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Evidence That the Mammary Fat Pad Mediates the Action of Growth Hormone in Mammary Gland Development

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ABSTRACT

Recent evidence from our laboratory suggests that GH and insulin-like growth factor I (IGF-I) mediate glandular mammary development together with estrogen. It has also been well established that both stromal and epithelial elements must interact for mammary glandular development to occur. To determine whether the effect of GH is mediated by the stromal or epithelial tissue, we set up the following experiment. Bovine GH (bGH; 100 μg) or BSA (as a control), without or with estradiol (E_2), was injected ip into sexually immature female rats that were hypophysectomized and oophorectomized. Mammary glands and subscapular fat pads were removed from the animals. The mammary glands were divided into two parts: a gland-free fat pad and remaining glandular tissue. The end point of bGH activity was induction of IGF-I messenger RNA (mRNA). This was determined quantitatively by solution hybridization and also by RT-PCR. We found that the effects of GH on stimulation of IGF-I mRNA in the gland-free mammary fat pad and the remainder of the mammary gland were similar (3.6- *vs.* 3.9-fold, respectively; $P < 0.001$). In both sorts of mammary tissue, bGH was found to synergize with E_2 in the induction of IGF-I mRNA (5.8- *vs.* 5.3-fold; $P < 0.001$). There was also an increase in IGF-I mRNA in subscapular fat pads in response to 100 μg bGH (5.3-fold; $P < 0.001$); however, no synergism between bGH and E_2 was found. These data indicate that bGH works as well on mammary stromal tissue as on tissue with glands and suggests that GH acts on the stromal compartment of the mammary gland to induce IGF-I mRNA and possibly IGF-I itself, which, in turn, causes differentiation of epithelial ducts into terminal end buds. These data also might explain why mammary epithelium is also able to differentiate in nonmammary fat pads when transplanted there. (*Endocrinology* **139:** 659–662, 1998)

PUBERTAL mammary development occurs in response to an increase in estradiol (E_2). However, mammary development cannot take place in the absence of the pituitary gland. The pituitary hormone necessary for mammary development is GH (1–5). In recent years, we found that GH acts on the mammary gland through specific GH receptors (4, 5) to induce differentiation of an immature ductal tree into more mature terminal end buds (TEBs) and alveolar structures. TEBs extend into the mammary fat pad and lead to further ductal morphogenesis. This process, which requires the synergy of GH and E_2, probably involves local production of insulin-like growth factor I (IGF-I) that mediates the action of GH (6, 7).

Stromal elements must be present for mammary epithelial elements to mature (8–11). Based on the facts that IGF-I can substitute for GH in mammary development in hypophysectomized rats, that GH induces IGF-I messenger RNA (mRNA) in mammary gland, and that GH causes differentiation of adipose cells (12–15), we have hypothesized that stromal elements within the mammary gland mediate at least some of the actions of GH in pubertal mammary development by stimulating IGF-I mRNA and the IGF-I protein within the stroma, which, in turn, act on glandular elements in a paracrine fashion.

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To test the part of this hypothesis that addresses the site of action of GH, we determined effects of GH on IGF-I mRNA production in three types of tissue: 1) gland-free mammary stromal tissue, 2) whole mammary glands from which the gland-free fat pads were removed, and 3) subscapular fat pads. The results are reported here.

Materials and Methods

Animals

Female Sprague-Dawley rats were hypophysectomized and oophorectomized at 21 days of age, as previously described (7). At 45 days of age, groups of 10 animals received either a single injection of bovine GH (bGH; 100 μg) or a saline control injection ip. Some animals were also given E_2 in SILASTIC brand capsules (Dow Corning, Midland, MI) implanted sc (16). After 12 h, the point at which maximal stimulation of IGF-I mRNA occurs (4), animals were killed, and both lumbar mammary glands were removed. The mammary glands were divided into the gland-free mammary fat pad (Fig. 1) and the remainder of the mammary gland containing the glandular epithelial elements. Figure 2 is a photomicrograph of a lumbar mammary gland with the mammary fat pad separated from the remainder of the gland. The subscapular fat pad was also removed.

mRNA isolation

Immediately after removal from the animals, the mammary gland and subscapular fat pad tissue were snap-frozen in liquid nitrogen. Total RNA was prepared by the acid guanidine phenol chloroform extraction method (17). RNA was used for solution hybridization and/or RT-PCR experiments.

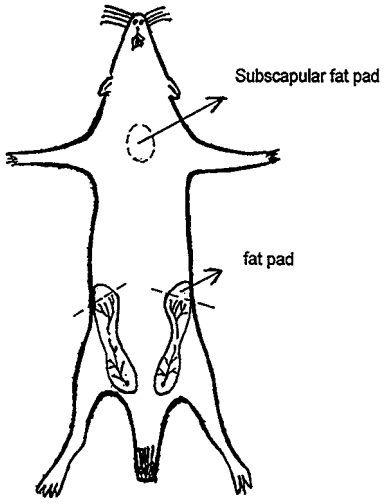

FIG. 1. Schematic of rat with lumbar mammary glands divided into the gland-free and gland-rich areas and the approximate location of the subscapular fat pad.

Solution hybridization

The full-length rat IGF-I complementary DNA (cDNA) fragment (18) was isolated by *Eco*RI digestion and subcloned into the *Eco*RI site of the vector pcDNA3 (Invitrogen, San Diego, CA) in the sense orientation with respect to the cytomegalovirus promoter. The resulting pcDNA3-IGF-I construct was used to synthesize sense and antisense IGF-I RNA probes using T7 and SP6 RNA polymerases in the presence of [^{32}P]UTP using the MaxiScript kit from Ambion (Austin, TX). Sense and antisense rat β-actin probes were prepared similarly. The RNA probes were purified by electrophoresis on a 6% acrylamide-8 M urea gel. Total cellular RNA (10 μg) was coprecipitated with 10^6 cpm radioactive probe. Solution hybridization was performed using the RPA II ribonuclease protection kit (Ambion). The resulting protected hybrids were electrophoresed through a 6% acrylamide-8 M urea sequencing gel and visualized by autoradiography of the dried gel.

RT-PCR

RT-PCR primers specific for rat (and human) IGF-I and β-actin were designed using the GCG prime software package. Both IGF-I and β-actin primer pairs were able to be used alone or in combination without effect on the yield of either PCR product. Furthermore, under the conditions of our assay, the results obtained by RT-PCR were semiquantitative, inasmuch as the relative ratios of IGF-I and β-actin mRNAs were very similar to the ratios determined by solution hybridization. For RT-PCR, RNA (1 μg) was reverse transcribed into cDNA using the Superscript preamplification kit (Life Technologies, Grand Island, NY). One tenth of the RT reaction was used for PCR in a total volume of 40 μl with primers for IGF-I and β-actin (12.5 pmol each) using *Taq* DNA polymerase (Perkin-Elmer, Norwalk, CT) and the buffer supplied by the manufacturer. All four deoxy (d)-NTPs were included at a final concentration of 20 μM together with the dCTP being included as [α-^{32}P]dCTP. PCR conditions were 10 cycles of 94 C for 30 sec, 52 C for 30 sec, and 72 C for 1 min. The PCR products were electrophoresed on a 1% agarose Tris-acetate EDTA (TAE) gel, excised with a scalpel blade, and counted by scintillation counting.

Results

We have previously shown that GH increases IGF-I mRNA in whole mammary glands (4), and that E_2 enhanced the effect of GH (7). In the present study we found that effects of bGH and E_2 were similar in both mammary gland fat pads and whole glands. bGH stimulated IGF-I mRNA in each of the tissue types studied. Figure 3 depicts the effects of bGH, bGH plus E_2, and E_2 alone compared to control values in gland-free mammary fat pads and whole mammary glands. GH significantly stimulated IGF-I mRNA, and E_2 enhanced that effect. That the effect was equal in gland-free mammary fat pads suggests that stromal tissue may mediate the effect of GH in the mammary gland.

Figure 4 compares effects of the above combinations of hormones on IGF-I mRNA production in subscapular fat pads and gland-free mammary fat pads. In the former, bGH had a more pronounced effect on IGF-I mRNA then in the latter, and there was no synergism with E_2.

The quantitative nature of the RT-PCR assay was confirmed by solution hybridization/ribonuclease protection. A representative solution hybridization gel shown in Fig. 5 validates the stimulatory effect of bGH on IGF-I mRNA production in whole mammary gland, gland-free mammary fat pads, and subscapular fat pads. The solution hybridization data also confirm the synergistic effects of E_2 in whole mammary gland and mammary fat pads and the nonsynergistic effects of E_2 in subscapular fat pads.

Discussion

The process of mammary development at puberty is dependent upon estrogen and GH (19). The fact that IGF-I mimics the action of GH in pubertal mammary development in hypophysectomized animals (6, 7) suggests that GH may act through IGF-I in this process. Although it has not been proven that IGF-I mediates this action of GH, growing evidence suggests that this is the case. IGF-I mRNA has been found in mammary gland by Murphy *et al.* (18), and we have shown that lactogenic and nonlactogenic GHs stimulate the production of IGF-I mRNA in mammary gland and that E_2 enhances that activity (7). These and other factors related to mesenchyme-epithelial interactions in mammary gland function have led us to hypothesize that GH stimulates IGF-I production in the stromal compartment of the mammary gland, and that IGF-I, in turn, acts on ductal elements to stimulate the formation of TEBs.

The importance of stromal-epithelial interactions from embryonic to adult stages of mammary gland growth and development has been the subject of intense interest for some time (8–11, 20, 21). Each stage of mammary glandular development from ductal branching through TEB development and later alveolar formation and milk protein production is dependent on the presence of the mammary fat pad. Sakakura has shown that different types of mesenchyme affect epithelial differentiation differently (10, 11, 21). Similarly, Cunha *et al.* have found that although adult mammary epithelia grow in response to heterotypic neonatal and embryonic mesenchymes, branching morphogenesis and the ability to form alveoli and produce milk are variable depending upon the type of mesenchyme (8). In contrast, Hoshino has found that the fat pad does not have to be of mammary origin to induce apparently normal development of transplanted mammary epithelial elements into pararenal and subscapular fat pads, indicating less specificity for the mesenchyme and more for epithelial tissues (22).

That both stromal and glandular elements of the mammary gland have receptors for estrogen and GH suggests that these hormones might act at either location. Our results are

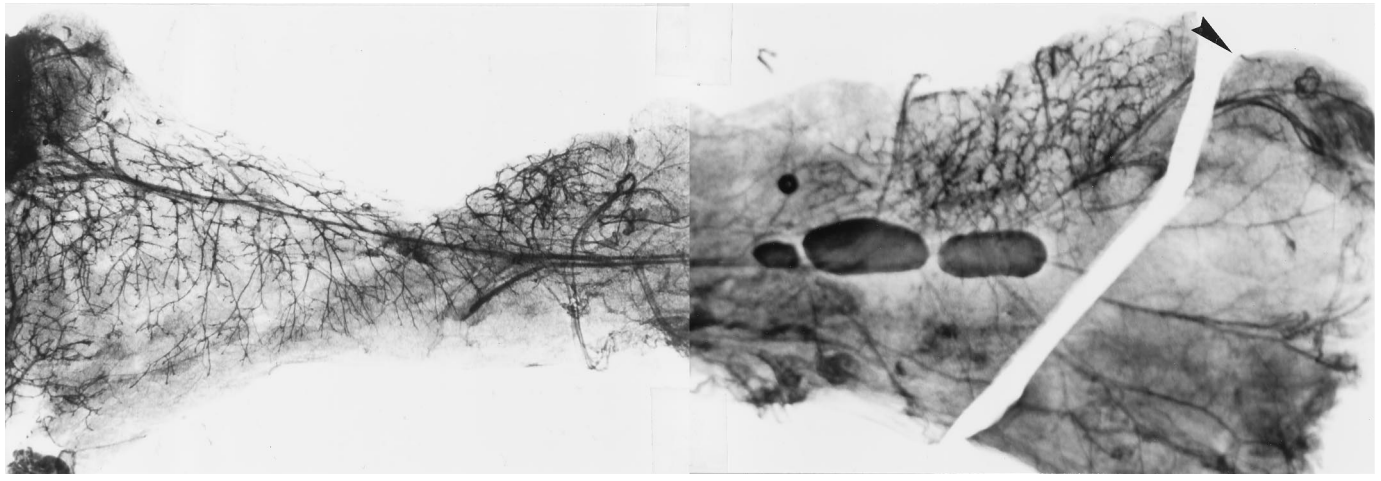

FIG. 2. Whole lumbar mammary gland removed from a 45-day-old female Sprague-Dawley rat that had been hypophysectomized and oophorectomized at 21 days of age. The mammary fat pad without glands (*arrow*) was separated from the rest of the gland and then photographed adjacent to the area from which it was removed. Magnification, ×1.9.

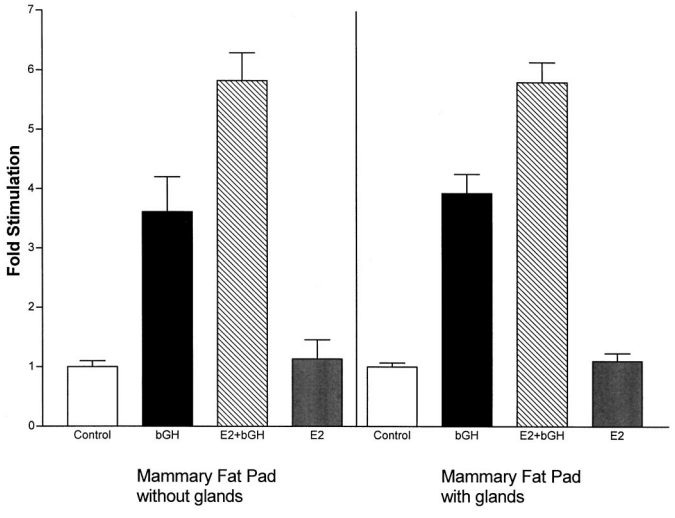

FIG. 3. Effects of bGH alone, bGH plus E_2, or E_2 alone on the production of IGF-I mRNA in mammary gland fat pads without or with glandular tissue. Results are expressed as fold stimulation over the control value.

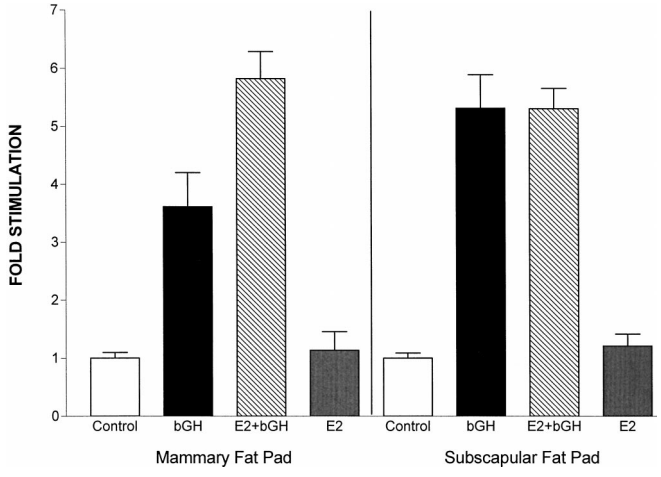

FIG. 4. Effects of bGH, bGH plus E_2, or E_2 alone on the production of IGF-I mRNA in mammary *vs.* subscapular fat pads. IGF-I mRNA levels are expressed as fold stimulation over the control value.

more consistent with the major action of GH being on the fat tissue rather than on the glandular tissue. In the first place, the effect of bGH on IGF-I mRNA production was equal in both gland-free and gland-rich compartments. If the major effect of bGH was on the glands themselves, one might have expected greater production of IGF-I mRNA in the gland-rich sections of the mammary gland. Secondly, the glandular epithelial elements in the so-called gland-rich areas occupy a relatively small proportion of the whole glands, with stromal tissues accounting for the majority. Thus, all or some of the observed bGH-induced increase in IGF-I mRNA might be taking place in the connective tissue. These results do not permit further speculation as to the potential effect of bGH on IGF-I mRNA production in glands, because isolated glands were not studied.

It has been known for some time that GH can stimulate IGF-I production in fat. Peter and colleagues (23) found that IGF-I mRNA was present in white adipose tissue of the rat in concentrations equal to those in liver. They also found that GH regulated not only IGF-I mRNA but also the IGF-I protein and IGF-binding protein-2, -3, -4, and -5 (23). Those studies support the likelihood that a major effect of GH in mammary development is on stromal tissue.

The significance of the synergy between E_2 and bGH in production of IGF-I may be of physiological importance but cannot explain the entire combined effect of these two hormones in mammary development, as even high concentrations of IGF-I do not cause full pubertal mammary development without additional E_2 (7).

Our findings that subscapular fat was affected by GH, but was not further affected by E_2, shows both similarities and differences between the two types of fat. That E_2 did not synergize with bGH in stimulating IGF-I mRNA production signifies a difference between the two fat pads, whereas the effect of GH is similar in both systems. The difference in the effects of E_2 might indicate an insensitivity to E_2 or might occur because the effect of bGH was maximal.

We believe that our findings support our hypothesis that

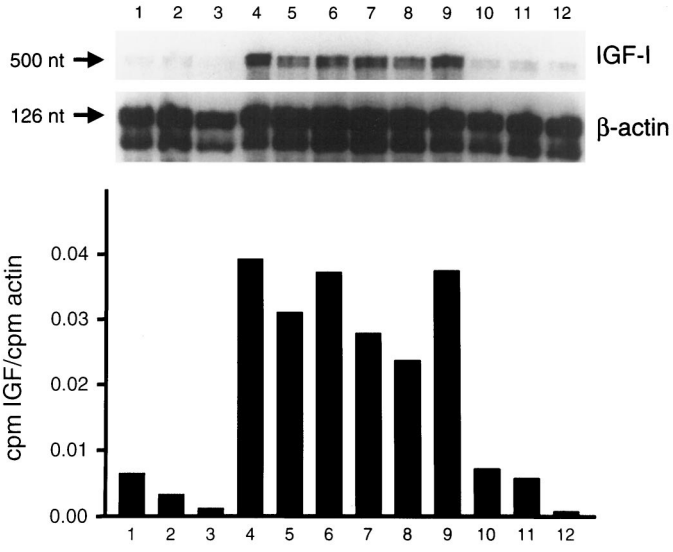

FIG. 5. Analysis of IGF-I mRNA and β-actin mRNA expression by solution hybridization in mammary fat pads with and without glandular tissue and subscapular fat pads treated with control vehicle (lanes 1–3), bGH (lanes 4–6), bGH plus E_2 (lanes 7–9), or E_2 alone (lanes 10–12). Protected fragments are shown on *top* of the figure, and the ratio of the counts in the IGF-I and actin bands are shown on the *bottom*.

GH acts on the fat pad in the mammary gland to induce IGF-I mRNA. That this cascade includes actual production of IGF-I protein and that this growth factor acts by paracrine means to induce glandular formation and differentiation await further proof.

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