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Acidic and Basic Fibroblast Growth Factor mRNA and Protein in Mouse Mammary Glands

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Previous studies suggest that members of the fibroblast growth factor (FGF) family are mitogenic to mammary epithelium. In order to determine expression of acidic and basic FGF (aFGF and bFGF) during mammary development, mice were euthanized as virgins, early pregnant, mid-pregnant, late-pregnant, or during early lactation. Mammary expression of both aFGF and bFGF mRNA increased through pregnancy. Acidic FGF mRNA continued to increase during early lactation, but basic FGF message level decreased drastically during early lactation. Western blots probed with anti-aFGF showed four immunoreactive bands approx 30, 48, 52, and 55-kDa in size. The 30-, 48-, and 55-kDa bands for aFGF were expressed at low levels during virgin and early pregnant stages but were more prominent during the later stages. The 52-kDa band was high during the virgin and early pregnant stages and low in mid-pregnancy through early lactation. Blots probed with anti-bFGF showed two bands approx 30 and 55 kDa in size. Both bands increased through early-pregnancy, but during late-pregnancy there was a decrease in immunoreactive protein levels, which remained low during early lactation. Experiments to determine where FGF mRNAs are produced in the mammary gland suggest that both FGFs may be produced in the stroma, leading to the hypothesis that aFGF and bFGF are stromally produced growth factors and probably act on the epithelial component of the gland in a paracrine fashion.

Key Words: Mammary gland; acidic fibroblast growth factor (aFGF); basic fibroblast growth factor (bFGF).

Introduction

The complex process whereby mammary epithelial cells develop and differentiate into secretory cells capable of synthesizing and secreting milk involves a series of growth and developmental changes that occur over a relatively long period of time. This process is driven by the interaction of many hormonal factors. Although the systemic importance of many hormones such as growth hormone, progesterone, prolactin, and estrogen in the growth and development of the mammary gland has been well documented (Bresciani, 1971; Haslam, 1987), recent studies suggest that other growth factors may also have a role in the regulation of mammary growth and differentiation (Topper and Freeman, 1980; Imagawa et al., 1990; Oka et al., 1991; Imagawa et al., 1994). These growth factors can either be produced locally in the mammary gland and act in a paracrine/autocrine fashion, or they may be produced elsewhere and act in an endocrine manner. As suggested by Oka et al. (1991), there exists the possibility that production of certain growth factors in the mammary gland may be hormone regulated and these growth factors may be mediating the effects of the hormones that regulate their expression.

Fibroblast growth factors (FGFs) are a class of heparin-binding protein mitogens that induce division of a wide variety of cultured cells derived from embryonic mesoderm and the neuroectoderm (Gospodarowicz et al., 1987). The FGFs have been shown to induce DNA synthesis, cell migration, blood vessel growth and dermal wound closure (Montesano et al., 1986). Both acidic and basic fibroblast growth factor (aFGF and bFGF, respectively, also referred to as FGF-1 and FGF-2 or HBGF-1 and HBGF-2, i.e., heparin-binding growth factor 1 and 2, respectively) have been well documented in the literature as being potent angiogenic and wound healing agents, promoting proliferation of all cell types involved in wound healing; (capillary endothelial cells, vascular smooth muscle cells, fibroblasts, chondrocytes, and myoblasts) both in vivo and in vitro (Buntrock et al., 1982a,b; Baird et al., 1985). Like other angiogenic agents of physiological importance, the FGFs are chemotactic for vascular endothelial cells. They are also known to induce expression of plasminogen activators and collagenases, which are proteolytic enzymes that would be expected to mediate tissue remodelling by the endothelial cells in vivo (Thomas and Gimenez-Gallego, 1986; Folkman and Klagsbrun 1987). Since mammary gland development involves angiogenesis, aFGF and bFGF

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might be potentially important in regulating mammary gland development.

The mitogenic effects of FGF, particularly bFGF, on mammary epithelium is well known (Levay-Young et al., 1989). However, the source of FGFs for supporting mammary growth is unclear. FGFs have been identified in human breast tissue in myoepithelial cells (Gomm et al., 1991). Other studies have shown that aFGF and bFGF are expressed in myoepithelial cells and in epithelial cells undergoing differentiation into myoepithelial cells (Barraclough et al., 1990; Fernig et al., 1990). Recently, Coleman-Krnacik and Rosen (1994) reported that aFGF and bFGF mRNA levels decline during pregnancy and drop further during lactation. They also reported that most of the aFGF gene expression was in the luminal eplithelial cells and expression of bFGF was in the mammary stroma.

Results

Expression of aFGF and bFGF at Different Stages of Mammary Gland Development

Northern blot analysis indicated that mammary aFGF mRNA was a single band of 4.8 kb. Quantitation of Northern blots by computer-assisted image analysis indicated that aFGF messenger RNA level increased slightly during early, mid and late pregnancy relative to the nonpregnant controls (Fig. 1). There was a substantial increase in aFGF message level during early lactation (fourfold over control and twofold over late pregnancy).

Basic FGF mRNA was present as two bands of 3.7 and 7.0 kb. Results for both bands were combined in the analysis since they varied similarly. Basic FGF messenger RNA level showed a slight increase in early and mid-pregnancy relative to the controls (virgins). There was a significant increase in bFGF message level during late-pregnancy (sevenfold over control), and a precipitous fall in bFGF mRNA level during early lactation, the level being similar to the control (Fig. 2).

As a control for our Northern analysis, we used a probe against GAPDH mRNA. GAPDH mRNA level was similar in magnitude during the different physiological stages, and varied considerably less than did aFGF or bFGF (Fig. 2). Using this control enables us to ascertain the fact that there is equal loading of total RNA in all the lanes during gel electrophoresis. This ensures that the differences we see in the autoradiograms is specific and not owing to unequal RNA loading per lane.

Expression of aFGF and bFGF Proteins During the Various Stages of Development

In order to determine whether aFGF and bFGF protein levels follow similar or different patterns compared to mRNA levels, we performed Western analysis on total protein samples, probing with primary antibodies against acidic and basic FGF. The polyclonal antibody against

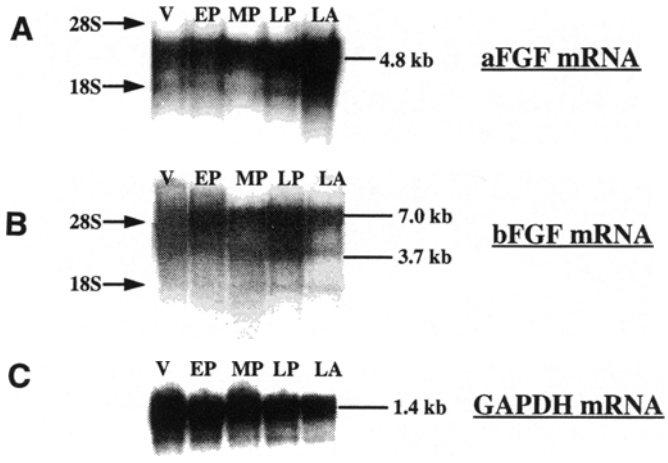

Fig. 1. Autoradiograms of aFGF **(A)**, bFGF **(B)**, and GAPDH mRNA **(C)** during the different physiological stages: virgin (V), early pregnant (EP), mid pregnant (MP), late pregnant (LP), and 5-d lactating (LA) mice. Northern analysis detected one mRNA band of size 4.8 kb for aFGF, two mRNA bands of sizes 3.7 and 7.0 kb for bFGF, and a single mRNA band of size 1.4 kb for GAPDH.

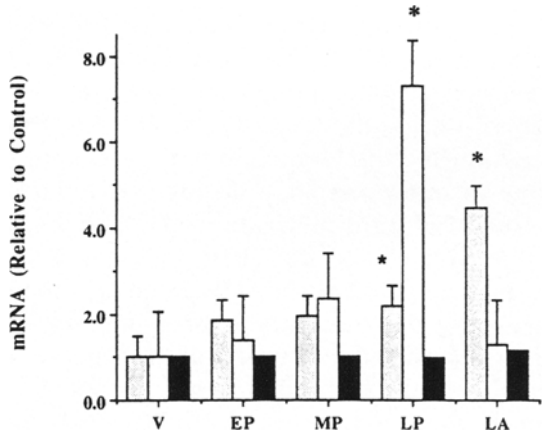

Fig. 2. Expression of mRNA for acidic(▨) and basic (□) fibroblast growth factors (aFGF, bFGF) and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (■) during the different physiological stages: virgin (V), early pregnant (EP), mid pregnant (MP), late pregnant (LP), and 5-d lactating (LA) mice. Autoradiographic bands from hybridizations were quantitated by densitometry using Collagen. For bFGF, the two bands varied similarly, so the results were combined in the statistical analysis. Values normalized so that virgin = 1.0. Each value represents the mean ± SEM (n = 7–8).

aFGF protein detected four immunoreactive bands of sizes approx 30, 48, 52, and 55 kDa (Fig. 3A). The 30-, 48-, and 55-kDa bands followed a similar pattern of expression, being low during virgin and early pregnant stages but quite prominent during mid-pregnancy through early lactation. The 52-kDa band was high during the virgin and early pregnant stages and low in mid-pregnancy through early lactation.

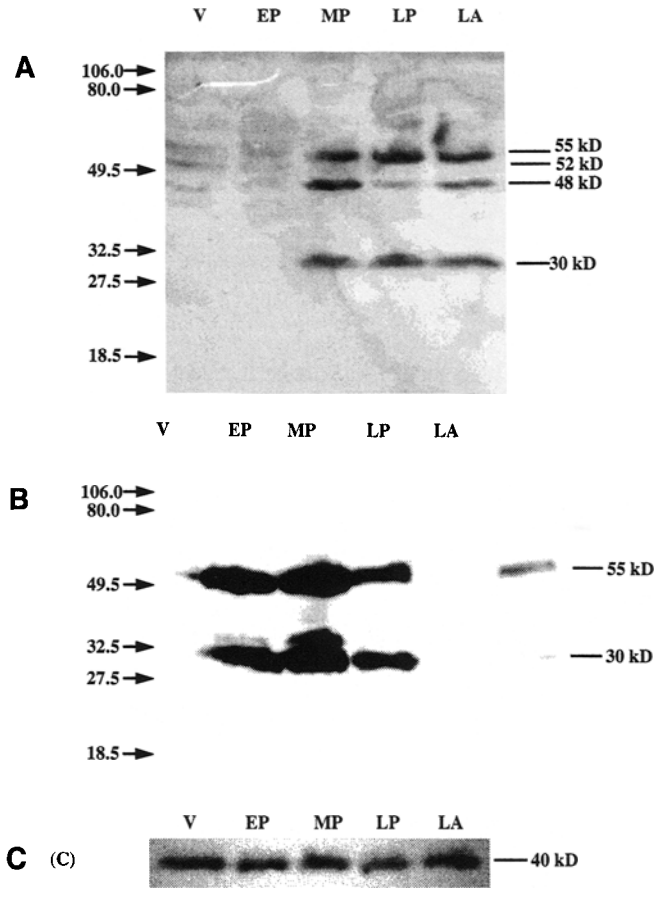

Fig. 3. (A) Western blot of aFGF protein during the different physiological stages (V, virgin; EP, early pregnancy; MP, mid pregnancy; LP, late pregnancy; LA, lactation). Western analysis using anti-aFGF revealed four immunoreactive bands corresponding to sizes approx 30, 48, 52, and 55 kDa. Prestained low range mol-wt markers (BioRad) have been indicated with arrows on the left of the photograph. They represent (from the bottom) 18.5, 2 7.5, 32.5, 49.5, 80.0, and 106.0 kDa, respectively. This blot is representative of four blots showing similar results. **(B)** Western blot of bFGF protein during the different physiological stages (V, virgin; EP, early pregnancy; MP, mid pregnancy; LP, late pregnancy; LA, lactation). Western analysis using anti-bFGF revealed two immunoreactive bands corresponding to sizes approx 30 and 55 kDa. Prestained low range mol-wt markers (Bio-Rad) have been indicated with arrows on the left of the photograph. They represent (from the bottom) 18.5, 27.5, 32.5, 49.5, 80.0, and 106.0 kDa, respectively. This blot is representative of four blots showing similar results. **(C)** Western blot of β-actin during the different physiological stages. Western analysis using anti- β-actin revealed one immunoreactive band corresponding to size approx 40 kDa.

The monoclonal antibody against bFGF protein recognized two bands of approx 30 (the 30-kDa band appears to be composed of two bands) and 55-kDa (Fig. 3B). Both the bands follow a similar pattern, which is different from that of bFGF mRNA. Both proteins increase through early pregnancy, but during late pregnancy there was a significant decrease in protein level. Protein levels remained low during early lactation. This is in contrast to bFGF mRNA, which showed highest levels of expression during late pregnancy and declined during early lactation.

Since we did not detect any bands of the size for native aFGF/bFGF proteins (i.e., 16–18 kDa respectively), to demonstrate that the immunoreactive bands are actually owing to specific interaction of antibodies with either acidic- or basic FGF- containing proteins, we did three different types of control. For the first control, we performed Western analysis, where the primary antibody was excluded from the protocol. Such blots showed no bands, suggesting that the bands we observed in all other blots by incubation with primary antibody against bFGF and aFGF are specific for those antibodies, respectively. In the second type of control, we added both native aFGF and bFGF to our Western gels, immunoblotted the proteins, and then probed the blots with the antibodies against either aFGF or bFGF. In the blots probed with anti-aFGF, only one immunoreactive band for native aFGF of the size of approx 16 kDa was detectable, and no band for native bFGF was detected (for blots probed with anti-bFGF, only one immunoreactive band for bFGF of approx 18 kDa was detected), indicating that the antibodies are capable of recognizing native aFGF/bFGF, and the reason why we don't detect the native proteins in the mammary gland is because they are not present in the mammary gland owing to reasons yet unknown to us. In our third control, we preabsorbed the antibodies for either aFGF or bFGF with native aFGF and bFGF, respectively, and then used the preabsorbed antibodies for our Western analysis. None of the bands observed previously were detectable in these Western blots, again suggesting that the bands that we are detecting in the mammary gland, are indeed specifically owing to aFGF or bFGF containing proteins.

The monoclonal antibody against β-actin recognized one immunoreactive band of approx 40 kDa (Fig 3C). The protein appears fairly uniform in expression across all lanes, indicating that the differences we were detecting in our Western blots for aFGF and bFGF were specific, and not owing to different amounts of protein loaded in each lane.

Expression of aFGF and bFGF mRNA in the Gland-Free Fat-Pad Mice

Acidic and basic FGF mRNA expression in the intact mammary glands of virgin mice and that in the gland-free fat pads of virgin mice are similar. Acidic FGF mRNA expression in the glands of intact mice mated and sacrificed on d 5 of lactation and that in the gland-free mice mated and sacrificed on d 5 of lactation were essentially the same. Similar results were obtained with bFGF mRNA expression when intact mice were compared with the gland-free fat-pad mice. These results support the hypothesis that aFGF and bFGF are produced in the stroma, and that stromal aFGF and bFGF production is developmentally regulated (Fig. 4A,B).

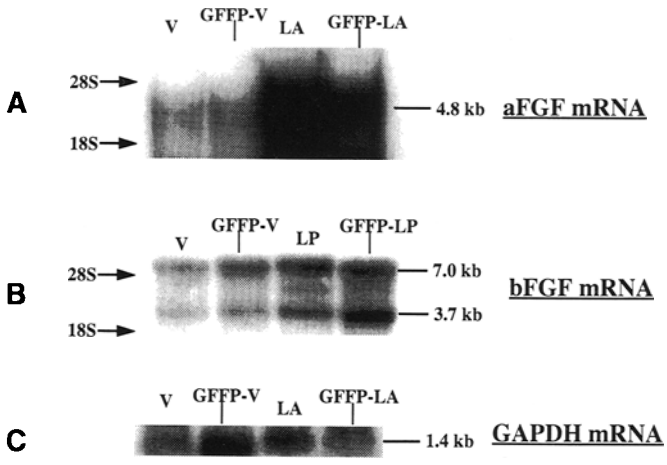

Fig. 4. (A) Northern blot of aFGF mRNA levels in the gland-free fat pad studies (V, virgin; GFFP-V, gland-free fat pad virgins; LA, lactation; GFFP-LA, gland-free fat pad lactating mice). This blot is representative of three blots showing similar results. **(B)** Northern blot of bFGF mRNA levels in the gland-free fat pad studies (V, virgin; GFFP-V, gland-free fat pad virgins; LP, late pregnancy; GFFP-LP, gland-free fat pad late pregnancy mice). This blot is representative of three blots showing similar results. **(C)** Northern blot of GAPDH mRNA for the gland-free fat pad studies (V, virgin; GFFP-V, gland-free fat pad virgins; LA, lactation; GFFP-LA, gland-free fat pad lactating mice).

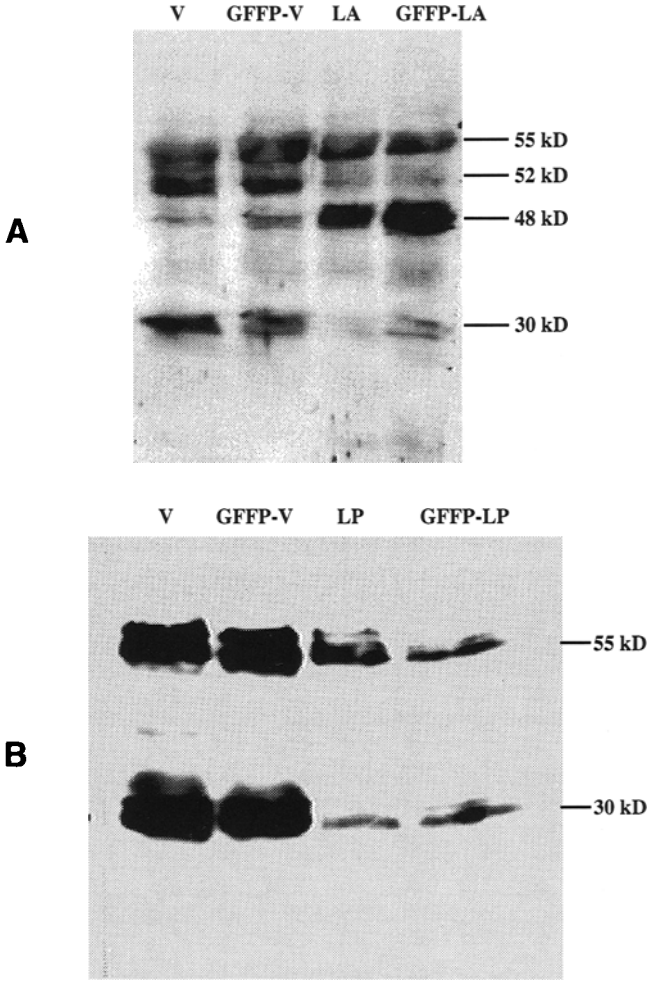

Fig. 5. (A) Western blot of aFGF protein levels in the gland-free fat pad studies (V, virgin; GFFP-V, gland-free fat pad virgins; LA, lactation; GFFP-LA, gland-free fat pad lactating mice). This blot is representative of three blots showing similar results. **(B)** Western blot of bFGF protein levels in the gland-free fat pad studies (V, virgin; GFFP-V, gland-free fat pad virgins; LP, late pregnancy; GFFP-LP, gland-free fat pad late pregnancy mice). This blot is representative of three blots showing similar results.

Expression of aFGF and bFGF Proteins in the Gland-Free Fat Pad Mice

In order to determine whether aFGF and bFGF protein levels in the gland-free fat pad studies were similar to the mRNA patterns observed in the physiological stages, we performed Western analysis on total protein samples prepared from glands free of the epithelial component (i.e., deepithelialized fat pads, also referred to as gland-free fat pads). The polyclonal antibody against aFGF protein detected four immunoreactive bands (Fig. 5A) identical in size to those detected in the physiological stages (30, 48, 52, and 55 kDa). The 48-, 52-, and 55-kDa bands showed consistent results when compared to the Western blots from physiological stages. The 30-kDa band appeared to be regulated too, but the results obtained were not consistent. The monoclonal antibody against bFGF protein recognized the same two bands of approx 30 and 55 kDa (Fig. 5B). Expression of the two bands followed a similar pattern, which was different from the corresponding mRNA pattern. Both bands were higher in the control virgin and the gland-free virgin mice compared to late pregnant and gland-free late pregnant mice.

Discussion

The growth and development of the mammary gland occurs in distinct stages that are governed by sexual maturity and the reproductive status of the animal. A variety of growth factors have been proposed to be produced in the mammary gland and may act as local regulators of growth and/or differentiation (Topper and Freeman, 1980; Borellini and Oka, 1989; Imagawa et al., 1990; 1994). Our results suggest that acidic and basic FGF are developmentally regulated in the mammary gland. Our results from the gland-free fat pad studies suggest that acidic and basic FGF are produced in the stroma, and that stromal production is developmentally regulated. However, definite proof of the site (i.e., which cell type actually produces it) of acidic and basic FGF mRNA production await *in situ* localization. Furthermore, although these results indicate that acidic and basic FGF are produced in the stoma, they do not rule out additional production in the epithelium or myoepithelium.

It is important to note that there are some differences in our results and those from Coleman-Krnacik and Rosen (1994). The first most important difference between their work and ours is that they detected two aFGF mRNAs of

sizes 4.7 and 3.4 kb, whereas we detect only one mRNA band for aFGF of size 4.8 kb. The pDH15 aFGF cDNA probe that we used was derived from human brain stem and has been reported to detect a single 4.8-kb mRNA (Jaye et al., 1986; Libermann et al., 1987). A messenger RNA band for aFGF corresponding to a size of 4.8 kb has also been previously reported in a number of tissues such as the brain, retina, pituitary (Thomas et al., 1984; Lobb and Fett, 1984; Bohlen et al., 1985; Baird et al., 1985; Alterio et al., 1988), rat mammary fibroblast cell line (Barraclough et al., 1990), rat, bovine, and human kidney (Gautshi-Sova et al., 1987; Wang et al., 1989; Zhang et al., 1991) and bovine and human vascular smooth muscle cells (Weich et al., 1990). For bFGF mRNA, we have reported the presence of two bands corresponding to sizes 3.7 and 7.0 kb, while they have reported only one bFGF nnRNA corresponding to size of 5.9 kb. The pJJ11-1 bFGF cDNA probe that we used was derived from the bovine pituitary and has been reported to detect two bands of sizes 3.7 and 7.0 kb, respectively (Abraham et al., 1986). Messenger RNA bands for bFGF corresponding to identical sizes have been previously reported in a number of tissues such as brain, pituitary, retina, corpus luteum, adrenal gland, kidney, placenta, prostrate, thymus, bone, immune system, and various tumors (reviewed in Gospodarowicz et al., 1986; Baird et al., 1986; Lobb et al., 1986; for additional references *see* Schweigerer et al., 1987a,b; Ernfors et al., 1990), in bovine and human vascular smooth muscle cells (Weich et al., 1990), in normal human fibroblasts (Sternfeld et al., 1988), and in rat mammary epithelial cell lines derived from malignant metastatic tumors (normal rat mammary epithelial cells did not possess any bFGF mRNA; Fernig et al., 1993). Northern blot analysis of mRNA extracted from irradiated bovine aortic endothelial cells (BAECs) hybridized with the pJJ11-1 cDNA probe (the same probe that we used in our studies) also revealed two mRNA bands corresponding to sizes 3.7 and 7.0 kb (Haimovitz-Friedman et al., 1991). The size discrepancy between 4.8 and 4.7 kb for aFGF mRNA is within the range of error for agarose gel electrophoresis, but the total absence of the 3.4-kb aFGF mRNA band or two completely different size bands for bFGF mRNA in our studies could be owing to a couple of reasons. First, the source and length of cDNA probes used for both the studies are very different. We used human aFGF and bovine bFGF cDNA probes, and our probes were generated by restriction digestion, whereas they used full length mouse cDNA probes for their studies. Second, we used nick translation for our Northern analysis, while they used random priming.

Also, they reported FGF-1 and FGF-2 (aFGF and bFGF, respectively) mRNA levels to decline during pregnancy and drop further during lactation. What we report in this paper is that aFGF mRNA level increases slightly during pregnancy, and a significant increase occurs during early lactation. Basic FGF mRNA level showed a slight increase in early and mid-pregnancy relative to the controls (virgins). There was a significant increase in bFGF message levels during late-pregnancy, but there was a precipitous decline in bFGF mRNA level during early lactation. Some of the reasons to account for the differences between our results and the results of Coleman-Krnacik and Rosen's group could be: (1) differences in the stage of estrous cycle for mice within each experimental group; (2) we used total RNA for our Northern analysis, whereas they used poly(A)$^+$ RNA for all their analysis; and (3) different strains of mice used, since we used ND/4 mice for all our studies, whereas they used ICR or BALB/c mice. Also, for the lactating stage, they used 10-d lactating mice, whereas we used 5-d lactating mice for all of our studies.

Another important difference between the work reported by Coleman-Krnacik and Rosen (1994) and our study is that they attributed the majority of FGF-1 gene expression to be in the luminal epithelial cells, whereas FGF-2 gene expression was reported in the mammary stroma and possibly myoepithelial cells, while our results suggest that both aFGF and bFGF are produced in the stroma. However, our results do not rule out the possibility of epithelial as well as stromal production of the FGFs. To determine the site of FGF expression, they fractionated the virgin glands to separate the epithelial cells from the stromal component. The two fractions were then assayed by quantitative RT-PCR for their relative levels of FGF-1 and FGF-2 expression. The purification procedure used in their protocol does not completely rule out contamination of the epithelial fraction by the stromal cells and vice versa. This might account for the differences in their results from ours, although our localization experiments are preliminary, and accurate localization of the FGFs awaits *in situ* hybridization studies.

The results obtained from Western analysis are rather intriguing. Although the sizes of native aFGF and bFGF have been reported to be 16 and 18 kDa, respectively (Gospodarowicz et al., 1987), other proteins of higher molecular weights have been reported as well. For example, from cultures of normal human dermal fibroblasts, three molecular weight forms of the bFGF protein (18.0, 23.0, and 26.6 kDa) and three molecular weight forms of aFGF protein (18.4, 19.2, and 28.6 kDa) were detected using Western blot analysis (Root and Shipley, 1991). There has been another instance where the native protein was not detected, but higher molecular weight forms of the same were detected. In a study using rat testicular spermatocytes and round spermatids, bFGF as determined by Western blot analysis and immunoprecipitation was determined to be 30.0, 27.0, and 24.0 kDa (In Suk Han et al., 1993). A 25-kDa form of bFGF in guinea pig brain (Moscatelli et al., 1987), 22.0-, 24.0-, 27.0-, and 29-kDa forms in rat brain and pituitary (Doble et al., 1990; Presta et al., 1988); and 24-, 30–33-, and 46-kDa forms in the adrenal gland (Grothe et al., 1990) have also been reported till date. Data suggests that the 25-kDa form of bFGF isolated from guinea pig brains is owing to methylation of arginine residues (Burgess et al., 1991).

The 30-, 48-, and 55-kDa bands for aFGF detected in our studies followed a similar pattern of expression, being low during virgin and early pregnant stages but high during mid-pregnancy through early lactation. The 52-kDa band followed a different pattern from the 30-, 48-, and 55-kDa bands, being high during virgin and early pregnant stages and low during mid-pregnancy through early lactation. The reason for the 52-kDa band to follow a pattern of expression different from the other three bands is unclear.

There are several possible explanations to account for the difference in the patterns of expression of aFGF and bFGF mRNA and proteins. For aFGF, although there is a dramatic increase in mRNA levels during early lactation, there is a dramatic increase in immunoreactive protein in the mid- and late-pregnant stages. The reason why the protein does not follow the pattern of the mRNA, we do not know. There does exist the possibility that the polyclonal antibody against aFGF is not recognizing a particular form of the protein (maybe owing to a confirmational change) that should have been detected more prominently during pregnancy. For the bFGF proteins, both the 30- and 55-kDa bands followed a similar pattern, which was different from that of bFGF mRNA. Both proteins increased through early-pregnancy, but during late pregnancy (during which bFGF message levels were the highest) there was a decrease in protein levels, which continued to remain low during early lactation. The decrease in the two bands for bFGF during late pregnancy may be owing to the fact that translation of bFGF protein does not occur at a comparable rate during this stage of mammary gland development. It could also be that the bFGF protein is relatively unstable during late pregnancy. Another interesting speculation is that bFGF may undergo receptor-mediated internalization during late pregnancy.

Although our Northern analysis indicated that aFGF mRNA appears as a single transcript of 4.8 kb and bFGF mRNA appears as two transcripts of sizes 3.7 and 7.0 kb, the number of proteins appearing on our Western blots is two for bFGF (of sizes approx 30 and 55 kDa) and four for aFGF (of sizes approx 30, 48, 52, and 55 kDa). The size of native bFGF has been reported to be about 16 kDa (Gospodarowicz et al., 1985; Esch et al., 1985) and that of aFGF is approx in the range of 16–18 kDa (Gimenez-Gallego et al., 1985). Speculations on some of the reasons for diverse sizes of FGFs include the following:

1. Alternative splicing reactions could give rise to the different sized proteins;
2. Modification reactions (e.g., glycosylation, phosphorylation, methylation, etc.) are responsible for the production of the different sized proteins (Gospodarowicz et al., 1987);
3. Alternative start sites for translation (non-AUG codons) could also give rise to these different sized proteins (Florkiewicz and Sommer, 1989); and
4. The higher forms could also be precursor forms of acidic and basic FGF that are sequentially truncated to give rise to the native proteins.

Milk and colostrum contain a variety of proteins, peptides, and steroids that possess biological activity (Baumrucker and Blum, 1993). Growth factor activities reported include EGF, TGF-α, TGF-β, NGF, and MDGF-1 (Grosvenor et al., 1992). So far, nothing has been reported of the presence of aFGF or bFGF in murine milk. Preliminary studies in our laboratory indicate that, in addition to the high molecular weight bands, anti-bFGF also recognized a band of approx 15–18 kDa in murine milk samples. Also, anti-aFGF detected a band in the range of 16–18 kDa in mouse milk in addition to the high-mol-wt bands (data not shown). This is interesting because none of the low-mol-wt proteins (of similar size reported for the native aFGF and bFGF proteins) appeared in the whole gland protein samples from early lactation. The most likely reason for that to have happened is that the amount of milk in the whole gland sample was not sufficient to allow detection of the low-mol-wt proteins. This observation might be significant considering the fact that the high-mol-wt forms probably give rise to the native proteins by sequential truncations occuring specifically in milk.

In summary, our results suggest that aFGF and bFGF are developmentally regulated in the mammary gland. Our results also suggest stromal production of acidic and basic FGF, which probably act on the epithelial component via paracrine mechanisms.

Materials and Methods

In order to determine the patterns of expression of basic and acidic FGF during mammary gland development, ND/4 mice (Harlan-Sprague Dawley, Indianapolis, IN) were euthanized at five different stages—virgins (9–10 wk of age), early pregnancy (d 3–4 of pregnancy), mid-pregnancy (d 10–11 of pregnancy), late-pregnancy (d 17–18 of pregnancy), and early lactation (d 5 of lactation) by ip injection of 5 mg pentobarbitol, and both inguinal mammae were excised for analysis. Immediately upon removal, the glands were rapidly frozen in liquid nitrogen and stored at −70°C for Northern and Western analysis. All animal procedures were approved by the local IACUC.

Northern Blot Hybridization

Mammary tissue was homogenized in guanidium isothiocyanate and extracted with phenolchloroform as previously described (Chomzinski and Sacchi, 1987). Ten micrograms of total RNA were separated on a 1.2% denaturing agarose gel and transferred to Zeta probe membrane (Bio-Rad, Hercules, CA) by capillary transfer. Blots were dried and UV crosslinked for 8 min. The human aFGF-cDNA probe (pDH15) was obtained from American Type Culture Collection (Rockville, MD). The

860-bp fragment generated by *Eco*RI and *Hind*III digestion of the plasmid was utilized for detecting aFGF mRNA. The bovine bFGF-cDNA probe (PJJ11-1) was obtained from Dr. Judith Abraham (Abraham et al., 1986). The 1.05-kb fragment generated by *Nco*I digestion of the plasmid was utilized for detecting bFGF mRNA. The human glyceraldehyde-3- phosphate dehydrogenase (GAPDH) cDNA probe (pHcGAP) was also obtained from the American Type Culture Collection. The 780-bp fragment generated by *Pst*I and *Xba*I digestion of the plasmid was utilized for detecting the GAPDH mRNA. The probes were labeled with (^{32}P)dCTP by nick translation and free dCTP was removed using Sephadex Nick Columns (Pharmacia, Piscataway, NJ). The range of specific activity was between $1.3 \times 10^7 - 1.8 \times 10^7$ and $1.2 \times 10^8 - 0.2 \times 10^8$ cpm/μg DNA for all the probes. Blots were probed as previously described (Fenton and Sheffield, 1993) except that hybridization was at 55°C for aFGF and 42°C for bFGF and GAPDH. Blots were then dried and exposed to preflashed Fuji X-ray film (Fisher Scientific, Chicago, IL) with intensifying screens at −70°C. Bands were quantitated by densitometry using Collage (Fotodyne, New Berlin, WI).

Western Analysis

Glands were weighed and SDS loading buffer (Laemmli, 1970) was added (1 mL/200 mg gland wet weight). Glands were heated for 20 min in a steam bath, and 80 μL of the solution were electrophoresed on a 15% polyacrylamide gel under reducing conditions (Laemmli, 1970). Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) as described by Towbin et al. (1979). Membranes were blocked in phosphate buffered saline (PBS, 0.01*M* phosphate buffer, 0.0027*M* KCl, 0.137*M* NaCl, pH 7.4 at 25°C) and 3% Tween-20 (PBS-T) supplemented with 2% BSA (bovine serum albumin) overnight at 4°C. The blots were then incubated in fresh PBS-T containing 2% BSA for 2 h along with the primary antibodies (2 μg primary antibody/1 mL PBS-T). The primary antibody against aFGF was a polyclonal antibovine aFGF antibody (UBI, Lake Placid, NY), and the primary antibody against bFGF was a monoclonal antibovine bFGF antibody (UBI). As a control, blots were incubated with monoclonal antimouse β-actin primary antibody (Sigma, St. Louis, MO). Blots were washed six times for 5 min each in PBS-T containing 0.1% BSA and incubated with peroxidase-conjugated goat antirabbit IgG for the aFGF primary antibody (Sigma) or peroxidase-conjugated sheep antimouse IgC; for the bFGF/β-actin primary antibody (Sigma) at a concentration of 1 μg secondary antibody/mL PBS-T containing 2% BSA for 30 min. Blots were washed again six times, for 5 min in PBS-T containing 0.1% BSA, and aFGF/bFGF/β-actin proteins were detected using enhanced chemiluminescence (ECL) as described by the manufacturer (Dupont NEN, Boston, MA).

Gland-Free Fat-Pad Studies

For these studies, ND/4 mice were obtained from Harlan-Sprague Dawley when they were 3 wk of age, and the epithelial component of the gland was surgically removed as described by De Ome et al. (1959). The mice were then allowed to grow to 9–10 wk of age when they were either sacrificed as virgins or mated and euthanized during late pregnancy or early lactation. For assessing bFGF mRNA, we chose late pregnancy since our Northern analysis detected highest levels of bFGF mRNA at late pregnancy. For studies of aFGF mRNA, we chose the lactational stage since our Northern analysis detected highest levels of aFGF mRNA during early lactation. Control mice that had intact glands were also euthanized at similar stages of development to enable us to compare levels of aFGF or bFGF mRNA expression with respect to mice with deepithelialized glands and those with intact glands. Northern and Western analysis was performed as described.

Statistical Analysis

Each experiment was replicated using 7–8 mice per group. Data were analyzed as a randomized complete block design with fixed treatment effects and random block effects. The data were analyzed by a two factor analysis of variance. Values were considered to be significantly different when the P-value was ≤ 0.05 with the Dunnett's *t*-test. The statistical analysis was performed using Statview 512 (Brainpower, Inc., Calabasas, CA).

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