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Evidence for the involvement of the submandibular gland epidermal growth factor in mouse mammary tumorigenesis

(mammary tumor growth/sialoadenectomy)

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ABSTRACT **The submandibular gland is a rich source of epidermal growth factor (EGF) in mice. The concentration of EGF in the gland of virgin female mice of C3H/HeN strain increased as much as 9-fold from the age of 30 to 52 weeks. During this period, the incidence of mammary tumor in virgin females increased markedly to a maximal level of 62.5% (n = 48) at 52 weeks of age. Removal of the submandibular gland (sialoadenectomy) of virgin mice 14–22 weeks old reduced the tumor incidence to 12.8% (n = 39) at the age of 52 weeks and also increased the latency period of mammary tumor development as much as 14 weeks when compared to that of normal mice. Long-term treatment of sialoadenectomized virgin mice with EGF (5 μg per mouse every other day) increased the tumor incidence to 33.3%. Moreover, sialoadenectomy of mammary tumor-bearing animals caused a rapid and sustained cessation of tumor growth, but EGF administration (5 μg per mouse per day) quickly restored the rate of tumor growth to a normal level. These results indicate that submandibular gland EGF plays a crucial role in mouse mammary tumorigenesis.**

Epidermal growth factor (EGF), a single-chain polypeptide consisting of 53 amino acid residues, is produced by the mouse submandibular gland (1). EGF has diverse biological actions both *in vivo* and *in vitro*, influencing proliferation, differentiation, and functional activities of various types of cells (1, 2). EGF has been shown to stimulate proliferation of mouse mammary epithelial cells (3–6), whereas it inhibits functional differentiation of these cells (5, 6). Our recent studies involving pregestational sialoadenectomy of female mice have indicated that EGF plays a physiological role in the development of the mammary gland during pregnancy (7, 8).

In recent years, several lines of evidence have implicated EGF in the processes of neoplastic transformation. It has been shown that EGF stimulates cell proliferation in cultured tumor cells (1, 9, 10), enhances the carcinogenicity of methylcholanthrene in mouse skin tumor (11), and potentiates the tumorigenicity of Kirsten sarcoma virus in rat ovarian granulosa cells (12). EGF has also been shown to share amino acid sequence homology with transforming growth factor (13, 14). More recently, it was reported that the EGF receptor has a strong amino acid homology with the product of an oncogene, avian erythroblastosis virus (15).

Earlier studies have established that certain strains of mice, such as C3H, have a high incidence of mammary tumors (16, 17). Formation of these tumors has been shown to be related to a virus that is transmitted by the milk of females to the young (16, 17). Because of accessibility to palpation and predictable frequency, this mammary tumor system provides an invaluable tool in various aspects of cancer research.

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Based on our previous studies of EGF in the development of the mouse mammary gland (5–8) and the findings that EGF mimics the effect of the tumor promotor phorbol ester in this system (18), it was of interest to assess a possible role of the submandibular gland EGF in mouse mammary tumorigenesis. In the present study, we have taken several experimental approaches such as sialoadenectomy and EGF replacement to examine the role of EGF in the processes of mammary neoplasia *in vivo*. Our data indicate that submandibular gland EGF plays a critical role in the growth of mammary tumors in mice.

MATERIALS AND METHODS

Materials were purchased as follows: mouse EGF (receptor grade) and rabbit anti-EGF antiserum used for radioimmunoassay was from Collaborative Research (Waltham, MA), ^{125}I-labeled EGF was from New England Nuclear, goat anti-rabbit IgG was from Miles. Mouse EGF used for injection was from Bethesda Research Laboratories.

Immature female mice of C3H/HeN strain were obtained from the Animal Breeding Facility (National Institutes of Health). Mice were maintained under controlled air and temperature (25°C) as well as under 12 hr of light (0800–2000 hr) and 12 hr of darkness (2000–0800 hr). Animals had free access to food and water at all times.

Radioimmunoassay. The radioimmunoassay of EGF in submandibular extracts was performed by a liquid-phase double-antibody method as follows: 100 μl of diluted submandibular extracts or unlabeled EGF standards ranging from 0.05 to 20 ng was mixed with 100 μl of phosphate-buffered saline (P_i/NaCl) containing ^{125}I-labeled EGF with ≈15,000 cpm, 1% bovine serum albumin, and 0.1% sodium azide (buffer A); 100 μl of rabbit anti-EGF antiserum diluted 1:50,000 with buffer A containing 1% normal rabbit serum was then added. The reaction mixtures were incubated for 3 days at 4°C. Antibody-bound and free ^{125}I-labeled EGF were separated by adding 200 μl of 7% goat anti-rabbit antiserum and were incubated at 4°C for an additional 24 hr. The subsequent precipitate was collected by centrifugation at 8700 × g for 5 min and washed three times in buffer A to remove unbound radioactivity. Radioactivity in the precipitate was measured in a well-type γ counter. The standard curve was prepared with 10 different amounts of mouse EGF.

Submandibular gland extracts were prepared by homogenizing the tissues in 20 vol of cold P_i/NaCl (wt/vol) using a Polytron homogenizer. The homogenates were centrifuged at 12,000 × g for 20 min at 4°C, and aliquots of the supernatant were removed and stored at −20°C until assayed. Submandibular gland extracts were diluted with buffer A and assayed in duplicate using two dilutions.

For the determination of the mammary tumor incidence in virgin female mice, 93 age-matched animals were randomly

Abbreviation: EGF, epidermal growth factor.

divided into three groups. One group received sialoadenectomy at the age of either 14 weeks (n = 21) or 22 weeks (n = 18). The second group consisted of 6 mice that were sialoadenectomized at 14 weeks of age and given EGF. EGF was administered subcutaneously at a dose of 5 μg per mouse in 0.1 ml of normal saline every other day up to 52 weeks of age. The third group, consisting of mice sham-operated at 14 weeks or 22 weeks of age (n = 48), served as control. Occurrence of mammary tumors in these groups of mice was examined by routine palpation until the animals were 52 weeks old. The size, number, and location of mammary tumors were monitored throughout the experiment. The size of tumors was determined by measuring them in two dimensions using a vernier caliper, and it is expressed as a product of length (cm) times width (cm).

Histological examination was carried out by tissue biopsy of mammary tumor and normal glands. Tissue fragments were fixed in 10% formaldehyde, embedded in paraffin wax, and cut in 5-μm-thick sections. Each section was stained with hematoxylin and eosin.

RESULTS

As shown in Fig. 1, the concentration of EGF in the submandibular gland of virgin female mice remained virtually constant at a level of 46 ng per mg of tissue from the age of 8–24 weeks. However, the glandular EGF level began to increase at 30 weeks of age and reached a plateau level (400 ng per mg of tissue) at 40 weeks of age. This level was maintained up to 52 weeks of age.

Virgin female mice of C3H strain have a high incidence of mammary tumors during the latter half of the first year of life (16, 17). To examine the possible physiological role of the submandibular EGF in mammary tumorigenesis, sialoadenectomy (removal of the submandibular gland) was performed on virgin females 14 or 22 weeks old, and the incidence of mammary tumors was examined up to 52 weeks of age. No apparent signs of abnormality were found in sialoadenectomized animals during the entire experimental period, as judged by their body-weight gain, uptake of food and water, and gross appearance.

Fig. 2 shows the incidence of mammary tumor as a function of age in normal (control) and sialoadenectomized virgin mice. Mammary tumors in normal females were first detected at 31 weeks of age, and thereafter the tumor incidence increased progressively with age. The most rapid increase occurred between 42 to 46 weeks of age. The tumor incidence reached a plateau level at 52 weeks of age, which amounted to 62.5%. This value is in good agreement with that reported previously (16). It is noteworthy that the temporal pattern of mammary tumor incidence closely follows the increase in the submandibular gland EGF that occurs after 30 weeks of age (Fig. 1). By contrast, the incidence of mammary tumor was greatly reduced in sialoadenectomized mice: it was only 12.8% at 52 weeks of age. No difference in the tumor incidence was observed in mice sialoadenectomized at the age of 14 or 22 weeks. Moreover, the latency period of tumor incidence was prolonged as much as 14 weeks in sialoadenectomized mice when compared with that of normal females. Mice that were sialoadenectomized at 14 weeks of age and then given EGF treatment (5 μg per mouse) every other day up to the age of 52 weeks had a 33.3% of incidence of mammary tumor (data not shown). This was significantly higher than that in sialoadenectomized mice not given EGF ($P < 0.05$).

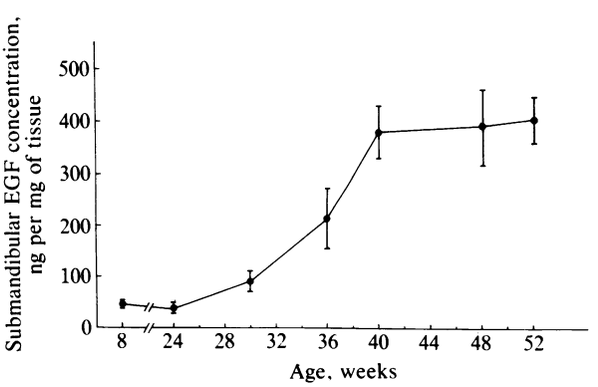

FIG. 1. EGF concentration in submandibular glands of virgin mice at various ages. Each point denotes the mean ± SEM of 4–6 mice. EGF levels were significantly increased at 30 weeks ($P < 0.05$), at 36 weeks ($P < 0.02$), and at 40–52 weeks ($P < 0.001$) when compared with those at 24 weeks of age.

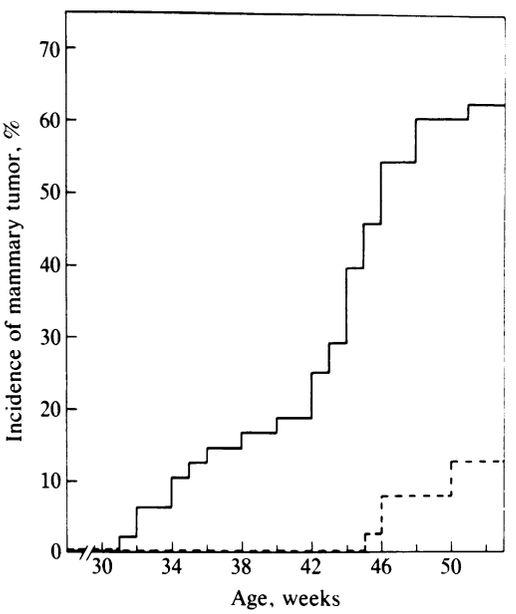

FIG. 2. Mammary tumor incidence in normal and sialoadenectomized virgin female mice at various ages. The tumor incidence was monitored by using 48 normal virgin (—) and 39 sialoadenectomized virgin mice (---) over a period of 52 weeks.

In the study described above, we also found that in the control group the number of mammary tumors per mouse varied from 1 to 5 with an average of 1.6, whereas sialoadenectomized tumor-bearing mice usually had only 1 tumor per mouse. In addition, the maximal growth rate of tumors in normal mice was roughly twice as fast as that in sialoadenectomized mice (data not shown). Autopsy examination revealed no macroscopic sign of metastasis of mammary tumor in either group of animals.

Histological examination of mammary tumors found in normal female mice indicated that they are composed of small cuboidal epithelial cells (Fig. 3*A*). The glandular pattern was greatly distorted and the amount of stroma was relatively small. These features resemble type B adenocarcinoma, which is common in C3H strain of mice (17). The appearance of the tumors found in sialoadenectomized mice indicated a somewhat more organized pattern of ductal and glandular structure (Fig. 3*B*). In addition, these tumors contained more stromal elements (Fig. 3*B*). Fig. 3 *C* and *D* are sections of normal mammary glands of control and sialoadenectomized mice, respectively, at 52 weeks of age. The glands contain largely ductal epithelial cells surrounded by fat cells. The mammary epithelial cells in these glands show virtually no sign of mitotic or secretory activities.

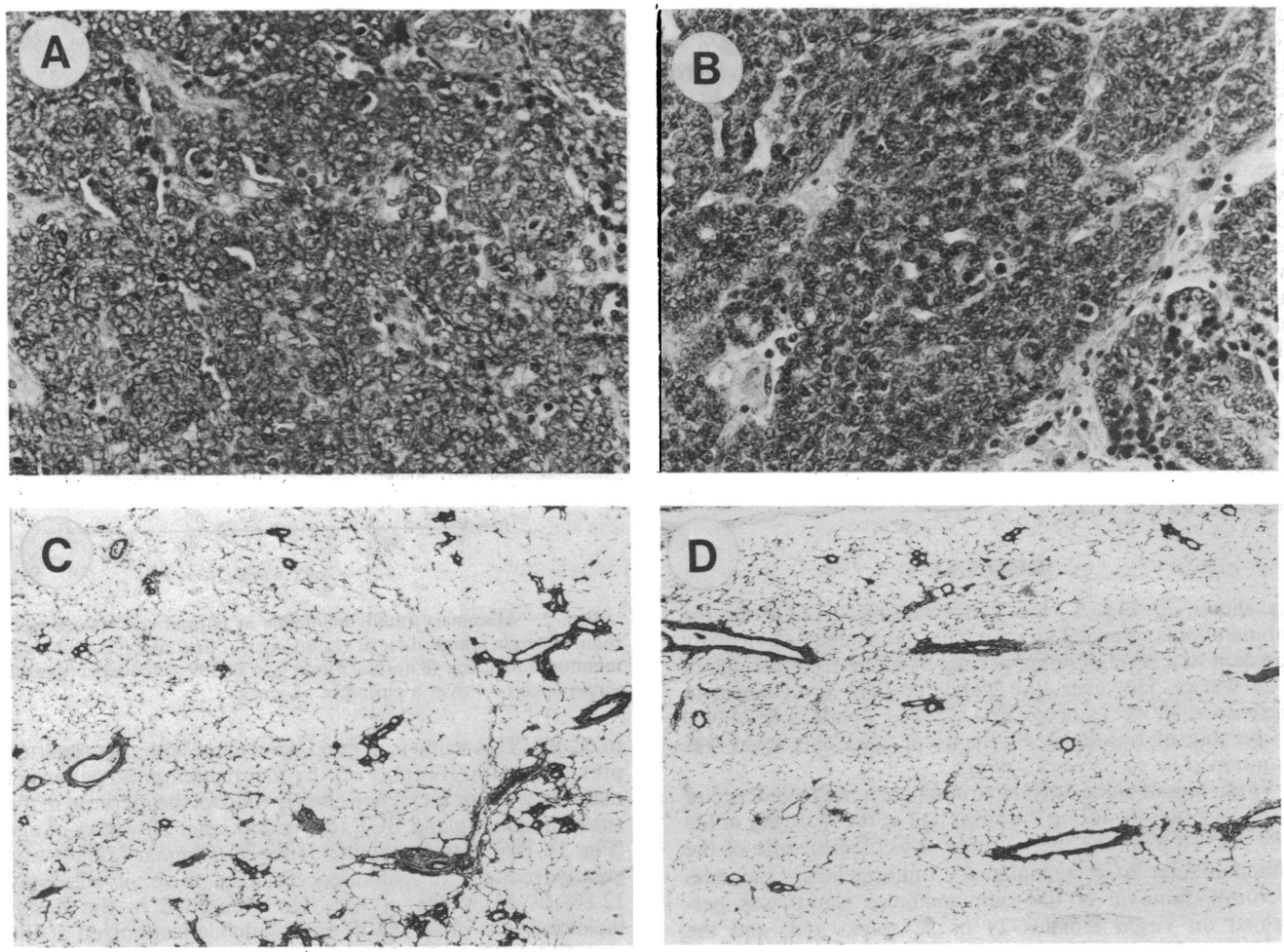

FIG. 3. Histological sections of mammary tumors in control mice (*A*) and sialoadenectomized mice (*B*), and mammary tissues from tumor-free control (*C*) and sialoadenectomized mice (*D*). (*A* and *B*, ×255; *C* and *D*, ×40.)

The results presented above demonstrate that long-term sialoadenectomy substantially decreased the incidence of the mouse mammary tumor. As an extension of these studies, it became of interest to examine the short-term effect of sialoadenectomy on the growth of the tumors. The results of such experiments are shown in Fig. 4. Normal virgin females bearing mammary tumors were divided into two groups. One group served as control; the others were sialoadenectomized on the 20th day after the detection of tumor. It is seen that mammary tumors in the control group grew extensively during 58 days, whereas sialoadenectomy completely prevented further growth of the tumor. Moreover, when EGF was administered daily to those sialoadenectomized mice at later times, the tumor quickly resumed its growth at a rate similar to that in the control group over the ensuing 30 days. Other experiments not presented here indicated that when sialoadenectomy was performed on tumor-bearing mice at different times, it invariably inhibited the growth of mammary tumor for a period of 30–60 days.

DISCUSSION

It has been well established that the C3H strain of mice has a high incidence of mammary tumors during the later part of life (16, 17). Earlier studies demonstrated the importance of an agent in the milk of the females, which is transmitted to the young (16, 17). This agent has been shown in later studies to be a virus (16, 17). It has been recognized that the etiology of mammary tumor is complex, involving viral, genetic, and humoral factors (16, 17, 19). It is generally thought that the virus modifies the responsiveness of the mammary tissue, so that with a favorable genetic constitution and the proper hormonal stimuli, some mammary epithelial cells develop into mammary tumor (19).

The submandibular gland has been shown to serve as a major source of EGF in mice (1, 2). Earlier studies (3–6) have indicated that EGF exerts profound biological actions on mouse mammary epithelial cells by affecting their proliferation and functional differentiation in *in vitro* systems. It has been also shown (5) that normal mouse mammary epithelial cells possess specific EGF receptors. Recently, we have presented several lines of evidence indicating that the submandibular gland EGF is involved in the development of the mouse mammary gland during pregnancy (7, 8). As an extension of our studies to assess the physiological role of submandibular gland EGF on mammary gland development, the present investigation was aimed at evaluating its possible involvement in mammary tumorigenesis in C3H/HeN mice.

The examination of EGF concentrations in the submandibular gland of virgin female mice at various ages revealed a marked sustained increase occurring after 30 weeks of age. Our recent studies indicate that this increase occurs as a result of ovarian dysfunction in aging postreproductive females (unpublished data). It is noteworthy that the increase in submandibular EGF closely preceded the appearance of mammary tumor, which was first detected at 31 weeks of age followed by a rapid increase from 42 to 46 weeks of age. The tumor incidence in normal females reached a plateau level by

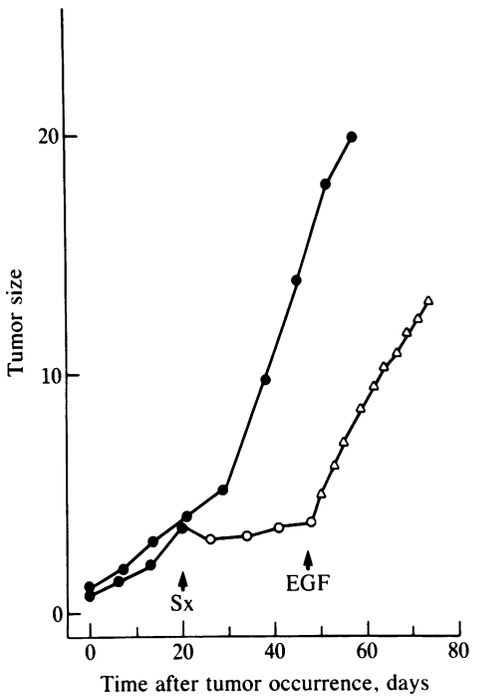

FIG. 4. The effect of sialoadenectomy (Sx) and EGF replacement on mammary tumor growth. Normal female mice bearing mammary tumors were either sham-operated or sialoadenectomized, or they were sialoadenectomized and subsequently treated with EGF (5 μg per mouse per day) at the times indicated by arrows. The tumor growth was monitored daily: ●, control mice; ○, sialoadenectomized mice; △, sialoadenectomized and EGF-treated mice. The data represent typical patterns of tumor growth in these groups of mice that were obtained in 12 other experiments.

52 weeks of age, as reported (16). Earlier studies indicate that the growth of the mammary tumor occurs in the absence of functioning ovary (17).

The surgical removal of an endocrine organ and appropriate replacement therapy using a given organ-specific agent have been used as a tool in the field of endocrinology to evaluate the physiological function of the organ and its product. In the present study, sialoadenectomy and EGF treatment were used to assess the role of submandibular EGF in mammary tumorigenesis. The results indicate that sialoadenectomy of young animals 14–22 weeks old markedly decreases the incidence of mammary tumors occurring at later times of life. Thus, at the age of 1 year, nearly 90% of sialoadenectomized mice were free of tumors, whereas control mice exhibited a maximal tumor incidence of 63%. Moreover, EGF replacement therapy has been shown here to increase the tumor incidence significantly in sialoadenectomized mice. Because of practical limitations, the EGF treatment used in this study was given every other day at a dose of 5 μg per mouse. However, it may be possible to enhance the effect of EGF on tumor incidence by more frequent injections.

The present study also showed that a small number of sialoadenectomized mice developed mammary tumors during the first year of life. However, these tumors had a much longer period of latency and a slower rate of growth when compared with the tumors in control mice. These findings may account, at least in part, for the difference in tumor incidence found between control and sialoadenectomized mice. In addition, since EGF is implicated in the development of the normal mammary gland (7, 8), it is possible that the delay in tumor onset in sialoadenectomized mice results from the retarded growth of mammary tissue and/or the decreased induction of progenitors of mammary tumors in the gland. It remains to be determined whether low mammary tumor incidence in sialoadenectomized mice is due to a decrease in transformation of cells or whether it reflects slower growth of the same number of transformed cells in the absence of EGF. Our preliminary data indicate that mammary tumor incidence in sialoadenectomized mice increases to 28% at 66 weeks of age, suggesting that tumors may grow more slowly in these animals. On the other hand, it is possible that mammary tumors in sialoadenectomized and control mice are different with regard to origin, cell type, etiological agents, and their dependency on EGF. Histological studies of these tumors suggest some difference in the structural pattern and cytological appearance of tumor cells. It would be of interest to examine this possibility by adapting these tumors in a cell culture system and isolating clonal cell lines.

The data presented here revealed another striking effect of sialoadenectomy on mammary tumors. It was shown that sialoadenectomy of tumor-bearing animals caused rapid and sustained cessation of the growth of mammary tumors. Moreover, this effect of sialoadenectomy was found to be reversed by EGF treatment. Our preliminary experiments indicate that the removal of the parotid glands, another salivary organ, is ineffective in preventing tumor growth. These results indicate that the growth of mammary tumor is dependent on EGF.

Based on the data presented in this paper, we propose that submandibular gland EGF plays a crucial role in mammary tumorigenesis. Although the precise mode of its involvement is unclear at present, the data presented here and in earlier studies (3–8, 10, 18) indicate that EGF serves as a mitogen for mammary tumor cells. In addition, the present results suggest a possible means of developing procedures to prevent and treat mouse mammary tumors by manipulating the production and/or action of submandibular gland EGF.

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