Growth hormone treatment induces mammary gland hyperplasia in aging primates

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The decline of growth hormone (GH) and insulin-like growth factor I (IGF-I) production during aging has been likened to the decrease in gonadal steroids in menopause. The repletion of GH/IGF-I levels in aging individuals is suggested to restore the lean tissue anabolism characteristic of youth[1]. In addition to anabolic effects on musculoskeletal tissues, GH also stimulates mammary glandular growth in some species[2-4], although its effects on primate mammary growth remain unclear[5]. Some clinical observations implicate GH in human mammary growth, for example, gynecomastia occurs in some children treated with GH (ref. 6), and tall stature and acromegaly are associated with an increased incidence of breast cancer[7,8]. To investigate the effects of GH/IGF-I augmentation on mammary tissue in a model relevant to aging humans, we treated aged female rhesus monkeys with GH, IGF-I, GH + IGF-I or saline diluent for 7 weeks. IGF-I treatment was associated with a twofold increase, GH with a three- to fourfold increase, and GH + IGF-I with a four- to fivefold increase in mammary glandular size and epithelial proliferation index. These mitogenic effects were directly correlated with circulating GH and IGF-I levels, suggesting that either GH or its downstream effector IGF-I stimulates primate mammary epithelial proliferation.

Female monkeys aged 16–20 years were treated by Alzet pump infusion as described[9]. After 7 weeks, mammary tissue was harvested for histological analysis, and serum was collected for hormonal assays. Increased mammary tissue abundance was grossly obvious in most monkeys in the active treatment groups, especially those receiving GH or GH + IGF-I. To accurately assess glandular as opposed to mammary connective tissue growth, we analyzed the glands morphometrically under direct microscopic visualization.

Mean GH and IGF-I serum levels and mammary glandular growth indices in the different treatment groups are compared in Table 1. IGF-I levels were progressively increased in the IGF-I, GH and GH + IGF-I treatment groups, although because of substantial variation between animals, the increase reached statistical significance only in the last-mentioned group. The number and size of mammary gland lobules also showed progressive increments, reaching statistical significance in the GH and GH + IGF-I groups. Mammary epithelial cell proliferation was assessed by immunodetection of the Ki67 antigen, which is a nonhistone protein associated with the nuclear matrix selectively expressed in proliferating cells[10]. Few Ki67-positive nuclei were detected in glands from diluent-treated animals, whereas IGF-I, GH and GH + IGF-I treatment groups demonstrated progressively increasing proportions of Ki67-positive nuclei (Table 1), which were localized predominantly in terminal ductule epithelial cells (Fig. 1). The increases in mammary epithelial proliferation index were directly proportional to the increases in glandular size in each group, that is, approximately twofold in the group that received IGF-I alone, three- to fourfold in the GH group and four- to fivefold in the GH + IGF-I group, suggesting that augmentation in glandular growth was primarily due to increased proliferation.

Mean estradiol (E2) and progesterone (P4) levels were not different in the four treatment groups [E2 (pg/ml)/P4 (ng/ml): Sal,164 ± 26/0.9 ± 0.1; IGF-I, 182 ± 32/1.04 ± 0.5; GH, 167 ± 18/1.63 ± 0.9; GH + IGF-I, 125 ± 15/0.87 ± 0.5]. A correlative analysis comparing the Ki67 labeling index and serum levels of E2, P4, IGF-I and GH for individual animals is shown in Fig. 2. The Ki67 index was significantly correlated with IGF-I and GH levels ($P = 0.013$ and 0.003, respectively), but not with E2 or P4 levels.

To evaluate the potential for direct GH action on mammary growth, we used *in situ* hybridization histochemistry to identify mammary cells expressing GH receptor (GHR) or prolactin receptor (PRLR) transcripts (Fig. 3), since human GH activates both these receptors[11]. GHR mRNA was not detected in mammary epithelium but was abundant in mammary adipose tissue (Fig. 3, *c* and *d*). PRLR mRNA was, however, concentrated in mammary epithelium (Fig. 3, *e* and *f*). These are the first data on the comparative cellular localization of gene expression for

Table 1 Growth factor levels, mammary morphometry and proliferation (Ki67) index

	Saline	IGF-I	GH	GH+IGF-I
GH (ng/ml)	0.4 ± 0.2	0.5 ± 0.3	12.9 ± 3.8[a]	16.2 ± 6.5[a]
IGF-I (ng/ml)	185.0 ± 36.0	322.6 ± 66.4	432.7 ± 105.8	905.8 ± 260.7[b]
Lobule number	27.0 ± 19.7	73.9 ± 14.6	114.0 ± 14.0[c]	138.9 ± 29.55[a]
Lobule size (mm²)	0.07 ± 0.03	0.14 ± 0.04	0.22 ± 0.03[c]	0.27 ± 0.05[a]
Ki67 index (%)	7.28 ± 3.93	15.3 ± 1.96	30.14 ± 5.26[d]	32.98 ± 1.35[e]

Data are expressed as means ± s.e.m. for five animals in each group.
[a]$P < 0.05$ vs. saline and IGF-I groups.
[b]$P < 0.04$ vs. all other groups.
[c]$P < 0.04$ vs. saline groups.
[d]$P = 0.0003$ vs. saline and 0.009 vs. IGF-I groups.
[e]$P = 0.0001$ vs. saline and 0.003 vs. IGF-I groups.

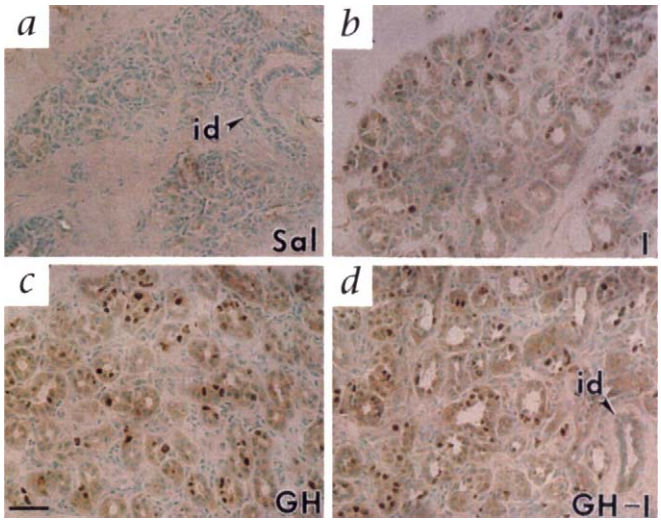

Fig. 1 Mammary epithelial cell proliferation demonstrated by Ki67 immunostaining. A proliferation-specific nuclear matrix protein is detected by the Ki67 antibody and DAB, yielding a brown nuclear stain in positive cells. Representative sections from animals treated with saline (a), IGF-I (b), GH (c) and GH + IGF-I (d) are shown. id, intralobular duct. Scale bar, 50 μm.

these two homologous receptors in primate mammary tissue. The expression of GHR in adipocytes is consistent with GH's well-established lipolytic role, and the expression of PRLR in mammary epithelium fits with prolactin's known lactogenic role. Thus, if GH had direct mitogenic effects on primate mammary epithelium in our study, they must have been mediated by the PRLR, activation of which has been implicated in mammary epithelial proliferation[4,12,13] and tumorigenesis[14]. Another theoretical possibility is that GH acts directly on mammary adipose tissue to stimulate the release of a paracrine effector, which may then act on mammary epithelium.

Although most of GH's somatic growth-promoting effects in humans appear to be due to GH-induced increases in circulating IGF-I levels[15], in rodents GH has also been shown to stimulate local IGF-I production in various tissues, including mammary gland[3]. To determine whether GH-induced local IGF-I production could be implicated in primate mammary growth, we examined mammary IGF-I mRNA expression in the different treatment groups. IGF-I mRNA was localized in stromal cells clustered around intra- and interlobular ducts (suggesting expression by myoepithelial cells, Fig. 3, *g* and *h*) in a pattern identical to that reported in humans[16]. IGF-I mRNA was detected in the diluent- and IGF-I-treated animals, but was more abundant in the GH-treated groups, suggesting that GH enhances local IGF-I production in the primate mammary gland. Although local IGF-I mRNA levels were significantly correlated with circulating GH levels (Fig. 4*b*), they were not correlated with the degree of local epithelial proliferation ($P = 0.174$; Fig. 4*b*). This observation is consistent with recent data showing that mice overexpressing IGF-I in mammary glands did not demonstrate increased mammary epithelial proliferation[17,18]. Completing the picture, we found that IGF-I receptor mRNA is abundant in mammary epithelium (Fig. 3, *i* and *j*).

The present study provides the first experimental evidence that GH treatment augments primate mammary growth. A previous study in juvenile hypophysectomized monkeys examined the effects of GH added to E2 treatment ($n = 3$) and noted an approximately twofold enhancement of E2's growth-promoting effects, which did not, however, appear statistically significant[5]. Potential explanations for the greater effect seen in the present study include the prior normal mammary development and pituitary function of our monkeys, as well as a possible permissive role of progesterone in mammary proliferation. In addition, we studied a much larger number of animals. As to

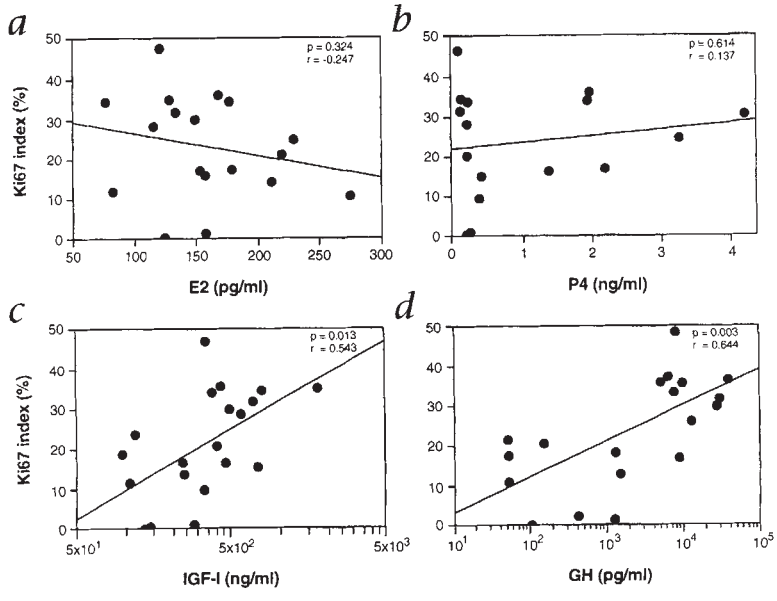

Fig. 2 Correlation analyses between circulating hormone levels and mammary proliferation (Ki67) index. GH and IGF-I values are plotted on a log scale.

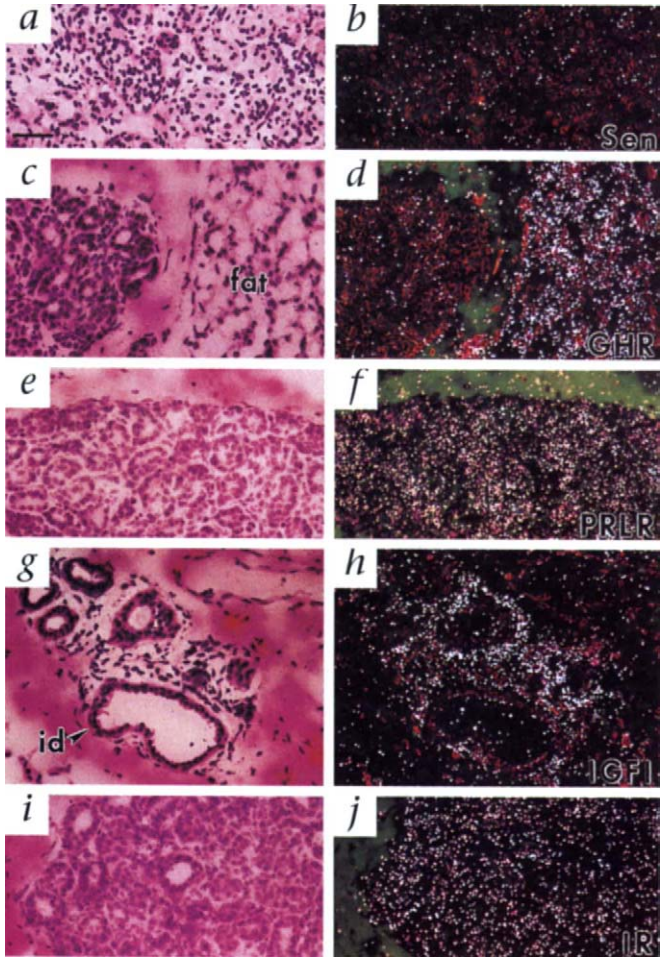

Fig. 3 Cellular pattern of gene expression for GH receptor (GHR, *c* and *d*), prolactin receptor (PRLR, *e* and *f*), IGF-I (*g* and *h*) and IGF-I receptor (*i* and *j*) in primate mammary tissue shown by *in situ* hybridization histochemistry. Sense RNA probe hybridization shows little signal (*a* and *b*). GHR RNA is concentrated in adipose tissue, whereas PRLR and IGF-I receptor RNAs are localized in glandular epithelium. IGF-I mRNA is localized in stromal cells concentrated around interlobular ducts (id). Scale bar, 50 μm.

the mechanism or site of GH's action, the present data suggest that GH may stimulate proliferation directly by acting through the epithelial PRLR, or indirectly, by increasing circulating IGF-I, which then acts through its cognate receptor in mammary epithelium. The latter possibility seems most likely, since some animals in the IGF-I treatment group had significant epithelial proliferation despite relatively suppressed GH levels.

Supplementation of aging individuals with GH is advocated to promote IGF-I's anabolic effects on bone and muscle mass, but IGF-I also has potent effects on the female reproductive system, for example, IGF-I appears to enhance E2's proliferative effects on primate uterine tissue[19]. In fact, some animals in the present study also demonstrated endometrial hyperplasia (O.O.A. *et al.*, unpublished data). Given the association between IGF-I receptor activation and human breast cancer[20], the present data showing mammary epithelial hyperplasia in primates treated with GH or IGF-I suggest that caution be used in the treatment of menopausal women who may be at increased risk for breast cancer. Although some animals in this study attained GH/IGF-I levels in the acromegalic range, the correlation between mammary hyperplasia and IGF-I level extended across a broad range of concentrations, with mitogenic effects apparent at levels considered physiological, at least for young adults (250–500 ng/ml, Fig. 2*c*).

The repletion of GH/IGF-I in young and middle-aged adults with GH deficiency (for example, due to pituitary ablation) has beneficial effects on musculoskeletal tissues[21] although its value in older patients, particularly those with GH deficiency acquired late in life, is uncertain[22]. The GH dose in GH-deficient adults is typically lower than that used in this study, which was patterned after the regimen used to treat adults with catabolic states such as AIDS who are not GH deficient[23]. It remains to be determined whether GH-deficient patients should receive lifelong GH treatment and what IGF-I levels should be targeted, especially as they age, since GH and IGF-I normally decline with aging. At present, it is not clear what constitute "physiological" GH/IGF-I levels for the aging population, and it seems possible, as a matter of speculation, that the decline in GH/IGF-I occurring during these years may actually be protective for tissues that have accumulated many years of oxidative or genetic damage.

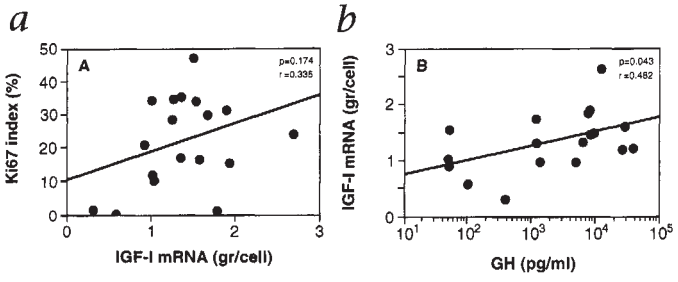

Fig. 4 Correlation between mammary IGF-I mRNA levels and mammary epithelial proliferation (*a*) and circulating GH levels (*b*).

Methods

Animals. Twenty female rhesus monkeys 16–20 years of age were used in accordance with a protocol approved by NIDDK and NICHD animal care and use committees. The animals were randomly assigned to four treatment groups (n = 5 each) receiving subcutaneous infusions by Alzet minipump: (1) saline diluent, (2) IGF-I at 120 μg/kg per day, (3) GH at 100 μg/kg per day, (4) both GH and IGF-I as previously described[9]. Tissue and serum were obtained after 7 weeks of treatment. Serum E2 and P4 levels were measured by radioimmunoassay (RIA) at Hazleton Laboratories (Vienna, Virginia). IGF-I levels were measured by RIA, and GH levels were measured by ELISA at the Genentech Laboratories (South San Francisco, CA). Mammary tissue was snap-frozen, and 10-μm cryostat sections were cut through the entire mass of glandular tissue for each breast.

Morphometry. Six tissue sections spaced 200 μm apart spanning the greatest diameter of each mammary gland were analyzed for lobular number and size. The number of lobules in each section was counted under low magnification microscopy. Lobular size was determined by image analysis using NIH Image v1.57 software. Individual lobules were outlined using cursor control. The mean of the areas of the ten largest lobules in each section was determined for each animal, and these values were pooled to obtain group means.

Proliferation index. Immunohistochemistry for Ki67 using a Boehringer-Mannheim antibody was performed in fresh-frozen tissue sections as previously described[19]. Ki67-positive epithelial nuclei were scored at ×200 magnification and expressed as percent of total epithelial nuclei. At least 200 cells were counted in each of three to six sections for each animal.

***In situ* hybridization.** The protocols for synthesizing ^{35}S-labeled RNA sense and antisense probes and performing *in situ* hybridization on frozen sec-

tions have been described[19]. IGF-I mRNA levels were quantified by counting silver grains per stromal cell at ×400. Counts were obtained for two or three sections per animal, which were hybridized, exposed and developed in a single batch. Background signal obtained from counting grains over stromal cells in sense probe hybridized sections was subtracted and group means obtained.

Statistical analysis. Data are expressed as means ± s.e.m. Differences among means were compared among the four treatment groups using one-way ANOVA followed by Fisher's least significant difference tests. Correlation analysis was performed using simple linear regression.

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