Selective breeding and improved management have had major effects in increasing peak milk yields but relatively little effect on lactation persistency. In ruminants, cell loss appears to be largely responsible for the decline in milk yield. Little is known about the longevity of individual cells, but, in lactating dairy cows, few epithelial cells are in the S phase (DNA synthesis) of the cell cycle.

The IGF and epidermal growth factor families are direct mitogens, stimulating DNA synthesis in cultures of ruminant mammary epithelial cells. Receptors that mediate the effects of these growth factors, the type 1 IGF receptor and the epidermal growth factor receptor, respectively, are present at similar levels in membranes prepared from the mammary glands of nonpregnant and pregnant sheep. Binding capacity falls by parturition and remains low during lactation. These findings suggest that the drive to mammary development in pregnancy comes from control of growth factors, and, in the case of IGF, modulating binding proteins, a control exerted by hormones, which, in general, are not themselves mitogens. A paracrine or autocrine mode of action and, therefore, local growth factor synthesis, are more likely to be important than systemic concentrations of growth factor. Stimulatory growth factors produced locally by the mammary gland include IGF-I, IGF-II, transforming growth factor-α, and amphiregulin. More information is needed on the control of stimulatory and inhibitory growth factors and on how growth factors control the cell cycle. Knowledge of these processes could result in strategies to improve lactation persistency by increasing secretory cell renewal or reducing cell loss during lactation.

**Key words:** growth factors, growth factor receptors, mammary growth

**Abbreviation key:** EGF = epidermal growth factor, IGFBP = IGF-binding protein, $K_d$ = dissociation constant, TGF = transforming growth factor.

For all species, milk yield follows the lactation curve, increasing to peak yield and then declining to weaning or the cessation of milking. The underlying cellular determinants of the lactation curve, however, vary among species (39). For dairy cows, genetic selection has greatly increased two interrelated factors, peak yield and lactation persistency, defined as the change of yield with time in midlactation. Broster and Broster (2) calculated that peak yield of dairy cows accounts for 66 to 80% of the variance in total yield compared with 8 to 12% for persistency. Peak yield is in turn determined by secretory cell number and by secretory activity per cell. Studies of goats by Knight and Wilde (40) show that parenchyma cells increase in number during pregnancy and into early lactation. Between parturition and peak lactation, secretory cells hypertrophy and become more fully differentiated. After peak, cell loss is largely responsible for decline in milk yield, but activity per cell is maintained. These results are in contrast to those for rats in which decline in milk yield is associated with maintained total DNA and a decline in secretory activity per cell (39).

A reduced requirement for dairy herd replacements as the size of dairy herds falls and consumer pressure against intensive rearing of calves for veal are among factors that may put a premium on extending the length of economically viable lactations. A better understanding of how cell number in the mammary gland is controlled may lead to strategies for prolonging lactation, not only by increasing peak yield, which appears to have been the major success of selective breeding and improved management, but also by reducing net loss of mammary cells. The objective of this paper is to review what is known about the drives and limitations to mammary growth, using as far as possible examples drawn from ruminants, and, in particular, to examine the roles of stimulatory growth factors acting via the type 1 IGF receptor and the epidermal growth factor (EGF) receptor.
GROWTH FACTOR RECEPTORS IN THE RUMINANT MAMMARY GLAND

IGF Receptors

Two membrane receptors specifically recognizing IGF are known (35). The type 1 IGF receptor is a heterotetramer with a high degree of homology with the insulin receptor; this receptor consists of two extracellular α subunits, each linked to a β subunit. The α subunits contain the ligand-binding domain, and the β subunits contain the transmembrane domain and the intracellular tyrosine kinase catalytic domain. The type 1 receptor binds IGF-I with a dissociation constant \( K_d \) of about 1 nM, has an approximately 10-fold lower affinity for IGF-II, and has a 100 to 1000-fold lower affinity for insulin. The insulin receptor has high affinity for insulin, some 100-fold lower affinity for IGF-I, and a still lower affinity for IGF-II. The type 1 IGF receptor is the only IGF receptor that has been shown unequivocally to mediate signaling functions. Hybrid IGF and insulin receptors have been recognized and transmit a signal in vitro. Stimulated by IGF-I, they function as type 1 IGF receptors in cell lines, but their physiological relevance is not known (64).

The type 2 IGF receptor is quite different, a large (~300 kDa) monomeric transmembrane glycoprotein that also binds mannose 6-phosphate at a separate but potentially interacting binding site. This receptor binds IGF-II with high affinity (reported \( K_d \) range was 0.017 to 0.7 nM), has a 500-fold lower affinity for IGF-I, and does not bind insulin. Binding of IGF-II to the receptor for IGF-II and mannose 6-phosphate causes internalization and degradation of the growth factor; thus, the receptor function may be mainly a scavenging system removing IGF-II from the extracellular environment (35), but also possibly to fulfill other biological functions, including the mediation of mitogenic effects of IGF-II, as indicated for human breast cancer cell lines (47).

Both type 1 and type 2 IGF receptors have been detected in the normal mammary gland of several species, including sheep (13) and cows (12). The number of type 2 binding sites is always higher, about 3- to 4-fold higher in sheep (13). For dairy cows, Hadsell et al. (25) found no significant change in binding affinity for IGF to mammary microsomes across pregnancy and lactation. Numbers of binding sites for IGF-II did not change, but IGF-I binding declined between d 150 prepartum and term (250 d), rose at parturition, and then declined slowly to d 411 of lactation. For sheep, \( K_d \) decreased (affinity increased) for IGF-I binding to mammary microsomes in lactation, and binding capacity declined (Figure 1). Mammary microsomes prepared from nonpregnant sheep or sheep that were pregnant for 40, 75, or 110 to 120 d bound 1005 ± 113 fmol of IGF-I/mg of membrane protein \( (n = 19) \), but the binding capacity fell to about half by parturition and remained low throughout lactation (80).

EGF Receptor

The EGF receptor is a 170-kDa transmembrane glycoprotein, encoded in man by the c-erb-B1 protooncogene and containing an intracellular domain with tyrosine kinase activity (74). A family of structurally related growth factors—including EGF itself, transforming growth factor (TGF)-α, amphiregulin, and heparin-binding EGF (46)—interacts with this receptor. Analysis of binding often yields two classes of binding sites: high and low affinity. Mitogenic stimulation involves ligand-induced dimerization and activation of the intracellular tyrosine kinase domain (74).

The studies of EGF receptor binding have been carried out in cows, using radiolabeled EGF (70), and sheep, using TGF-α (49). For both species, a single class of high affinity binding sites were found. For adult sheep in all physiological states (nonpregnant, pregnant, and lactating) the apparent dissociation constant was similar \( (K_d = 24.3 ± 2.7 \text{ nM}; n = 23) \). The number of binding sites, however, was lower in

![Figure 1](image-url)
SYMPOSIUM: CONTROL OF MAMMARY CELL GROWTH

Figure 2. Binding of IGF-I-labeled transforming growth factor-α to microsomes prepared from the mammary glands of sheep: effect of physiological state on binding capacity, expressed as femtomoles per milligram of membrane protein. Values are means (±SE); number of sheep is shown within each bar. Means were lower (P < 0.005) during late pregnancy and lactation. Data from Moorby et al. (49).

late pregnant and lactating sheep (14.1 ± 2.4 fmol/mg of protein; n = 10) than in nonpregnant sheep or in sheep at 10 or 15 wk of pregnancy (43.0 ± 5.9 fmol/mg of protein; n = 13; Figure 2).

Figure 3. Effect of IGF-I (○), IGF-II (●), or des-(1-3) IGF-I (▲) on DNA synthesis, measured by incorporation of [3H]thymidine, into mammary epithelial cells grown on collagen and prepared from 17-wk pregnant sheep (n = 3). Values are means, expressed as the percentage of maximum response in each experiment. For clarity, standard error is shown for des-(1-3) IGF only.

ACTIONS OF GROWTH FACTORS ON THE MAMMARY GLAND

Mitogenic Effects of IGF

A number of reports document the stimulatory effects of IGF-I on DNA synthesis or on the increase in cell number in vitro in the ruminant mammary gland. Using tissue slices from prepartum and lactating cows, Baumrucker and Stemberger (1) stimulated DNA synthesis with IGF-I and found by autoradiography that ductal epithelial, secretory alveolar epithelial, and myoepithelial cells all showed incorporation of [3H]thymidine. Increases in DNA synthesis or cell number in response to IGF-I are shown by cultures in collagen gels in serum-free medium of undifferentiated bovine mammary cells (66), by cells from pregnant cows (48), and by ovine mammary cells on collagen gels (78). In striking contrast to mammary cells of virgin mice (30), the addition of EGF is not required to enable ruminant mammary epithelial cells to respond to IGF-I. The IGF-II also shows a dose-dependent, stimulatory effect on mammary epithelial cells in culture (Figure 3). As would be expected from its lower affinity for the type I IGF receptor, IGF-II is less potent than IGF-I, and, in undifferentiated bovine mammary cells, produced a lower maximal effect (51). A natural variant of IGF-I, des-(1-3) IGF-I, lacking the N-terminal tripeptide, is present in bovine colostrum (20) and has reduced affinity for IGF binding proteins (16). In ruminant mammary cells, des-(1-3) IGF-I shows either a similar (51) (Figure 3) or an increased (48) potency, by comparison with intact IGF-I, possibly depending on the type and extent of inhibitory IGF-binding protein (IGFBP) accumulation in the particular culture system. Yamamoto and Murphy (83) have recently shown that des-(1-3) IGF-I can be generated in serum by an acid protease at neutral pH, which possibly represents a regulated step in mammary development.

Digestion methods using collagenase and hyaluronidase are generally used to generate clumps of mammary epithelial cells for inoculation into (30, 48, 51) or onto (78, 79) type 1 collagen gels. The cell types in the resultant cultures are predominantly cuboidal, luminal epithelial cells, and some spindle-shaped cells are thought to be myoepithelial (79). By allowing cells to grow out from sheep mammary explants and subculturing them on plastic in Dulbecco’s modified Eagle’s medium and medium 199 (1:1, vol/vol) containing 10% fetal calf serum, we obtained...
cultures of myoepithelial cells (19). Cell identity was confirmed by immunohistochemistry using antibodies to vimentin, α-smooth muscle actin, and α-actinin. The mitogenic response to growth factors by these cells was tested by inducing DNA synthesis in confluent, serum-depleted cultures (8). Insulin-like growth factor-I stimulated DNA synthesis about 5-fold; the effect was maximal at 100 ng/ml, which was similar to effects of IGF-I on DNA synthesis by predominantly luminal mammary epithelial cells grown on collagen (Figure 4). For mammary development, it is clearly vital that luminal and myoepithelial cells show coordinated development.

Studies of the dose dependency of IGF effects on DNA synthesis, using epithelial cell clumps prepared from sheep that were in different physiological states, suggest that sensitivity to IGF is not markedly different for nonpregnant or pregnant sheep (78). There are, however, big effects on the time course of the response (78). Mammary cells from nonpregnant or early pregnant sheep do not show a significant stimulation of DNA synthesis by IGF-I until d 3 to 4 of culture. Growth curves generated from labeling indices determined by autoradiography showed a lag phase of about 30 h before nuclear labeling. From about 10 wk of pregnancy, the response to IGF-I is more rapid, becoming statistically significant by d 2 in culture.

Other IGF Effects

In vitro studies (51, 66) have shown that IGF has no direct galactopoietic activity and that, unlike insulin, IGF has little activity in supporting milk protein synthesis stimulated by prolactin. However, close arterial infusion via the external pudic artery of IGF-I (55) or IGF-II (53) into the mammary gland of goats increased milk yield. Mammary blood flow was also increased. The effect was quite rapid, becoming significant within 2 to 4 h, and was confined to the infused gland, suggesting a direct action. Stimulation of mammary blood flow by IGF-I has been demonstrated in dry, nonlactating goats (I. R. Fleet, 1995, personal communication), supporting the view that the effect on blood flow is a cause and not a consequence of the increase in milk yield.

Recent work (26, 65) indicated that, distinct from its mitogenic activity, but acting via the type 1 IGF receptor, IGF-I can act as a survival factor, inhibiting apoptosis induced in cultured cells. Although not yet demonstrated for the mammary gland, this aspect of IGF activity is potentially of major importance in maintaining the cell population in the lactating mammary gland and deserves study.

Figure 4. Effect of IGF-I (upper panel) or transforming growth factor-α (lower panel) on DNA synthesis, measured by incorporation of [3H]thymidine, in serum-free confluent cultures of myoepithelial cells derived by explant outgrowth from two 15-wk pregnant sheep (•, •). n = Number of independent experiments.

Sources of the IGF Family

Controversy still exists concerning the relative importance of systemic and local production of IGF in influencing the mammary gland. The liver is the major source of circulating IGF, and both IGF-I (56) and IGF-II (54) are transported across mammary epithelium into milk. Although when compared with liver and also other reproductive tissues such as uterus (71, 72) the expression of IGF in the mam-
Mammary gland is quite low, its physiological significance should not be underestimated. The potential for an autocrine or paracrine mode of action was demonstrated by Romagnolo et al. (60) by cotransfecting MAC-T cells derived from bovine mammary gland with cDNA for an ovine pre pro IGF-I.

Mammary expression of IGF-I has been reported in cows (24) and pigs (42), as well as in rodents (61) and messenger RNA for IGF-II detected in pig mammary gland (42). For pigs, messenger RNA levels of both IGF-I and IGF-II were, moreover, developmentally regulated, declining after d 45 of pregnancy and falling further coincident with the onset of differentiation. The mammary tissue content of IGF, of which both IGF-I and IGF-I1 were, moreover, developmentally regulated, declining after d 45 of pregnancy and falling further coincident with the onset of differentiation. The molar concentration of IGF-I1 in pregnancy, although expression is unaffected by in vivo treatment with either growth hormone or placental lactogen I, and placental lactogen II (15). The sheep mammary gland expresses messenger RNA for IGF-I1 in pregnancy, although expression is unaffected by in vivo treatment with either growth hormone or placental lactogen (38). In lactating ewes, the IGF-I message in liver increases but is reduced in mammary gland to very low levels (38). Such findings indicate that IGFBP may play a role in mammary development and function, but the nature of that role remains to be determined.

**Mitogenic Effects Via the EGF Receptor**

Compared with IGF action on mammary gland, growth factors acting via the EGF receptor have been less studied, especially for ruminants. Zurfluh et al. (84) cultured mammary epithelial cells from 150-d pregnant cows in collagen gels and found dose-dependent effects of human EGF and synthetic bovine TGF-α in the presence of 3% fetal calf serum and IGF-I (76 ng/ml) on number of cells measured by DNA content. Maximum effects were obtained with 5 to 10 ng/ml of each growth factor, but TGF-α increased DNA content more. In serum-free medium, Peri et al. (51) found that EGF (20 ng/ml) stimulated DNA synthesis in collagen gel cultures of mammary gland epithelium from two of three calves and had an additive effect with IGF-I, IGF-II, and des-(1–3) IGF-I.

We have studied the effects of human recombinant EGF and TGF-α on DNA synthesis in sheep mammary epithelial cells that were cultured on collagen gels (49). Although EGF competed effectively with 125I-labeled TGF-α for binding sites on mammary microsomes, it failed to stimulate DNA synthesis significantly. Synthesis of DNA was increased about 2-fold by TGF-α; response was maximal at 10 ng/ml. As with the response to IGF in mammary epithelial cells derived from sheep in different physiological conditions, potentiating IGF action [see (35) for review]. Suggested mechanisms include association with the extracellular matrix or the cell surface to facilitate IGF transfer to receptors.

The IGFBP are present in milk (14), and bovine mammary tissue releases IGFBP (5). Mammary cells derived from pregnant, nonlactating heifers and grown in collagen gels in serum-free medium show secretion of IGFBP-2 and IGFBP-3 that is IGF-I inducible (48). Similarly, mouse mammary epithelial cells release IGFBP-3 and a 29-kDa IGFBP, both showing regulation in vitro by IGF and by three lactogenic hormones, mouse prolactin, placental lactogen I, and placental lactogen II. The IGFBP may play a role in mammary development and function, but the nature of that role remains to be determined.

**IGFBP**

Very little IGF is free in circulation or in extracellular space. Most IGF is bound to high affinity binding proteins, which are thought to act as transport proteins, to prolong half-life, and to modulate biological activity in various ways (35). Six IGFBP have been cloned and sequenced. The mechanisms by which IGFBP may influence IGF bioactivity are the subject of intensive investigation. Initially IGFBP were thought to function purely as inhibitors. More recently, using purified proteins in cell culture, it has been shown that IGFBP-1, -2, -3, and -5 can, under specific experimental conditions, potentiate IGF action [see (35) for review]. Suggested mechanisms include association with the extracellular matrix or the cell surface to facilitate IGF transfer to receptors.

Mitogenic Effects Via the EGF Receptor

Compared with IGF action on mammary gland, growth factors acting via the EGF receptor have been less studied, especially for ruminants. Zurfluh et al. (84) cultured mammary epithelial cells from 150-d pregnant cows in collagen gels and found dose-dependent effects of human EGF and synthetic bovine TGF-α in the presence of 3% fetal calf serum and IGF-I (76 ng/ml) on number of cells measured by DNA content. Maximum effects were obtained with 5 to 10 ng/ml of each growth factor, but TGF-α increased DNA content more. In serum-free medium, Peri et al. (51) found that EGF (20 ng/ml) stimulated DNA synthesis in collagen gel cultures of mammary gland epithelium from two of three calves and had an additive effect with IGF-I, IGF-II, and des-(1–3) IGF-I.

We have studied the effects of human recombinant EGF and TGF-α on DNA synthesis in sheep mammary epithelial cells that were cultured on collagen gels (49). Although EGF competed effectively with 125I-labeled TGF-α for binding sites on mammary microsomes, it failed to stimulate DNA synthesis significantly. Synthesis of DNA was increased about 2-fold by TGF-α; response was maximal at 10 ng/ml. As with the response to IGF in mammary epithelial cells derived from sheep in different physiological
states, the lag phase of the response to TGF-α was much longer when cells were from nonpregnant, early pregnant, or lactating sheep; the dose-response curves were not affected. We have also demonstrated a stimulatory effect of TGF-α on DNA synthesis in confluent cultures of sheep myoepithelial cells (19) (Figure 4).

**Sources of EGF-Like Peptides**

There is no evidence that EGF is expressed in ruminants, but, in cows (84) as in other species (17), messenger RNA for TGF-α has been detected in the mammary gland. We have also very recently (J. A. Taylor, S. Lennard, and I. A. Forsyth, 1995, unpublished results) obtained evidence using reverse transcription-polymerase chain reaction and Western blotting that the sheep mammary gland expresses amphiregulin, another member of the EGF family. The primers for reverse transcription-polymerase chain reaction were based on the coding sequences for human, rat, hamster, and mouse amphiregulin (also known as schwannoma-derived growth factor). The antibody for Western blotting was raised against human recombinant amphiregulin.

Unlike EGF and TGF-α, amphiregulin is a heparin-binding growth factor, and its biological activity is blocked by heparin, which prevents interaction with the EGF receptor (7). We (18) have found that DNA synthesis in mammary epithelial cells from sheep is inhibited by heparin and that this inhibition can be partially or wholly overcome by TGF-α, depending on stage of pregnancy. Similar results have been reported (43) for human mammary epithelial cells and provide supporting evidence that amphiregulin functions as a local autocrine and paracrine regulator in the mammary gland.

Because of its heparin-binding properties, mature amphiregulin may be sequestered on the cell surface or in the extracellular matrix. Amphiregulin mitotic signaling in the human breast cancer cell line, MCF-10A, is dependent on extracellular heparan sulfate glycosaminoglycan (33). Another interesting feature of human amphiregulin that distinguishes it from EGF and TGF-α is the presence in the NH₂ domain of two putative nuclear targeting sequences, Arg-Lys-Lys. Site-directed mutagenesis of the domain in rat amphiregulin abolishes its mitogenic properties (37). Localization of amphiregulin to the nucleus has been shown in immunocytochemical studies (36).

All members of this growth factor family are synthesized as transmembrane glycoproteins; the EGF-like sequence is external to the plasma membrane. The mechanisms involved in the release of the mature peptides are not well understood, but a kallikrein has been detected in the mammary gland of lactating mice (51), and this serine protease may be involved in releasing EGF into mouse milk. Juxtacrine interactions can also occur between cells that have transmembrane precursors and adjacent cells expressing EGF receptors (46).

**Interaction Between IGF-I and TGF-α**

The mitogenic response of bovine (51) and ovine (49) mammary epithelial cells to IGF-I is enhanced by TGF-α. This interaction appears to be additive (49). However, when sheep mammary cells were cultured in the presence of heparin, which blocks amphiregulin action via the EGF receptor, there was evidence of synergism between IGF-I and TGF-α, especially in cells prepared from sheep before midpregnancy (J. A. Taylor and I. A. Forsyth, 1995, unpublished results).

**Other Growth Factors**

A number of other factors, both stimulatory and inhibitory, are produced by or act on mammary cells (17). Their physiological significance is often poorly understood and, in most cases, has not yet been studied in any ruminant. Stimulatory growth factors include platelet-derived growth factor, which we have found to stimulate DNA synthesis in sheep myoepithelial cells (I. A. Forsyth and M. Villa, 1994, unpublished results), and the fibroblast (or heparin-binding) growth factor family (17, 73).

Mammary-derived growth inhibitor is isolated from lactating bovine mammary gland and is a member of a family of proteins binding hydrophobic ligands and including fatty acid-binding proteins. Its role in the mammary gland appears to be as a differentiation factor (41). Members of the TGF-β superfamily are strongly implicated as developmental modulators in mammary development. For mice, implantation of slow-release pellets of TGF-β1 inhibits ductal elongation in virgin females (59) and suppresses casein synthesis in explants from pregnant mice (58), but has little effect during early pregnancy (11). The targeting of TGF-β1 expression to the mouse mammary gland under the influence of the whey acidic protein promoter expressed during pregnancy results in inhibition of lobuloalveolar development and lactation suppression (32). There is increased expression through pregnancy in mice of TGF-β2 and TGF-β3 and reduced expression in lactation of all three isoforms. Expression has also been shown in the bovine mammary gland (45).
GROWTH FACTOR ACTION IN THE CELL CYCLE

As discussed previously, primary cultures of sheep mammary epithelial cells cultured on collagen gels show a long lag phase before initiating DNA synthesis, except when the cells are derived from sheep after about 10 wk of pregnancy. Synthesis of DNA is stimulated, but its time course is not altered by either IGF (78) or TGF-α (49), which has been interpreted by us as an indication that, like fibroblasts, mammary epithelial cells may require sequential growth factor action to progress through the cell cycle, the series of regulated events through which proliferating cells pass [see (50) for review].

Four major phases are recognized in the cell cycle: mitosis (M phase), a presynthetic gap phase (G1), S phase when DNA is replicated, and a second gap phase (G2) in which preparation is made for mitosis. From G1, cells may leave the cell cycle and pass into a nonproliferating state, G0. Nonproliferating cells in G0 may be able to return to the cell cycle and resume growth or may, as terminally differentiated cells, have growth arrested irreversibly. There is still no consensus as to how these considerations apply to the mammary gland. We do not have clear information on the longevity of secretory epithelial cells or the degree of cell turnover in lactation. From electron microscopy it is clear that fully differentiated secretory epithelial cells can divide [(21) and Figure 5], although division may be a rare event. For mice and rats, some 40% of the increase in total DNA in the mammary gland occurs after parturition (39), and mammary growth can be experimentally induced in lactating goats (40), showing the potential for increasing or maintaining the cell population during lactation.

Normal cells generally require more than one growth factor for cell cycle progression. In vitro studies of fibroblasts show that IGF-I acts in late G1, stimulating these cells into the S phase. Platelet-derived growth factor and EGF have somewhat overlapping functions, stimulating fibroblasts to leave G0 and traverse the early part of G1 (50). The mechanisms that underlie these requirements are starting to be understood. Progression through the cell cycle is dependent on activation of protein kinases that are specific for serine and threonine, termed the cyclin-dependent protein kinases (or cdk). Activity of these kinases is regulated by accessory proteins, termed cyclins, of which nine have so far been identified. Different cyclin and cyclin-dependent kinase complexes appear to function in different parts of the cell cycle. In human osteosarcoma cells, IGF-I stimulates cyclin-dependent kinases and cyclins (22). Most critically, IGF-I increases cyclin-D1 messenger RNA and protein expression early in G1 when IGF-I stimulates mitogenesis in these cells. It will be important to determine in similar detail the requirements of mammary epithelial cells to understand the drive to mammary development in pregnancy and the constraints on cell proliferation in lactation.

ENDOCRINE CONTROL OF MAMMARY DEVELOPMENT

Classic studies of rodents determined the hormones that drive postnatal mammary development. Estriadiol, adrenal corticoids, and somatotropin are implicated in allometric mammary growth in peripubertal females, but, during pregnancy, estrogen, corticoids, and progesterone are critical, acting together with prolactin and somatotropin or equivalent activities from the placenta (44). Similarly for goats, mammary growth is not stimulated by exogenous or endogenous steroids in the absence of the pituitary (10) or in the absence of both pituitary and placenta (3). There is still considerable uncertainty and conflicting evidence about how hormones bring about mammary growth.

Evidence from the culture of rodent mammary epithelial cells in collagen gels (30) indicates that progesterone and prolactin could be direct mitogens, although similar results have not been reported for ruminant mammary cells. In contrast, there has been a general failure, using any species, to stimulate DNA synthesis or increase in cell number with either estradiol or somatotropin in isolated normal mammary cells. We still do not know how these clearly very important mamnogenic hormones act, but a number of potential mechanisms have been investigated.

Estrogens

For unbred females, the mammary gland shows cyclic changes in DNA content as steroid hormone concentrations change during estrous cycles (68). For the postpubertal goat or cow with intact pituitary, extensive udder development can be obtained with estrogen alone, although the presence of cystic alveoli and reduced total epithelial surface area is a consistent feature. Combining progesterone with hexoestrol in spayed virgin goats produced more normal alveolar structure (9). Despite striking successes for some individual animals, milk yields from steroid-induced lactations average only about 70% of postpartum yields, which strongly suggests some factor specific to pregnancy. The lack of a direct effect of estradiol is illustrated for ovine mammary epithelial cells grown
on collagen in Figure 6. This failure has led to suggestions that estrogens act indirectly, via either systemic or locally produced mediators. Evidence that endogenous estrogen acts locally and via the estrogen receptor in virgin mice has been obtained by implanting antiestrogens with no estrogenic activity (termed pure antiestrogens) into the mammary glands of 5- or 12-wk-old mice (67). Both ductal elongation and ductal branching were inhibited only in the vicinity of the implants. Ductal branching was reduced for both

Figure 5. a. Micrograph from a fully lactating rat mammary gland showing all three alveolar components, secretory cells, myoepithelial cell (M), and part of an intraepithelial lymphocyte (L). The secretory cells are sharply polarized with basal nucleus (N) and serried ranks of endoplasmic reticulum cisternae underpinning extensive Golgi areas (g) with numerous secretory vesicles containing casein. Small lipid droplets (*) are also present at the cell apex close to the alveolar lumen (A). The basement membrane of the alveolus is indicated by the small arrows. b. Shown is a dividing secretory cell from an alveolus close to that shown in part a. The cell is fully differentiated, as indicated by the numerous vesicles containing casein (large arrowheads) and large lipid droplet (*), but the endoplasmic reticulum is divided into small segments with no particular location (small arrowheads) rather than continuous sheets. The Golgi area is reduced to small aggregates of vesicles (g) that are scattered through the largely nonpolarized cell. The cell is in late anaphase, and the separated chromosomes (C) are about to reform into two daughter nuclei. Scale bar = 1 μm for both a and b; ×7500. (Photograph by courtesy of F.B.P. Wooding.)
immature (5 wk) and mature (12 wk) female mice, suggesting a local role for estrogens in ductal maintenance as well as duct elongation. Autoradiography showed that the labeling index was suppressed in end buds and ducts, but that stromal DNA synthesis was unaffected. Synthesis of messenger RNA for the progesterone receptor is regulated by estradiol via its receptor and was reduced in antiestrogen-implanted mammary glands.

Whether the effects of estrogen are purely local during pregnancy of mice and whether local estrogen effects occur in other species are unknown. Earlier studies by Haslam (29) suggested that estrogen effects are local for immature mice, but that, for adult mice, systemic effects become important. A possibility for local action is that estrogen acts via its receptors in stromal cells (28). In mice, estrogen-induced epithelial cell proliferation in vivo is preceded by labeling in stromal adipocytes (28), but this labeling does not occur in heifers (82). A role for epithelial-stromal interaction, however, is suggested by the failure of isolated epithelial cells to respond to estradiol; mixed cell populations or explants do show modest increases in DNA synthesis (28, 57, 81). Vanderboom and Sheffield (75) have shown that pretreatment of a normal mouse mammary gland cell line with estradiol increased the response and sensitivity of the cells to EGF, which was associated with an increase in high affinity EGF receptors. Preliminary experiments (J. A. Taylor and I. A. Forsyth, 1995, unpublished results) suggest a similar effect in primary cultures of sheep mammary cells. However, numbers of EGF-binding sites do not change significantly in the mammary gland of sheep during pregnancy (Figure 2). Another potential local mediator of estrogen action is TGF-α, because, as shown by transient transfection experiments in estrogen-responsive human breast cancer cells, the 5' flanking region of the TGF-α gene contains an estrogen-responsive element (63).

In immature, hypophysectomized, ovariectomized rats, local implants of estradiol, which are ineffective alone, enhanced IGF-I effects on mammary development and also enhanced the effect of locally administered somatotropin on the messenger RNA content of IGF-I in mammary gland (61).

The Growth Hormone and Prolactin Gene Family

A major problem in understanding the undoubted effect of growth hormone on the growth and function of the mammary gland has been the general failure to detect receptors in mammary tissue by the usual ligand-binding methods. Studies (23) have detected messenger RNA for the growth hormone receptor in several species, including dairy cows, but whether the receptor protein is expressed is unknown. Exogenous prolactin does not stimulate mammary growth in cows, whether given locally (6) or systemically (4), which appears to conflict with results for goats, in which prolactin suppression with bromocriptine prevented udder development in response to estradiol and progesterone (27). Effect of somatotropin given locally by intramammary infusion (6) is limited and effect is greater of bovine placental lactogen administered systemically to heifers primed with steroid (4). Locally administered IGF-I and a much larger dose of EGF were also stimulatory (6). Because the placental lactogen was given systemically, this study does not address its site of action, but does suggest that the growth hormone receptor, or a distinct placental lactogen receptor, was involved. Concentrations of IGF-I, either systemically or in mammary secretions, were not affected.

In hypophysectomized, immature rats, implants of somatotropin, but not prolactin, into the mammary gland stimulate local IGF-I messenger RNA production, and the effects of somatotropin can be mimicked...
by IGF-I or more potently by des-(1-3) IGF-I (62). This result strongly indicates the importance of paracrine or autocrine regulation rather than systemic regulation.

CONCLUSIONS

Factors that control the number of epithelial cells in the mammary gland are clearly complex. The role of growth factors acting via the type 1 IGF receptor and the EGF receptor has been particularly considered, together with their possible modes of interaction with systemic hormones that influence mammary growth. Improved knowledge strongly indicates that growth factors influence the control machinery of the cell cycle, both positively (IGF-I) (22) and negatively (TGF-β) (52). How hormones, which are able to drive mammary development, control the growth factors that may mediate their actions is still largely unknown.

The mammary gland of nonpregnant females apparently is able to show a growth response, as indicated by short-term changes that take place during the estrous cycle and by the presence in the nonpregnant mammary gland of receptor populations that are not markedly different from those found during pregnancy, including type 1 IGF receptors (Figure 1), EGF receptors (Figure 2), and receptors for estrogen and progesterone (28, 69). Mammary growth during pregnancy is exponential, but the highest labeling indices are found in the first half of pregnancy. As with many other biological systems, positive feedback systems probably lead to the sustained mammary development during pregnancy, which is limited ultimately by the size of the mammary fat pad. The size of the fat pad appears to be regulated independently of penetration of the fat pad by parenchyma (34). The proliferative capacity of the functional gland is strictly limited, but not totally eliminated, which may in part be due to reduced receptors but is probably also governed by inhibitory factors and other aspects of the differentiated state (73). A better understanding of control of the cell cycle in the mammary gland may enable alteration of the balance between cell loss and cell gain to improve lactation persistency.

REFERENCES


