Contribution of insulin-like growth factor (IGF)-I and IGF-binding protein-3 to mitogenic activity in bovine mammary extracts and serum

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Abstract

Peripubertal development of the mammary gland is probably 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. An aqueous 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-³H]thymidine

incorporation as a measure of DNA synthesis. Addition of mammary extracts stimulated DNA synthesis 545% compared with 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 the addition of rhIGFBP-3 at, respectively, 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 probably play a critical role in prepubertal development of the bovine mammary gland.

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Introduction

Regulation of mammary growth and development is accomplished by a complex interplay of circulating hormones with locally 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 probably 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 (IGF)-I (Akers 1985, Kleinberg 1997). The mitogenic effect of IGF-I on mammary explants (Baumrucker & 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 (Campbell *et al.* 1991, McGrath *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 (McGrath *et al.* 1991, Romagnolo *et al.* 1994). 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 recombinant human (rh) 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 (molecular weight=7646 Da) was purchased from Bachem AG (Bubendorf, Switzerland). Glycosylated recombinant human IGFBP-3 (molecular weight=47 000 Da) was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Rabbit anti-human IGF-I polyclonal antibody (immunoglobulin G (IgG) fraction) was purchased from Austral Biologicals (San Ramon, CA, USA). 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 were obtained from Sigma (St Louis, MO, USA). Collagenase (type II; 273 U/mg) was purchased from Worthington Biochemical Corp. (Freehold, NJ, USA). Fetal bovine serum (FBS) was purchased from Life Technologies (Gaithersburg, MD, USA). Basal medium was prepared by dissolving sodium bicarbonate (24 mM) in water containing M-199 powder with Earle's salts, L-glutamine and Hepes buffer (Sigma, St Louis, MO, USA), medium was sterile filtered prior to adding sterile bovine serum albumin (2.6 g/l), transferrin (5 mg/l), reduced glutathione (1 mg/l), soybean trypsin inhibitor (1 mg/l), bovine insulin (10 µg/l), selenium (1 µg/l) and antibiotic solution (0.2%) containing penicillin (50 000 IU/l) and streptomycin (50 mg/l) were added to the medium. [Methyl-³H]thymidine (specific activity 3.03 TBq/mmol) was obtained from Amersham International (Birkørød, 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 days 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 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 days of age) essentially according to Shamay *et al.* (1988). Within 10 min of slaughter, mammary parenchyma was excised aseptically from the outer margin of the 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 tissue, for 10 min in a 37°C shaking water bath. Tissue pieces were allowed to precipitate 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 tissue) and insulin (67 µg/g tissue), at a ratio of 10 ml medium per gram tissue, for 5 h in a 37°C shaking water bath. Organoids were isolated by filtering the suspension through 200 µm Nitex (Tetbo Co., Buancliff manoa, NY, USA) and centrifuging the filtrate for 10 min at 300 g. Organoids were suspended in basal medium and allowed to precipitate 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 ($10 \times \text{M}-199$ 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 basal medium was added. Cells were cultured for 5 days at 37°C in air: CO_2 (95:5). Because treatments 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- ^3H]thymidine was determined essentially according to Shamay *et al.* (1988). On day 3 of culture, $50 \mu\text{l}$ basal medium containing 20 mCi/l [methyl- ^3H]thymidine were added to each well. After 24 h incubation, media were aspirated from the wells and collagen gels were transferred to microcentrifuge tubes. Gels were dissolved by the addition of $150 \mu\text{l}$ 25% acetic acid and subsequent incubation for 20–30 min at 37°C . Cells were precipitated by centrifugation for 10 min at 500 g . Following aspiration of the supernatant, $750 \mu\text{l}$ 4% perchloric acid (PCA) were added and tubes were centrifuged as above. Subsequent washes were performed similarly with $750 \mu\text{l}$ 80% ethanol, 100% ethanol and 4% PCA. The final supernatant was aspirated and cells were hydrolyzed with $500 \mu\text{l}$ 6% PCA at 80°C for 1 h. The suspension was transferred to scintillation vials, mixed with 5 ml 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 ($\mu\text{g/l}$) of rhIGFBP-3 be slightly more than sixfold greater than that of IGF-I (47:7.6). For experiments utilizing addition of recombinant IGF-I, a concentration of $50 \mu\text{g/l}$ was utilized because this consistently

corresponds with maximal responses in prior reports (Purup *et al.* 1995). Thus cultures containing $50 \mu\text{g/l}$ IGF-I were supplemented with $400 \mu\text{g/l}$ 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 in serum obtained from three prepubertal heifers, were determined by an ELISA (OCTEIA IGF-I; ImmunoDiagnostic Systems Limited, Tyne and Wear, UK) that had been validated previously (Norup & Vestergaard 1996).

Ligand blotting

Concentrations of IGFBPs in mammary extracts were evaluated by Western ligand blotting (Hossenlopp *et al.* 1986). Samples ($200 \mu\text{g}$ protein; $17\text{--}31 \mu\text{l}$ 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.

[^{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 & Wilkins (1984). [^{125}I]IGF-I ($3.9 \times 10^6 \text{ c.p.m.}$; specific activity $77 \mu\text{Ci}/\mu\text{g}$) was incubated in 0.1 M Dulbecco's phosphate-buffered saline, pH 7.5 ($400 \mu\text{l}$ total) (Life Technologies, Grand Island, NY, USA) 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, USA) followed by SDS-PAGE. Following a 2-h preincubation period, samples ($0.08\text{--}6.25 \text{ mg}$ protein/ l) were treated with $4 \mu\text{l}$ 25 mM DSS in DMSO (final concentration 0.25 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 0.02% 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 percentage 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). Significance of effects of factors added to media (in comparison with basal medium) was tested by analysis of variance followed by Dunnett's multiple comparison test. Comparisons with $P < 0.05$ were considered to be significantly different. Values are presented as least squares means (LS means) $\pm$ S.E.M. 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. Organoids cultured with heifer serum consistently displayed a stellate appearance with broad outgrowths. In contrast, organoids cultured with mammary extracts, rhIGF-I, or basal medium alone showed distinct needle-like projections. Figure 1A illustrates the dose-dependent effects on DNA synthesis of rhIGF-I in concentrations ranging from 3.125 to 100 µg/l during the final 24 h of a 4-day treatment period ($P < 0.05$ at all concentrations compared with basal medium (0)). Similar to previous experiments, maximal stimulation of DNA synthesis was provided by approximately 25 µg/l IGF-I. Addition of 10% FBS stimulated DNA synthesis by 30% ($169\,979 \pm 24\,600$ d.p.m., $P < 0.05$). The response to basal medium and 10% FBS treatments did not differ according to position within the experiment ($P > 0.05$), 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 ($P < 0.05$) (Fig. 1B), with a dose-dependent response apparent over a range from 2 to 10% serum. As serum IGF-I concentration was 107 µg/l, the increased DNA synthesis was apparent at an IGF-I concentration of 4.3 µg/l in the medium (4% serum).

Figure 1 Effect of various concentrations of (A) rhIGF-I (µg/l), (B) percent prepubertal heifer serum, or (C) percent mammary extract in culture medium on DNA synthesis (d.p.m.) in primary cultures of bovine mammary epithelial organoids. In A, B and C, the zero (0) level represents basal medium. Values are LS means $\pm$ S.E.M. for cultures performed in triplicate.

Mammary extracts, on the other hand, stimulated cell proliferation much more potently than serum or IGF-I alone, yielding a mitogenic response more than twice that induced by the highest IGF-I concentration tested (Fig. 1C). The dose-dependent response was different from basal medium at all levels ($P < 0.05$). 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 µg/l; thus, maximal cell proliferation

was achieved at an IGF-I concentration of less than 3 µg/l in the medium. Of the IGFBP detected in mammary extracts, IGFBP-3 comprised 75% of the total, with lesser amounts of IGFBP-2 (7%), a 28-kDa species (9%) and a 24-kDa 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 µg/l 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 approximately half-maximal responses. Addition of a low concentration of rhIGFBP-3 (40 µg/l) inhibited DNA synthesis in basal medium by 26% ($P<0.05$), while addition of a higher concentration of rhIGFBP-3 (800 µg/l) reduced DNA synthesis in basal medium by 21% ($P>0.05$) (Fig. 2A). Addition of monoclonal (mAb) or polyclonal (pAb) IGF-I antibodies to cultures in basal medium did not show a consistent effect on DNA synthesis (Fig. 2B,C). Addition of a high concentration (2000 µg/l) of a non-immune IgG fraction inhibited DNA synthesis in response to basal medium ($P<0.05$) whereas a low concentration (12.5 µg/l) had no effect ($P>0.05$) (Fig. 2D).

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 µg/l. Similarly, antibody concentrations were 62.5, 125, 250, 500, 1000 and 2000 µg/l. When added to medium supplemented with 50 µg/l IGF-I, equimolar and greater concentrations of rhIGFBP-3 reduced DNA synthesis to the level of basal medium ($P<0.05$) (Fig. 3). Monoclonal and polyclonal antibodies negated approximately 25% of the mitogenic activity of IGF-I when added at equimolar and greater concentrations ($P>0.05$).

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 (Fig. 4A) in medium with 5% serum. Corresponding concentrations of rhIGFBP-3 in media were 20, 40, 80 and 160 µg/l. Antibody concentrations were 50, 100, 200 and 400 µg/l. Addition of rhIGFBP-3 elicited a dose-related complete inhibition of the mitogenic activity at half the molar concentration of IGF-I and above ($P<0.05$). Addition of IGF-I antibodies or a non-immune IgG fraction did not significantly inhibit the mitogenic activity of serum ($P>0.05$).

Similarly, antibodies and rhIGFBP-3 were added with 5% mammary extracts at similar calculated antibody or

Figure 2 Effect of (A) rhIGFBP-3, (B) monoclonal (+mAb) and (C) polyclonal (+pAb) IGF-I antibodies, and (D) a non-immune globulin (+IgG) on DNA synthesis in basal medium (0). Concentrations of added factors shown represent the lowest and highest amounts used in cultures. Values are LS means $\pm$ S.E.M. for cultures performed in triplicate.

Figure 3 Inhibition of DNA synthesis (d.p.m.) in media containing rhIGF-I alone (50 µg/l) 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 ratios. The horizontal dotted line represents DNA synthesis in basal medium without IGF-I addition. Values are LS means ± S.E.M. for cultures performed in triplicate.

rhIGFBP-3:IGF-I molar ratios as those given above (Fig. 4B). Corresponding concentrations of rhIGFBP-3 in media were 5, 10, 20 and 40 µg/l. Antibody concentrations were 12.5, 25, 50 and 100 µg/l. Addition of rhIGFBP-3 inhibited cell proliferation at all levels ($P < 0.05$). 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 (82% inhibition) times the molar concentration of IGF-I. Addition of IGF-I antibodies inhibited up to one half of the mitogenic activity in mammary extracts.

[¹²⁵I]IGF-I binding studies

The relative binding activity of rhIGFBP-3 and the IGF-I antibody (pAb) for [¹²⁵I]IGF-I was determined following a 2-h incubation, corresponding to time of treatment media addition to cell cultures. Complexes of rhIGFBP-3 and [¹²⁵I]IGF-I or antibody and [¹²⁵I]IGF-I were covalently bound using the cross-linking agent disuccinimidyl suberate and separated from free [¹²⁵I]IGF-I by SDS-PAGE. Samples containing rhIGFBP-3 and [¹²⁵I]IGF-I exhibited one band corresponding to the protein complex that was not evident in a sample containing only [¹²⁵I]IGF-I, and one lower molecular weight band corresponding to free [¹²⁵I]IGF-I. 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 [¹²⁵I]IGF-I band. At an IGFBP-3:IGF-I molar ratio of 1/8, 1/2, 1, and 2, the amount of bound [¹²⁵I]IGF-I was, respectively, 4.4, 18.0,

Figure 4 Inhibition of DNA synthesis (d.p.m.) in media containing (A) 5% heifer serum or (B) 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 ratios. The horizontal dotted line represents DNA synthesis in basal medium without 5% extracts added. Values are LS means ± S.E.M. for cultures performed in triplicate.

37.9, and 70.4% of the total [¹²⁵I]IGF-I available. Samples containing the IGF-I antibody and [¹²⁵I]IGF-I did not show any bands corresponding to complexes of the antibody and [¹²⁵I]IGF-I; only free [¹²⁵I]IGF-I was detected. However, the presence of low amounts of an [¹²⁵I]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 the presence of rhIGFBP-3 or the IGF-I antibody. These bands probably resulted from nonspecific binding of [¹²⁵I]IGF-I, possibly with bovine serum albumin in the [¹²⁵I]IGF-I stock buffer. Regardless, the bands represented less than 2% of total counts in the incubation mixtures.

Discussion

Development of the mammary gland is probably influenced by multiple growth factors and their synergistic or antagonistic interactions (Oka *et al.* 1991, Forsyth *et al.* 1998). Many of these growth factors originate in the

mammary gland and may act in an autocrine, juxtacrine or paracrine manner. Such factors also derive from other tissues and may 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 transforming growth factor- α (Zurfluh *et al.* 1990, Koff & 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 the insulin concentrations in individual mammary extracts composing the pooled stock in this experiment 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 IGF-I antibodies were considerably less potent inhibitors of IGF-I bioactivity. Binding studies 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 (M Weber, S Purup & R M Akers, unpublished observations), 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, which 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 the 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, our recent data (Weber *et al.* 1998) demonstrate precocious alveolar bud formation in mammary glands of mouse mammary tumor virus (MMTV)-IGF-I transgenic mice, providing 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 μ g/l) was approximately one third of that in serum (107 μ g/l), 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 do provide a considerable portion of the mitogenic activity available in serum and mammary tissue extract. 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.

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; furthermore, mRNA and protein for IGFBP-3 are more abundant than those for IGFBP-1 or IGFBP-2 in prepubertal bovine mammary tissue (Weber *et al.* 1996). The major difference in IGFBP profile between mammary extracts and serum of prepubertal heifers fed at a moderate feeding level is a two times higher relative abundance of IGFBP-2 in serum (22%) compared with mammary extracts (10%) (M Vestergaard, S Purup & K Sejrsen, unpublished observations). 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.* 1999). 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 & Clemmons 1995). 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 may also be attributed in part to relative concentrations of different IGFBPs. In our study, 10% heifer serum stimulated DNA synthesis to a greater degree than 10% FBS. Analysis of IGFBPs 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 the 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 & 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 the ultimate selection of one exhibiting high activity (S 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.

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. The morphological appearance of growth observed during the culture period resembled the stellate and needle-like appearance of outgrowths described earlier (Shamay & 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 probably 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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