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Hormonal Regulation of Acidic and Basic Fibroblast Growth Factor Production and Expression in Mouse Mammary Gland

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Based on previous results showing developmental regulation of aFGF and bFGF, we evaluated the effect of various hormones on aFGF and bFGF mRNA levels, in mammary gland. Northern blots indicated that estrogen alone increased aFGF but had no effect on bFGF messenger RNA level. Progesterone alone increased aFGF and bFGF mRNA levels. Estrogen and progesterone together increased aFGF mRNA level in mammary gland, but the increase was no greater than that caused by either hormone alone. However, the combination of estrogen and progesterone had no effect on bFGF message level. PRL or GH, when administered with estrogen and progesterone, increased aFGF, but did not have any effect on bFGF message level. However, when PRL and GH were administered together with estrogen and progesterone, they increased bFGF messenger RNA level. Ovarian steroid withdrawal increased aFGF, but did not have any effect on bFGF mRNA accumulation. PRL alone (in the absence of estrogen and progesterone) decreased aFGF, whereas it had no effect on bFGF message level. Hydrocortisone alone decreased aFGF, but increased bFGF mRNA level. However, PRL and hydrocortisone increased aFGF, but did not have any significant effect on bFGF message level. In the overall model, during growth of the mammary gland, ovarian steroids cause an increase in aFGF mRNA. During lactogenesis, ovarian steroid withdrawal causes an increase in aFGF messenger RNA levels. Lactogenic hormones together cause a further increase in aFGF message levels.

Key Words: Acidic fibroblast growth factor (aFGF); basic fibroblast growth factor, (bFGF); mammary gland; development; angiogenesis; pregnancy; lactation.

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Introduction

The classical endocrine factors controlling mammary gland growth and differentiation are well described (Topper and Freeman, 1980; Oka et al., 1991; Imagawa et al., 1994). During pregnancy, mammary growth involves a dramatic increase in the branching of ducts, as well as the formation of numerous alveoli. Previous studies indicate that periods of maximum proliferation of the mammary gland during pregnancy correlate with periods of high levels of progesterone and prolactin or placental lactogen production from the ovaries, anterior pituitary, and placenta (Bresciani, 1971). Estrogen (E) stimulates ductal development, and progesterone (P) regulates lobuloalveolar development (Imagawa et al., 1990). Estrogen has also been reported to modulate the level of progesterone receptors in the pregnant mammary gland (Haslam, 1987). Prolactin (PRL) appears to fulfill functions essential to secretory cell proliferation and differentiation during late pregnancy and lactation (Dilly, 1971; Oka and Topper, 1972). The function of growth hormone (GH) appears to be its role in the preferential partitioning of nutrients required for the growing mammary gland during pregnancy (Bchini et al., 1991). The classical lactogenic hormones (hormones essential for the induction of lactation) are adrenal steroids (hydrocortisone or cortisol, depending on the species) and PRL, (Forysth, 1986; 1991). Although the systemic importance of the aforementioned hormones have been well documented, recent studies suggest that many growth factors may also have a role in the regulation of mammary gland growth and differentiation (Oka et al., 1991). The importance of all these different growth factors on making gland development reported to date raised the possibility that acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF) might also play critical roles in mammary gland development.

Fibroblast growth factors (FGFs) are potent regulators of cell proliferation, differentiation, and function. FGFs are also referred to as heparin binding growth factors (HBGFs) owing to their ability to bind heparin (Gospodarowicz et al., 1987). While these factors were initially discovered

as fibroblast mitogenic factors, hence their name, their roles are far more extensive. In fact, not all FGF members are mitogenic for fibroblasts. The FGF family currently consists of nine members that usually show 30–50% sequence identity at the amino acid level and conservation of the position of two cysteine residues (Baird, 1994). The factors are designated FGF1 through FGF9, although they also have other names.

Acidic and basic FGF are prototype members of the FGF family, so named as a result of their differing isoelectric points. They have similar molecular weights and spectra of biological activities, and they show approx 55% amino acid identity. Acidic and basic FGF have been isolated from a variety of sources (Gospodarowicz et al., 1987). One of the important properties of aFGF and bFGF are as potent angiogenic agents, playing crucial roles in normal development, in the maintenance of tissues, and in wound healing and repair (Whitby and Ferguson, 1991; Fallon et al., 1994; Cohn et al., 1995). They are chemotactic and mitogenic for endothelial cells in vitro, inducing the production of a variety of factors by endothelial cells that are involved in the breakdown of the basement membrane, and the migration of capillary endothelial cells into collagen matrices to form capillary-like tubes (Avraham et al., 1993).

Since mammary gland development during pregnancy and lactation involves angiogenesis (neovascularization or in other words, new blood vessel growth) to supply the growing gland with nutrients, and aFGF and bFGF are potent angiogenic agents of physiological importance (Folkman and Klagsbrun, 1987), aFGF and bFGF may play critical roles in mammary gland development. In addition, acidic and basic FGF were found to be mitogenic to mouse mammary epithelial cells (Levay-Young et al., 1989). Since FGFs are produced by endothelial cells, their presence in the mammary gland is to be expected. Basic FGF has been identified in normal human mammary gland by immunocytochemistry and immunoblotting of membrane preparations. It was found to be localized in the myoepithelial cells (Gomm et al., 1991). In another study, bFGF mRNA was found in fibroblast cells and in epithelial cells undergoing differentiation to myoepithelial cells (Barraclough et al., 1990; Fernig et al., 1990). We hypothesized that aFGF and bFGF are developmentally regulated in the mammary gland; our results supported this hypothesis (Chakravorti and Sheffield, 1993, 1994). The studies reported here were conducted to define the ability of specific endocrine factors to regulate intramammary expression of aFGF and bFGF.

Results

FGF Expression During Estrogen and Progesterone Time-Course Experiment

Northern blot analysis indicated that mammary aFGF mRNA was a single band of 4.8 kb and mammary bFGF mRNA comprised of two bands of 3.7 and 7.0 kb, respectively. Both the bFGF bands varied together and therefore, results for both the bands were combined in the analysis. Quantitation of Northern blots indicated that long-term E + P (estrogen and progesterone) treatment caused an increase in the aFGF message level after as little as 2 d of treatment. However, E + P did not affect bFGF mRNA expression (Fig. 1).

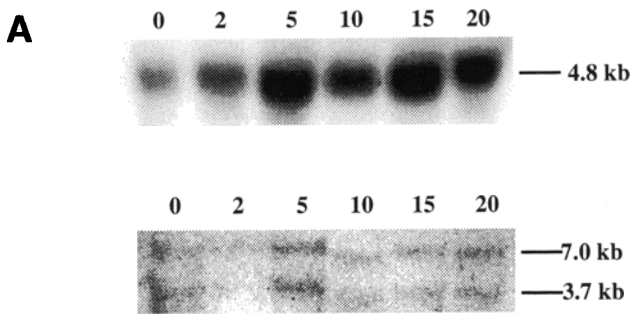

Fig. 1A. Autoradiograms of aFGF (top) and bFGF (bottom) mRNA expression in response to estrogen and progesterone (E + P) injections for various times. Northern blot analysis revealed a single mRNA species for aFGF corresponding to a size of 4.8 kb, and two mRNA bands of sizes corresponding to 3.7 and 7.0 kb, for bFGF.

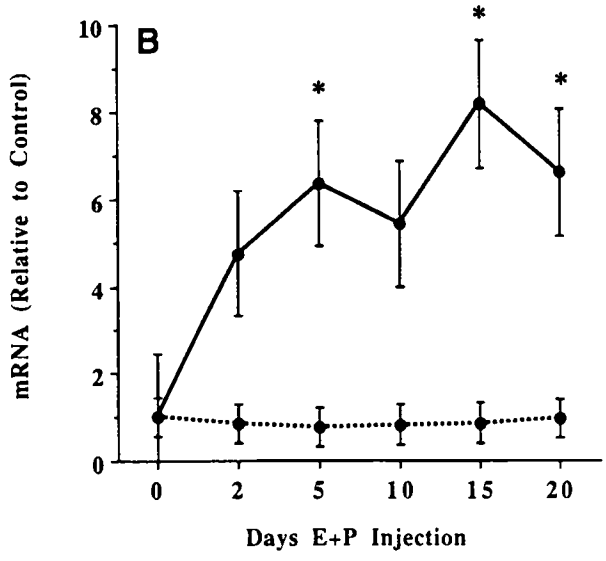

Fig. 1B. Acidic (—) and Basic (---) FGF mRNA Expression during Estrogen and Progesterone Time Course Experiment. Autoradiographic bands from Northern hybridizations were quantitated by densitometry using Collage. For bFGF, the two bands varied similarly, so the results were combined in the statistical analysis. Each value represents the mean + SEM (n = 3 for aFGF and bFGF).

Effect of Estrogen and Progesterone on FGF mRNA Levels

The objective of this particular experiment was to determine the individual effect of estrogen or progesterone on FGF mRNA levels, and compare the results with the effect

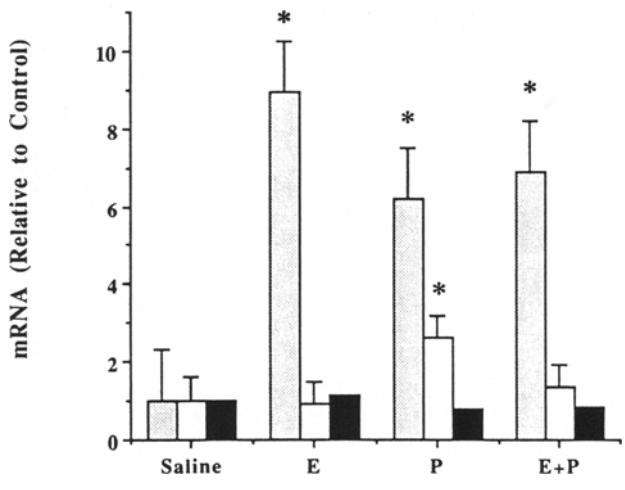

Fig. 2. Effect of estrogen and progesterone on expression of acidic (▨) and basic (□) fibroblast growth factors (aFGF, bFGF) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (■) mRNA in mouse mammary tissue. Mice were ovariectomized at 6 wk of age, allowed a 2 wk recovery period, and then injected with specific treatments for 10 d (saline, estrogen, E; progesterone, P; and estrogen + progesterone, E + P). Autoradiographic bands from Northern hybridizations were quantitated by densitometry using Collage. Each value represents the mean ± SEM (n = 3–5).

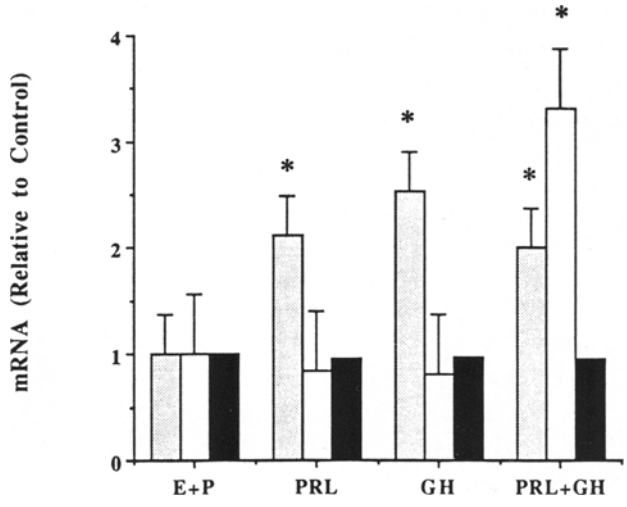

Fig. 3. Effect of mammogenic hormones on expression of acidic (▨) and basic (□) fibroblast growth factors (aFGF, bFGF) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (■) mRNA in mouse mammary tissue. Ovariectomized mice were primed with estrogen and progesterone at a concentration of 1 μg + 1 mg/d for 10 d. After the E + P priming, the mice were injected with specific hormones as indicated in Materials and Methods. Autoradiographic bands from Northern hybridizations were quantitated by densitometer using Collage. Each value represents the mean ± SEM (n = 6).

of estrogen and progesterone administered together. Estrogen or progesterone alone also increased aFGF message level relative to the control (saline treated). Administration of estrogen and progesterone together increased aFGF mRNA expression in mammary tissue, but the increase was no greater than that caused by either estrogen or progesterone alone (Fig. 2). Estrogen alone had no effect on bFGF mRNA expression relative to the control. Progesterone alone increased bFGF message level relative to the control. However, estrogen and progesterone together had no effect on bFGF messenger RNA level relative to saline treated mice (Fig. 2).

As a control for our Northern analysis, we used a probe against GAPDH mRNA. Figure 2 illustrates the results obtained by quantitating autoradiographic bands from hybridizations with the control probe. GAPDH messenger RNA level was similar in magnitude across all treatments. This control enables us to ascertain the fact that there is equal loading of total RNA in all the lanes during gel electrophoresis, and equal efficiency transfer before doing Northern hybridizations. This ensures that the differences observed in the autoradiograms are caused by different treatments and are not owing to unequal RNA loading per lane.

Effect of Mammogenic Hormones (E, P, PRL, and GH) on FGF Expression

PRL or GH, in combination with estrogen (E) and progesterone (P), increases aFGF messenger RNA level relative to E + P alone (Fig. 3). PRL and GH together also caused an increase in aFGF mRNA level relative to the E + P group. However, the increase caused by PRL and GH together was of the same magnitude as that caused by PRL or GH alone, when compared to the E + P group. Prolactin or growth hormone did not have any effect on bFGF mRNA level. However, PRL, GH, E, and P together caused a significant increase in bFGF message level relative to the E + P group. The results in fact suggest that PRL and GH have a synergistic effect on bFGF messenger RNA level (Fig. 3). None of the treatments examined had any measurable effect of expression of the GAPDH control mRNA (Fig. 3).

Effect of Lactogenic Hormones (Hydrocortisone and Prolactin) on FGF Expression

Estrogen and progesterone withdrawal increases aFGF mRNA level, as suggested by comparing the saline group with the E + P group (Fig. 4). It is important to mention here that the saline group was injected with E + P for 10 d (E + P priming) followed by E + P withdrawal with concomitant administration of only saline for the next 2 d. The E + P group was primed with E + P for 10 d, after which the mice were injected with E + P for another 2 d. Therefore, comparison of the saline group with the E + P group indicates the effect of the 2-d E + P withdrawal, on acidic and basic FGF mRNA levels. Prolactin (PRL) or hydrocortisone (F) alone decreased aFGF mRNA level relative to the saline group. Prolactin and hydrocortisone together caused an increase in aFGF message level relative to the saline group. The action of PRL + F on aFGF message level appears to be synergistic, relative to the effect of PRL or F alone.

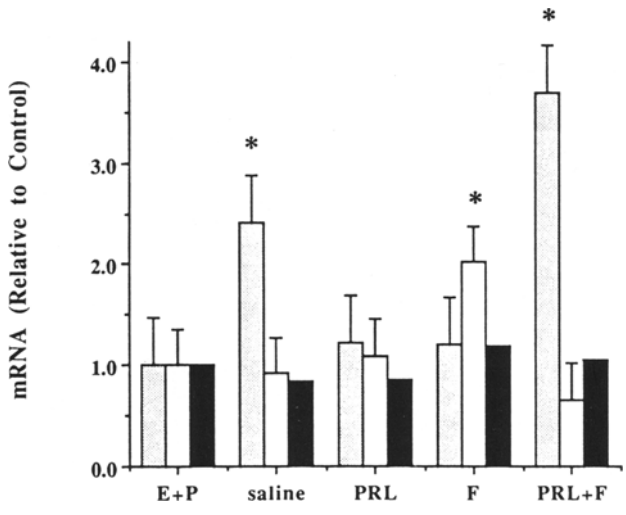

Fig. 4. Effect of lactogenic hormones on expression of acidic (□) and basic (□) fibroblast growth factors (aFGF, bFGF) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (■) mRNA in mouse mammary tissue. Mice were primed for 10 d with estrogen + progesterone, and then injected with the specific hormones for 2 d, as indicated in Materials and Methods. Autoradiographic bands from Northern hybridizations were quantitated by densitometry using Collage. Each value represents the mean ± SEM (n = 5–6).

Prolactin alone did not have any effect on bFGF mRNA level relative to the saline group. Hydrocortisone alone appeared to cause an increase in bFGF message level relative to the saline group. PRL + F together caused a decrease in bFGF messenger RNA level relative to the group treated with only hydrocortisone. However, PRL+F together did not have any effect relative to the saline group (Fig. 4).

Level of the GAPDH control mRNA was not measurably different among treatment groups (Fig. 4).

Discussion

There is accumulating evidence that growth factors can play an important role in the mammary gland (Oka et al., 1991). The growth, morphogenesis, and differentiation of the epithelium, synthesis and maintenance of the epithelial-stromal interface, and vascularization of the gland, can all potentially be regulated by growth factors. Based on available evidence (Oka et al., 1991), it can be speculated that growth factors will differ in localization within the gland, in the timing of expression and in biological efficacy. It has already become clear that the regulation of mammary parenchymal growth and differentiation is modulated by the stroma, which plays a role in the formation of the ECM, regulates epithelial sensitivity to hormones, and is a source of growth factors. The stroma as well as the epithelium can be a target for mammogenic and lactogenic hormones. These hormones are the primary regulators of mammary gland growth and differentiation that occurs during pregnancy and lactation. Growth factors, under the regulation of these specific hormones, are likely to participate in events resulting in the secretor function of the gland, but such interactions among hormones and growth factors have not yet been defined. The fact that growth factors are multifunctional presents opportunities for these factors to simultaneously regulate both the epithelial and stromal compartments, thus acting as coordinating factors at the whole gland level. Growth factors can act either on their own, or their action might be regulated by hormones in the mammary gland. A fascinating aspect of growth factor action is their ability to integrate diverse functions such as epithelial cell proliferation, basement membrane formation, epithelial morphogenesis, and angiogenesis in the mammary gland (Imagawa et al., 1994).

Basic and acidic FGF, prototype members of the FGF family of growth factors, have been reported to be ECM-associated growth factors (Vlodavsky et al., 1991). As stated before, aFGF and bFGF has been found to be mitogenic to mouse mammary epithelial cells (Levay-Young et al., 1989) and has been localized in normal human mammary gland by immunocytochemistry and immunoblotting of membrane preparations (Gomm et al., 1991). Amongst the action of some other growth factors in the mammary gland, it is interesting to note that while TGF-β promotes matrix synthesis and stabilization and inhibits mammary epithelial cell proliferation and angiogenesis, the FGFs antagonize the effects of TGF-β by promoting matrix degradation, mammary epithelial cell proliferation, and angiogenesis. Both of these families of growth factors are known to act on the matrix (autocrine) as well as mammary epithelial cells (paracrine) and probably reflect a balance between extracellular and cellular actions that can be mutually interactive (Bissel) and Hall, 1987).

Our results suggest that estrogen and progesterone alone increase aFGF mRNA as does their combined action, although the magnitude of increase is quite similar, relative to the control, i.e., saline-treated mice (Fig. 2). On the other hand, administration of estrogen alone does not affect bFGF messenger RNA level but progesterone increases bFGF message level. However, the combined administration of E + P had no effect on bFGF mRNA level (Fig. 2), suggesting an antagonism between estrogen and progesterone mediated pathways on bFGF message level. The effect of progesterone on bFGF mRNA is interesting, since, in the absence of estrogen, the mammary gland epithelium has few progesterone receptors. However, the mammary gland stroma contains substantial progesterone receptors not induced by estrogen (Haslam, 1987).

The results obtained from mammogenic and lactogenic experiments were very interesting. It is a well-established fact that the mammary gland undergoes growth during pregnancy, and that mammogenic hormones (E, P, PRL, GH) are responsible for this phase of mammary growth. Physiologically, we observed that aFGF and bFGF mRNA increased during pregnancy (Chakravorti and Sheffield, 1993, 1994). We hypothesized that the increase in FGF

mRNA we saw during pregnancy was being caused by mammogenic hormones. The results reported here (Fig. 3 A, B) indicate that PRL and GH, together with estrogen and progesterone, increase aFGF and bFGF expression. This finding suggests that prolactin and growth hormone are responsible for the increase in aFGF and bFGF mRNA levels during pregnancy.

Preliminary results indicated that aFGF m)RNA continues to increase during early lactation, whereas bFGF mRNA decreases during this phase, relative to late-pregnancy (Chakravorti and Sheffield, 1993, 1994). Figure 4 demonstrates that E + P withdrawal (comparing E + P group with the saline group) increases aFGF mRNA. PRL and hydrocortisone (F) together cause a further increase in aFGF mRNA, but do not cause any significant effect on bFGF message level (comparing PRL + F group with the saline group). These results suggest that the increase in aFGF mRNA level during early lactation might be caused by the net effect of E + P withdrawal along with the presence of the lactogenic hormones, prolactin and hydrocortisone. For bFGF (Fig. 4), E + P withdrawal along with the presence of PRL + F does not appear to be adequate to account for the decline observed during early lactation. Perhaps, the withdrawal of some other hormone is responsible for the dramatic decrease in bFGF message level during early lactation, relative to late pregnancy. A likely candidate for being responsible for the decrease in bFGF messenger RNA level during early lactation could be mouse placental lactogen II (mPL-II). The hormone profile for mPL-I and mPL-II during gestation has been characterized (Colosi et al., 1986). Since mPL-II is present in the serum at highest concentrations during late pregnancy and diminishes drastically on parturition, it could be responsible for the high level of bFGF mRNA seen during late pregnancy and a significant decrease in bFGF mRNA during early lactation (level being similar to virgins) when no mPL-II is present (the situation being similar to that in a virgin).

In the overall model, during growth of the mammary gland, estrogen and progesterone together cause an increase in aFGF mRNA. PRL and GH administered together cause a further increase in aFGF and bFGF mRNA. During lactogenesis, estrogen and progesterone withdrawal causes an increase in aFGF mRNA level. Hydrocortisone and prolactin together cause a further increase in aFGF message level.

Materials and Methods

Animals

ND/4 mice (Harlan-Sprague Dawley, Indianapolis, IN) were used for all of our experiments. In all studies, mice were ovariectomized at 6 wk of age and allowed a 2-wk recovery period. For the duration of each study, the mice were given ad libitum access to standard laboratory diets and water. All animal procedures were conducted as approved by the local IACUC.

Estrogen and Progesterone Time-Course Experiment

Ovariectomized mice received hormonal injections as per the following schedule:

Saline (control) for 20 d;
Saline for 18 d, followed by E + P (1 μg + 1 mg/d) for 2 d;
Saline for 15 d, followed by E + P (1 μg + 1 mg/d) for 5 d;
Saline for 10 d, followed by E + P (1 μg + 1 mg/d) for 10 d;
Saline for 5 d, followed by E + P (1 μg + 1 mg/d) for 15 d; and
E + P (1 μg + 1 mg/d) for 20 d.

The saline control was 150 m*M* NaCl + 1 mg/mL gum arabic. E + P was suspended to a concentration of 10 mg + 10 μg/mL in 150 m*M* NaCl + 1 mg/mL gum arabic. Treatments were administered by sc injection (0.1 mL/d). Treatments are referred to by the duration of E + P injections. After the 20 d of hormonal injections, the mice were euthanized, and the inguinal mammary glands were excised and frozen at –70°C for Northern analysis.

Effects of Estrogen and Progesterone on aFGF and bFGF Expression

Ovariectomized mice were treated for 10 d, once daily, as per the following schedule:

Saline (control);
Estrogen (1 μg/d, for 10 d);
Progesterone (1 mg/d, for 10 d); and
Estrogen and progesterone (1 μg + 1 mg/d, for 10 d).

Effect of Mammogenic Hormones (E, P, GH, and PRL) on FGF Expression

Ovariectomized mice were primed with 17 β-estradiol and progesterone (Sigma, St. Louis, MO) at a dose of 1 μg and 1 mg/d, administered sc, for a period of 10 d. Following E + P priming, the mice received one of the following treatments, once daily, for 2 d:

Estrogen + progesterone (1 μg + 1 mg);
Estrogen + progesterone + ovine prolactin (250 μg, NIH);
Estrogen + progesterone + bovine growth hormone (250 μg, NIH); and
Estrogen + progesterone + ovine prolactin (250 μg, NIH) + bovine growth hormone (250 μg, NIH).

Effect of Lactogenic Hormones (Hydrocortisone and Prolactin) on FGF Expression

Ovariectomized mice were primed with 17 β-estradiol and progesterone (Sigma) at a dose of 1 μg and 1 mg/d, administered sc, for a period of 10 d. Following E + P priming, the mice received one of the following hormonal injections daily, for 2 d (hormones administered sc):

Saline;
Estrogen + progesterone (1 μg + 1 mg)
Prolactin (250 μg, NIH)
Hydrocortisone (250 μg, Sigma, St. Louis, MO)
Prolactin (250 μg, NIH) + hydrocortisone (250 μg, Sigma, St. Louis, MO)

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 upward 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 (G,APDH) 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 $0.2 \times 10^7 - 1.5 \times 10^7$ and $0.4 \times 10^8 - 1.2 \times 10^8$ 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 (Fischer, Chicago, IL) with intensifying screens at –70°C. Relative intensities of bands were quantitated by Collages (Fotodyne, New Berlin, WI).

Statistical Analysis

Each experiment was replicated using 5–6 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 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).

Acknowledgments

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