fractions (data not shown). All lanes displayed two minor bands corresponding to high molecular weights that were not consistently affected by presence of rhIGFBP-3 or the IGF-I antibody (not shown). These bands likely resulted from nonspecific binding of [^{125}I]IGF-I, possibly with bovine serum albumin in the [^{125}I]IGF-I stock buffer. Regardless, the bands represented less than 2% of total counts in the incubation mixtures.

DISCUSSION

This experiment investigated the potential contribution of IGF-I and IGFBP-3 to the mitogenic activity in serum and mammary tissue extracts. In collagen gel cultures of primary undifferentiated mammary epithelial cells, rhIGFBP-3 abrogated the mitogenic effects of added IGF-I. Further, rhIGFBP-3 negated approximately 35% of the mitogenic

activity in extracts of mammary parenchyma and abolished that in serum, when added in equimolar concentrations with IGF-I.

Development of the mammary gland is likely influenced by multiple growth factors and their synergistic or antagonistic interactions (Oka et al., 1991). Many of these growth factors likely originate in the mammary gland and act in an autocrine, juxtacrine or paracrine manner. Such factors may also derive from other tissues and reach the mammary gland via the circulation. In this experiment, mammary tissue extract was more mitogenic than heifer serum at a 10% concentration in the culture medium, stimulating DNA synthesis by 618% and 70%, respectively. This indicates that the bovine mammary gland is indeed a rich source of growth factors for mammary epithelium, as suggested by Sandowski et al. (1993). Of these factors, a potent mitogen for undifferentiated bovine mammary epithelial cells is IGF-I, which stimulated DNA synthesis approximately twofold in this study. In addition, the bovine mammary gland produces mRNA for TGF-α (Zurfluh et al., 1990, Koff and Plaut, 1995) which stimulates DNA synthesis in clonal (Woodward et al., 1994) as well as primary bovine mammary epithelial cells (Zurfluh et al., 1990). Also, expression of mRNA for IGF-II is developmentally regulated in mammary tissue of ewe lambs (Hovey et al., 1998) although IGF-II is considerably less mitogenic than IGF-I in vitro (Peri et al., 1992). Insulin alone can also stimulate proliferation of bovine undifferentiated mammary cells, although it is effective only at pharmacological concentrations (Shamay et al., 1988) and likely acts through the type I IGF receptor. In the present experiment, the insulin concentrations in individual mammary extracts composing the pooled stock were undetectable. From the present data,

it is impossible to ascertain how much of this mitogenic activity in serum or extracts becomes directly available to mammary cells. Regardless, IGFs clearly contribute a portion of the mitogenic activity present, as equimolar concentrations of rhIGFBP-3 could inhibit approximately one third of the DNA synthesis stimulated by serum or extracts presumably by binding IGF-I, and thereby reducing its access to type I IGF receptors on mammary cells. The effects of the IGF-I antibodies were not previously characterized in this culture system and were considerably less potent inhibitors of IGF-I bioactivity. Binding studies in this experiment support the conclusion that this difference in inhibition is at least partially explained by the greater binding of rhIGFBP-3 to IGF-I. Similar to our results, a neutralizing monoclonal antibody to IGF-I was incapable of inhibiting IGF-I-induced [^{3}H]thymidine incorporation in porcine granulosa cell cultures (Mondschein et al., 1989). The authors attributed this unexpected result in part to the moderate stimulation of [^{3}H]thymidine incorporation by IGF-I alone. In our case, bovine primary mammary epithelial cells also secrete IGFBP-3 and IGFBP-2 in response to addition of IGF-I (Figure 3), which in cultures may compete with the added antibody for binding of available IGF-I. In this study, serum stimulated only a modest increase in [^{3}H]thymidine incorporation, that was not inhibited by addition of the IGF-I antibodies. Along with the inherent difficulty in demonstrating inhibition of a slight growth response, it is possible that the IGF-I in the serum pool was less biologically available due to presence of IGFBPs.

Similar to our present and previous results (Purup et al., 1995), Shamay et al. (1988) and Peri et al. (1992) showed that IGF-I at a low concentration is highly mitogenic

for bovine undifferentiated mammary epithelial cells. Mammary IGF-I expression is low in comparison with the liver and other tissues (Forsyth, 1996) but is potentially more meaningful in mammary development than systemic concentrations of IGF-I. For example, the ability of locally available IGF-I to stimulate cell proliferation has been demonstrated in vivo (Ruan et al., 1992). Moreover, our recent data (Chapter 2; Weber et al., 1998) demonstrate precocious alveolar bud formation in mammary glands of MMTV-IGF-I transgenic mice, providing further evidence that modulation of local mammary IGF-I can markedly alter prepubertal mammary development. In this study, the concentration of IGF-I in mammary extract (34.0 ng/ml) was approximately one third of that in serum (107 ng/ml), yet the extract was nearly 20 times more mitogenic than serum. Interestingly, rhIGFBP-3 significantly reduced DNA synthesis in response to both serum and extracts. This suggests that the IGFs indeed provide a considerable portion of the mitogenic activity available in serum and mammary tissue extract, either individually or through interactions with other growth factors. In ovarian granulosa cells, it has been suggested that the primary role of the IGFs is to amplify the actions of other hormones (Mondschein et al., 1989). Thus, the extent of IGF-I effects may be determined in large part by the influence of co-expressed factors in the mammary gland. Our observations of the dramatic inhibition by IGFBP-3 of the mitogenic response to mammary extracts and serum containing low concentrations of IGF-I would support a similar principle for mammary IGF-I actions. Similarly, Waksman et al., (1991) demonstrated that added IGF-I synergizes strongly with other factors in mammary extract to stimulate DNA synthesis.

Such a dramatic difference in mitogenic activity between serum and mammary extracts may also derive from varying IGFBP profiles, or a lower concentration of free IGF-I in serum. The IGF-I molecule displays high-affinity binding to IGFBPs, which regulate its bioactivity in a fashion that is not well characterized. Of these, IGFBP-3 is the predominant IGFBP in the circulation; further, mRNA and protein for IGFBP-3 in prepubertal bovine mammary tissue are more abundant than that for IGFBP-1 or IGFBP-2 (Weber et al., 1996). The increased mitogenic activity of des-3-IGF-I, which has a greatly reduced affinity for IGFBPs, relative to IGF-I suggests that association with IGFBPs is inhibitory (McGrath et al., 1991). The major difference in IGFBP profile between mammary extracts and serum of prepubertal heifers fed at a low feeding level is a three times higher relative abundance of IGFBP-2 in serum (22%) compared with mammary extracts (7%) (Chapter 6). The relationship of IGFBPs in serum and mammary tissue extracts from prepubertal heifers with DNA synthesis in primary cell cultures has been evaluated (Purup et al., 1998). Synthesis of DNA induced by 5% serum was negatively correlated with serum IGFBP-2 and positively correlated with serum IGFBP-3 and IGF-I. In multiple cell lines, IGFBP-2 has been shown to inhibit IGF-dependent effects on cell growth as shown by addition of IGFBP-2 to cultures (Jones and Clemmons, 1995). Further, serum IGFBP-2 levels are elevated in response to somatotropin deficiency or in fasting situations, particularly in the case of protein restriction (Collett-Solberg and Cohen, 1996). In contrast, DNA synthesis in response to 5% mammary extract was negatively correlated with IGFBP-3, while no significant correlation was observed between the growth response and IGFBP-2 or IGF-I. These

results support the suggestion of Jones and Clemmons (1995) that circulating IGFBP-3 may stimulate IGF-I activity, while IGFBP-3 in mammary tissue may inhibit IGF-I activity.

Differences in mitogenic activity between serum preparations also may be attributed in part to relative concentrations of different IGFBP. In our study, 10% heifer serum stimulated DNA synthesis to a greater degree than 10% FBS. Analysis of IGFBP was performed using serum from a representative set of heifers similar in age and body weight as well as rearing regimen to the three prepubertal heifers that supplied serum for these in vitro experiments. A higher proportion of total IGFBP consisted of IGFBP-3 (72%) compared with that in FBS (13%). On the other hand, IGFBP-2 was more abundant in FBS (82%) compared with the heifer serum (22%). Taken together with the above correlations for serum IGFBP with DNA synthesis, these data indicate that the amounts of IGFBP-3 and IGFBP-2 present in serum preparations can contribute to different growth responses. For example, the mitogenic activity present in our IGF-I and FBS treatments appeared to differ from that reported by Shamay et al. (1988). In their study, IGF-I induced cell proliferation of only 25-40% of that achieved by addition of 10% FBS. In contrast, our results showed that maximal IGF-I stimulation was twice that induced by 10% FBS and were more in line with the comparatively greater IGF-I stimulation reported by Baumrucker and Stemberger (1989) using prepartum and lactating mammary tissue. However, this difference with the results of Shamay et al. (1988) may also be related to the authors' comparison of the mitogenic activity of various FBS stocks and ultimate selection of one exhibiting high activity (Purup, personal

communication). Clearly, the IGFBP present in serum in addition to IGF-I may contribute to a balance of positive and negative growth regulation.

Interactions between the stromal and epithelial compartments can dramatically affect development of the mammary gland (Cunha and Hom, 1996). Thus, the observation that mammary synthesis of IGFBP-3 mRNA is modulated by physiological stage in the pig (Lee et al., 1993) supports the possibility of local regulation of bovine mammary development by the IGFs. The presence of transforming growth factor-ß1 (TGF-ß1) has recently been confirmed in both serum and mammary extract from heifers (Purup et al., 1998); its higher concentration in serum relative to extract potentially contributes to the difference noted in mitogenic activity in this study. Interestingly, evidence from cancer research suggests an IGF-independent role of IGFBP-3 in TGF-ß induced cell growth inhibition of breast cancer cells (Oh et al., 1995) and TGF-ß1 induced apoptosis in prostate cancer cells (Rajah et al., 1997).

Fibroblasts were not observed in culture experiments using our previously frozen cell preparations, lending further support to the earlier justification of this culture system for studying growth regulation of bovine mammary epithelium. Moreover, the ultrastructural organization of the cultured epithelial organoids observed resembles that in vivo (Waksman et al., 1991). The morphological appearance of growth observed during the culture period (Figure 1) resembled the stellate and needle-like appearance of outgrowths described earlier (Shamay and Gertler, 1986). Interestingly, only organoids cultured with serum displayed the former phenotype, whereas added IGF-I and mammary extracts both induced distinct needle-like extensions from organoids. Taken together

with the dramatic inhibitory effect of rhIGFBP-3 on DNA synthesis in response to mammary extracts, this observation indicates that IGF-I may supply a considerable portion of the mitogenic activity in mammary extracts.

In conclusion, these results suggest that the mammary gland is a rich source of mitogenic activity for developing mammary epithelium, in addition to the growth factors transported across mammary epithelium that originate from other body tissues. Insulin-like growth factor-I contributes significantly to mitogenic activity from both systemic and mammary sources, together with its multiple interactions with other growth factors. Regardless of the source of the mitogenic activity, local mammary synthesis of IGFBP-3 likely influences the growth response of the developing mammary epithelium to IGF-I and other mitogens. The physiological regulation of IGFBP-3 synthesis and the interactions that may exist between IGFBP-3 and other growth factors in the mammary gland remain unclear.

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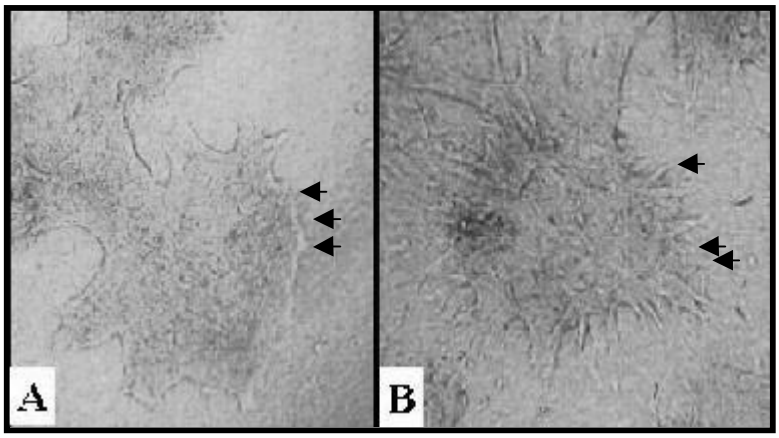

Figure 1. Representative images of primary mammary organoids in culture. In the presence of 5% heifer serum (panel A), primary organoids consistently grew with a smooth, even border and displayed a stellate appearance. In contrast, cells grown with 5% mammary extract (panel B) showed distinct needle-like projections. Arrows in the figure indicate either the broad extensions (panel A) or the needle-like projections (panel B) observed in culture. Each arrow equals 30 µm.

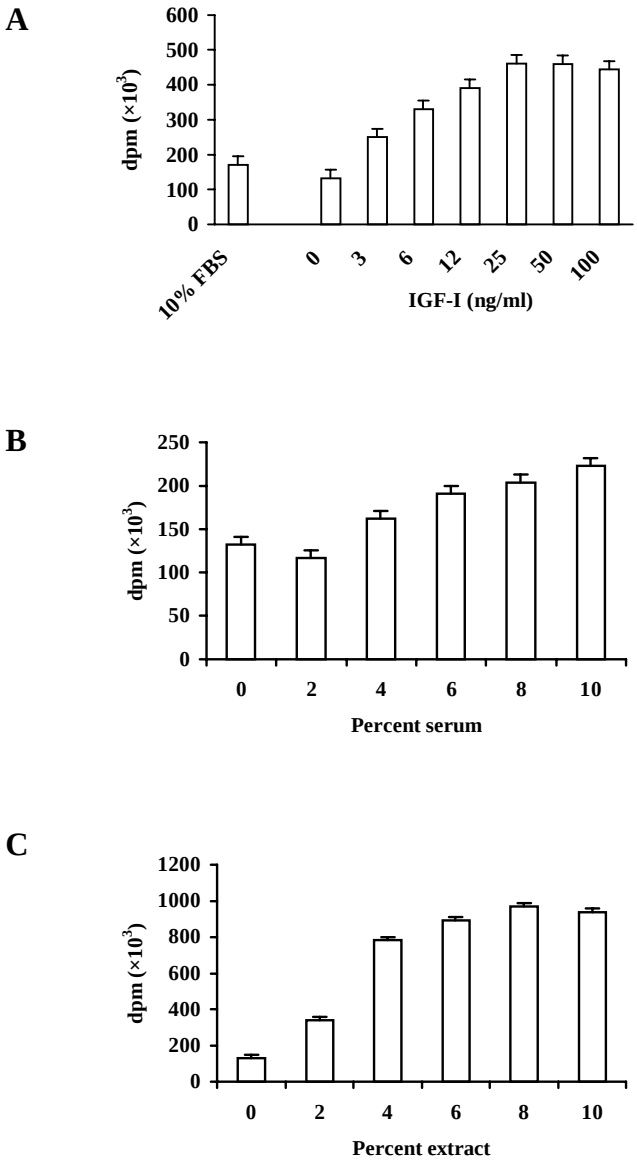

Figure 2. Effect of various concentrations of (A) rhIGF-I (ng/ml) and 10% FBS, (B) percent prepubertal heifer serum, or (C) percent mammary extract in culture medium on [^{3}H]thymidine incorporation (dpm) in primary cultures of bovine mammary epithelial organoids. In A, B and C, the 0 level represents basal medium. Values are LS means ± SEM for cultures performed in triplicate.

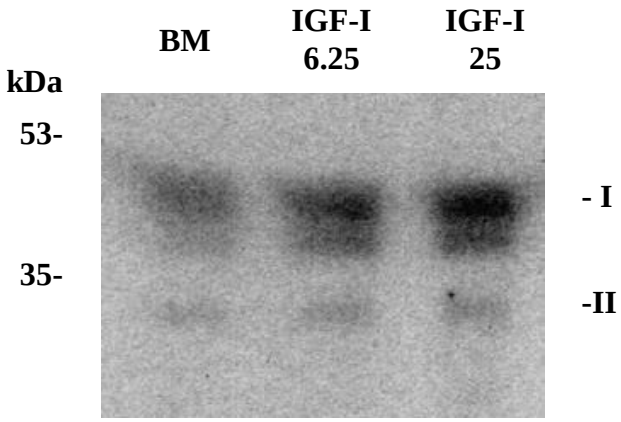

Figure 3. Western ligand blot analysis of conditioned media from primary cultures of bovine mammary epithelial organoids after 2 d in culture. Media (500 µl) was collected from treatments of basal medium (BM) and media containing 6.25 and 25 ng/ml IGF-I performed in triplicate, pooled, and lyophilized. Samples were reconstituted in 100 µl. For each sample, 50 µl was analyzed by SDS-PAGE, blotted to nitrocellulose, and probed with [^{125}I]-IGF-I. Two bands corresponding to (I) 39-43 kD IGFBP-3 and (II) 32 kD IGFBP-2 were detected in conditioned media. Blots were exposed for 30 d.

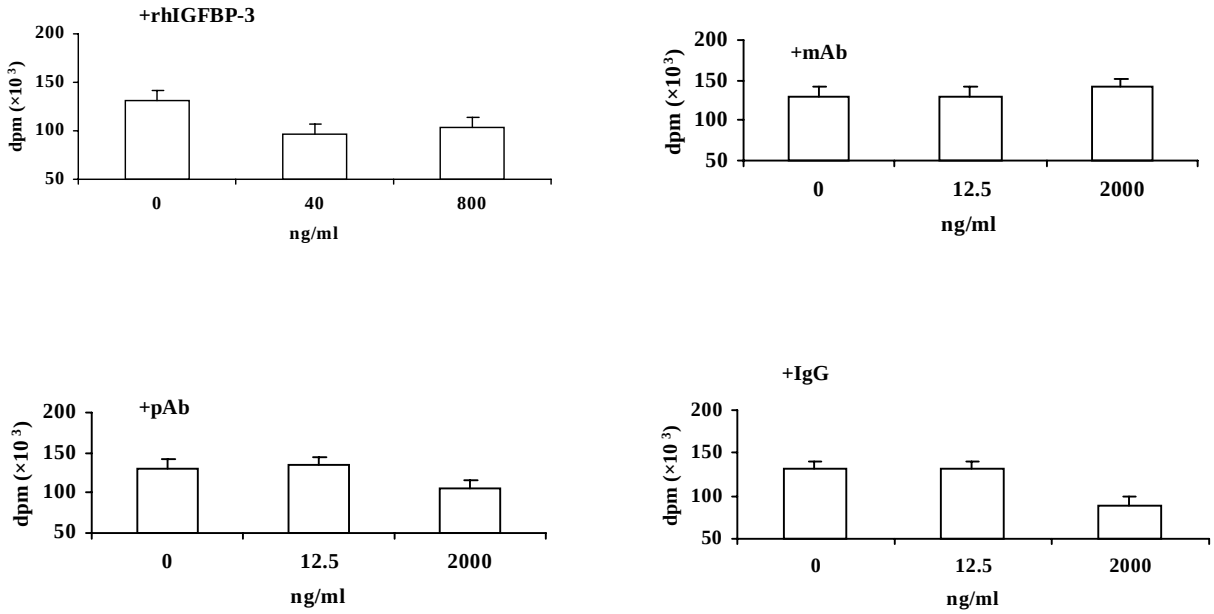

Figure 4. Effect of rhIGFBP-3, monoclonal (+mAb) and polyclonal (+pAb) IGF-I antibodies, and a non-immune globulin (+IgG) on [^{3}H]thymidine incorporation in primary cultures of bovine mammary epithelial organoids in response to basal medium (0). Concentrations of added factors shown represent the lowest and highest amounts used in cultures. Values are LSmeans ± SEM for cultures performed in triplicate.

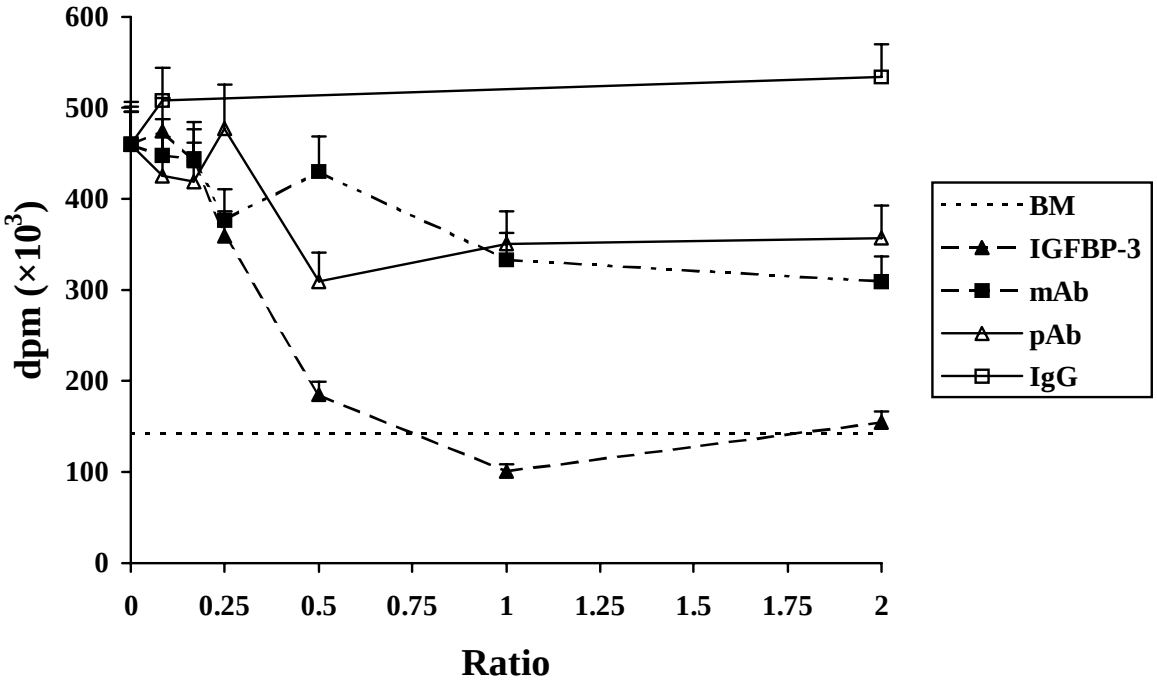

Figure 5. Inhibition of [^{3}H]thymidine incorporation (dpm) in primary cultures of bovine mammary epithelial organoids in response to media containing rhIGF-I alone (50 ng/ml) or with rhIGFBP-3, monoclonal (mAb) or polyclonal (pAb) antibodies against IGF-I, or a non-immune globulin (IgG). Concentrations are given as molar Ab/IGF-I, IGFBP-3/IGF-I or IgG/IGF-I ratio. The horizontal dotted line represents [^{3}H]thymidine incorporation in basal medium (BM) without IGF-I addition. Values are LS means ± SEM for cultures performed in triplicate.

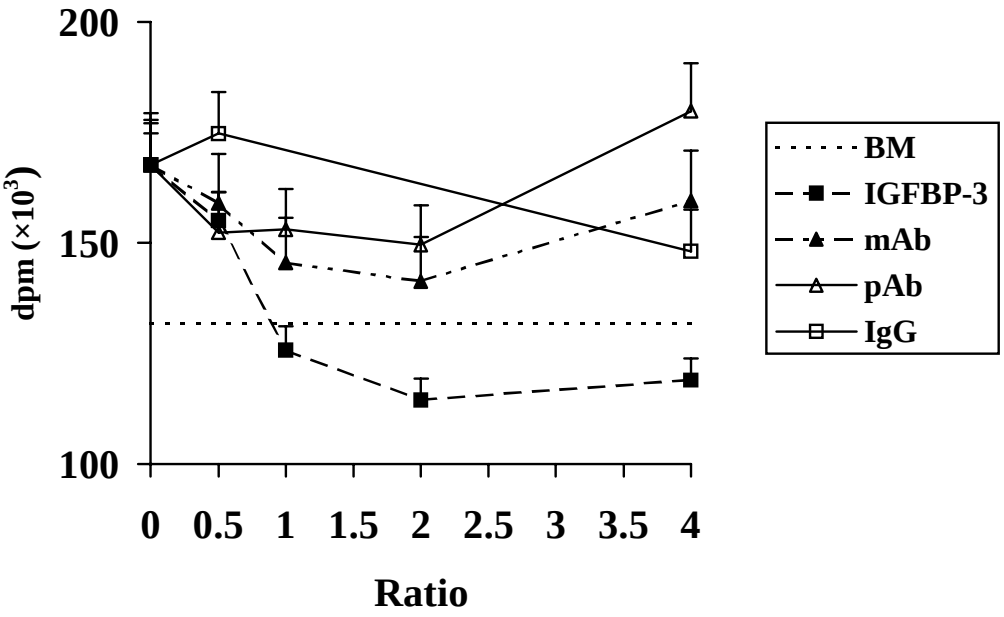

Figure 6. Inhibition of [^{3}H]thymidine incorporation (dpm) in primary cultures of bovine mammary epithelial organoids in response to media containing 5% heifer serum alone or with rhIGFBP-3, monoclonal (mAb) or polyclonal (pAb) antibodies against IGF-I, or a non-immune globulin (IgG). Concentrations are given as molar IGFBP-3/IGF-I, Ab/IGF-I or IgG/IGF-I ratio. The horizontal dotted line represents [^{3}H]thymidine incorporation in basal medium (BM) without 5% serum added. Values are LSmeans ± SEM for cultures performed in triplicate.

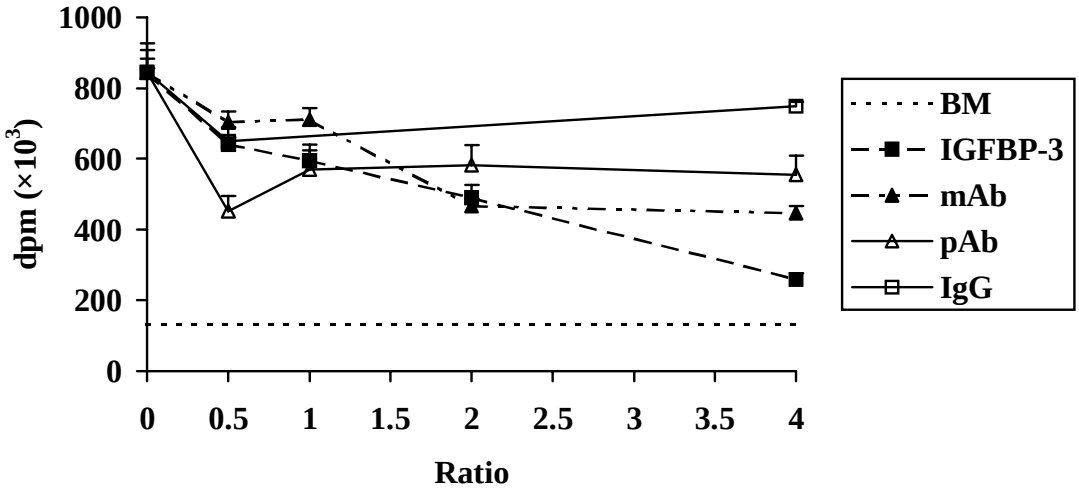

Figure 7. Inhibition of [^{3}H]thymidine incorporation (dpm) in primary cultures of bovine mammary epithelial organoids in response to media containing 5% mammary extracts alone or with rhIGFBP-3, monoclonal (mAb) or polyclonal (pAb) antibodies against IGF-I, or a non-immune globulin (IgG). Concentrations are given as molar Ab/IGF-I, IGFBP-3/IGF-I or IgG/IGF-I ratio. The horizontal dotted line represents [^{3}H]thymidine in basal medium (BM) without 5% extracts added. Values are LS means ± SEM for cultures performed in triplicate.

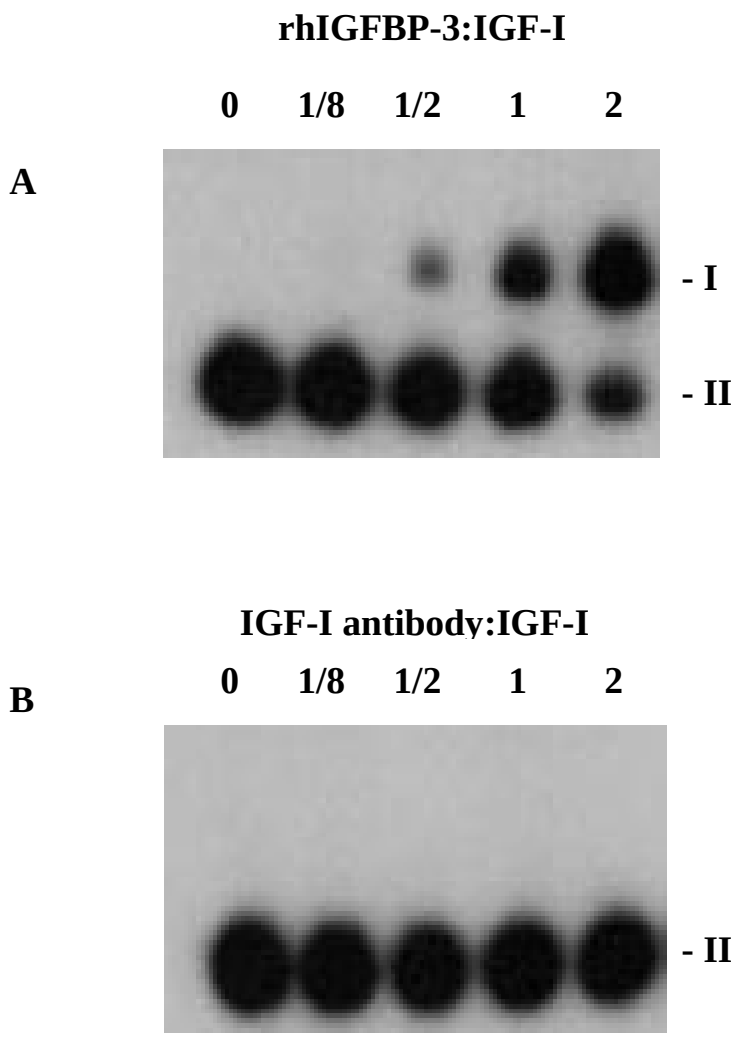

Figure 8. Relative binding activity of increasing concentrations (molar ratios of IGFBP-3 or IGF-I antibody:IGF-I) of rhIGFBP-3 (A) and a polyclonal IGF-I antibody (B) with [^{125}I]IGF-I following a 2 h incubation. Complexes of rhIGFBP-3 and [^{125}I]IGF-I or antibody and [^{125}I]IGF-I were covalently bound using the cross-linking agent disuccinimidyl suberate and separated from free [^{125}I]IGF-I by SDS-PAGE under reducing conditions. Samples containing rhIGFBP-3 and [^{125}I]IGF-I exhibited one band (I) corresponding to the protein complex and one lower molecular weight band corresponding to free [^{125}I]IGF-I (II). Samples containing the IGF-I antibody and [^{125}I]IGF-I showed only one band corresponding to free [^{125}I]IGF-I (II).

APPENDIX A

Appendix A. Table 1. Analysis of variance for the effect of extracts of milk from control or transgenic mice on [^{3}H]thymidine incorporation into MAC-T cells.

Source of Variation	df	Mean Square	F Value	Pr > F
Transgene	1	47362133.33	69.58	.0001
Concentration	5	47329723.02	69.53	.0001
Error	41	27909818.167		

Appendix A. Table 2. Analysis of variance for the effect of transgene presence on [^{3}H]thymidine incorporation/μg DNA of mammary explants from 60 d virgin control and line 29 mice.

Source of Variation	df	Mean Square	F Value	Pr > F
Block	5	348.22	1.70	.2881
Transgene	1	81.95	0.40	.5553
Error	5	205.32		

Appendix A. Table 3. Analysis of variance for the effect of transgene presence on [^{3}H]thymidine incorporation/μg DNA of mammary explants from 60 d virgin control and line 15 mice.

Source of Variation	df	Mean Square	F Value	Pr > F
Block	5	167.33	2.03	.2284
Transgene	1	741.43	8.98	.0302
Error	5	82.58		

Appendix A. Table 4. Analysis of variance for the effect of transgene presence on [^{3}H]thymidine incorporation/μg DNA of mammary explants from 90 d virgin control and line 29 mice.

Source of Variation	df	Mean Square	F Value	Pr > F
Block	5	332.57	.37	.8495
Transgene	1	7.47	.01	.9308
Error	5	558.83		

Appendix A. Table 5. Analysis of variance for the effect of transgene presence on [^{3}H]thymidine incorporation/μg DNA of mammary explants from 90 d virgin control and line 15 mice.

Source of Variation	df	Mean Square	F Value	Pr > F
Block	5	347.90	.59	.7094
Transgene	1	734.29	1.25	.3138
Error	5	585.97		

Appendix A. Table 6. Analysis of variance for the effect of transgene presence on [^{3}H]thymidine incorporation/μg DNA of mammary explants from 12 d pregnant control and line 29 mice.

Source of Variation	df	Mean Square	F Value	Pr > F
Block	5	932.072	.07	.9942
Transgene	1	886.04	.07	.8051
Error	5	13092.99		

Appendix A. Table 7. Analysis of variance for the effect of transgene presence on [^{3}H]thymidine incorporation/μg DNA of mammary explants from 12 d pregnant control and line 15 mice.

Source of Variation	df	Mean Square	F Value	Pr > F
Block	5	10314.46	4.14	.0724
Transgene	1	19676.11	7.90	.0375
Error	5	12446.59		

APPENDIX B

Appendix B. Table 1. Repeated measures analysis of variance for the effect of transgene presence on total IGF-I concentrations in skim milk from CD-1 or transgenic line 29 mice (n = 3 or 4) on d 4, 8, 12, and 16 lactation.

Source of Variation	df	Mean Square	F Value	Pr > F
Transgene	1	906049.7	9.98	.0251
Error	5	90793.6		

Appendix B. Table 2. Repeated measures analysis of variance for the effect of transgene presence on native IGF-I concentrations in skim milk from CD-1 or transgenic line 29 mice (n = 3 or 4) on d 4, 8, 12, and 16 lactation.

Source of Variation	df	Mean Square	F Value	Pr > F
Transgene	1	806736.00	10.91	.0214
Error	5	73911.4		

Appendix B. Table 3. Analysis of variance for the effect of transgene presence on [^{3}H]thymidine incorporation/μg DNA of mammary explants from d 4 lactating control and line 29 mice.

Source of Variation	df	Mean Square	F Value	Pr > F
Block	5	447.49	1.31	.3862
Transgene	1	6948.38	20.39	.0063
Error	5	340.80		

Appendix B. Table 4. Analysis of variance for the effect of transgene presence on [^{3}H]thymidine incorporation/μg DNA of mammary explants from d 4 lactating control and line 15 mice.

Source of Variation	df	Mean Square	F Value	Pr > F
Block	5	665.29	2.46	.1725
Transgene	1	95.46	.35	.5781
Error	5	270.11		

Appendix B. Table 5. Analysis of variance for the effect of transgene presence on [^{3}H]thymidine incorporation/μg DNA of mammary explants from d 14 lactating control and line 15 mice.

Source of Variation	df	Mean Square	F Value	Pr > F
Block	5	182.49	2.45	.1742
Transgene	1	2.80	0.04	.8540
Error	5	74.58		

Appendix B. Table 6. Analysis of variance for the effect of transgene presence on [^{3}H]thymidine incorporation/μg DNA of mammary explants from 12 d pregnant control and line 29 mice.

Source of Variation	df	Mean Square	F Value	Pr > F
Block	5	25.45	.41	.8235
Transgene	1	3.16	.05	.8300
Error	5	61.75		

Appendix B. Table 7. Analysis of variance for the effect of transgene presence on average daily gain (g/d) of fostered nontransgenic CD-1 litters suckling CD-1 or transgenic line 15 dams (n = 6/line).

Source of Variation	df	Mean Square	F Value	Pr > F
Block	11	0.47	.47	.8836
Transgene	1	2.65	2.68	.1328
Error	10	.99		

Appendix B. Table 8. Analysis of variance for the effect of transgene presence on average daily gain (g/d) of fostered nontransgenic CD-1 litters suckling CD-1 or transgenic line 29 dams (n = 6/line).

Source of Variation	df	Mean Square	F Value	Pr > F
Block	5	.33	4.70	.0573
Transgene	1	6.15	86.60	.0002
Error	5	.07		

APPENDIX C

Appendix C. Table 1. Analysis of variance for the effect of feeding level on [^{3}H]thymidine incorporation into primary cultures of bovine mammary epithelial organoids in response to extracts of mammary tissue from placebo-treated heifers, for cultures performed in triplicate using two different cell preparations (heifer).

Source of Variation	df	Mean Square	F Value	Pr > F
Block	5	7.91	7.50	.0010
Feeding level	1	2.12	2.01	.1771
bST	1	.00	.00	.9654
Feeding level × bST	1	33.36	31.62	.0001
Block × Feeding level	5	.93	.88	.5176
Block × bST	5	2.14	2.03	.1325
Block × Feeding level × bST	5	3.35	3.17	.0376
Heifer	1	27.46	26.03	.0001
Heifer × Block	5	6.50	6.16	.0027
Heifer × Feeding level	1	.53	.50	.4898
Heifer × bST	1	1.22	1.15	.2998
Heifer × Feeding level × bST	1	11.29	10.70	.0052
Error	15	1.06		

APPENDIX D

Appendix D. Table 1. Analysis of variance for the effect of feeding level and bST on abundance of IGF-I mRNA in mammary tissue from prepubertal heifers.

Source of Variation	df	Mean Square	F Value	Pr > F
Block	4	.51	2.69	.0825
Feeding level	1	.18	.96	.3473
bST	1	.12	.65	.4347
Feeding level × bST	1	.72	3.78	.0757
Error	12	.19		

Appendix D. Table 2. Analysis of variance for the effect of feeding level and bST on abundance of IGFBP-3 mRNA in mammary tissue from prepubertal heifers.

Source of Variation	df	Mean Square	F Value	Pr > F
Block	5	26.30	5.26	.0055
Feeding level	1	.18	.23	.6395
bST	1	.20	.26	.6193
Feeding level × bST	1	1.77	2.24	.1551
Error	15	.19		

Appendix D. Table 3. Analysis of variance for the effect of feeding level and bST on abundance of IGFBP-2 mRNA in mammary tissue from prepubertal heifers.

Source of Variation	df	Mean Square	F Value	Pr > F
Block	5	116864.19	30.69	.0001
Feeding level	1	37.77	.01	.9220
bST	1	26.72	.01	.9343
Feeding level × bST	1	5024.50	1.32	.2687
Error	15	3807.83		

Appendix D. Table 4. Analysis of variance for the effect of feeding level and bST on abundance of IGFBP-1 mRNA in mammary tissue from prepubertal heifers.

Source of Variation	df	Mean Square	F Value	Pr > F
Block	5	23.92	.37	.8600
Feeding level	1	327.26	5.09	.0394
bST	1	5.24	.08	.7793
Feeding level × bST	1	.01	.00	.9924
Error	15	64.30		

Appendix D. Table 5. Analysis of variance for the effect of feeding level and bST on abundance of bST receptor mRNA in mammary tissue from prepubertal heifers.

Source of Variation	df	Mean Square	F Value	Pr > F
Block	5	13868.11	28.17	.0001
Feeding level	1	.07	.00	.9906
bST	1	1867.94	3.79	.0704
Feeding level × bST	1	1286.51	2.61	.1268
Error	15	492.26		

Appendix D. Table 6. Analysis of variance for the effect of feeding level and bST on abundance of IGF-I receptor mRNA in mammary tissue from prepubertal heifers.

Source of Variation	df	Mean Square	F Value	Pr > F
Block	3	3.92	21.69	.0002
Feeding level	1	1.67	9.24	.0140
bST	1	.10	.54	.4822
Feeding level × bST	1	.64	3.54	.0926
Error	15	.18		

Appendix D. Table 7. Analysis of variance for the effect of feeding level and bST on abundance of IGF-I protein in mammary tissue from prepubertal heifers.

Source of Variation	df	Mean Square	F Value	Pr > F
Block	5	32.87	1.35	.2969
Feeding level	1	619.15	25.44	.0001
bST	1	366.60	15.06	.0015
Feeding level × bST	1	246.40	10.12	.0062
Error	15	24.34		

Appendix D. Table 8. Analysis of variance for the effect of feeding level and bST on abundance of IGFBP-3 protein in mammary tissue from prepubertal heifers.

Source of Variation	df	Mean Square	F Value	Pr > F
Block	5	1070507.38	3.02	.0442
Feeding level	1	1081626.04	3.05	.1012
bST	1	1090987.04	3.08	.0999
Feeding level × bST	1	501415.04	1.41	.2530
Error	15	354777.18		

Appendix D. Table 9. Analysis of variance for the effect of feeding level and bST on abundance of IGFBP-2 protein in mammary tissue from prepubertal heifers.

Source of Variation	df	Mean Square	F Value	Pr > F
Block	5	5667.98	1.55	.2350
Feeding level	1	41251.04	11.25	.0044
bST	1	2625.04	.72	.4108
Feeding level × bST	1	6176.04	1.68	.2140
Error	15	3667.71		

Appendix D. Table 10. Analysis of variance for the effect of feeding level and bST on abundance of IGFBP-1 protein in mammary tissue from prepubertal heifers.

Source of Variation	df	Mean Square	F Value	Pr > F
Block	5	35895.74	5.92	.0032
Feeding level	1	51.04	.01	.9281
bST	1	5370.04	.89	.3617
Feeding level × bST	1	782.04	.13	.7245
Error	15	6065.61		

Appendix D. Table 11. Analysis of variance for the effect of feeding level and bST on abundance of IGFBP-4 protein in mammary tissue from prepubertal heifers.

Source of Variation	df	Mean Square	F Value	Pr > F
Block	5	50371.14	7.78	.0009
Feeding level	1	50508.38	7.80	.0137
bST	1	12105.04	1.87	.1918
Feeding level × bST	1	4401.04	.68	.4227
Error	15	6477.45		

APPENDIX E

Appendix E. Table 1. Analysis of variance for the effect of IGF-I addition to basal treatment media on [^{3}H]thymidine incorporation into primary cultures of bovine mammary epithelial organoids.

Source of Variation	df	Mean Square	F Value	Pr > F
Treatment	6	98014775450	41.03	.0001
Error	23	2389108746		

Appendix E. Table 2. Analysis of variance for the effect of serum addition to basal treatment media on [^{3}H]thymidine incorporation into primary cultures of bovine mammary epithelial organoids.

Source of Variation	df	Mean Square	F Value	Pr > F
Treatment	5	7221518920	28.65	.0001
Error	21	252069447		

Appendix E. Table 3. Analysis of variance for the effect of extract addition to basal treatment media on [^{3}H]thymidine incorporation into primary cultures of bovine mammary epithelial organoids.

Source of Variation	df	Mean Square	F Value	Pr > F
Treatment	5	730162887000	745.03	.0001
Error	21	980665133.3		

Appendix E. Table 4. Analysis of variance for the effect of rhIGFBP-3 addition to basal treatment media on [^{3}H]thymidine incorporation into primary cultures of bovine mammary epithelial organoids.

Source of Variation	df	Mean Square	F Value	Pr > F
Treatment	2	1918598868	6.13	.0113
Error	15	312836896		

Appendix E. Table 5. Analysis of variance for the effect of monoclonal IGF-I antibody addition to basal treatment media on [^{3}H]thymidine incorporation into primary cultures of bovine mammary epithelial organoids.

Source of Variation	df	Mean Square	F Value	Pr > F
Treatment	4	1334532213	3.62	.0235
Error	19	368710887.5		

Appendix E. Table 6. Analysis of variance for the effect of polyclonal IGF-I antibody addition to basal treatment media on [^{3}H]thymidine incorporation into primary cultures of bovine mammary epithelial organoids.

Source of Variation	df	Mean Square	F Value	Pr > F
Treatment	4	1189143182	4.08	.0148
Error	19	291150875.1		

Appendix E. Table 7. Analysis of variance for the effect of non-immune globulin addition to basal treatment media on [^{3}H]thymidine incorporation into primary cultures of bovine mammary epithelial organoids.

Source of Variation	df	Mean Square	F Value	Pr > F
Treatment	4	1965685809	6.48	.0018
Error	19	303577482		

Appendix E. Table 8. Analysis of variance for the effect of rhIGFBP-3 addition to treatment media containing 50 ng/ml IGF-I on [^{3}H]thymidine incorporation into primary cultures of bovine mammary epithelial organoids.

Source of Variation	df	Mean Square	F Value	Pr > F
Treatment	6	76428481000	38.62	.0001
Error	14	1978872810		

Appendix E. Table 9. Analysis of variance for the effect of monoclonal IGF-I antibody addition to treatment media containing 50 ng/ml IGF-I on [^{3}H]thymidine incorporation into primary cultures of bovine mammary epithelial organoids.

Source of Variation	df	Mean Square	F Value	Pr > F
Treatment	6	11015365000	3.24	.0327
Error	14	3397082020		

Appendix E. Table 10. Analysis of variance for the effect of non-immune globulin addition to treatment media containing 50 ng/ml IGF-I on [^{3}H]thymidine incorporation into primary cultures of bovine mammary epithelial organoids.

Source of Variation	df	Mean Square	F Value	Pr > F
Treatment	2	1780892690	.43	.6708
Error	6	4170368037		

Appendix E. Table 11. Analysis of variance for the effect of rhIGFBP-3 addition to treatment media containing 5% heifer serum on [^{3}H]thymidine incorporation into primary cultures of bovine mammary epithelial organoids.

Source of Variation	df	Mean Square	F Value	Pr > F
Treatment	5	1388470184	12.99	.0007
Error	9	106888727.1		

Appendix E. Table 12. Analysis of variance for the effect of monoclonal IGF-I antibody addition to treatment media containing 5% heifer serum on [^{3}H]thymidine incorporation into primary cultures of bovine mammary epithelial organoids.

Source of Variation	df	Mean Square	F Value	Pr > F
Treatment	4	356414817.8	1.25	.3525
Error	10	285975210.5		

Appendix E. Table 13. Analysis of variance for the effect of polyclonal IGF-I antibody addition to treatment media containing 5% heifer serum on [^{3}H]thymidine incorporation into primary cultures of bovine mammary epithelial organoids.

Source of Variation	df	Mean Square	F Value	Pr > F
Treatment	4	503232372	2.02	.1676
Error	10	249263673.2		

Appendix E. Table 14. Analysis of variance for the effect of non-immune globulin addition to treatment media containing 5% heifer serum on [^{3}H]thymidine incorporation into primary cultures of bovine mammary epithelial organoids.

Source of Variation	df	Mean Square	F Value	Pr > F
Treatment	2	574379059	2.16	.1967
Error	6	266067524.8		

Appendix E. Table 15. Analysis of variance for the effect of rhIGFBP-3 addition to treatment media containing 5% mammary extracts on [^{3}H]thymidine incorporation into primary cultures of bovine mammary epithelial organoids.

Source of Variation	df	Mean Square	F Value	Pr > F
Treatment	4	117655790000	24.06	.0001
Error	9	4890204452		

Appendix E. Table 16. Analysis of variance for the effect of monoclonal IGF-I antibody addition to treatment media containing 5% mammary extracts on [^{3}H]thymidine incorporation into primary cultures of bovine mammary epithelial organoids.

Source of Variation	df	Mean Square	F Value	Pr > F
Treatment	4	75057027000	23.90	.0002
Error	8	3140039646		

Appendix E. Table 17. Analysis of variance for the effect of polyclonal IGF-I antibody addition to treatment media containing 5% mammary extracts on [^{3}H]thymidine incorporation into primary cultures of bovine mammary epithelial organoids.

Source of Variation	df	Mean Square	F Value	Pr > F
Treatment	4	59403130000	6.75	.0085
Error	9	8797225301		

Appendix E. Table 18. Analysis of variance for the effect of non-immune globulin addition to treatment media containing 5% mammary extracts on [^{3}H]thymidine incorporation into primary cultures of bovine mammary epithelial organoids.

Source of Variation	df	Mean Square	F Value	Pr > F
Treatment	2	23404603000	57.99	.0003
Error	5	403592687.8		

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EDUCATION

Doctor of Philosophy, Virginia Polytechnic Institute and State University, expected completion November, 1998
Dr. R. Michael Akers, advisor
Major: Animal Science/ Dairy; Molecular and Cellular Biology and Biotechnology Option
Dissertation: The Role of Insulin-like Growth Factor-I and IGF-Binding Proteins in Mammary Gland Development

Master of Science, Virginia Polytechnic Institute and State University, August 1995
Dr. R. Michael Akers and Dr. Frank C. Gwazdauskas, co-advisors
Major: Dairy Science
Thesis: Overexpression of Ovine Insulin-like Growth Factor-I (IGF-I) in the Mammary Glands of Transgenic Mice

Bachelor of Science, cum laude, Michigan State University, December 1992
Major: Animal Science

RESEARCH EXPERIENCE

Virginia Polytechnic Institute and State University; January 1993 to present
Graduate Research Assistant

- perform pronuclear microinjections of mouse embryos
- clone sequences into plasmid, transform bacteria and isolate sequence for microinjection
- analyze tissue samples by polymerase chain reaction and Southern blotting for transgene presence
- screen tissue samples for gene expression by Northern blotting and reverse transcriptase-polymerase chain reaction
- prepare and analyze milk, blood, and tissue samples by radioimmunoassay
- isolate and culture primary bovine mammary organoids
- prepare and evaluate mouse mammary gland whole mounts
- share in supervision of students conducting undergraduate research
- demonstrate procedure for preparation and culture of primary bovine mammary organoids

RESEARCH EXPERIENCE (CONT'D)

Danish Institute of Agricultural Sciences, Foulum, Denmark; October 1995 to May 1996, September 1996 to December 1996
Graduate Research Assistant

- isolate and culture primary bovine mammary organoids in collagen gels
- demonstrate Northern blotting technique for analysis of gene expression in tissue samples
- dissect mammary parenchyma and stroma portions from heifer mammary glands

Technical University; Berlin, Germany, June to September 1992
Research Assistant

- perform feedstuff and forage fiber analyses
- evaluate milk sample composition

Michigan State University, East Lansing; September 1989 to December 1992
Laboratory Assistant

- conduct radioimmunoassays for blood hormone analysis
- assist in catheterization and maintenance of infusion catheters
- collect tissue, milk and blood samples for biochemical analyses and radioimmunoassay

TEACHING EXPERIENCE

Virginia Polytechnic Institute and State University; 1994 to present
Graduate Teaching Assistant

- co-instruct lecture and laboratory sections of a 4-credit undergraduate Anatomy and Physiology course utilizing an internet homepage; received student rating of 4.4 (4=very good; 5=excellent) (ALS 2304), 1997
- instruct laboratory sections of Anatomy and Physiology course (ALS 2304), 1994, 1995, 1998

Coach, Dairy Cattle Judging Team II - 1993, 1994, 1997

- instruct undergraduate students in dairy cattle evaluation, development of decision making skills and oral communication
- supervise judging team at national intercollegiate competition

Ad-hoc Presentations

- Advanced Placement Biology class, 1997: Dissect ewes for evaluation of gross anatomy with high school students
- Dairy Science Recruiting Day, 1997: Conduct mock class to describe mammary gland development and function for high school students and parents
- Virginia 4-H/FFA Field Day, 1993, 1994, 1998: Evaluate dairy cattle classes with other committee members and score oral reasons given by 4-H and FFA participants
- Virginia 4-H/FFA Dairy Judging Workshop, 1994, 1997, 1998: Describe the basics of dairy cattle evaluation and preparation for giving effective oral reasons

PUBLICATIONS

Full Papers

Weber, M.S., P.L. Boyle, B.A. Corl, F.C. Gwazdauskas, E.A. Wong, and R.M. Akers. (1998) Overexpression of insulin-like growth factor-I (IGF-I) in mammary glands of transgenic mice stimulates alveolar bud development. Endocrine 8:251-259.

Weber, M.S., S. Purup, M. Vestergaard, S.E. Ellis, J. Søndergaard, R.M. Akers and K. Sejrsen. (1998) Contribution of insulin-like growth factor-I (IGF-I) and IGF-binding protein-3 (IGFBP-3) to the mitogenic activity in mammary tissue of prepubertal dairy heifers. J. Endocrinology (submitted).

Purup, S., Vestergaard, M., Weber, M.S., Plaut, K., Akers, R.M. and K. Sejrsen. (1998) Local regulation of pubertal mammary growth in heifers. J. Animal Sci. (accepted).

Presentations

Weber, M.S., B.A. Corl, P.L. Boyle, E.A. Wong, F.C. Gwazdauskas, and R.M. Akers. (1998) Expression of ovine insulin-like growth factor-I (IGF-I) stimulates alveolar bud development in mammary glands of transgenic mice. Proc. Keystone Symposia on Breast and Prostate Cancer, Feb 21-26, Copper Mountain, CO (Abstract no. 230).

Weber, M.S., S. Purup, M. Vestergaard, K. Sejrsen, and R.M. Akers. (1997) Contribution of insulin-like growth factor-I (IGF-I) to the mitogenic activity in mammary tissue of prepubertal dairy heifers. J. Dairy Sci. 80: (Suppl 1, page 205).

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Weber, M.S., S. Purup, M. Vestergaard, R.M. Akers, and K. Sejrsen. (1996) Effects of feeding level and exogenous bST on mitogenic activity of mammary gland extracts in mammary epithelial cell cultures. Proc. Third Joint EAAP/ASAS Workshop on the Biology of Lactation in Farm Animals, Lillehammer, Norway. Livestock Production Sci. 50:168.

Weber, M.S., S. Purup, K. Sejrsen, and R.M. Akers. (1996) Effects of feeding level and exogenous bST on mammary IGF-I and IGFBP-3 expression in prepubertal dairy heifers. J. Dairy Sci. 79: (Suppl. 1, page 261).

Weber, M.S., A. VanHoff, F.C. Gwazdauskas, E.A. Wong, and R.M. Akers. (1996) Overexpression of ovine insulin-like growth factor-I in the mammary glands of transgenic mice. J. Dairy Sci. 79: (Suppl. 1, page 130).

HONORS AND ACHIEVEMENTS

Gamma Sigma Delta Honor Society of Agriculture
Outstanding Graduate Teaching Assistant Award, Virginia Tech Dairy Club - 1998
Competitive Graduate Research Development Proposal Recipient - 1996, 1997
American Dairy Science Association Annual Meeting Graduate Paper Presentation, 3rd Place
American Dairy Science Association (Midwest) Undergraduate Paper Presentation, 1st Place
Dairy Shrine
National Intercollegiate Dairy Judging Competition, 1st Place Oral Reasons, 3rd Place Overall
Michigan State University College of Agriculture and Natural Resources Ambassador,
Student Senate Representative, Dairy Club, Alpha Zeta Honorary Agricultural Fraternity,
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