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HIGH FREQUENCY OF MAMMARY ADENOCARCINOMAS IN METALLOTHIONEIN PROMOTER-HUMAN GROWTH HORMONE TRANSGENIC MICE CREATED FROM TWO DIFFERENT STRAINS OF MICE

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Summary—Transgenic mice were developed by injecting a mouse metallothionein promoter-human growth hormone (Mt-hGH) gene fragment into the pronucleus of C57BlxDBA/2J-f_2 or C57BlxCBA-f_2 one cell embryos. Six founder animals with the C57BlxDBA genetic background grew 1.3–2.2 times larger than littermate controls and had higher levels of hGH in plasma (4.6–279 mU/l). Three of the four female transgenic founders developed malignant papillar adenocarcinomas of mammary origin at 27–43 weeks of age. One male transgenic founder was successfully mated and two of three female transgenic offsprings developed mammary tumors. To examine if the tumor induction was dependent on the strain of mice used the experiments were repeated using animals with different genetic background. Fourteen female hGH transgenic mice from five founder animals were generated using C57BlxCBA-f_2 mice. Thirteen of the animals had elevated levels of hGH in plasma (7–1960 mU/l) and grew larger than control animals. Nine of the animals developed mammary adenocarcinomas. Four of the hGH expressing animals did not demonstrate macroscopic tumor formation but have not yet been analyzed histologically. The present study suggests that markedly elevated endogenous levels of GH cause mammary carcinoma in hGH transgenic mice. The present animal model might prove useful for studying molecular mechanisms involved in the development of hormonally induced mammary tumors.

INTRODUCTION

Growth hormone (GH) is an important regulator of somatic body growth but its possible role in pathological growth (e.g. tumor formation) is less clear. Patients suffering from acromegaly, a disease in the human with an abnormal increase in production of GH, develop in addition to increased body growth malignant disorders more frequently than the normal population [1–3]. A general increase of malignant tumors [1, 3], an overrepresentation of tumors in the female mammary gland [2] and in the colon [4] have been reported. However, other investigators have not been able to demonstrate a significant increase in number of deaths due to malignant diseases in patients with acromegaly [5].

Transgenic mice are a useful tool for studying the effect of chronically elevated levels of GH. Palmiter and co-workers [6–9] have used transgenic animals in a series of experiments to clarify the role of GH in regulation of somatic growth. In addition, GH transgenic animals have been reported to develop hepatocellular carcinoma [10] as well as other pathological lesions [11]. Mammary tumors show a high frequency in patients with elevated serum GH levels. Female human-GH (hGH) transgenic mice develop mammary adenocarcinomas at a high incidence [12]. However, other investigators [11] failed to demonstrate mammary tumors in GH transgenic animals. Induction of mammary tumors in mice is a multifactorial process involving retroviruses (e.g. mouse mammary tumor virus; MMTV), hormones [13, 14], (proto-)oncogenes [15–18] and probably several other as yet unidentified factors. One difference between studies showing an induction of mammary carcinomas in hGH transgenic mice and those failing to demonstrate this phenomenon is the strain of mice used. The aim of this study was to compare induction of mammary carcinomas in transgenic mice with different genetic

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backgrounds. We used a mouse metallothionein I promoter-hGH (Mt-hGH) fusion gene to obtain high levels of GH in plasma [7]. We now report that mammary gland tumors occur with a high frequency in GH transgenic mice generated from the C57BlxDBA or C57BlxCBA strain.

EXPERIMENTAL

Generation of transgenic animals

A BstEII–NdeI fragment was isolated from the plasmid Mt-hGH 111 [7] and used for injection. This DNA fragment contains the mouse metallothionein I promoter linked to a genomic sequence encoding hGH. The isolated fragment was injected into the pronucleus of C57Bl/6JxDBA/2J-f_2 or C57Bl/6JxCBA-f_2 embryos obtained after pregnant mare's serum gonadotropin/human chorion gonadotropin treatment for superovulation of the mice. The C57Bl/6JxDBA/2J- and the C57Bl/6JxCBA-f_1 animals were obtained from Bomholtgård Breeding and Research Centre Ltd (Ry, Denmark). The injections were performed using Narishigi hydraulic micromanipulators and a Nikon inverted microscope [19]. After injection, the embryos were implanted in pseudopregnant f_1 recipients of the same strain as the embryos. Mice that had integrated the GH gene were identified with dot blot analysis of DNA from tail biopsy specimens obtained 3 weeks after birth of the animals using a ^{32}P-labeled PvuII fragment of the hGH gene as a probe. Positive results were confirmed with Southern blot analysis using the same probe.

Measurement of hGH

The concentration of hGH in serum was determined using a hGH RIA kit (Pharmacia Diagnostics AB, Uppsala, Sweden). The antibody did not react with rat GH.

Histology

Dissection of the animals and histological examination of the tumours were performed at the National Veterinary Institute (Uppsala, Sweden). The tissues were fixed in formalin (pH 7.4) for 24 h at room temperature and stained with hematoxylin/eosin.

Statistical analysis

Chi-squared analysis was used to compare the incidence of tumors in Mt-hGH transgenic animals with that in normal animals. $P < 0.01$ was considered statistically significant.

RESULTS

To generate Mt-hGH transgenic animals with the C57Bl/6JxDBA/2J genetic background 480 injected C57Bl/6JxDBA/2J-f_2 embryos were implanted into 23 C57Bl/6Jx/DBA/2J-f_1 foster mothers resulting in 44 newborn mice. Six mice were identified as carrying Mt-hGH sequences (founder animals) using dot blot analysis and the presence of the injected DNA was confirmed with Southern blot analysis. Mt-hGH transgenic mice with the C57Bl/6JxCBA genetic background were created by implantation of 303 injected C57Bl/6JxCBA-f_2 embryos into 15 C57Bl/6JxCBA-f_1 foster mothers. Fifty-two pups were born and 7 founder animals were identified using dot blot and Southern blot analysis. Of this strain only female animals were included in this study.

Macroscopic examination

C57Bl/6JxDBA/2J. The weight of the 6 founder animals was between 130 and 220% of littermate controls (Table 1). Palpable mammary tumors were detected in 3 of the 4 female transgenic founder mice at 27–43 weeks of age whereas none was found in the control group consisting of 42 female mice older than 43 weeks

Table 1. Sex, weight, hGH levels, age at discovery of the tumors, presence of mammary tumors and pregnancies in 4 female founder mice of the strain C57BlxDBA

Founder	Weight[a] (%)	hGH[b] (mU/l)	Age[c] (weeks)	Phenotype	Pregnancy
4 Fo	220	279	43	Mammary adenocarcinoma	Yes
35 Fo	200	111	27	Mammary adenocarcinoma	Yes
36 Fo	170	40	27	Mammary adenocarcinoma	No
38 Fo	140	22	41	N/A	No

[a]Weight at 8 weeks of age expressed as percent of the weight of normal mice of same age.
[b]hGH determined in serum by RIA.
[c]Age at which the mouse was sacrificed.

Table 2. Sex, weight, hGH levels, age at discovery of the tumors, presence of mammary tumors and pregnancies in the female offsprings of 1 of the founders (37 Fo) C57BlxDBA-f_2-embryos were used to generate the transgenic animals

Number	Weight[a] (%)	hGH[b] (mU/l)	Age[c] (weeks)	Phenotype	Pregnancy
37F1.2	190	9.8	37	Normal	No
37F1.3	180	39	40	Mammary adenocarcinoma	No
37F1.4			30	Mammary adenocarcinoma	No

[a]Weight at 8 weeks of age expressed as percent of the weight of normal mice of same age.
[b]hGH determined in serum by RIA.
[c]Age at which the mouse was sacrificed.

and of the same genetic background ($P < 0.01$ vs control group). The female transgenic mouse that did not develop a palpable mammary tumor showed a low concentration of circulating hGH (22 mU/l) and the smallest increase in body growth among the female mice (140% weight of littermate controls). This mouse was not examined histologically. One male founder was successfully mated with a normal C57Bl6JxDBA/2J female and received 4 transgenic offsprings. Two of three female animals developed mammary tumors (Table 2). Both virgin and previously pregnant mice developed tumors.

C57Bl/6JxCBA. Fourteen female Mt-hGH transgenic mice were generated from 5 different founders. Thirteen of them had elevated levels of hGH in serum (7–1960 mU/l) and grew larger than control mice (135–240% weight of control mice; Table 3). Nine of these mice developed palpable mammary tumors at 29–55 weeks of age (Table 3). Both previously pregnant and virgin mice were affected. As controls served 24 female mice older than 40 weeks and of the same genetic background. None of the control mice showed any palpable mammary tumor. In the male transgenic mice no palpable tumors were discovered (data not shown).

Determinations of hGH serum levels

The expression of the integrated Mt-hGH gene was evaluated by measuring the serum levels of hGH by RIA. The level of hGH varied between 4.6 and 1960 mU/l serum in the transgenic animals (Tables 1–3). In serum from normal mice the average serum level was 1.9 ± 0.42 mU/l (mean $\pm$ SEM, $n = 38$).

Tumor histology

The mammary tumors were malignant papillar adenocarcinomas with necrotic and cystic parts (Figs 1 and 2). Two of the mice with mammary tumors showed tumor nodules in different parts of the mammary glands. One tumor was a solid, less differentiated mammary carcinoma and the other a papillar adenocarcinoma. The two tumors in this mouse are therefore suspected to be of separate origins. The lungs of this mouse contained solid and acinar metastatic adenocarcinomas. Another mouse displayed metastasis to the regional lymphnodes. Five of the control mice were

Table 3. Weight, hGH levels, age at discovery of the tumors, presence of mammary tumors and pregnancies in 12 female mice of the strain C57BlxCBA

Animal	Weight[a] (%)	hGH[b] (mU/l)	Age[c] (weeks)	Mammary adenocarcinoma	Pregnancy
57F1 6	185	470	33	Yes	No
57F1.8	170	377	55	Yes	No
59F2.1	240	1140	29	Yes	No
59F2.2	220	1395	37	Yes	No
59F2.4	140	7	45	Yes	Yes
59F2.10	230	1670	29	Yes	No
59F2.11	210	1545	34	Not macroscopic	No
59F2.13	250	1960	29	Yes	No
60FO	105	0	58	Not macroscopic	No
62FO	135	67	54	Not macroscopic	No
63FO	225	249	43	Yes	Yes
63F1.1	175	410	30	Not macroscopic	No
63F1.4	175	290	48	Yes	No
63F1.5	155	572	48	Not macroscopic	No

[a]Weight at 8 weeks of age expressed as percent of the weight of normal mice of same age.
[b]hGH determined in serum by RIA.
[c]Age at which the mouse was sacrificed.

Fig. 1. A papillar adenocarcinoma in the mammary gland The micrograph shows an area displaying necrotic zones (N) and cholsterol clefts. 32 × magnification.

sacrificed and sent for histological examination. No tumor formation in the mammary gland could be demonstrated (data not shown).

DISCUSSION

The present study demonstrates that Mt-hGH transgenic female mice created from mice from two different strains and with elevated plasma levels of hGH develop mammary tumors at high frequency. Only female transgenic mice developed tumors and the disposition to develop tumors was not dependent on previous pregnancies.

Human female patients with acromegaly have a high incidence of mammary tumors [2] and 40% of breast cancer patients have elevated GH levels [20]. Because hGH can bind both to somatogenic GH-receptors and lactogenic prolactin (PRL) receptors [21], it is not clear

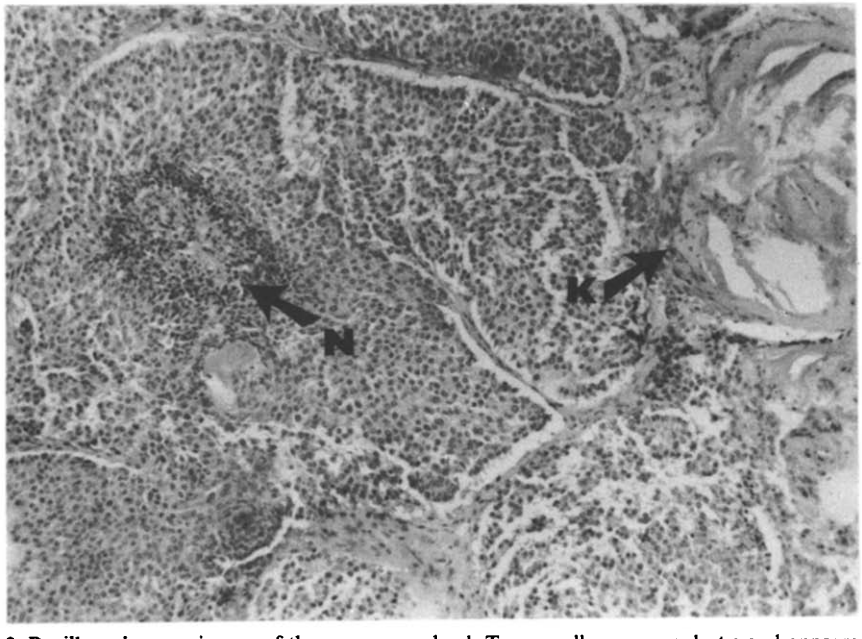

Fig. 2. Papillar adenocarcinoma of the mammary gland Tumor cells were anaplastic and appeared in a solid arrangement. Squamos metaplasia with keratinization (K) and necrosis (N) were abundant 128 × magnification

whether GH- or PRL-receptors are activated or a combination of both. Several findings are consistent with the notion that the effect of GH on the mammary gland is mediated via somatogenic GH-receptors. GH-receptor-specific mRNA has recently been found in lactating bovine mammary gland [22] and in rat mammary gland [23] although binding of GH to somatogenic mammary GH-receptors have been difficult to demonstrate [24]. Growth of the rat mammary gland was more stimulated by hGH and rat GH (which do not bind to PRL-receptors) than by hPRL or rat PRL [25]. Furthermore, regression of chemically induced mammary tumors in rats was seen after suppression of GH [26].

Some studies support the hypothesis that PRL is important for development of mammary tumors. PRL receptors are present in up to 50% of human breast tumors (for references see [27]) and pituitary isografts, which are not under the control of the hypothalamus and secrete increased amounts of PRL, can substitute for pregnancy when pregnancy dependent mammary tumors are chemically induced (for references see [14]). However, it has not been possible to demonstrate increased levels of PRL in patients with mammary tumors and attempts to depress the levels of PRL to induce tumor remission have given inconsistent results. GH may influence the mammary gland directly, by increasing the levels of estradiol or progesterone secretion from the ovaries or by increasing the levels of insulin-like growth factor 1 (IGF-1) production from the liver. Whether the ovaries are necessary for tumor induction in the GH transgenic animals or if the serum levels of estradiol, progesterone or IGF-1 are increased have not been examined in the present study.

An increased frequency of mammary tumors was not observed in previous investigations employing the same Mt-hGH construct [7, 11]. the reason for this discrepancy might be that a strain of mice of different genetic background (C57Bl or C57BlxSJL) was used in this previous study. We have tested two different strains of mice in this study and both of them develop mammary tumors at a high incidence. An obvious genetic difference between different strains of mice of interest for studies of mammary tumors, is the presence of mouse mammary tumour virus (MMTV). This retrovirus integrates near the *int*-1 or *int*-2 genes and induces mammary tumours by activating them [28]. This has been shown by expressing *int*-1 [16] or *int*-2 [18] in the mammary gland of transgenic mice which leads to tumor formation and hyperplasia, respectively. The DBA strain has a tumorigenic insertion of MMTV on chromosome 7 [29] which also harbors the *int*-2 gene [19]. C57Bl has a low incidence of spontaneous mammary tumors as has the f_1-hybrid of C57BlxDBA/2f (for references see [13]). The CBA strain contains MMTV integrations on chromosome 6, 12 and 16 [29] whereas *int*-1 is located on chromosome 15 and *int*-2 on chromosome 7 [19]. Because the C57BlxCBA hGH transgenic mice develop mammary tumors as frequently as the C57BlxDBA hGH transgenic mice, MMTV activation of the *int*-1 or *int*-2 genes may not be responsible for tumor formation in the GH transgenic mice. At physiological plasma levels GH is not likely to induce mammary tumors. Our data indicate that animals with high serum GH levels and large body size develop macroscopic mammary adenocarcinomas more often than transgenic animals with low serum GH levels. The present study demonstrate that hGH transgenic animals frequently develop mammary adenocarcinomas in genetically disposed animals. The GH transgenic animals might serve as a useful model for hormonally induced breast cancer.

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