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Mitogenic Responsiveness of Caprine Mammary Epithelial Cells to Endocrine and Cytokine Factors

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Mammary cell mitogenic responsiveness was evaluated with an established nontransformed caprine mammary epithelial cell line (CMEC). As expected, the cells responded to insulin-like growth factor 1 (IGF-1), insulin, and hydrocortisone with an increased number of cells after 3 and 5 d in culture. In combination, insulin and hydrocortisone augmented each other. A proliferation response was also observed for transforming growth factor-α (TGF-α), and epidermal growth factor (EGF), but not for fibroblast growth factor a (FGFa), FGFb, IGF-2, bovine somatotropin, or prolactin. Comparison of mitogenic potential for these growth factors with cells grown on plastic substratum indicated that hydrocortisone was most potent, followed by TGF-α in inducing a proliferative response measured by ^{3}H-thymidine incorporation assay. Hydrocortisone augmented proliferation by 149% and TGF-α stimulated proliferation by 126% relative to the media control ($p < 0.01$). EGF, which binds to the same receptor as TGF-α in other species, induced a modest 35% increase in proliferation. Comparison of culture conditions with plastic, fibronectin, and type I collagen suggests that extracellular matrix/stroma influences the magnitude and effective concentration for cytokine-mediated growth response. Studies on responsiveness to ovarian steroids estradiol 17-β (E2) and progesterone (P4) showed a modest proliferation response to E2 only in combination with triiodo-L-thyronine (T3), and no response to P4 or T3 either alone or in combination when grown on plastic.

Key Words: Proliferation; mammary gland; cytokines; hormones; cell growth.

Introduction

In vivo studies over the last 75 years have provided a concise description of the nutritional and hormonal factors involved in mammary gland development and function. However, in vitro experiments with ruminant mammary epithelial cells cultured with known mammogenic endocrine factors, such as estrogen, progesterone, glucocorticoids, prolactin, somatotropin, and placental lactogen suggest that these factors have little to no direct effect on epithelial cell proliferation *(1–3)*. The data imply an indirect role of hormone action mediated through stimulation of or interaction with "growth factors." Many of the growth factors are cytokines, produced and acting locally to elicit a proliferation response by autocrine or paracrine action. Recent studies have focused on the influence of growth factors and their collaboration in hormonal regulation of mammary development and lactation *(4,5)*. To date, at least 50 bioactive substances consisting of proteins, peptides, and steroids have been found to be present in colostrum and normal milk (reviewed in *6*). Although many of the growth factors found in mammary secretions are passed to the suckling neonate to provide developmental support, many also act as mediators of mammary gland development and function. These include: insulin-like growth factors (IGF-1 and IGF-2), epidermal growth factor (EGF), mesenchyme-derived growth factor (MDGF), platelet-derived growth factor (PDGF), fibroblast growth factors (FGFa and FGFb), mammary-derived growth inhibitor (MDGI) and transforming growth factor α and β (TGF-α and β). Many of these factors function as cytokines with either direct action on the parenchyma or indirect modulation of stromal cells, which subsequently influence epithelial function. Therefore, understanding mammary epithelium modulation is important not only for disease mechanisms, but also for a more thorough understanding of glandular physiology.

In vitro studies with established cell lines and primary cultures of bovine mammary epithelial cells have produced conflicting data regarding responsiveness to endocrine and cytokine mitogens. The bovine mammary alveolar cell-large T-antigen (MAC-T) cell line has been shown to have a proliferative response to IGF-1 and insulin *(7,8)*. It is nonresponsive to pituitary and ovarian hormones as well as EGF. In the same experiment, bovine primary epithelial cultures were also nonresponsive to EGF, but the murine NmuMG mammary cell line was. Others have shown proliferation response to EGF/TGF-α by bovine primary cul-

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Table 1
Growth Factors Supports CMEC Proliferation/Survival in the Presence of Serum

	Viable cell number, 10^4		
Treatment	Day 1	Day 3	Day 5
Control	3.26 ± 0.41^a	4.48 ± 1.12^a	3.61 ± 0.70^a
Insulin and Hydrocortisone	4.32 ± 0.74^b	11.41 ± 1.15^b	21.18 ± 1.52^b
Insulin (10 μg/mL)	$3.54 \pm 0.61^{a,b}$	7.95 ± 1.19^c	12.12 ± 0.97^c
Hydrocortisone (5 μg/mL)	$3.89 \pm 0.57^{a,b}$	9.22 ± 1.16^b	12.87 ± 1.19^c
IGF-1 (100 ng/mL)	$3.81 \pm 0.74^{a,b}$	7.17 ± 0.99^c	10.32 ± 0.70^c

^{a,b,c}Values within the same columns with different superscripts are significantly different at $P < 0.05$.

Control media consisted of DME/F-12 culture media without insulin and hydrocortisone with 2% FBS. Cells were cultured for 3 d in control media with 5% FBS prior to being plated at 5×10^4 cells/4 cm^2/well^{-1} at day 0.

Cell viability for treatments and control were >98% on all days tested.

Values represent the mean ± SD of 4 independent experiments.

ture epithelial cells *(9,10)*. The more recently established clonally derived bovine cell lines BME-UV1 and BME-UV2 are distinctive in their mitogenic responsiveness *(11)*. BME-UV1 cells respond to IGF-1 and EGF where IGF-1 was able to augment proliferation greater than EGF. BME-UV2 cells showed no response to either IGF-1 or EGF. Both ruminant cell lines (MAC-T and BME-UV) depend on the stable integration of SV40 large T antigen for culture establishment. The random insertion of the expression plasmid may contribute to phenotypic variability, and since SV40 large T-antigen is known to modulate pRB and p53, the cell cycle and apoptotic pathways may be affected. As such, the role of EGF/TGF-α remains unclear in ruminant mammary gland biology, although they are well-known mitogens for rodent and human mammary epithelial cells.

We have described the establishment and characterization of a spontaneously immortalized caprine mammary epithelial cell line (CMEC-UCONN) *(12)*. These cells show functional differentiation when grown on plastic substratum with the expression of lactation-specific proteins preferentially in cells that form dome-like structures and morphologic differentiation with the formation of duct-like and acini-like structures when grown within a collagen matrix. As a further characterization of this cell line and to elucidate the mitogenic potential of various known in vivo endocrine and cytokine factors, we examined proliferation responses by direct cell counts or by ^{3}H-thymidine incorporation assay.

Results

CMECs Respond to Insulin, IGF-1, and Hydrocortisone with an Increase in the Number of Cells

Proliferation responses were assayed by direct viable cell count (Table 1). From previous experiments, we observed that cells continued to proliferate for 3 d after insulin and hydrocortisone were removed. Therefore, to maximize the sensitivity of the proliferative response assay, cell were cultured in growth media without insulin and hydrocortisone for 3 d prior to the experiments. Optimization of the proliferation assays included cell number, length of treatments, and basal serum concentration allowing for no growth without loss of cell viability (not shown). The greatest difference in treatments was observed when cells were cultured with a combination of insulin and hydrocortisone. After 5 d in culture, the combination of the two resulted in a 70% increase in the number of cells compared to insulin or hydrocortisone alone, a 100% increase compared to IGF-1 alone, and a 480% increase compared to the control media. Cell viability assays by trypan blue exclusion for all treatments and negative control were >98% for all days tested. This demonstrates that CMEC proliferation and culture adaptation are dependent on growth factor stimulation. Furthermore, insulin and hydrocortisone had an additive effect on increasing the cell number, corroborating independent stimulatory pathways for each.

TGF-α and Hydrocortisone Are Strongly Mitogenic for CMECs

Comparative assessment of mitogenic activity of various known or potential mammogenic factors on CMEC proliferation as measured by ^{3}H-thymidine incorporation indicated that TGF-α and hydrocortisone had a greater effect on proliferation than insulin, IGF-1, or EGF (Fig. 1). Both insulin and IGF-1 were modest stimulators of proliferation with a mean percent change from control of 41 (SD +/– 14.0) and 42.7 (SD +/– 11.8), respectively, at maximum responsiveness. EGF was slightly less mitogenic with a change of 34.8% (SD +/– 5.9). TGF-α caused a greater than fourfold increase in proliferation compared to EGF with a mean change from control of 125.7% (SD +/– 29.7). Hydrocortisone was a potent mitogen, inducing an increase of 149.5% (SD +/– 24.6) from the control and with insulin was additive, with a response of 196.7% (SD +/– 28.5), compared to a calculated/anticipated combined response of 190.5%. All proliferation responses were dose-dependent

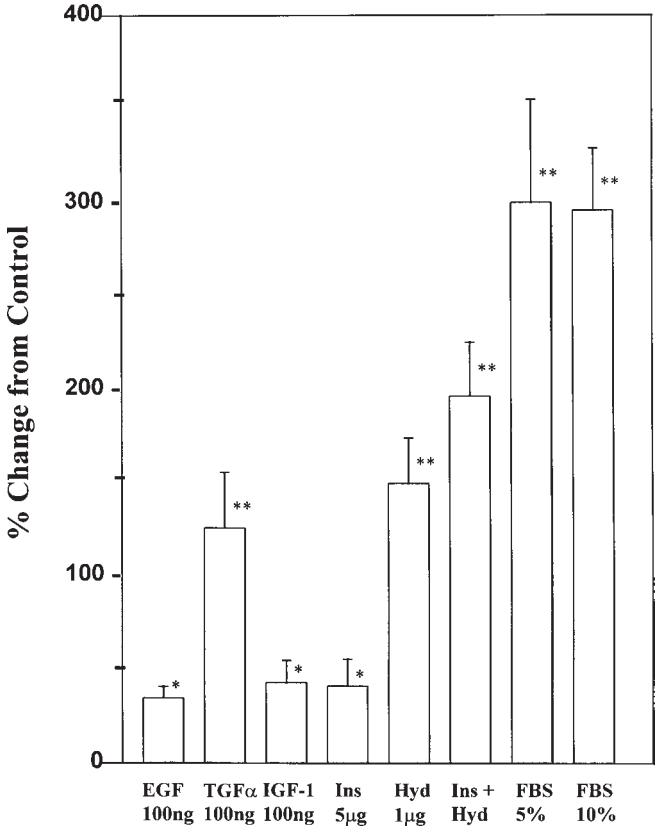

Fig. 1. Comparative proliferation response to endocrine and cytokine factors of caprine mammary epithelial cells grown on plastic substratum in DME/F-12 supplemented with 0.5% FBS determined by ^{3}H-thymidine incorporation assay. Data represent peak/plateau response. All responses were dose-dependent and linear. Concentrations are per milliliter of culture. Ins, insulin; Hyd, hydrocortisone. Data represent mean +/– SD of a minimum 4 independent experiments. *$p < 0.05$, **$p < 0.01$ compared to media control.

and linear for treatment concentrations tested (data not shown). Maximum proliferation responses at peak/plateau are described relative to the response for cultures containing 5% fetal bovine serum (FBS), which usually resulted in a mean 300.4% (SD +/– 54.7) change from control and ranged from 8000 to 20,000 counts per minute (cpm)/well. Culture in growth media with 5% FBS was not statistically significant from cells cultured with 10% FBS for these experiments. Various parameters were evaluated to optimize the proliferation assay, including cell number, length of incubations/treatments, length of ^{3}H-thymidine incorporation, and culture media supplements. Although we were able to observe cell growth in serum-free media (0.1% bovine serum albumin [BSA]) with the various mitogens (not shown), more consistent responses and responses of greater magnitude occurred with background media supplemented with 0.5% FBS. Experiments with bovine prolactin (bPRL), bovine somatotropin (bST), IGF-2, FGFa, and FGFb showed no significant proliferative response.

Culture with Fibronectin or Type I Bovine Tail Collagen Augments TGF-α and IGF-1 Proliferation Response Relative to Culture on Plastic Substratum Alone

Although supra-physiologic concentrations of insulin were able to support proliferation similar to hydrocortisone in the presence of 2% FBS after 5 d in culture (Table 1), insulin and IGF-1 were only moderately mitogenic by ^{3}H-thymidine incorporation assay with 0.5% FBS. We evaluated the influence of extracellular matrix/stromal proteins on proliferation. To emphasize the effect of matrix on cytokine responses, the data are presented as relative to the positive control (5% FBS) when cultured on plastic, on collagen, or with fibronectin since basal level of ^{3}H-thymidine incorporation and amplitude of maximum response were affected by the culture condition (Fig. 2). TGF-α at 100 ng/mL of culture induced a peak change of 76.3% (SD +/– 7.5) of the maximum response compared to a peak 52.5% (SD +/– 6.2) for IGF-1 (10–100 ng/mL) and 42.8 (SD +/– 3.4) for media control. These responses are proportional to those described in Fig. 1. With the addition of fibronectin, there is little change in the amplitude of peak responsiveness to TGF-α, 79% (SD +/– 4.7), ($p > 0.05$) (not significant). However, the effective dose required for mitogenic activity that resulted in a significant proliferative response was reduced from 1 to 0.01 ng/mL relative to the media control with the addition of fibronectin ($p < 0.05$). A more dramatic effect is observed for the IGF-1 dose–response with fibronectin. At 0.01 ng/mL, a significant increase in proliferation is observed with a plateau trend to 100 ng/mL with a 64.7% (SD +/– 7.1) of maximum response compared to media control of 36.7% (SD +/– 8.4) ($p < 0.01$), but still less than the maximum effect of TGF-α. With increasingly complex substratum, the magnitude and sensitivity of the effective cytokine concentration are augmented. Cells cultured on bovine tail collagen showed a peak response to 10 ng/mL of TGF-α with a 90% (SD +/– 12.3) maximum response. IGF-1 at 100 ng/mL showed a peak response of 79.3% (SD +/– 15.1) compared to 19.7% (SD +/– 3.2) of maximum response for media. Proliferation of the cells grown on collagen was augmented by sixfold when compared to plastic substratum for IGF-1.

CMECs Proliferate in Response to Estradiol When Combined with Triiodothyronine

Previous studies have shown responsiveness of ovine mammary explants to estradiol when cultured in phenol red-free media *(13)*, and since both estrogens and progesterone are well known in vivo mammogenic factors, we further characterize the proliferative capacity of CMECs by examining their responsiveness to ovarian steroids (Fig. 3). Cells were cultured in 5% charcoal-stripped FBS (CSFBS) for one passage, and during the experiment, in 0.5% CSFBS in phenol red-free media with or without 3 p*M* T3 as negative control. Responses are described as percent

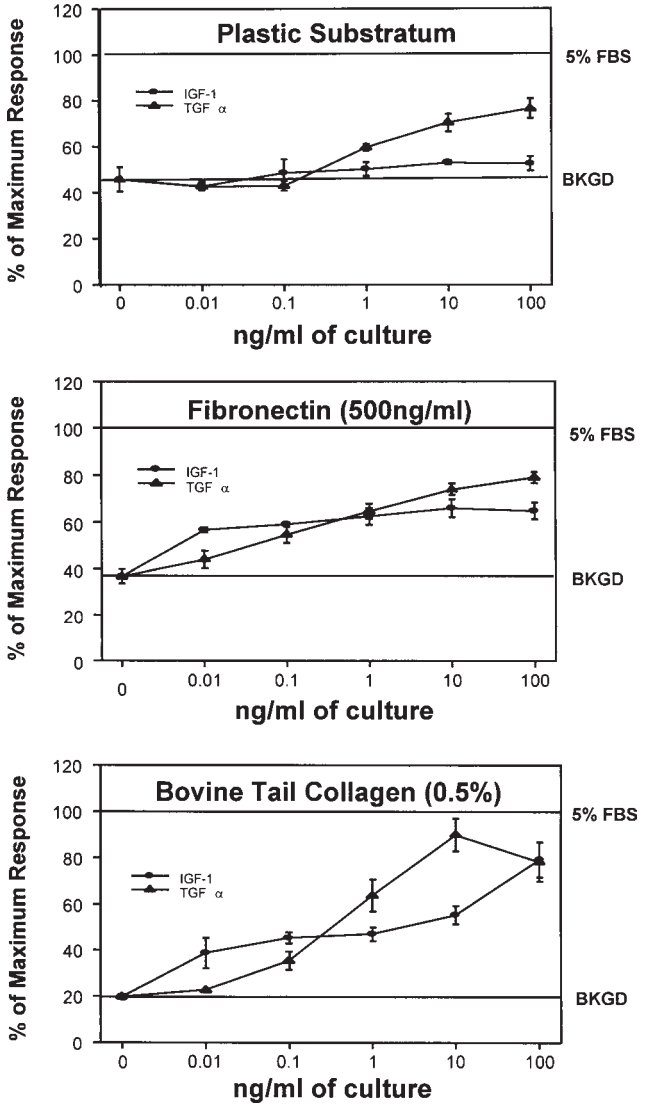

Fig. 2. Comparison of the effects of culture substratum on caprine mammary epithelial cell proliferative response to IGF-1 and TGF-α determined by ^{3}H-thymidine incorporation assay: Percent of maximum response induced by DME/F-12 with 5% FBS and background response induced by DME/F-12 with 0.5% FBS, BKGD. Data represent mean +/– SD of a minimum 4 independent experiments.

change from negative control. Individually, β-estradiol (E2), progesterone (P4), and triiodio-L-thyronine (T3) had no significant effect on proliferation. However, when the cells were cultured with a combination of E2 and T3, an E2 dose-dependent proliferation response was observed with a maximum 49.4% change from control with 1 n*M* of E2 combined with 3 p*M* of T3.

Discussion

We describe the proliferative responsiveness of caprine mammary epithelial cells to endocrine and cytokine factors using an established cell line. The interaction of growth factors with the mammary epithelium is not well understood in dairy animals, in particular, the role of EGF/TGF-α, which has produced conflicting data depending on the experimental model (explant culture, primary or established cell line culture) and the species (bovine, ovine, rodent, or human). For economic reasons, the majority of ruminant mammary gland biology has been dedicated to bovine species. The caprine mammary gland is not necessarily representative of ruminants, since important physiologic differences from the bovine have been described. The caprine mammary gland epithelium retains its proliferative capacity during the lactation cycle *(14,15)*, which is distinct from the bovine in which an involution period is required between successive lactations for maximum productivity *(16)*. Early stages of goat mammary gland regression is associated with a decrease in the number of parenchymal cells *(17)* rather than a loss of differentiated function as in the bovine *(18)*. As such, the mitogenic responsiveness of the caprine mammary gland may differ from that of the bovine regarding the potency of response to a particular endocrine or cytokine factor, but also regarding which factors modulate a proliferative or differentiation response.

The CMEC used in this study has been maintained in insulin (5 μg/mL) and hydrocortisone (1 μg/mL) with 5% FBS for over 80 passages. The supplemented media formula was chosen based on the metabolic and growth-promoting activity of supra-physiologic concentrations of insulin. Insulin, acting through its own receptor *(1)* and with 100-fold less affinity, acting through the IGF-1 receptor *(7)*, is a well-known mediator of mammary epithelial cell proliferation and a cell-survival factor *(19)*. IGF-1 modulation of proliferation functions in part as a progression factor allowing for advancement through the G_0/G_1 stage of the cell cycle *(20)*. Similarly, hydrocortisone has been shown to stimulate proliferation of mammary epithelial cells *(21)*.

In this study, CMEC responsiveness to insulin and IGF-1 was demonstrated in the presence of 2% FBS. A significant increase in cell number was observed after 3 and 5 d in culture, which was similar to the effect of hydrocortisone (Table 1). However, mitogenic responsiveness in low-serum (0.5%) or serum-free (not shown) media suggested that the effects of insulin and IGF-1 were, at best, modest compared to hydrocortisone and TGF-α, which were strongly mitogenic (Fig. 1). In part, these observations are in contradiction to the potent proliferative response to IGFs and insulin observed with MAC-T cells grown on plastic substratum in low serum (1–1.5% FBS) or serum free media *(7,8)*. Other studies using ovine primary dispersion mammary cell cultures described a potent mitogenic response to insulin and IGF-1. In these experiments, while not comparing substratum, the responses were assayed with fibronectin and collagen *(1)*. In our studies, despite using different preparations of insulin (bovine or human and Sigma or Intergen), different sources of rh IGF-1 (Promega, Madison, WI or R&D Systems, Minneapolis, MN) and various

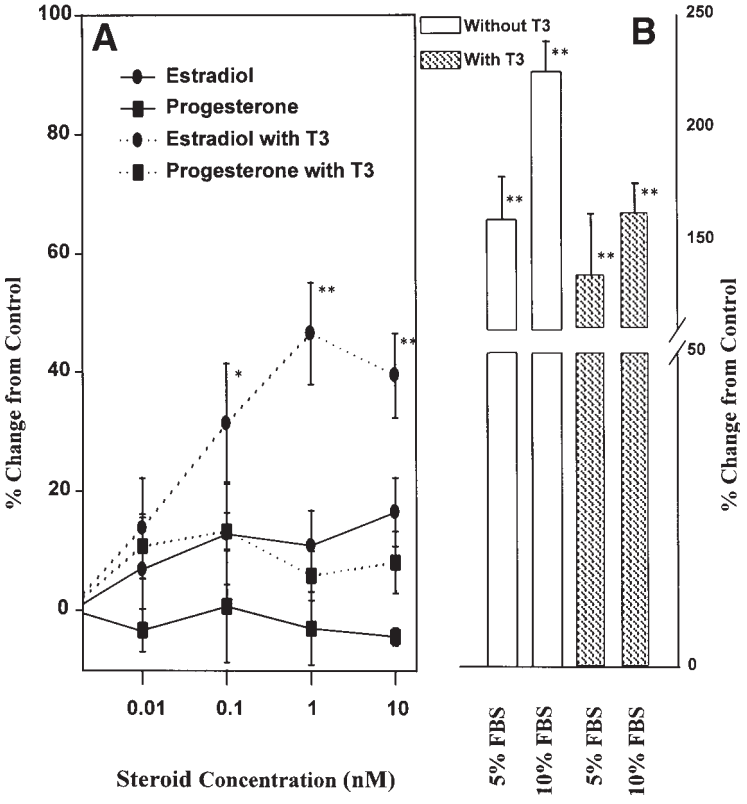

Fig. 3. Proliferative response of caprine mammary epithelial cells cultured in DME/F-12 with 1% charcoal stripped FBS with or without triiodothyronine (T3) as negative media control to **(A)**, ovarian steroids; **(B)** positive controls with or without T3 (2 ng/mL of culture). Data represent mean +/– SD of a minimum three independent experiments. $*p < 0.05$, $**p < 0.01$ compared to media control.

culture conditions, we did not observe a strong proliferative response to these factors for cells grown on plastic substratum. However, cells cultured with fibronectin or on collagen gels showed an augmented responsiveness to IGF-1 compared to cells cultured on plastic substratum (Fig. 2). These responses were compared with TGF-α using a cell surface receptor-mediated pathway rather than the cytosolic steroid receptor transcription factor-mediated pathway (hydrocortisone) as a more physiologically relevant comparison for substratum influence. The mitogenic activity of TGF-α was augmented on more complex substratum, but not to the same degree as that of IGF-1. These data suggest that the responsiveness to insulin and IGF-1 is influenced by serum factors that may include soluble basement membrane components (i.e., fibronectin) and the substratum (i.e., collagen). Furthermore, these data suggest that CMECs represent a more physiologically relevant in vitro model than similar cell lines immortalized with SV40-LTA, a known dis-regulator of cell-cycle control pathways, which may affect the normal ability of IGFs to act as progression or mitogenic factors.

We have also been able to corroborate with this in vitro established cell line model the mitogenic activity of TGF-α observed with primary ovine cell cultures *(22)* and its greater mitogenic potency compared to EGF described with bovine mammary cells *(9,10)*. TGF-α consistently induced a strong mitogenic response, second only to hydrocortisone, and up to fourfold higher than the response for EGF with cells grown on plastic substratum (Fig. 2). Increasing evidence from in vivo and in vitro studies suggests TGF-α acting through the EGF receptor *(23)* plays a role in ruminant mammary gland development and lactation *(2,22,24)*. Although an EGF homolog has not been described for ruminants, the EGF receptor has been described in the bovine species *(25)*. The greater mitogenic activity of TGF-α relative to EGF may be explained by the differential processing of these ligands with the EGF receptor or the degree of phylogenetic conservation of these cytokines. On interaction with the EGF receptor, EGF is directly routed to a degradation pathway, whereas TGF-α interaction with the EGF receptor allows receptor recycling prior to degradation *(26)*. This suggests signal amplification may be modulated at the plasma membrane level, which would be particularly important for secreting/lactating cells that have a high degree of membrane turnover. It has also been suggested that the difference in cellular responses to the two ligands may be related to different affinities to the receptor or activation of other secondary mediators by the variation in the binding domains. Characterization of TGF-α has shown a high degree of conservation between species. The bovine sequence differs from that of the human by two amino acids and from the rat sequence by four *(9)*. Con-

versely, EGF appears not to be well conserved, since the human sequence differs from murine EGF by 16 amino acids *(27)*. The low conservation of EGF implies a similar divergence in the ruminant species, either with an as yet unidentified homolog or because TGF-α functions in ruminants as EGF does in other species.

Ovarian steroids are well known in vivo mammogenic endocrine factors supporting glandular development during the periparturient period. Studies using tissue explants from hormonally primed prepubertal heifer calves suggest that the proliferation response of mammary epithelial cells to ovarian steroids may be different in ruminants from than of rodents *(28)*. Murine studies have demonstrated the requirement for the stromal fibroblast–epithelial cell interaction for responsiveness to estradiol by epithelial cells *(29)*. Mammary epithelial cells showed no responsiveness to estradiol unless cocultured with mammary fibroblasts. Fibroblasts support the epithelial cell response by production of extracellular matrix/stromal proteins and by estradiol-dependent upregulated expression of progesterone receptors by the epithelial cells. Furthermore, epithelial cell responses depended on metabolically activated fibroblasts in close proximity to the epithelial cells, suggestive of paracrine-mediated cytokine involvement. Rodent studies have also demonstrated the influence of adipocytes and the mammary fat pad in modulation of the epithelial cell response to ovarian steroids. Estrogen-dependent proliferation of mouse mammary epithelial cells has been associated with the preceding proliferation of adipocytes in the surrounding ducts *(30)*. These data suggest an indirect role for ovarian steroid action on mammary gland development. Although similar studies in the ruminant are lacking, evidence suggests that stromal cell proliferation prior to an epithelial cell response is not necessary *(28)*. However, responsiveness to estrogen and progesterone is likely modulated by the stroma, and secondary paracrine- or autocrine-acting cytokines or endocrine factors. Although steroid and thyroid hormones are neither structurally nor biosynthetically related, their receptors share a common structure and mechanism of action. The steroid/thyroid hormone receptor action is dependent on ligand–receptor interaction, which induces an allosteric change allowing the hormone–receptor complex to act as a transcription factor that binds to the promoter region of a target gene *(31)*. Recent studies describe the upregulated expression of estrogen receptor by triiodothyronine *(32)* and the thyroid hormone-enhanced proliferative response to estradiol in breast carcinoma *(33)*. Similarly, we have observed a modest, but significant proliferation response with a combination of T3 and estradiol for cells cultured in 1% charcoal-stripped FBS (Fig. 3). However, this combination did not induce a significant proliferation response for cells cultured in 0.1% bovine serum albumin (BSA) (not shown). For breast carcinoma cell proliferation, the augmented responsiveness was associated with crosstalk between nuclear receptors and their signal transduction pathways, and not with IGF-1 growth stimulation. Others have shown that T3 with estradiol upregulates the production of IGF-1 and IGF binding protein-3 *(34)*, suggesting an indirect mode for induction of proliferation. Estradiol has been shown to synergize the responsiveness to insulin/IGF-1 and EGF in the MCF-7 breast cancer cell line *(35)*. Our data suggest an indirect mechanism for estradiol-T3-induced proliferation, since only in the presence of serum/growth factors were we able to observe a proliferation response.

Using the CMEC as being representative of the goat mammary gland confirms the mitogenic activity of TGF-α and its greater potency compared to EGF. The data also indicate that the growth-promoting activity of IGF-1 in goat nontransformed mammary epithelial cells is dependent on substratum and serum factors. The response to estrogen occurs only with the addition of T3 and requires serum factors, presumably inducing growth through autocrine- or paracrine-acting cytokines. This study suggests that CMECs are a physiologically relevant in vitro model to study mammary gland development.

Materials and Methods

Cells and Culture Conditions

The CMEC established by our laboratory was used in all experiments *(12)*. The cell line was originally derived from biopsy of a lactating (114 d postparturition) Anglo-Nubian *(Capra hircus)* goat mammary gland. The established culture shows no evidence of a transformed phenotype, and maintains anchorage dependence and contact inhibition. Cells were routinely cultured in phenol red-free DME/F-12 media (Sigma Chemical Co., St. Louis, MO) supplemented with 2.2 mg/mL sodium bicarbonate, 5 m*M* sodium acetate, 5 μg/mL holo-transferrin (Sigma), 0.5 m*M* ethanolamine (Fisher Scientific Co., Pittsburgh, PA), 10 μg/mL bovine insulin (Intergen Co., Purchase, NY), 5 μg/mL hydrocortisone (Fisher), 5% FBS, (Rehatuin, Intergen Co.,), 100 U/mL penicillin-G, and 100 μg/mL streptomycin sulfate (culture media). Cultures were incubated at 37°C, with saturated humidity and 5% CO_2. Prior to confluence, media were aseptically siphoned, and cells were detached with 0.05% trypsin/0.04% ethylenediaminetetraacetic acid (EDTA), washed three times with modified Hanks' balanced salt solution (HBSS), and passaged at a threefold dilution.

Proliferation Response to Growth Factors

Responsiveness was assayed by either viable cell count and/or by incorporation of ^{3}H-thymidine. Based on previous findings that insulin and hydrocortisone had a 3-d carryover effect on cell proliferation subsequent to their removal, for cell count experiments, CMECs were subcultured in growth media with 5% FBS omitting insulin and hydrocortisone for 3 d in 75-cm^2 tissue-culture flasks. The cells were then plated at 5×10^4/2 mL/well^{-1} in 12-well flat-bottom tissue-culture plates (Falcon, Becton Dickinson &

Co., Lincoln Park, NJ) with media containing 2% FBS with insulin (10 μg/mL) and/or hydrocortisone (5 μg/mL), or recombinant human insulin-like growth factor-1 (rh-IGF-1) (100 ng/mL), (R&D Systems, Minneapolis, MN). After 1, 3, and 5 d, the media were aspirated, cells detached with trypsin/EDTA, and counted by the trypan blue exclusion method.

For ^{3}H-thymidine incorporation experiments, CMECs were plated at 5×10^3 cells/well/100 μL^{-1} in flat-bottom 96-well tissue-culture plates (Falcon) in media with 5% FBS, without insulin and hydrocortisone. After 24 h, media were aspirated and replaced with maintenance media consisting of 0.1% BSA without insulin and hydrocortisone, and changed daily for 2 d. Test samples were incubated for 72 h with media containing 0.5% FBS without insulin and hydrocortisone. Positive controls consist of 5 or 10% FBS, and background controls were those without test sample in appropriate media. Growth factors evaluated for mitogenic induction included recombinant human-transforming growth factor alpha (rh-TGF-α), epidermal growth factor (rh-EGF), insulin-like growth factor 2 (rh-IGF-2), fibroblast growth factor acidic and basic (rh-FGFa and rh-FGFb) (United States Biochemical Corp., Cleveland, OH), insulin-like growth factor 1 (rh-IGF-1) (R&D Systems) (for all cytokines; 150–0.01 ng/mL of culture), bPRL, bST (USDA Beltsville, MD) (10–0.01 μg/mL of culture), E2, P4 (100–0.01 n*M*), and T3 (2 ng/mL of culture) (Sigma). Culture of cells with extracellular matrix/stromal proteins is similar to those described above. Fibronectin (Sigma) was added at a final concentration of 500 ng/mL of culture prior to plating of the cells. Calf tail type I collagen solution was prepared as previously described *(36)*. Thirty microliters per well of neutral collagen were added, allowed to polymerize at 37°C, and then equilibrated overnight with media. Assay for responsiveness to ovarian steroids was the same as described above, except cells were cultured for one passage and in the experiment, with 1% CSFBS, which was stripped of steroids by a 2–4°C overnight incubation with dextran-coated charcoal (Sigma). Eighteen hours prior to harvesting, cells were pulsed with [methyl-^{3}H] thymidine (925 GBq/mmol, Amersham Pharmacia Biotech, Piscataway, NJ), 0.5 μCi/well. CMECs were detached with trypsin/EDTA or 0.01% collagenase (Sigma), and H_2O was lysed and harvested onto glass fiber filters using a cell harvester (Inotech Biosystems International Inc., Lansing, MI). ^{3}H-thymidine incorporation was assayed with a scintillation counter.

Statistical Analysis

Proliferation response as determined by ^{3}H-thymidine incorporation assay was described as percent change = [(experimental – control)/control] × 100, where all hormone/cytokine/serum treatments (experimental) were compared to media control. Percent of maximum response is calculated based on the positive control of 5% FBS. Significance is determined by analysis of variance (ANOVA) followed by Student's two-tailed *t*-test for unpaired comparison using SigmaStat/SigmaPlot software (Jandel Scientific Software, San Rafael, CA).

Acknowledgments

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