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Inhibitory effect of sialoadenectomy on hepatocellular tumourigenesis in male mice induced by 3′-methyl-4-dimethylaminoazobenzene

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Abstract Male C57BL/6×DS-F_1 mice that had been treated with 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) neonatally were subjected to sialoadenectomy (removal of the submandibular salivary glands) 60 days after birth. The development of adenomatous nodules and carcinomas in the livers of these mice was compared with the livers of males without sialoadenectomy. The incidence of adenomatous nodules in sialoadenectomized males at 6, 8, 10, and 12 months was significantly lower than that in sham-operated animals. Carcinomas were found in the livers of both sham-operated and sialoadenectomized males that were 10 and 12 months old and were less frequent in sialoadenectomized males although the difference was significant only at the age of 12 months. Sialoadenectomy decreased the serum concentration of epidermal growth factor (EGF) by about 40%. The present results indicate that sialoadenectomy inhibits the development of hepatocellular tumours induced by 3′-Me-DAB in male mice, an effect which may be caused by decrease in the serum level of EGF.

Key words Liver · Carcinogenesis
Submandibular salivary gland · Sialoadenectomy
Epidermal growth factor

Introduction

Epidermal growth factor (EGF), a polypeptide with a molecular weight of 6045, has been shown to exert diverse biological actions on a variety of cells [3, 5]. Moreover, exogenously administered or endogenous EGF has been shown to accelerate carcinogen-induced tumourigenesis in mouse skin [18] and hamster pancreas [4] and spontaneous tumourigenesis in mouse mammary gland [7, 11].

The liver has many EGF receptors [5], and EGF has a variety of effects in the liver, both in vivo and in vitro, including stimulation of DNA synthesis, ornithine decarboxylase activity, phospholipid methyltransferase activity, monovalent ion transport, alanine uptake, and inhibition of glucagon-stimulated amino acid uptake [5, 13]. Recent studies using transforming growth factor-α (TGF-α) transgenic mice have shown that overexpressed TGF-α, which binds to the EGF receptors and shares similar biological activities with EGF [5], enhances the liver tumorigenicity of various carcinogens or oncogenes [19, 20].

In mice, the submandibular gland is a major source of EGF [3, 5, 8], and sialoadenectomy (removal of the submandibular salivary glands) of mice decreases serum EGF [11, 15, 16, 26]. Sialoadenectomy in mice thus provides a technique which may be used to reveal the effect of endogenous EGF on liver tumourigenesis and we investigated the effect of this procedure on liver tumourigenesis induced in mice by neonatally administered 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB).

Materials and methods

Male C57BL/6×DS-F_1 mice bred in our laboratory were used. They were kept at 25° C under controlled lighting (12 h light/12 h darkness) and allowed free access to water and pellet food.

3′-Me-DAB (ICN Pharmaceuticals, Plainview, N.Y., USA) was suspended in an aqueous solution of 0.7% (w/v) gelatin at a concentration of 10 mg/ml, and 0.05 ml of the suspension was injected intraperitoneally into mice 10, 12, 14, 16 and 18 days old.

Male mice that were 60 days old and that had been treated with 3′-Me-DAB neonatally as described above were divided into two groups; one group was sialoadenectomized, and the other group was given a sham operation. Mice were sialoadenectomized under pentobarbital sodium anaesthesia. For histological examination of the liver, 36–78 mice from each group were killed at 6, 8, 10 or 12 months of age after weighing and the liver was promptly removed.

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For assay of serum EGF, blood was obtained from 19 or 12 mice of each group at the age of 6 months, and 10 intact male mice of the same age that had not received neonatal 3′-Me-DAB treatment and sialoadenectomy. Blood was taken from the inferior vena cava of mice under pentobarbital sodium anaesthesia.

The liver was fixed in Zamboni's solution and cut into 4-mm-thick serial strips. A thin section of each strip was stained with haematoxylin and eosin, and all sections were examined for nodular lesions (adenomatous nodules and carcinomas). An adenomatous nodule of hepatocellular origin in the liver was defined with reference to previous reports [6, 12, 22] as described previously [25] as a mixture of eosinophilic, basophilic, vacuolated and foamy hepatocytes in various proportions that compressed the adjacent parenchyma, but did not contain a trabecular structure. A hepatocellular carcinoma was defined as a nodular lesion with a trabecular structure as described previously [25].

All sections of the liver were examined, and numbers of adenomatous nodules and carcinomas were counted, with care taken to count an adenomatous nodule or a carcinoma found in two or more adjacent sections as one lesion.

Photographs of all sections of the liver were taken at ×4 magnification, and areas of adenomatous nodules and carcinomas were measured using a PIAS, LA-5000 personal image analyser (PIAS, Tokyo, Japan). When one adenomatous nodule or carcinoma was found in two or more adjacent sections, the largest area found in these sections was regarded as the area to record.

Serum was obtained by centrifugation of blood at ×1,000 g for 10 min., and stored at −80° C until assay of EGF. The serum concentration of EGF was determined with a mouse EGF radioimmunoassay kit (Amersham International, Buckinghamshire, England) using a preparation of mouse EGF (Wako Pure Chemical Industries, Osaka, Japan) as a standard according to the manufacturer's instructions. The intra- and inter-assay coefficients of variation were 5.1 and 10.2%, respectively.

Statistical analyses were carried out by the chi-square test or Student's *t*-test. A *P* value below 0.05 was regarded as significant.

Results

Male mice that had been treated with 3′-Me-DAB neonatally were sialoadenectomized at the age of 60 days, and the development of adenomatous nodules and carcinomas in their liver was compared with that in the liver of sham-operated males that had been treated with 3′-Me-DAB neonatally. Figure 1 shows the body weight of the sialoadenectomized and sham-operated mice. Sialoadenectomy decreased the body weight of the mice by about 10% at the ages of 6, 8, 10 and 12 months. The incidences of adenomatous nodules in sialoadenectomized males were significantly lower than those in sham-operated males of the same ages (i.e. 9 vs 35%, 38 vs 67%, 43 vs 83%, and 63 vs 98%, respectively; Fig. 2). Carcinomas were found in the liver of both sham-operated and sialoadenectomized males at the ages of 10 and 12 months. All carcinomas were histologically similar to adenomatous nodules, but had a trabecular pattern. The incidences of carcinomas in sialoadenectomized males were lower than those in sham-operated males at the ages of 10 and 12 months (4 vs 17%, and 10 vs 29%, respectively), although the difference was significant only at the age of 12 months (Fig. 2). There was no significant difference between the numbers of adenomatous nodules per mouse in sham-operated and sialoadenectomized males except at the age of 8 months (Fig. 3). Only one carcinoma per mouse was found in both sham-operated and sialoadenectomized males at the ages of 10 and 12 months.

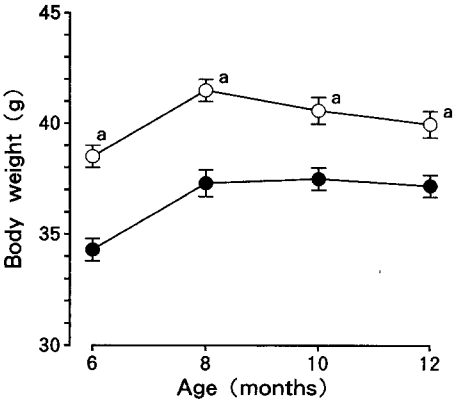

Fig. 1 Effect of sialoadenectomy on the body weight of mice. Male mice were treated with 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) neonatally. One group of mice was sialoadenectomized at the age of 60 days (●—●), and the other was not (○—○). Points are means ± SE for 36–78 mice. [a] Significant difference from the value for sialoadenectomized mice by Student's *t*-test ($P<0.01$)

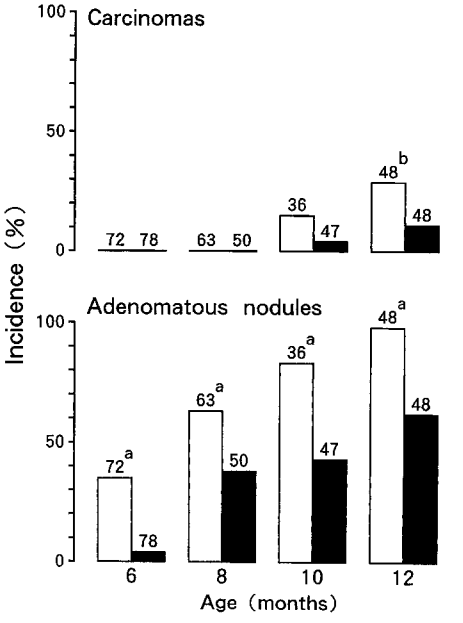

Fig. 2 Effects of sialoadenectomy on the incidences of adenomatous nodules and carcinomas. Male mice were treated with 3′-Me-DAB neonatally. One group of mice was sialoadenectomized at the age of 60 days (■), and the other was not (□). Mice were killed at the age of 6, 8, 10 or 12 months. Numbers above columns indicate numbers of mice examined. Significant difference from the value for sialoadenectomized mice by the chi-square test ([a] $P<0.01$, [b] $P<0.05$)

Figures 4 and 5 shows histograms of the areas of all adenomatous nodules and carcinomas that developed in sham-operated and sialoadenectomized mice. The areas of adenomatous nodules were more frequently smaller in sialoadenectomized mice than in sham-operated mice at the ages of 8 and 10 months, but less frequently so at the

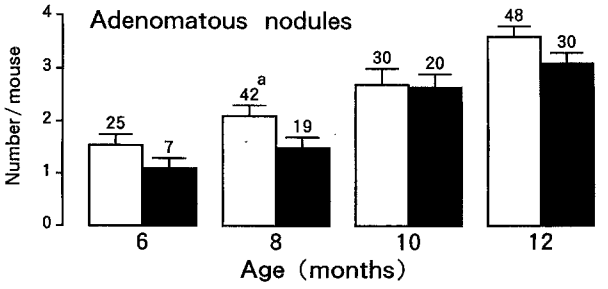

Fig. 3 Effect of sialoadenectomy on numbers of adenomatous nodules per mouse. Numbers of adenomatous nodules in the livers of the same sham-operated (□) and sialoadenectomized (■) mice as for Fig. 2 are presented. *Columns* and *bars* represent means + SE, and numbers above columns indicate numbers of mice. [a] Significant difference from the value for sialoadenectomized mice by Student's *t*-test ($P<0.05$)

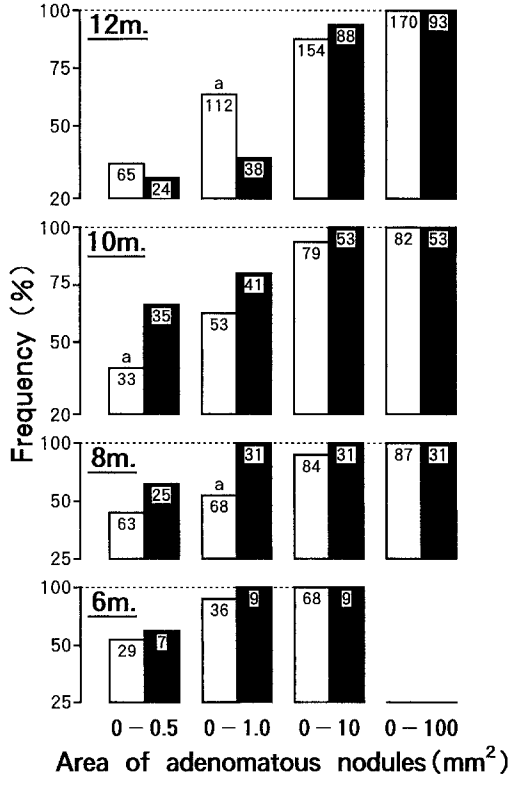

Fig. 4 Effect of sialoadenectomy on the size distribution of areas of adenomatous nodules. The areas of all adenomatous nodules in the same sham-operated (□) and sialoadenectomized (■) mice shown in Fig. 2 were measured. Numbers in columns indicate numbers of adenomatous nodules. [a] Significant difference from the value for sialoadenectomized mice by the chi-square test ($P<0.05$)

age of 12 months (Fig. 4). There were no significant differences between the size distributions of the areas of carcinomas in sialoadenectomized and sham-operated mice (Fig. 5).

Table 1 shows the effect of sialoadenectomy on the serum concentration of EGF, which was not affected by neonatal treatment of male mice with 3′-Me-DAB but was significantly decreased (about 40%) by sialoadenectomy.

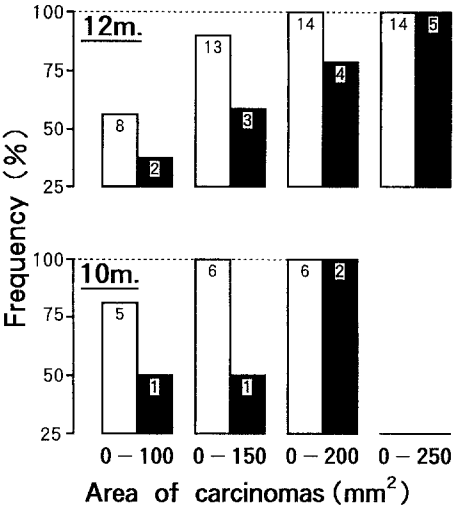

Fig. 5 Effect of sialoadenectomy on the size distribution of areas of carcinomas. Areas of all carcinomas in the same sham-operated (□) and sialoadenectomized (■) mice as for in Fig. 2 were measured. Numbers in columns indicate numbers of carcinomas

Table 1 Effect of sialoadenectomy on the serum concentration of epidermal growth factor (EGF). The serum concentration of EGF was assayed at the age of 6 months. Numbers of mice are indicated in parentheses

Treatment with 3′-Me-DAB	Sialoadenectomy	Serum concentration of EGF (ng/ml)[a]
+	+	2.89±0.17 (19)
+	−	4.80±0.18* (12)
−	−	4.51±0.23* (10)

[a] Means ± SE
* Significant difference from the value for sialoadenectomized mice by Student's *t*-test ($P<0.01$)

Discussion

The serum EGF levels of male mice with or without neonatal treatment with 3′-Me-DAB were comparable to those previously reported [15–17], and sialoadenectomy decreased the serum EGF levels of male mice significantly, consistent with previous reports [15, 16, 26]. Sialoadenectomy also decreased the incidences of adenomatous nodules and carcinomas. Thus, these results suggest that EGF secreted from the submandibular salivary glands enhances tumourigenesis in mouse liver induced by 3′-Me-DAB. Like us Takagi et al. [20] reported that overexpression of TGF-α in TGF-α transgenic mice accelerates the development of both adenomas and carcinomas in the liver induced by carcinogens. However, since sialoadenectomy reduced the body weight by about 10%, we cannot exclude the possibility that its effect on the well-being of the mice was related to its effect in decreasing the incidence of the liver tumours. This possibility could be tested by treating sialoadenectomized mice with EGF.

From our results on the size distribution of areas of adenomatous nodules, we found no conclusive evidence

that sialoadenectomy results in smaller adenomatous nodules than those in sham-operated mice, or that it had a significant effect on the size distribution of carcinomas. Thus our results suggest that sialoadenectomy had little effect on the growth of adenomatous nodules or carcinomas. However sialoadenectomy apparently suppressed the incidences of adenomatous nodules and carcinomas in mice at all ages. The histology of carcinomas was similar to that of adenomatous nodules except for a trabecular structure, suggesting that carcinomas arise from adenomatous nodules. Thus, it is likely that in our system for carcinogen-induced tumourigenesis in mouse liver, sialoadenectomy suppressed the development of adenomatous nodules from initiated hepatocytes and of carcinomas from adenomatous nodules, although it had little effect on the growth of adenomatous nodules and carcinomas.

There are several reports that androgen secreted from the testes promotes liver tumourigenesis induced by various carcinogens in both mice and rats [10, 14, 21, 23, 24]. Moreover, it has been shown that in mice the promoting effect of androgen is not due to its direct action on hepatocytes initiated by carcinogens [10], and that in rats the effect is due to indirect actions on tissues other than the liver, such as the pituitary and thyroid glands [21]. In a previous study on tumourigenesis in mouse liver induced by 3′-Me-DAB, we found that orchiectomy of males resulted in decreases in incidence of both adenomatous nodules and carcinomas [25]. Since orchiectomy reduces the serum level of EGF and the content of EGF in the submandibular salivary gland in mice [2, 16], this gland may be one of the sites through which androgen exerts its promoting effect on liver tumourigenesis in mice. This is supported by several studies which show that androgen increases EGF receptors in the liver [1, 9, 16].

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