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Short Communication

Keratinocyte Growth Factor Is a Growth Factor for Mammary Epithelium *In Vivo*

The Mammary Epithelium of Lactating Rats Is Resistant to the Proliferative Action of Keratinocyte Growth Factor

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Keratinocyte growth factor (KGF) is a member of the fibroblast growth factor (FGF) family. KGF is secreted by stromal cells and affects epithelial but not mesenchymal cell proliferation. KGF injected intravenously was found to cause dramatic proliferation of mammary epithelium in the mammary glands of rats. KGF causes ductal neogenesis and intraductal epithelial hyperplasia but not lobular differentiation in nulliparous female rats. KGF causes ductal and lobular epithelial hyperplasia in male rats. KGF causes proliferation of ductal and acinar cells in the mammary glands of pregnant rats. On the other hand, the ductal epithelium of lactating postpartum rats is resistant to the proliferative action of KGF. The mammary glands of lactating rats did not express less KGF receptor mRNA than the glands of pregnant rats, suggesting that the resistance of the ductal epithelium to KGF during lactation is not related to KGF receptor mRNA down-regulation. The mammary glands of both pregnant and postpartum lactating rats express KGF mRNA with more KGF present in the glands of lactating rats. In conclusion, the KGF and KGF receptor genes are expressed in rat mammary glands and recombinant KGF is a potent growth factor for mammary epithelium. (Am J Pathol 1994, 144:862–868)

Keratinocyte growth factor (KGF), also known as FGF-7, is a member of the fibroblast growth factor (FGF) family that is secreted by stromal cells and acts on epithelial cells[1] via a specific splice variant of the FGF receptor,[2,3] which is distinct from other known FGF receptors. KGF affects the proliferation of a variety of epithelia both *in vitro* and *in vivo*.[4–6] KGF, unlike other molecules in the FGF family, does not appear to cause mesenchymal stromal cell proliferation. The purpose of this study is to report the potent *in vivo* proliferative effect of recombinant KGF on the mammary epithelium of female and male rats.

Materials and Methods

Lewis rats weighing approximately 250 g received daily bolus injections of 5 mg/kg human KGF. The recombinant human KGF was purified to homogeneity, was 94% homologous to rat KGF, and stimulates both human and rodent keratinocytes equally well. Female rats received intraperitoneal injections and male rats

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received intravenous injections via the dorsal penile vein. After 1 to 7 days of daily injection the rats were killed, the mammary glands were paraffin embedded, and histological sections were stained with hematoxylin and eosin. Control rats received injections of saline. Whole mount preparations of the mammary glands were prepared by hematoxylin staining of formalin-fixed cleared mammary fat pads.[7]

Total RNA from mammary glands of pregnant (gestation day 20) and postpartum day 6 lactating rats was isolated as described by Chomczynski and Sacchi.[8] RNase protection of KGF and KGF receptor (KGFR) transcripts was performed using DNA cloned into the transcription vectors pGEM4Z and pSP72, respectively (Promega, Madison, WI). The KGFR[9] antisense transcript corresponded to bases 1270 to 1417 (GenBank accession number M63503). This region of KGFR sequence was determined to be identical in mice and rats (R. Biltz, unpublished data). The KGF antisense transcript corresponded to bases 132 to 336 (1, EMBL accession number X56551).[1] The vectors were linearized and antisense transcript synthesized *in vitro* using SP6 or T7 RNA polymerase and ^{32}Pr-UTP (800 Ci/mmol; New England Nuclear, Boston, MA; 1 Ci = 37 GBq). The full-length RNA probe was purified from an 8% polyacrylamide/7 M urea gel. After the protocol of Ambion RPA II kit 1410 (Austin, TX), triplicate samples of 50 μg of total cellular RNA were hybridized at 45 C overnight with 10^5 cpm of labeled antisense probe. RNase digestion was performed for 40 minutes with a 1:500 dilution of Ambion solution R. The RNA:RNA hybrids were precipitated then resuspended and separated on an 8% polyacrylamide/7 M urea gel. Signal from protected fragments was quantified on a Molecular Dynamics PhosphorImager (Sunnyvale, CA) and averaged over the triplicate sample points. To quantify the amount of message in tissue samples, unlabeled sense RNA corresponding to the labeled antisense probe was synthesized, purified over a G50 spin column, quantified by $OD_{260\ nm}$, and used as a standard in the hybridization assay. The equivalent picograms of message per 50 μg of total RNA was calculated by comparison of each tissue RNA protected band signal to one derived from hybridization to the known standard of sense RNA. The values obtained were then normalized using the ratio of the probe size to the full-length message.

Results

The normal mammary gland of the nulliparous female rat is composed of adipose tissue and sparsely interspersed ducts (Figure 1A). The ducts are lined by

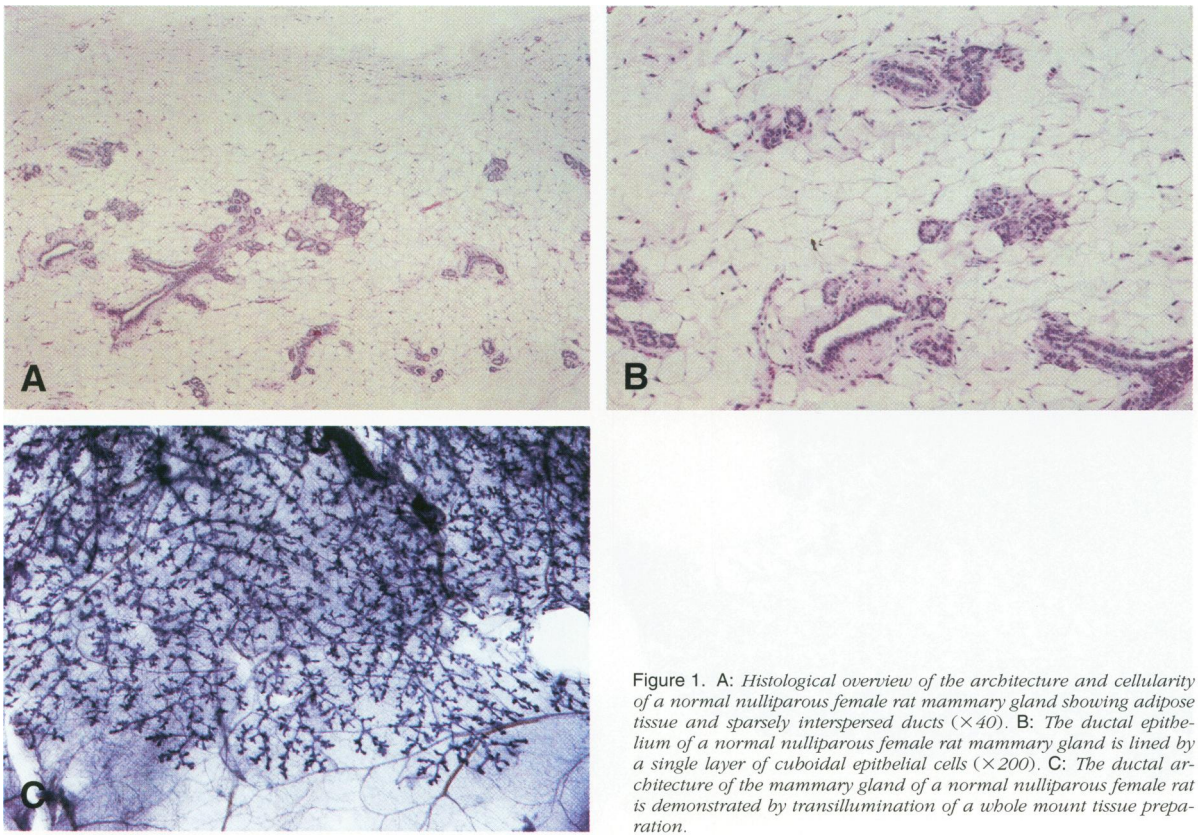

Figure 1. A: *Histological overview of the architecture and cellularity of a normal nulliparous female rat mammary gland showing adipose tissue and sparsely interspersed ducts* (×40). B: *The ductal epithelium of a normal nulliparous female rat mammary gland is lined by a single layer of cuboidal epithelial cells* (×200). C: *The ductal architecture of the mammary gland of a normal nulliparous female rat is demonstrated by transillumination of a whole mount tissue preparation.*

a mitotically inactive single cell layer of cuboidal epithelial cells (Figure 1B). The ductal architecture of the normal nulliparous female rat mammary gland (Figure 1C) is well demonstrated in transilluminated whole mount tissue preparations. Daily intraperitoneal injection of KGF for 3 consecutive days caused an increase in the number of ducts and in the architectural complexity of the ducts (Figure 2A). The mammary ductal epithelium was hyperplastic as evidenced by stratification of the ductal epithelial lining cells (Figure 2B) and the presence of numerous mitoses. The growth of new ducts was documented by whole mount tissue preparation (Figure 2C). After 1 week of daily intraperitoneal injections of KGF in female rats, an even more striking proliferation of the mammary epithelium is observed so that the mammary glands, normally inconspicuous to the naked eye, are grossly enlarged to many-fold their normal size. The cellular basis for the gross hypertrophy of the glands is histologically found to reside in the growth of new ducts (Figure 3A) lined by a mitotically active epithelium so hyperplastic as to completely compromise the lumina of many ducts (Figure 3B). Lobular, acinar, or lactational differentiation of the mammary epithelium was not observed.

The normal mammary glands of the male rat are composed of adipose tissue, interspersed ducts, and, somewhat surprisingly, lobular tissue. Daily intravenous injection of KGF for 1 week caused ductal and lobular hyperplasia (Figure 4A). The ducts were lined with hyperplastic epithelium indistinguishable from that seen lining the ducts of female rats treated with KGF for 1 week. However, in contrast to the mammary gland of the KGF-treated nulliparous female the gland of the male rat also showed large areas of lobular or acinar epithelial cell growth. In contrast to the basophilic cytoplasm of the hyperplastic ductal cells, the cytoplasm of the hyperplastic acini was eosinophilic (Figure 4A). The acinar cells demonstrate lactational differentiation as evidenced by cytoplasmic vacuolization and immunohistologically detectable expression of lactalbumin (Figure 4B).

The mammary gland of the normal pregnant female rat is enlarged due to acinar differentiation (Figure 5A) in preparation for lactation. The ducts of the normal pregnant female rat are lined by a single layer of cuboidal epithelium (Figure 5A). Intraperitoneal injection of KGF for 3 days (injections on days 17, 18, and 19 of gestation followed by death on day 20) caused ductal epithelial hyperplasia (Figure 5, B and C)

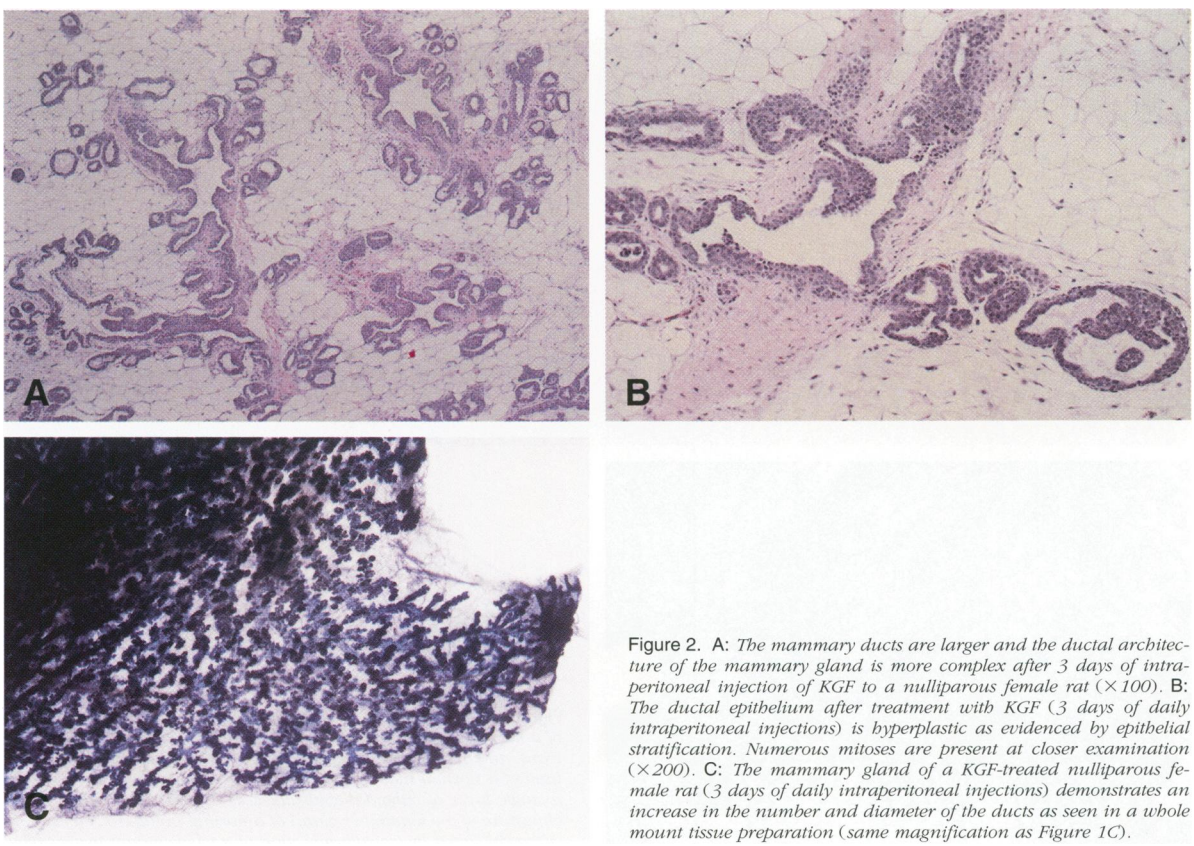

Figure 2. A: *The mammary ducts are larger and the ductal architecture of the mammary gland is more complex after 3 days of intraperitoneal injection of KGF to a nulliparous female rat (×100).* B: *The ductal epithelium after treatment with KGF (3 days of daily intraperitoneal injections) is hyperplastic as evidenced by epithelial stratification. Numerous mitoses are present at closer examination (×200).* C: *The mammary gland of a KGF-treated nulliparous female rat (3 days of daily intraperitoneal injections) demonstrates an increase in the number and diameter of the ducts as seen in a whole mount tissue preparation (same magnification as Figure 1C).*

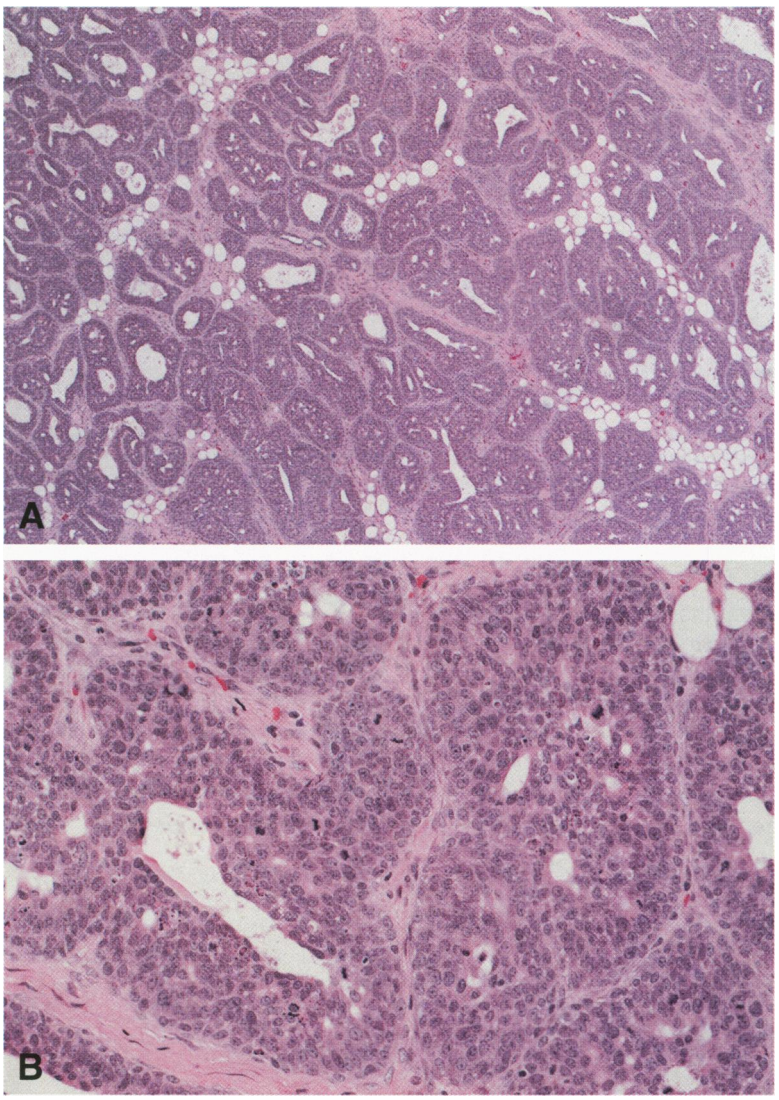

Figure 3. A: *A tremendous increase in the number of ducts is seen in a nulliparous female rat mammary gland after 1 week of daily intraperitoneal injections of KGF (×40).* **B:** *The ductal epithelial hyperplasia in a nulliparous female rat nearly obstructs the lumina of the ducts after 1 week of daily intraperitoneal injections of KGF. Note the many mitotic figures (×400).*

analogous to the ductal hyperplasia noted in nulliparous female rats. An increase in acinar mitoses was also noted in KGF-treated pregnant rats and was indicative of a proliferative action of KGF on the acinar epithelium.

The mammary ducts of the lactating postpartum female rat were lined with a single layer of epithelium and the majority of the breast tissue was composed of distended acini that secrete milk (Figure 6). Interestingly, KGF injected intraperitoneally in postpartum breastfeeding female rats for 3 days during the first postpartum week does not cause the obvious ductal epithelial hyperplasia and increase in acinar mitoses that occurs in pregnant rats. The ductal and acinar epithelium of actively lactating breasts thus appears resistant to the proliferative action of KGF.

The molecular basis for the resistance of the mammary epithelium of lactating mammary glands to KGF could be postulated to be at the level of KGF receptor down-regulation. KGF and KGFR mRNA were therefore quantitated in the mammary glands of day 20 pregnant and postpartum day 6 lactating mammary glands (Figure 7). KGF mRNA is present in the mammary glands of both pregnant and lactating rats, suggesting a role for endogenous KGF in the regulation of the growth of the mammary gland. KGF mRNA is increased in the postpartum lactating mammary gland compared with the mammary gland of late pregnancy. KGFR mRNA is also present within the mammary glands but no significant difference in KGFR levels was noted between pregnant and postpartum glands.

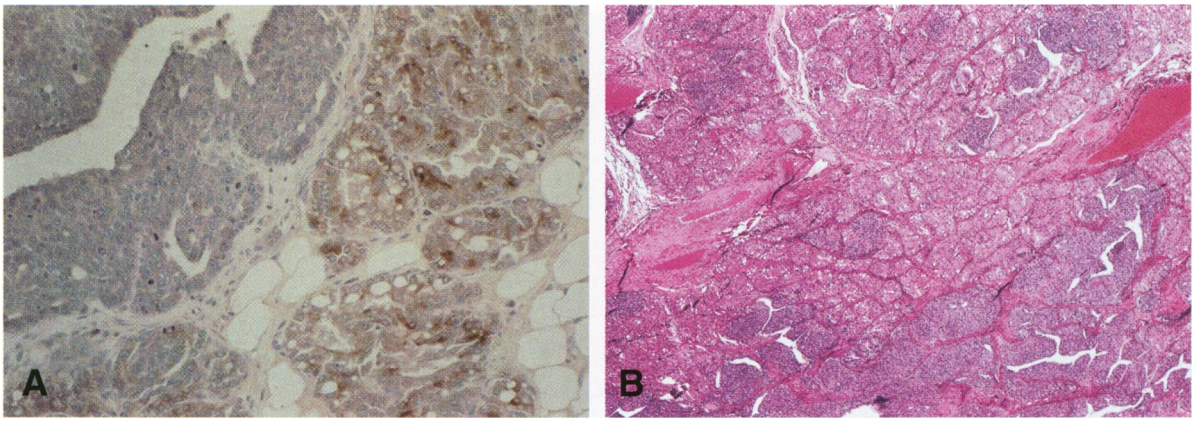

Figure 4. A: *The mammary gland of a male rat treated with KGF for 1 week shows ductal and acinar proliferation (×100).* B: *Lactalbumin, a protein that aids in the synthesis of lactose and is one of the main protein constituents of milk, is immunohistologically detectable as a brown precipitate within the lobular epithelium of a KGF-treated male. An increase in mitoses is noted within both the ductal and lobular epithelium (×400).*

Discussion

KGF is a potent growth factor for mammary epithelium *in vivo*. The interaction of other mammary growth and differentiation factors such as estrogens, progesterones, and prolactin with KGF will be an area of future study. Preliminary experiments in our laboratory with estrogen, progesterone, and KGF have shown that the proliferative effects of KGF in mice are very different than the *in vivo* effects of estrogen and progesterone either alone or in combination. Androgens are reported to stimulate KGF production by prostatic stromal cells[10] and the relationship between female steroid sex hormones, mammary stromal cells, and the secretion of KGF must be investigated. An androgen-induced growth factor that is a distinctive member of the FGF family has been shown to be an autocrine growth factor necessary for the proliferation of an androgen-dependent mouse mammary carci-

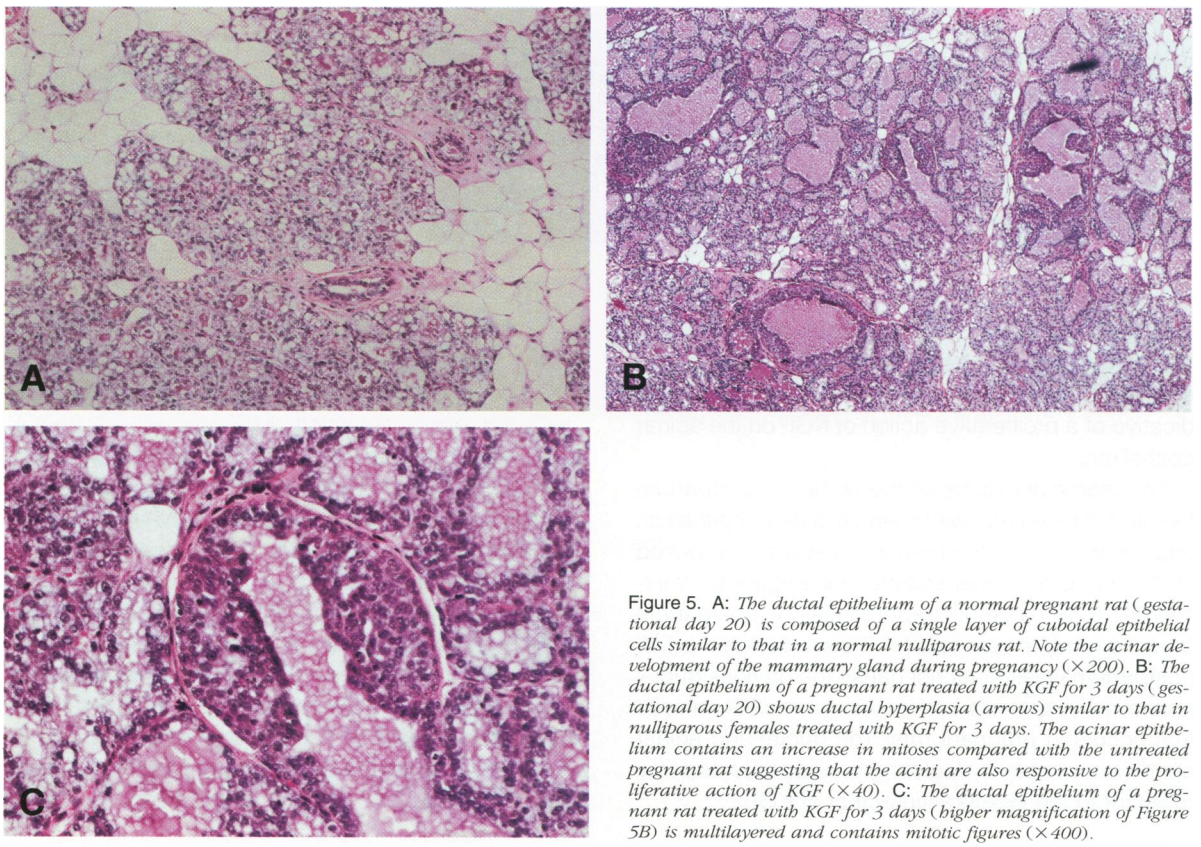

Figure 5. A: *The ductal epithelium of a normal pregnant rat (gestational day 20) is composed of a single layer of cuboidal epithelial cells similar to that in a normal nulliparous rat. Note the acinar development of the mammary gland during pregnancy (×200).* B: *The ductal epithelium of a pregnant rat treated with KGF for 3 days (gestational day 20) shows ductal hyperplasia (arrows) similar to that in nulliparous females treated with KGF for 3 days. The acinar epithelium contains an increase in mitoses compared with the untreated pregnant rat suggesting that the acini are also responsive to the proliferative action of KGF (×40).* C: *The ductal epithelium of a pregnant rat treated with KGF for 3 days (higher magnification of Figure 5B) is multilayered and contains mitotic figures (×400).*

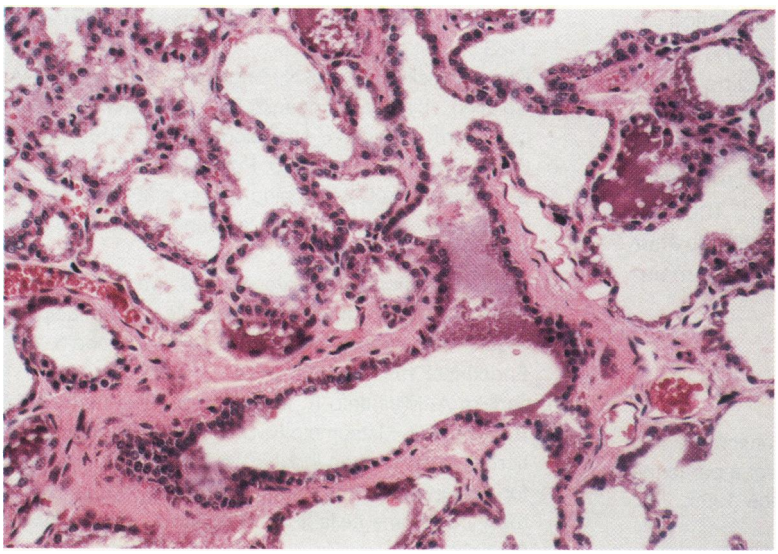

Figure 6. *The ductal and acinar epithelium of a lactating female rat treated with 3 days of KGF during the first week after delivery does not show any morphologically appreciable difference compared with control lactating rats (×400).*

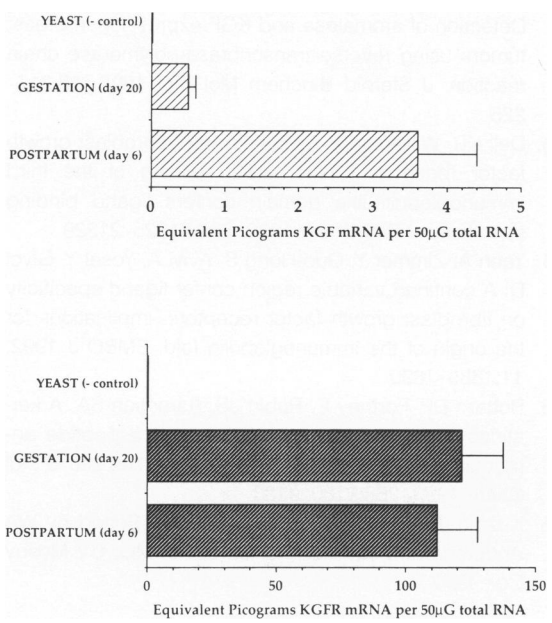

Figure 7. *KGF mRNA is increased in lactating mammary glands compared with the mammary glands of rats during the last days of pregnancy, suggesting a role for KGF in the maintenance of the lactational phenotype. No significant difference in KGFR mRNA levels is noted, suggesting that KGFR down-regulation is not the mechanism for the unresponsiveness of lactational mammary epithelium to recombinant KGF. RNase protection assays were performed as described and the average of triplicate samples with one standard deviation are presented. Yeast total RNA (Ambion, Austin, TX) served as a negative control for both KGF and KGFR messages. The yeast and the gestational day 20 mammary gland RNA both were below visual detection limits in the KGF RNase protection assay.*

noma cell line.[11] KGF mRNA has recently been demonstrated in normal human breast tissue and in 12 of 15 breast tumor samples using the reverse transcriptase polymerase chain reaction technique.[12] The presence of KGF mRNA in breast tumors considered in conjunction with our observations that KGF is present is nonneoplastic rat mammary glands and that KGF causes rampant proliferation of mammary epithelium suggests that KGF may be an autocrine or paracrine growth factor important in the regulation of the growth of normal and neoplastic mammary epithelium. Because KGF has only been detected in stromal cells, future investigations to determine which cells within the breast synthesize KGF will be of interest.

The elucidation of the third immunoglobulin-like domain of the FGF receptor family as the site of the KGF binding domain[13,14] with the development of synthetic receptor-derived peptides as KGF antagonists[15] suggests a possible therapeutic strategy by which to inhibit the growth of breast carcinoma. If KGF is found to be an endogenous growth factor for breast cancer, then antagonists to KGF may provide a new approach to the treatment of the most common form of cancer in women. The cellular localization of KGF and the KGFR in human breast cancers will be important to investigate. Infiltrating ductal mammary adenocarcinoma is characteristically enveloped by a desmoplastic stroma that has been postulated to represent a defensive host response to the carcinoma. The possibility that the stroma is a source of KGF that could contribute to rather than inhibit tumor growth can now be investigated.

The molecular basis for the resistance of lactating mammary epithelium to the proliferative action of systemically delivered KGF is not clear but does not appear related to KGFR mRNA down-regulation. Lactating rats do not exhibit a generalized resistance to the *in vivo* effects of KGF because other KGF-sensitive epithelia such as alveolar pneumocytes and

hepatocytes demonstrate marked proliferation in KGF-treated breastfeeding mother rats. The resistance of the ductal epithelium of breastfeeding rats to the action of KGF is of interest in regard to epidemiological observations that pregnancy in women decreases susceptibility to breast cancer and that dairy cows almost never develop breast cancer.[16] The finding of increased KGF mRNA in mammary glands after onset of lactation suggests a role for KGF in the maintenance of lactational differentiation.

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