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# Induction of adenocarcinoma containing myoepithelial cells in rat submandibular gland by 7,12-dimethylbenz(a)anthracene

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**Abstract** In an attempt to induce adenocarcinoma containing myoepithelial cells (MECs) in the rat submandibular gland, we injected 7,12-dimethylbenz(a)anthracene (DMBA) dissolved in acetone into the glands of rat pups at the age of 10 days. In both male and female pups, the glands, including their developing terminal secretory units, contained far greater numbers of cells positive for proliferating cell nuclear antigen (PCNA) than did adult glands. A single administration of 1% DMBA (0.05 ml/130 g b.w.) did not produce adenocarcinoma, but did induce occasional sarcomas, such as rhabdomyosarcoma and fibrosarcoma, in 2 months. Most glands regenerated with minimal scar formation. Microscopically, these glands were atypical in that they contained increased numbers of PCNA-positive cells, underdeveloped granular ducts, and striated ducts surrounded by MECs positive for alpha smooth muscle actin (αSMA). Though these features were also observed in the regenerated glands after acetone injection, the number of PCNA-positive cells was relatively high in the glands of DMBA-treated females, especially in the terminal secretory unit. The second DMBA injection at 10 weeks of age produced adenocarcinoma made up of αSMA-positive MECs and keratin 19-positive duct cells. Such MEC-associated adenocarcinoma was induced in the glands of more than half the female but not the male animals. Replacement of either of the double DMBA treatments with acetone, or DMBA treatment, single or double, of adult glands did not produce adenocarcinoma, but did produce sarcoma and squamous cell carcinoma. These results suggest that (1) at least two genetic mutations are necessary for induction of adenocarcinoma with MECs in the rat submandibular gland, (2) the mutation is efficiently introduced to pup glands whose terminal secretory units exhibit extreme proliferative activity, and (3) the second mutation is difficult to introduce in male glands, whose proliferative activity is relatively low, and/or transformed cells need some female hormone after the mutation to propagate.

**Keywords** 7,12-dimethylbenz(a)anthracene · Rat · Submandibular gland · Adenocarcinoma Myoepithelial cell

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## Introduction

Many attempts have been made to induce tumors in the salivary glands of laboratory species by means of chemical carcinogens, especially in submandibular glands of adult rats and mice. The most frequently used carcinogen is 7,12-dimethylbenz(a)anthracene (DMBA), because the chemical is the most potent and it has the shortest latency for carcinogenesis [17]. In previous studies that used DMBA squamous cell carcinoma and sarcomas, such as fibrosarcoma and rhabdomyosarcoma, were produced almost exclusively, both of which are rare in human salivary glands [6]. In 1996, however, Zaman et al. [20] produced adenocarcinoma, a malignancy common among humans, in the rat submandibular gland by repeated (6–7 times) injections of DMBA. This suggests that multiple genetic mutation is necessary for adenocarcinogenesis. Moreover, to achieve this, they lesioned the gland by partial excision before DMBA application. The defect in such a lesioned gland is restored by extensive proliferation of residual ducts [8] and thus seems to provide a good chance that the paren chymal cells will be genetically altered by the carcinogen.

The majority of adenocarcinomas in human salivary glands contain myoepithelial cells (MECs) as a component cell (e.g. [18]), and thus are believed to originate from the terminal secretory unit (acinus-intercalated duct), which is the main habitat of MECs. In this context, it is a pity that Zaman et al. [20] did not describe the characteristics of the DMBA-induced adenocarcino-

**Table 1** Monoclonal antibodies used for immunohistochemistry

| Antibody | Specificity | Dilution | Source |
|---|---|---|---|
| 1A4 | α-Smooth muscle actin | 1:1,000 | Dako, Glostrup, Denmark |
| CK-E3 | Keratin 19 | 1:1,000 | Sigma Chemical Co., St. Louis, Mo. |
| PC10 | Proliferating cell nuclear antigen | 1:4,000 | Dako |

ma and its component cells. During recovery from injury, residual ducts in the rat submandibular gland proliferate and give rise to two different cell lines: one line transforms into acinus-intercalated ducts and the other into striated ducts [8]. Therefore, adenocarcinoma containing MEC might generate if the carcinogen were introduced during active formation or proliferation of the terminal secretory unit. There is, however, no detailed study on the proliferative activity of the individual parenchymal elements during gland recovery.

The recovery process of an injured submandibular gland recapitulates the normal gland development [8]. Development of rat submandibular gland has been thoroughly investigated (e.g., [4, 9, 12]). The terminal secretory units start to develop at 15–16 days in utero and are established by puberty about 6 weeks after birth [4, 9]. Chang [2] and Alvares and Sesso [1] observed the rat submandibular gland cells following $^{3}$H-thymidine labeling during the early postnatal period. Though all the constituent cells proliferate actively, the developing terminal secretory unit exhibits a relatively higher rate of proliferation at around 1 week (during the late suckling period [16]). This rate of proliferation, however, decreases rapidly thereafter.

The aim of the present study was to induce adenocarcinoma with MEC in the rat submandibular gland. For this purpose, we used (1) glands of rat pups in the late suckling stage and (2) DMBA dissolved in acetone, a rapidly effective form [3]. These strategies were expected to allow the carcinogen to attack the constituent cells of terminal secretory units in the exact time slot in which they had higher proliferative activity. We also administered DMBA into adult glands for comparison.

## Materials and methods

A total of 112 male and female Wistar rats were purchased from Nihon Dohbutsu (Osaka, Japan). These included 78 pups at the age of 1 week and 34 young adults at the age of 7 weeks. They were housed in a room with controlled temperature and humidity, with lights on at 6 A.M. and off at 6 P.M. daily. Except for suckling periods, they were maintained on a standard laboratory diet and tap water ad libitum. All experiments were reviewed and approved by the Osaka University Faculty of Dentistry Intramural Animal Use and Care Committee prior to the study. All procedures were carried out at room temperature unless otherwise noted. The treatment and removal of salivary glands were done between 1 P.M. and 2 P.M. to normalize the influence of circadian rhythm on the proliferative activity of the gland cells [10, 15].

Rats were divided into three groups. The first group consisted of 6 pups and 6 adults (3 males and 3 females in each age group). The pups at the age of 10 days and the adults at the age of 8 weeks were killed by exsanguination from their abdominal aorta under deep anesthesia with chloroform, and the submandibular glands were removed.

The second group consisted of 24 pups (12 males and 12 females) and 18 adults (9 males and 9 females). The pups at the age of 10 days were anesthetized with ether, and the adults at the age of 8 weeks were anesthetized with chloral hydrate (20 mg/100 g b.w.; Wako Pure Chemical Industries, Osaka, Japan). The left submandibular gland of each animal was surgically exposed and injected with 1% DMBA in acetone (0.05 ml/130 g b.w.; Sigma Chemical Co., St. Louis, Mo.). The dose of DMBA was similar to that described previously [7, 20]. Six pups and 6 adults, 3 males and 3 females in each age group, received the same amount of acetone instead of DMBA and served as controls. Two months later, under anesthesia with chloroform, the animals were killed and the submandibular glands removed.

The third group consisted of 48 pups (24 males and 24 females) and 10 adults (5 males and 5 females). Among these, 26 pups (13 males and 13 females) were used as controls (see below). The rest of the animals were injected with DMBA, as described above. Two months later, they received an additional injection of the same amount of DMBA. The control animals were divided into three subgroups. In the first subgroup, both of the two DMBA treatments were substituted with treatments with the same volumes of acetone (Control A in Table 7). For the other two subgroups, only the second (Control B in Table 7) or the first (Control C in Table 7) DMBA injection was substituted with vehicle. These animals were killed from 3 to 4 months after the second injection, and the glands were removed.

The glands removed were cut into small tissue blocks and fixed in methacarn [11, 14] for 36 h. After fixation, the tissue blocks were processed according to Puchtler et al. [14] and embedded in paraffin. Serial sections (2–4 μm) were cut from each tissue block, mounted on silane-coated glass slides, deparaffinized and rehydrated to deionized water. One section from each series was stained with hematoxylin and eosin to assess the histology. The rest of sections were used for immunohistochemistry.

For immunohistochemistry, sections were treated for 30 min with 0.3% $H_2O_2$ to block endogenous peroxidase, rinsed in distilled water and then in 0.01 M phosphate-buffered saline (PBS; pH 7.2). The sections were preincubated for 30 min with 10% normal rabbit serum in PBS containing 1% bovine serum albumin (PBS-BSA) to block nonspecific binding. After the serum was wiped away, the sections were sequentially incubated with one of the primary monoclonal antibodies (Table 1) which had been diluted with PBS-BSA overnight at 4°C, with biotinylated rabbit anti-mouse immunoglobulins (1:500 in PBS-BSA containing 0.5% normal rat serum: Dako, Glostrup, Denmark) for 60 min, and with streptavidin-biotinylated horseradish peroxidase reagent (1:100 in PBS; Dako) for 30 min. Incubation for 4–5 min with 3,3'-diaminobenzidine tetrahydrochloride-$H_2O_2$ solution was carried out to visualize the immunoreaction sites. All the above steps were followed by at least three 10-min washes with PBS at 4°C. Sections were counterstained with methyl green, dehydrated, and coverslipped with Permount.

Negative controls for immunostaining were performed by substituting the primary antibody with PBS and normal mouse IgG (Miles Scientific, Naperville, Ill.).

For estimation of proliferating cells, a total of 1000 cells with and without PCNA-labeled nuclei were counted from a section of each gland sample. Microscopic image at 100× magnification was captured by a CCD camera (ICD-740; Olympus, Tokyo, Japan) and digitized by an image processor (VM-10; Olympus). On the digitized image, cell nuclei were counted using a digitizer tablet (SD-510 C; Wacom, Tokyo, Japan). The results were examined by an analysis of variance (ANOVA). Differences were considered statistically significant when $P<0.05$.

**Table 2** Percentage of PCNA-positive cells in normal submandibular gland of rat

| Animals | No. of animals | Mean percentage ± SD[a] | | |
|---|---|---|---|---|
| | | Total | Terminal secretory unit[b] | Duct[c] |
| 10 days old | 6 | 26.7±3.8 * | 31.4±3.1 * | 21.3±5.9 * |
| 8 weeks old | 6 | 6.0±0.6 | 7.3±0.7 | 4.6±0.8 |

*Significantly different ($P<0.05$)
[a] 1,000 cells were counted in a section of each rat
[b] Acinus and intercalated duct in the gland of an 8-week-old animal
[c] Developing intralobular and interlobular striated ducts in the gland of a 10-day-old animal, and granular, intralobular striated and interlobular striated ducts in that of an 8-week-old animal

**Table 3** Percentage of PCNA-positive cells in the right submandibular gland of rat which received a single DMBA injection into the left gland

| Animals | No. of animals | Mean percentage ± SD[c] | | |
|---|---|---|---|---|
| | | Total | Terminal secretory unit[d] | Duct[e] |
| 10 weeks old[a] | | | | |
| Male | 7 | 0.7±0.3 * | 0.8±0.4 * | 0.6±0.2 * |
| Female | 7 | 1.4±0.2 * * | 1.7±0.3 * * | 1.0±0.2 * * |
| 17 weeks old[b] | | | | |
| Male | 4 | 0.8±0.2 | 1.0±0.3 | 0.6±0.2 |
| Female | 5 | 0.7±0.0 | 0.8±0.1 | 0.6±0.1 |

*Significantly different ($P<0.05$)
[a] Animals received DMBA injections at the age of 10 days and were sacrificed 2 months later (at the age of 10 weeks). For cell count, glands of the animals listed in Table 4 were used
[b] Animals received DMBA injections at the age of 8 weeks and were sacrificed 2 months later (at the age of about 17 weeks). For cell count, glands of the animals listed in Table 4 were used
[c] 1,000 cells were counted in one section prepared from each rat at each stage
[d] Acinus and intercalated duct
[e] Granular, intralobular striated and interlobular striated ducts

## Results

### Normal submandibular gland

Submandibular glands of 10-day-old pups consisted of interlobular ducts, intralobular ducts and terminal tubules at the most proximal end (Fig. 1A). Acini were developing from the terminal tubules, and appeared as if they were budding out at the periphery of the tubules (arrows in Fig. 1A) [9]. The intralobular ducts were made up of intercalated ducts (future distal part of intercalated ducts) and larger ducts. Basal striations were frequently seen in the larger ducts, indicating development of striated ducts (Fig. 1A). Immunoreactivity for keratin 19 was seen in the duct cells (Fig. 1B), and that for αSMA was in the MECs residing at the periphery of the terminal tubules (Fig. 1C) [12]. The number of PCNA-positive cells was far greater than that in adult glands (cf. Fig. 1D, H; Table 2). The PCNA-positive cells were more numerous in the developing terminal secretory unit (acinus, terminal tubule and intercalated duct) than in the duct (Table 2). No apparent difference was detected between male and female animals in either gland morphology or immunostaining for keratin 19, αSMA and PCNA.

The glands of young adult rats at the age of 8 weeks were well developed and contained all the structural elements, i.e., acinus, intercalated duct, granular duct, striated duct and excretory duct (Fig. 1E). Terminal tubules had been transformed into proximal parts of the intercalated ducts and were no longer visible [9]. Granular ducts had developed from the proximal part of the intralobular striated ducts. These ducts become apparent between 5 and 6 weeks after birth, and continue to develop even after 4 months (cf. Figs. 1E, 2H) [9]. Keratin 19 immunoreactivity was observed in the intercalated, stri-

**Fig. 1A–H** Normal submandibular glands of female rats at the ages of **A–D** 10 days and **E–H** 8 weeks. Hematoxylin and eosin staining (**A**, **E**) or immunoperoxidase histochemistry for keratin 19 (**B**, **F**), αSMA (**C**, **G**) and PCNA (**D**, **H**) was followed by counterstaining with methyl green, *bars* 50 µm, ×300 **A**, **E** Terminal secretory units are actively developing at 10 days. Acini are developing from the terminal tubules (*arrows* in **A**), which per se will transform into proximal part of the intercalated duct. Also note the well-developed striated ducts and future distal part of the intercalated ducts. By 8 weeks, terminal secretory units have been established, and acini, intercalated ducts, granular ducts, striated ducts and excretory ducts are clearly identified. **B**, **F** Immunoreactivity for keratin 19 is seen in the luminal cells of the ducts. Granular duct cells are occasionally positive (*arrows* in **F**). **C**, **G** αSMA-positive MECs surround the terminal tubules at 10 days (**C**), while they are seen around the intercalated ducts and acini in the adult gland (**G**). **D**, **H** PCNA-positive cells are much more numerous at 10 days (**D**) than at 8 weeks (**H**). At 10 days, the positive cells are more abundant in the developing terminal secretory units. (*TT* terminal tubule, *ID* intercalated duct, *GD* granular duct, *SD* striated duct)

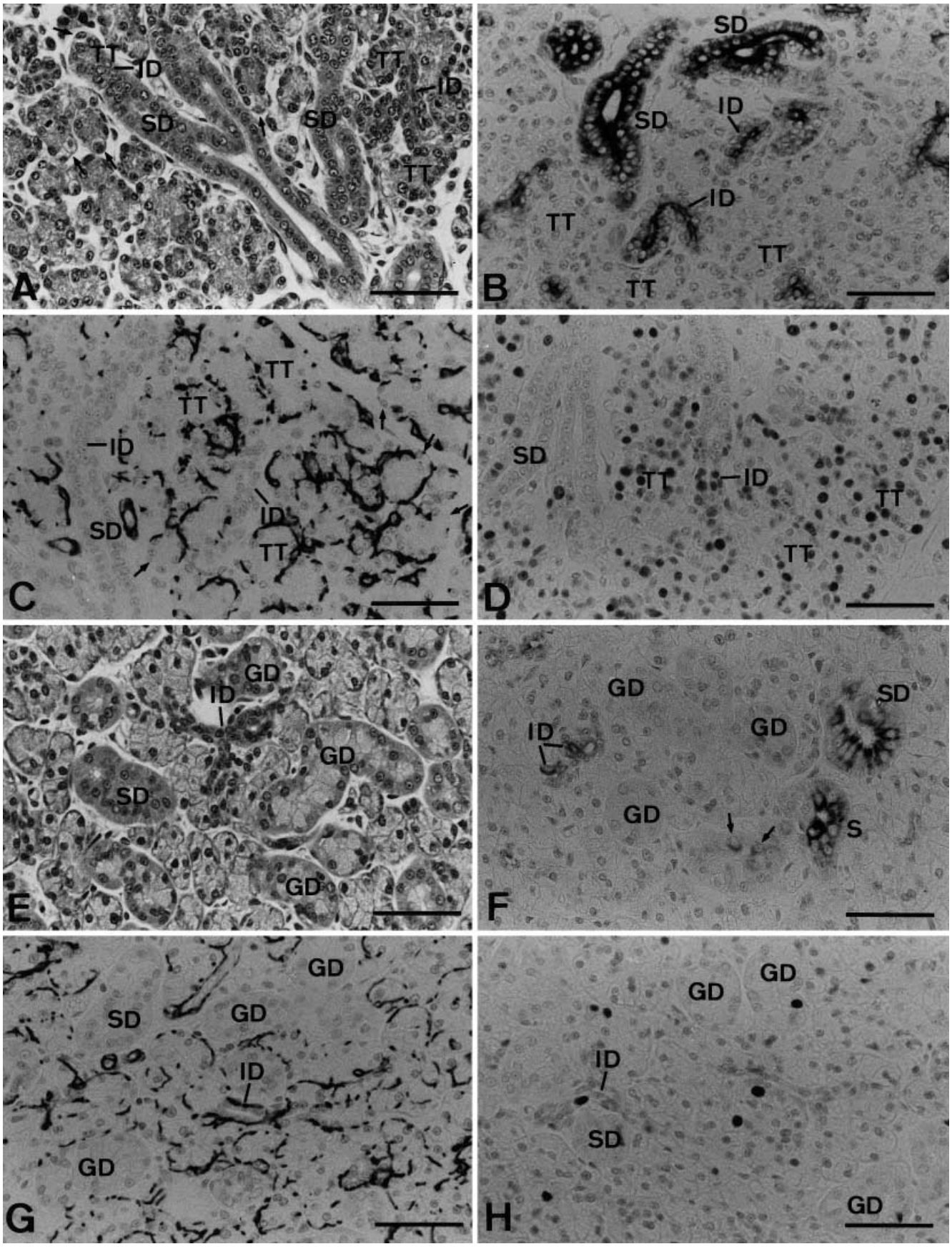
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**Table 4** Histopathology of rat submandibular glands 2 months after a single DMBA injection

| Animals | Tumor | Other gland histopathology |
|---|---|---|
| Males[a] | | |
| 9E11Bi1[b] | – | Atypical gland and regenerating tubular structure[d] |
| 9E11Bi2[b] | – | Atypical gland |
| 9E11Bi3[b] | – | Atypical gland |
| 9E21Bi1[b] | – | Atypical gland |
| 9E21Bi2[b] | – | Atypical gland |
| 9E21Bi3[b] | – | Atypical gland |
| 9E21Bi4[b] | – | Atypical gland |
| 9E21Bi5[b] | – | Atypical gland |
| 9E21Bi6 | Sarcoma | Degeneration |
| Females[a] | | |
| 8E11Ri1[b] | – | Atypical gland |
| 8E11Ri2[b] | – | Atypical gland |
| 8E11Ri3[b] | – | Atypical gland |
| 9E11Ri1 | Sarcoma | Gland not detected |
| 9E11Ri2 | – | Regenerating tubular structure[d] |
| 9E11Ri3[b] | – | Atypical gland |
| 9E21Ri1[b] | – | Atypical gland |
| 9E21Ri2[b] | – | Atypical gland |
| 9E21Ri3[b] | – | Atypical gland |
| Males[c] | | |
| 9E11Ba1 | – | Regenerating tubular structure[d] |
| 9E11Ba2[b] | – | Atypical gland[d] |
| 9E11Ba3 | – | Regenerating tubular structure[d] |
| 9E21Ba1[b] | – | Atypical gland[d] |
| 9E21Ba2[b] | – | Atypical gland and regenerating tubular structure[d] |
| 9E21Ba3[b] | – | Atypical gland[d] |
| Females[c] | | |
| 9E11Ra1[b] | – | Atypical gland[d] |
| 9E11Ra2[b] | – | Atypical gland[d] |
| 9E11Ra3 | Squamous cell carcinoma | Atypical gland and regenerating tubular structure[d] |
| 9E21Ra1[b] | – | Atypical gland and regenerating tubular structure[d] |
| 9E21Ra2[b] | – | Atypical gland[d] |
| 9E21Ra3[b] | – | Atypical gland[d] |

[a] Animals were 10 days old at the time of the DMBA injection
[b] PCNA-positive cell count was carried out in both uninjected and injected glands (Tables 3, 5)
[c] Animals were 8 weeks old at the time of the DMBA injection
[d] Also observed was a reparative connective tissue, which often contained regenerating muscle tissue and cyst lined with squamous epithelium

ated and excretory ducts (Fig. 1F). Occasional cells in the granular duct also expressed this immunoreactivity (arrows in Fig. 1F). MECs immunoreactive for αSMA surrounded the intercalated ducts and acini (Fig. 1G). PCNA-positive cells had decreased steeply (Fig. 1H; Table 2). Again, no gender difference was observed in either gland morphology or immunostaining for keratin 19, αSMA and PCNA.

We also examined contralateral untreated glands from the animals which received a single injection of DMBA. The pups were 10 weeks old and the adults about 17 weeks old at the time of examination. By the time the animals were 10 weeks of age, the granular ducts had become more conspicuous (Fig. 2H). The number of the PCNA-positive cells almost reached the minimal stable level (Table 3). At 10 weeks, granular ducts appeared somewhat more conspicuous in male than in female glands. Moreover, the PCNA-positive cells in 10-week-old female glands were slightly but significantly more numerous than in 10-week-old male glands and in 17-week female glands (Table 3).

## Submandibular gland after a single DMBA injection

Acetone caused almost immediate "fixation" of the glandular tissue [3]. In pups, the entire gland was bleached immediately after injection. All the injected glands ex-

**Fig. 2A–H** Tumors and submandibular glands after a single DMBA injection. The description for rats 9E11Ri1 (**A–C**), 9E11Bi1 (**D**), 9E11Ra3 (**E**) and 9E11Bi2 (**F–H**) appears in Table 4. Hematoxylin and eosin stain (**A–F**, **H**) and immunoperoxidase histochemistry for αSMA with methyl green counterstain (**G**), *bars* **A**, **E** 200 μm, ×75, **B**, **F–H** 50 μm, ×300, **C**, **D** 100 μm, ×150 **A**, **B** The sarcoma induced by DMBA is rhabdomyosarcoma. Note elongated and rounded malignant cells with deeply eosinophilic cytoplasm in which cross-striation is occasionally seen (*arrow* in **B**). **C** In places, tumor cells are spindle shaped and oriented in interlacing fascicles (herringbone pattern). **D** At the interface of glandular (*right*) and reparative connective (*left*) tissues regeneration is under way and small ducts are frequently encountered. **E** Squamous cell carcinoma has occurred in the wall of the epidermoid cyst in the reparative connective tissue. **F–H** Regenerated gland (**F**) is atypical, with poorly developed granular ducts, while the contralateral untreated gland appears normal (**H**). Granular ducts at this age (10 weeks after birth) are better developed than at 8 weeks (Fig. 1E). Regenerated gland is also atypical in that αSMA-positive MECs sometimes surround striated ducts (**G**). (*ID* intercalated duct, *GD* granular duct, *SD* striated duct)

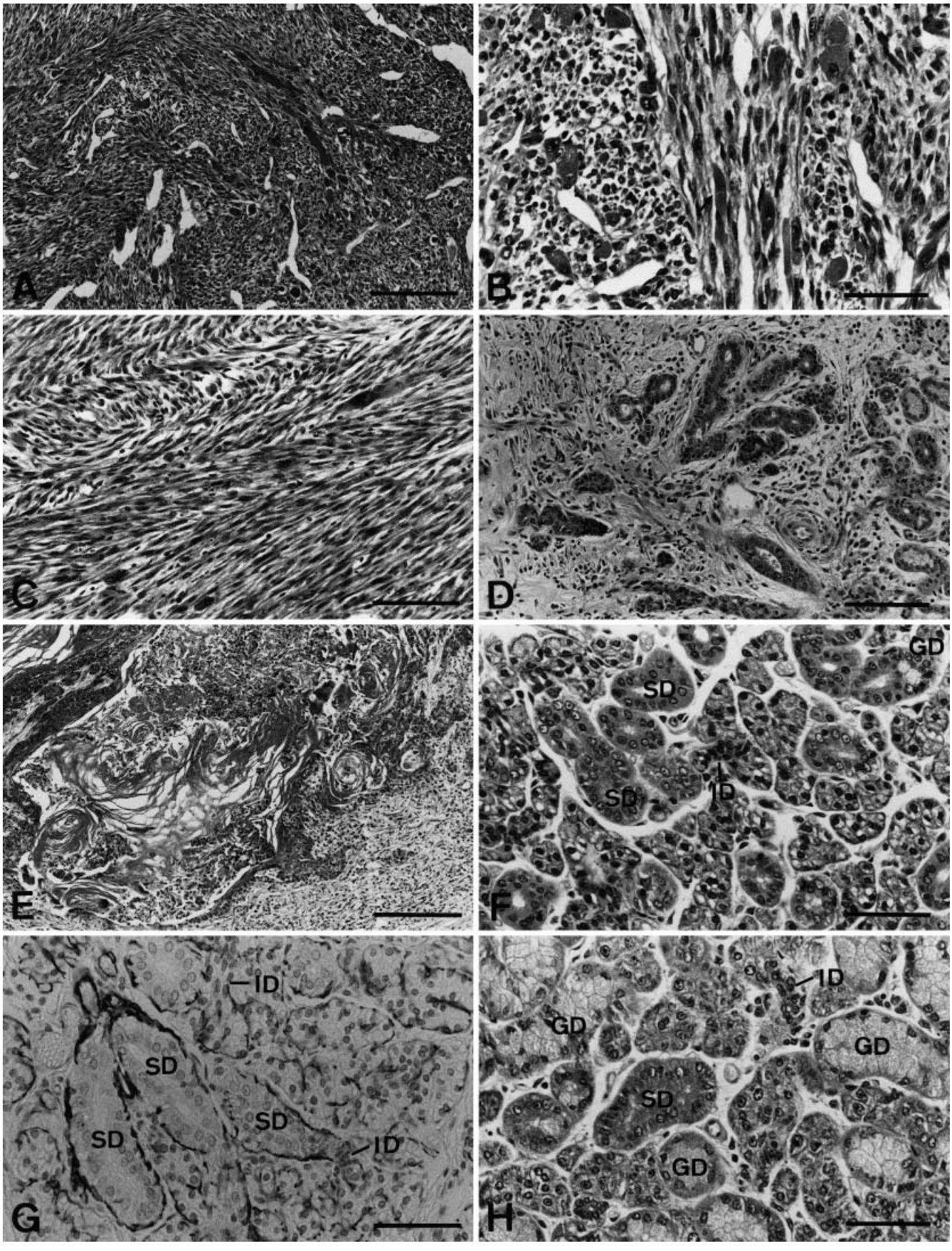
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**Table 5** Percentage of PCNA-positive cells in rat submandibular gland 2 months after a single injection of DMBA or acetone[a]

| Animals | No. of animals | Mean percentage ± SD[b] | | |
|---|---|---|---|---|
| | | Total | Terminal secretory unit[c] | Duct[d] |
| DMBA | | | | |
| Male | 7 | 1.6±1.0 * | 2.1±1.4 * | 1.1±0.7 |
| Female | 7 | 2.9±1.5 * | 4.0±2.1 * | 1.9±1.2 * |
| Acetone | 6 | 1.3±0.5 | 1.6±0.9 | 0.9±0.2 |

*Significantly different ($P<0.05$)

[a] Animals received DMBA injections at the age of 10 days and were sacrificed at the age of 10 weeks. For the cell count, glands of the animals listed in Table 4 were used

[b] 1,000 cells were counted in a section of each rat

[c] Acinus and intercalated duct

[d] Granular, intralobular striated and interlobular striated ducts

pressed swelling which was restored by about 1 month. Thereafter, a rapidly growing tumor appeared in 1 female pup (9E11Ri1 in Table 4) and, by 2 months, attained a size sufficient to disturb respiration and induce ulceration of the overlying skin. At this time, we decided to remove all the glands for gross and microscopic examinations. On gross examination, except for the one bearing the tumor, all the glands were smaller than the contralateral uninjected glands to a greater or lesser degree. Most of the neonatally (at 10 days) treated glands exhibited a normal appearance, while some were replaced partially or almost totally by white fibrous tissue. All the adult-treated glands were partially or almost totally replaced by white fibrous tissue.

Microscopically, the macroscopically detected tumor was diagnosed as a rhabdomyosarcoma (Fig. 2A, B). In some places, however, the tumor cells exhibited a herringbone growth pattern reminiscent of fibrosarcoma (Fig. 2C). The white fibrous tissue in other rats was a reparative connective tissue (Fig. 2D, E) and often contained regenerating skeletal muscle and cysts lined by squamous epithelium. Surrounded by the fibrous tissue in two animals, rhabdomyosarcoma and squamous cell carcinoma were detected (Fig. 2E) arising from the muscular tissue and the epidermoid cyst (9E21Bi6 and 9E11Ra3, respectively, in Table 4). Next to the fibrous tissue, there was regenerating glandular tissue, which was a collection of small ducts (Fig. 2D), and/or glands with atypical histology (see below). The macroscopically normal-looking glands of neonatally treated animals turned out to be atypical microscopically. In contrast to the contralateral normal glands, their granular ducts were underdeveloped (cf. Fig. 2F, H) and PCNA-positive cells were numerous (difference from the normal gland was significant, $P<0.05$; compare Tables 5 and 3). The MECs positive for αSMA sometimes extended from the acinus-intercalated duct to the intralobular and interlobular striated ducts (Fig. 2G). This atypical morphology was also observed in the glands treated with acetone instead of DMBA. We could not find any obvious difference in these atypical glands between males and females or between DMBA treatment and acetone treatment, except that the PCNA-positive cells were significantly more numerous in DMBA-treated female infants, especially in their terminal secretory units (Table 5; data from adult-treated animals not shown).

Because no obvious sign of adenocarcinoma development was observed after a single administration of DMBA, we decided to give an additional injection of the carcinogen.

## Submandibular gland after duplicate DMBA injections

In this experimental group, no apparent tumorous change was observed 2 months after the first DMBA injection. The second injection, however, was followed by tumor development in all animals. The earliest appearance of gross tumor was less than 2 months after the injection. These tumors were removed between 3 and 4 months after their appearance, when they were growing rapidly and inducing skin ulceration. Grossly the glands were replaced by tumorous nodules, which were mostly encapsulated. Metastatic foci were found in the lungs of three animals (8E12Ri3, 8E12Bi5 and 9E22Ba3 in Table 6).

Microscopically, all the tumorous nodules were composed mostly, if not entirely, of sarcoma (Table 6; Fig. 3A, G, J), suggesting that the rapid growth was attributed to this mesenchymal malignancy. Sarcoma was mainly rhabdomyosarcoma, but fibrosarcomatous foci were frequently observed. All the metastatic lesions in the lung were sarcomas (Table 6). In addition, squamous cell carcinoma and adenocarcinoma were found in some animals. Squamous cell carcinoma was found whether the treatment was started neonatally or in adult animals,

**Fig. 3A–L** Adenocarcinoma induced by duplicate DMBA injections. The description for rats 7E12Ri1 (**A–C**, **G–I**), 8E12Ri1 (**D–F**) and 7E12Ri2 (**J–L**) appears in Table 6. Hematoxylin and eosin stain (**A**, **D**, **G**, **J**). Immunoperoxidase histochemistry for keratin 19 (**B**, **E**, **H**, **K**) and αSMA (**C**, **F**, **I**, **L**) was followed by counterstaining with methyl green, *bars* **A**, **D**, **G**, **J** 100 μm, ×150, **B**, **C**, **E**, **F**, **H**, **I**, **K**, **L** 50 μm, ×300 **A**, **D**, **G**, **J** Tumor cells exhibit **A** tubular, **D** papillo-tubular, **G** cribriform, and **J** solid growth patterns. Adenocarcinoma is often intermingled with sarcoma (**A**, **G**, **J**). **B**, **E**, **H**, **K** Most tumor cells are immunoreactive for keratin 19. **C**, **F**, **I**, **L** αSMA-positive cells are seen in the periphery of each growth structure. In the cribriform structures, they surround some cystic spaces (**I**)

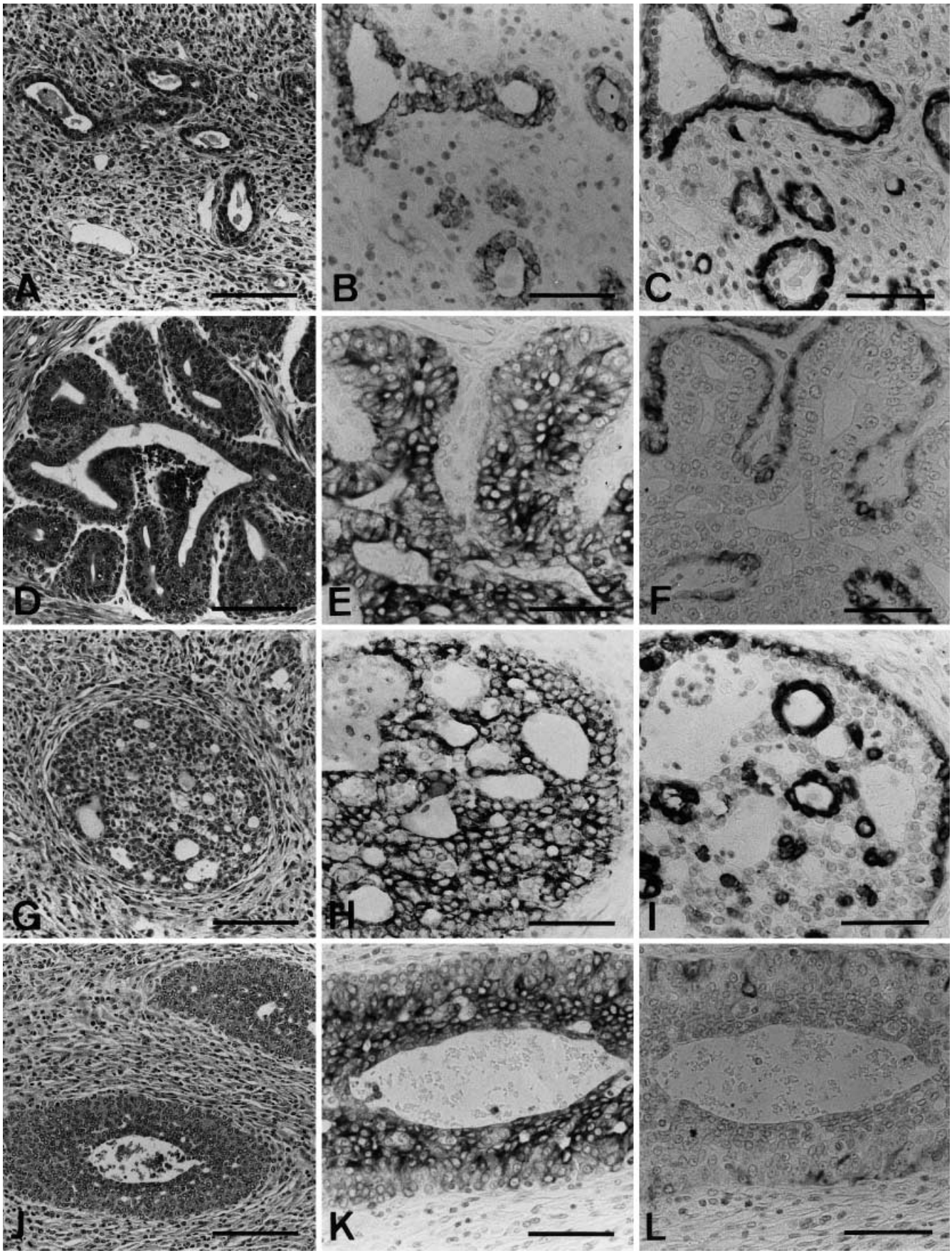
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**Table 6** Tumors induced in rat submandibular glands which received duplicate DMBA injection

| Animals | Adenocarcinoma | Squamous cell carcinoma | Sarcoma |
|---|---|---|---|
| Males[a] | | | |
| 7E12Bi1 | – | – | + |
| 7E12Bi2 | – | – | + |
| 7E12Bri1 | – | – | + |
| 7E12Bri2 | – | – | + |
| 7E22Wi | – | – | + |
| 7E22Bi2 | – | + | + |
| 7E22Bi3 | – | – | + |
| 8E12Bi1 | – | + | + |
| 8E12Bi2 | – | – | + |
| 8E12Bi4 | – | – | + |
| 8E12Bi5[b] | – | + | + |
| Females[a] | | | |
| 7E12Ri1 | + | – | + |
| 7E12Ri2 | + | + | + |
| 7E12Ri3 | – | – | + |
| 7E12Bli1 | + | – | + |
| 7E12Bi3 | – | – | + |
| 7E22Ri1 | – | – | + |
| 8E12Ri1 | + | + | + |
| 8E12Ri2 | – | – | + |
| 8E12Ri3[b] | – | + | + |
| 8E12Ri4 | + | – | + |
| 8E12Ri5 | + | – | + |
| Males[c] | | | |
| 9E22Ba1 | – | + | + |
| 9E22Ba2 | – | – | + |
| 9E22Ba3[b] | – | – | + |
| 9E22Ba4 | – | – | + |
| 9E22Ba5 | – | – | + |
| Females[c] | | | |
| 9E22Ra1 | – | – | + |
| 9E22Ra2 | – | – | + |
| 9E22Ra3 | – | – | + |
| 9E22Ra4 | – | – | + |
| 9E22Ra5 | – | + | + |

[a] Animals were 10 days old at the time of the first DMBA injection
[b] Animals expressed metastatic foci of sarcoma in the lungs
[c] Animals were 8 weeks old at the time of the first DMBA injection

whereas adenocarcinoma was seen only in the rats that had been treated neonatally (Table 6). Squamous cell carcinoma arose in both males and females, whereas adenocarcinoma occurred only in females (Table 6). Consequently adenocarcinoma was induced in the glands of more than half (6/11) of the neonatally treated female rats (Table 6). The adenocarcinoma was made up of variable mixture of tubular (Fig. 3A–C), papillary (Fig. 3D–F), cribriform (Fig. 3G–I) and solid (Fig. 3J–L) growth patterns of tumor cells. Immunohistochemically, tumor cells were mostly positive for keratin 19 (Fig. 3B, E, H, K). αSMA-positive cells were also observed distributed in the periphery of all the growth patterns (Fig. 3C, F, I, L). In the cribriform growth patterns, they were also found around some cystic spaces (Fig. 3I).

Table 7 shows the results of control experiments for the neonatally treated rats. When acetone was injected instead of DMBA (Control A in Table 7), the glands contained no tumor but did contain atypical glandular tissue with some scar tissue. When one of the DMBA injections was replaced by acetone injection, sarcoma and squamous cell carcinoma were formed in some animals (Controls B and C in Table 7). These malignancies appeared to occur with equal frequency in male and female animals.

## Discussion

This study is the first in which rat submandibular gland adenocarcinoma containing MECs was induced in a highly reproducible manner. Submandibular glands of rodents, especially rats and mice, have been most widely used for the study of experimental carcinogenesis (e.g., [3, 7, 17]). This is because they are easier to manipulate and more susceptible to chemical carcinogens than other salivary glands, i.e., sublingual and parotid glands [19]. As in previous studies, a single administration of DMBA did not produce adenocarcinoma in the adult. The present study extended the absence of adenocarcinoma genesis by the DMBA administration to neonatal rats. The only tumors produced were squamous cell carcinoma and sarcomas, such as rhabdomyosarcoma and fibrosarcoma.

**Table 7** Tumors induced in submandibular glands of control rats[a]

| Animals | Adenocarcinoma | Squamous cell carcinoma | Sarcoma |
|---|---|---|---|
| Control A[b] | | | |
| Males | | | |
| 8C12Bi1 | – | – | – |
| 8C12Bi2 | – | – | – |
| 8C12Bi3 | – | – | – |
| Females | | | |
| 8C12Ri1 | – | – | – |
| 8C12Ri2 | – | – | – |
| 8C12Ri3 | – | – | – |
| Control B[c] | | | |
| Males | | | |
| 8C22Bi1 | – | – | + |
| 8C22Bi2 | – | – | – |
| 8C22Bi3 | – | – | – |
| 8C22Bi4 | – | – | – |
| 8C22Bi5 | – | – | + |
| Females | | | |
| 8C22Ri1 | – | + | – |
| 8C22Ri2 | – | – | + |
| 8C22Ri3 | – | – | – |
| 8C22Ri4 | – | – | – |
| 8C22Ri5 | – | – | – |
| Control C[d] | | | |
| Males | | | |
| 8C32Bi1 | – | – | + |
| 8C32Bi2 | – | – | – |
| 8C32Bi3 | – | + | – |
| 8C32Bi4 | – | – | + |
| 8C32Bi5 | – | – | – |
| Females | | | |
| 8C32Ri1 | – | – | – |
| 8C32Ri2 | – | + | – |
| 8C32Ri3 | – | – | – |
| 8C32Ri4 | – | + | + |
| 8C32Ri5 | – | – | – |

[a] Animals were 10 days old at the time of the first injection
[b] Animals were given two injections of acetone
[c] Animals were given first an injection of DMBA and then an injection of acetone
[d] Animals were given first an injection of acetone and then an injection of DMBA

A single DMBA administration causes degeneration and necrosis followed by reparative response in both glandular and surrounding tissues. Squamous cell carcinoma arises from the wall of epidermoid cyst, which is derived from proliferating duct that has undergone squamous metaplasia, and sarcomas originate from reparative connective tissue and striated muscle close to the gland [3]. In the present study, squamous cell carcinoma and sarcoma were produced, but only occasionally. Most adult glands consisted of glandular, connective and muscular tissues which were in the course of active restoration. They often possessed epidermoid cyst. After injection of DMBA dissolved in acetone, both squamous cell carcinoma and sarcoma arise, with a peak incidence at about 100 days [7]. Therefore, these malignancies are assumed to occur more frequently with a latency that is longer than the presently used experimental period after a single injection.

In contrast, most of the neonatally treated glands had regenerated with little scar formation, probably due to their potent proliferative ability. These glands, however, showed some residual trace of the neonatal injury, and were atypical according to comparison with the contralateral normal glands. They were smaller and proliferated more actively at 10 weeks after birth. Their granular ducts were underdeveloped, and striated ducts were surrounded by MECs positive for αSMA. These features were not characteristic of DMBA treatment, however, but were also observed after acetone injection. Nevertheless, the extreme proliferative activity of developing glands would have provided a number of victims of the carcinogen, and introduced some genetic mutation necessary for carcinogenesis in the cells of the regenerated glands. This notion is supported by the fact that nonmutagenic acetone produced sarcoma and squamous cell carcinoma in these glands (Control B in Table 7). Application of acetone followed by DMBA did not produce adenocarcinoma with MEC either (Control C in Table 7). Therefore, genetic mutation introduced in the cells of the terminal secretory unit by a single dose of DMBA

was insufficient in itself for genuine adenocarcinoma generation. The second administration of DMBA appears to have supplemented the mutagenesis and led to adenocarcinoma generation. Multiple genetic mutation for adenocarcinogenesis has been also suggested previously [20].

The second DMBA injection induced adenocarcinoma in the glands of more than half the neonatally treated females. The adenocarcinoma consisted of αSMA-positive MECs and keratin 19-positive duct cells. As in human salivary gland adenocarcinomas, these two cell types combined to generate variable growth patterns, i.e., tubular, papillary, cribriform and solid growth patterns (reviewed in [6]). The duplicate DMBA injection also increased the incidence of sarcoma and squamous cell carcinoma. All the treated animals bore sarcomas with and without squamous cell carcinoma within 4 months after the second carcinogen injection. The high yield of sarcoma and squamous cell carcinoma might have been due to the experimental period, which was longer than the most frequent latent period of these malignancies (about 100 days) [7]. It is also likely that additional mutation augmented the malignant character and accelerated the growth of the tumors.

It is interesting to speculate on why the adenocarcinoma was induced in females but not in males. In the earlier study, adenocarcinoma was also produced only in female animals [20]. It is possible that the double mutation necessary for adenocarcinogenesis did not occur in males. Neither the present study nor the previous study [1] found any difference in either morphology or proliferative activity between male and female animals 10 days after birth. At the time of the second DMBA injection (10 weeks after birth), however, the proliferative activity was significantly higher in the female than in the male animals. This was particularly true for the terminal secretory units. Therefore, the additional mutagenesis by the second DMBA injection might well not have affected the glands in the male. It is also possible that transformed cells after the genetic mutation need some female hormone to propagate. Human salivary adenocarcinomas with MECs such as adenoid cystic carcinomas have been shown to bear receptors for estrogen and progesterone, and it has thus been suggested that they are dependent on some female hormone(s) [5, 13].

In conclusion, the results of the present study suggest that at least two genetic mutations are necessary for induction of adenocarcinoma with MECs in rat submandibular gland. We are now conducting a study to examine the genetic changes after the first and second administrations of DMBA in glands of both female and male pups. According to the method presented here, adenocarcinoma can easily be produced with MECs in the rat. It is expected that this experimental model provides important clues for understanding salivary gland carcinogenesis and development of a strategy for the prevention of salivary gland carcinomas.

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