Pergamon

Oral Oncol, Eur J Cancer, Vol. 31B, No. 1, pp. 27–31, 1995

0964–1955/95 $9.50 + 0.00

0964–1955(94)00035–2

Papers

Effect of Local Administration of Epidermal Growth Factor on 9,10-dimethyl-1,2-benzanthracene-induced Tumour Formation in Hamster Cheek Pouch

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The effect of local administration of epidermal growth factor (EGF) on 9,10-dimethyl-1,2-benzanthracene (DMBA)-induced tumour formation was investigated in a hamster cheek pouch carcinogenesis model. DMBA-treated hamsters underwent either sialoadenectomy (groups 1 and 2) or a sham operation (groups 3 and 4). Thereafter, EGF (groups 1 and 3) or vehicle (groups 2 and 4) was applied to the cheek pouches for 6 weeks. Fourteen weeks after the beginning of the experiment, the number of cheek pouch tumours was significantly greater in EGF-treated hamsters than in vehicle-treated hamsters, irrespective of whether the submandibular glands had been removed. The number of forestomach tumours, induced by DMBA application to the cheek pouches, was also increased by EGF. These results suggest that EGF applied from the luminal side of the mucosa stimulates tumour formation in the hamster cheek pouch and forestomach.

Keywords: epidermal growth factor, cheek pouch, forestomach, 9,10-dimethyl-1,2-benzanthracene, carcinogenesis

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INTRODUCTION

EPIDERMAL GROWTH factor (EGF), first isolated from mouse submandibular glands, is a well-characterised peptide growth factor [1, 2]. It stimulates the growth of various types of cells, including epithelial cells and fibroblasts. Parenteral and oral administration of EGF to animals inhibits gastric acid secretion and promotes the healing of wounds and gastroduodenal ulcers [1–7]. However, this factor has also been shown to play a role of carcinogenesis: removal of the submandibular gland, a rich source of EGF in mice, resulted in a reduction of the incidence of mammary tumours in these animals [8]. EGF has been shown to enhance the carcinogenic potential of methylcholanthrene in mouse skin and to stimulate the induction of mouse mammary tumours and hamster pancreatic and bronchial cancers [8–10]. The hamster cheek pouch model is the most reliable system for the induction of oral cancer [11–15]. Recently, we reported that sialoadenectomy, removal of the submandibular glands, reduced tumour induction in the 9,10-dimethyl-1,2-benzanthracene (DMBA)-treated hamster cheek pouch and that parenteral administration of urinary EGF reversed the effect of the sialoadenectomy [16].

The submandibular glands seem to be exocrine rather than endocrine organs for EGF [17–19]. Thus, the oral mucosa is generally coated with EGF-rich saliva. Although EGF administered parenterally has been shown to act as a tumour-enhancing factor in various animal models [8–10, 16], whether exocriated EGF plays a role in carcinogenesis remains unknown. If salivary EGF stimulates the development of tumours in the oral cavity, EGF applied from the luminal side of the mucosa to the hamster cheek pouch will increase the number of the DMBA-induced tumours, whereas sialoadenectomy will exert the opposite effect. To examine this possibility, we conducted an experiment in which DMBA-treated hamsters underwent sialoadenectomy or a sham operation and then EGF or vehicle was administered from the luminal side of the oral mucosa to the hamster cheek pouch. Since it has been shown that DMBA application to the hamster cheek pouch induced tumours in the forestomach simultan-

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Manuscript received 14 Dec. 1993; manuscript accepted 19 Sept. 1994.

eously [20], we examined the effect of EGF on tumour formation in the forestomach as well as cheek pouch.

MATERIALS AND METHODS

Animals and chemicals

Male Syrian hamsters, aged 5 weeks were purchased from Japan SLC, Inc. (Shizuoka, Japan). The hamsters were housed four to five per cage in an air-conditioned room at 24 ± 2°C with a 12-h light–dark cycle. They were given animal chow (CL-2; Clea Japan Inc., Tokyo, Japan) and tap water *ad libitum*. DMBA, purchased from Wako Pure Chemical Industries (Osaka, Japan), was dissolved in a 1:20 mixture of acetone and heavy mineral oil (Sigma Chemical Co., St. Louis, Missouri, U.S.A.). Human recombinant EGF (Wako) was dissolved in a vehicle consisting of 50% mineral oil, 10% ethanol and 40% 0.9% NaCl solution.

Experimental design

The experimental design is shown in Fig. 1. Hamsters were treated by painting both cheek pouches thrice weekly, for 6 weeks, with a 0.5% solution of DMBA dissolved in the acetone–heavy mineral oil mixture. One week later, hamsters in groups 1 and 2 underwent sialoadenectomy. A sham operation, vertical midline incision in the skin of the neck region, was performed on those in groups 3 and 4. One week after the operation, the hamsters were treated with test material by painting both cheek pouches thrice weekly for 6 weeks. The test materials were EGF (10 μg/body weight) for groups 1 and 3 and vehicle for groups 2 and 4.

Pathological examination

Fourteen weeks after the beginning of the experiment, the hamsters were killed by cardiac puncture performed when they were under general anaesthesia with pentobarbital. Their cheek pouches and stomachs were removed, opened longitudinally, washed in phosphate buffered saline, and stretched on pieces of cardboard. The mucosa was inspected and gross visible tumours (more than 1 mm in diameter) were counted and measured. Tumour volume was calculated by the formula of Attia and Weiss [21]: volume $= 0.4\ (ab^2)$, where a and b are perpendicular tumour diameters and b is the smaller. Cheek pouch tumours more than 2 mm in diameter were excised together with the surrounding mucosa; the remaining portions were cut into four pieces and subjected to histological examination. The forestomach was separated from the glandular stomach and cut into three pieces in such a way that the largest tumour could be represented in histological sections. After paraffin embedding, 4-μm sections were stained with hematoxylin and eosin for light microscopic examination. The cheek pouch lesions were histologically classified as hyperplasia, papilloma and squamous cell carcinoma, as described previously [11, 12, 16]. Cheek pouch tumours were examined for eosinophilia, this being defined as >10 eosinophils/$\times 200$ field [22].

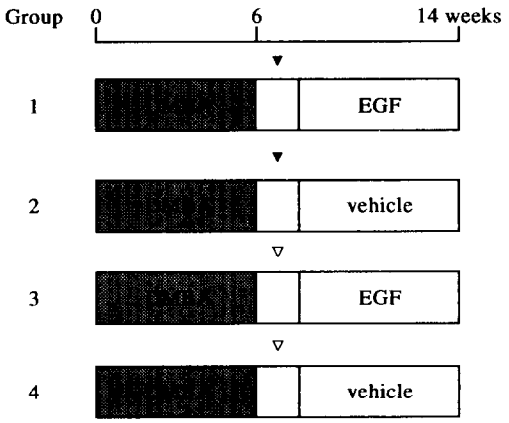

Fig. 1. Experimental design. All hamsters were treated by painting both cheek pouches thrice weekly for 6 weeks with 0.5% DMBA solution. One week later, hamsters in groups 1 and 2 underwent sialoadenectomy (▼) and a sham operation (▽) was performed in groups 3 and 4. Beginning 1 week after the operation, hamsters were treated by painting with test material thrice weekly for 6 weeks until the termination of the experiment. Test materials were groups 1 and 3, EGF (10 μg/kg body weight); groups 2 and 4, vehicle.

Statistical analysis

Statistical comparisons were carried out using unpaired Student's t-test for the number and volume of the tumours and χ^2 test for the incidence of carcinoma and tissue eosinophilia. A P value of 0.05 or less was considered significant.

RESULTS

Effects of local EGF administration

One hamster of group 4 was lost during the experiment. At the end of the experiment, the final mean body weights in groups 1–4 were 136 ± 13, 133 ± 17, 139 ± 11 and 135 ± 15 g, respectively. There were no significant differences among the experimental groups. Similarly, sialoadenectomy and EGF did not affect the final weights of liver and kidney in the hamsters (data not shown).

Gross cheek pouch tumours were demonstrated in all hamsters. When the number of the induced tumours was counted, the mean tumour number for each hamster was significantly greater in the EGF-treated hamsters (groups 1 and 3) than in the vehicle-treated animals (groups 2 and 4) ($P<0.05$), irrespective of whether the submandibular glands had been removed (Table 1). The tumour volume in the EGF-treated sialoadenectomised hamsters (group 1) was significantly larger than that of the vehicle-treated sialoadenectomised hamsters (group 2) ($P<0.05$). When the tumour number and volume were compared between the vehicle-treated sialoadenectomised hamsters (group 2) and the vehicle-treated normal hamsters (group 4), the latter animals developed more and larger tumours, but the difference was not significant.

At the end of the experiment, all of the hamsters, except for one in group 4, had developed tumours in the forestomachs. There were no differences among the experimental groups with regard to tumour incidence, but again, more and larger tumours were demonstrated in the forestomachs of the EGF-treated hamsters than in the vehicle-treated animals (Table 2). When the volumes of forestomach tumours were compared between group 2 and group 4 hamsters, a significant decrease of tumour volume was observed in the sialoadenectomised group.

Histologic examination of cheek pouches and forestomachs

All of the cheek pouch tumours more than 2 mm in diameter, a total of 285, were examined histologically and squamous cell carcinomas were demonstrated in 92–93% of

Table 1. Incidence, number and volume of cheek pouch tumours

Group	Treatment	No. of hamsters	No. of hamsters with tumours	No. of tumours/ hamster (mean ± S.D.)	Total tumour volume/ hamster (mm^3) (mean ± S.D.)
1	D + Sx + E	14	14	17.7 ± 10.7*	100.4 ± 90.9*
2	D + Sx + V	14	14	9.8 ± 4.7	47.7 ± 29.9
3	D + Sh + E	14	14	18.0 ± 7.1†	103.4 ± 78.9
4	D + Sh + V	13	13	11.9 ± 4.4	70.5 ± 51.4

D, DMBA; Sx, sialoadenectomy; E, EGF; V, vehicle; Sh, sham operation.
*$P < 0.05$ group 1 versus group 2.
†$P < 0.05$ group 3 versus group 4.

Table 2. Incidence, number and volume of forestomach tumours

Group	Treatment	No. of hamsters	No. of hamsters with tumours	No. of tumours/ hamster (mean ± S.D.)	Total tumour volume/ hamster (mm^3) (mean ± S.D.)
1	D + Sx + E	14	14	18.1 ± 11.7*	22.6 ± 18.5†
2	D + Sx + V	14	14	6.1 ± 2.8	7.8 ± 6.1‡
3	D + Sh + E	14	14	21.7 ± 11.2§	34.1 ± 25.8¶
4	D + Sh + V	13	12	8.9 ± 4.9	12.1 ± 4.0

D, DMBA; Sx, sialoadenectomy; E, EGF; V, vehicle; Sh, sham operation.
*$P < 0.001$ group 1 versus group 2.
†$P < 0.01$ group 1 versus group 2.
‡$P < 0.05$ group 2 versus group 4.
§$P < 0.001$ group 3 versus group 4.
¶$P < 0.01$ group 3 versus group 4.

Table 3. Proportion of carcinomas in cheek pouch tumours and incidence of tissue eosinophilia

Group	Treatment	No. of papillomas/ no. of tumours* (%)	No. of carcinomas/ no. of tumours* (%)	Papilloma: No. with eosinophilia/ no. examined (%)†	Carcinoma: No. with eosinophilia/ no. examined (%)†
1	D + Sx + E	32/82 (39)	50/82 (61)	8/24 (33)	17/34 (50)
2	D + Sx + V	20/52 (38)	32/52 (62)	5/19 (26)	11/26 (42)
3	D + Sh + E	36/83 (43)	47/83 (57)	6/26 (23)‡	17/33 (52)
4	D + Sh + V	27/68 (40)	41/68 (60)	5/25 (20)‡	16/30 (53)

D, DMBA; Sx, sialoadenectomy; E, EGF; V, vehicle; Sh, sham operation.
*Tumours more than 2 mm in diameter were examined histologically.
†Tumour specimens with sufficient surrounding mucosa for the evaluation of eosinophila were examined.
‡$P < 0.05$ papilloma versus carcinoma.

the hamsters in each experimental group. Most of the cheek pouch carcinomas were non-invasive and were classified as well-differentiated or moderately differentiated squamous cell carcinoma. The number of invasive squamous cell carcinomas in groups 1–4 were 1, 0, 3 and 3, respectively. When all 285 cheek pouch tumours were classified as either papilloma or squamous cell carcinoma, the percentage of carcinomas in each group ranged from 57 to 62% (Table 3). If we included only tumours 2–5 mm in diameter, the carcinoma percentages in groups 1–4 were 51% (36/70), 53% (25/47), 52% (39/75) and 54% (32/59), respectively; the differences were not significant. The incidence of squamous cell carcinoma in the forestomach was low, being 20% (3/15), 13% (2/15), 27% (4/15) and 21% (3/14), respectively in groups 1–4, and most of the forestomach tumours were classified as papilloma.

It has been suggested that transforming growth factor (TGF)-α mRNA-containing tissue eosinophils are associated with transformed cheek pouch epithelium [22]. When we examined the DMBA-induced tumours, we found that the infiltration of inflammatory cells, including eosinophils, was more prominent in large carcinomas. In the 217 tumours available for histological evaluation, tissue eosinophilia was demonstrated in 20–33% of the papillomas and in 42–53% of the carcinomas (Table 3), i.e. tissue eosinophilia was more frequent in carcinoma than in papilloma. However, there were no apparent differences among the experimental groups with regard to the incidence of tissue eosinophilia in papilloma and carcinoma.

DISCUSSION

The present study was conducted to demonstrate the tumour-enhancing effect of locally administered EGF, but we

also wanted to determine whether sialoadenectomy, removal of the submandibular glands, may retard the tumour development and whether EGF may affect the histological features of the tumours. To investigate these subjects, the hamsters were treated first with a 0.5% solution of DMBA for 6 weeks, based on the assumption that this treatment was sufficient to induce cheek pouch tumours after a latent period of several months [16], and then performed sialoadenectomy on 50% of the hamsters to reduce the amount of endogenous EGF, and the remaining hamsters underwent a sham operation. Thereafter, the bilateral cheek pouches of the hamsters were painted with EGF or vehicle. Fourteen weeks after the beginning of the experiment, as expected, all hamsters had developed cheek pouch tumours. When the numbers of the tumours were counted, it was found that more tumours were induced in the EGF-treated animals than in those treated with vehicle. EGF also increased the total tumour volume per hamster (group 1 versus 2). Thus, we concluded that EGF administered from the luminal side of the mucosa enhanced tumour induction in the cheek pouch and stimulated tumour growth. This cannot be ascribed to a general effect of EGF and/or to sialoadenectomy, since this procedure did not affect the body weight or the weights of liver and kidney.

We also found that the formation of forestomach tumours, which was induced by DMBA application to the cheek pouch, was also enhanced by locally administered EGF. At this site, the effect of EGF on tumour induction was more prominent than in the oral cavity. The mean tumour number and total tumour volume in the vehicle-treated sialoadenectomised hamsters (group 2) were lowest, whereas these in EGF-treated normal hamsters (group 3) were highest, indicating that both endogenous and exogenous EGF may contribute to tumour formation in the forestomach. Why the effect of EGF is more prominent in the forestomach than the cheek pouch is not known, but it could be explained in the following terms: EGF applied to both sides of the cheek pouches may be swallowed and retained in the forestomach at a higher concentration and for a longer period than in the oral cavity.

The mean number and mean volume of tumours in the sham-operated hamsters were usually higher than these values in the sialoadenectomised animals, indicating that sialoadenectomy retarded the development of both cheek pouch and forestomach tumours (Tables 2 and 3). However, the difference in tumour number between normal and sialoadenectomised hamsters was modest; there was a significant difference only in the volume of forestomach tumours (Table 2, group 2 versus 4). It appears that the effect of sialoadenectomy in the hamsters is not as dramatic as that seen in mouse mammary carcinogenesis [8], since the amounts of submandibular gland EGF seem to be lower in hamsters than in mice.

In this study, we also examined the effect of locally administered EGF on the histological features of the tumours. Previous studies have shown that the application of DMBA to the cheek pouch resulted in epithelial hyperplasia, papilloma and eventually squamous cell carcinoma [11, 12, 16], suggesting that most carcinomas occur in papillomas. If this were indeed the case, an agent could promote the malignant conversion of papilloma, then the relative number of carcinomas would be increased by that agent. In other words, the agent would increase the proportion of carcinomas in tumours of a defined size. When we examined all tumours 2–5 mm in diameter histologically, we found the percentage of squamous cell carcinoma in the tumours to be 51–54%; there were no significant differences among the groups. Thus, it appears that EGF applied to the cheek pouch does not accelerate the malignant conversion of papilloma.

Ghiabi *et al.* [22] demonstrated that the number of TGF-α mRNA-containing eosinophils associated with malignant epithelium was significantly higher than that associated with non-malignant cheek pouch epithelium. Since TGF-α, a single chain peptide related to EGF, is able to stimulate cell growth by binding to and activating the EGF-R [23], it is possible that TGF-α released from eosinophils is involved in the development of oral cancer. When we examined the incidence of tissue eosinophilia in the cheek pouch tumours, we found that eosinophilia occurred in 20–33% of the papillomas and in 42–53% of the squamous cell carcinomas (Table 3). Tissue eosinophilia thus does not seem to be associated with papilloma, but rather, with tumours at a more advanced stage. It is unlikely that eosinophils play an important role in papilloma formation, i.e. at the stage of tumour promotion.

Whether EGF can act from the luminal side of the mucosa has not been determined. Olsen *et al.* [19] indicated that EGF in physiological amounts was not absorbed in the rat gastrointestinal tract, although substantial amounts of intact protein were absorbed in newborn animals. Noguchi *et al.* [7] demonstrated that treatment of sialoadenectomised male mice with EGF in drinking water restored the rate of wound healing of the tongue to normal levels. Momose *et al.* [24] reported that instillation of urinary EGF instead of 0.9% NaCl solution into the transplanted bladder which had been treated with a single dose of *N*-methyl-*N*-nitrosourea increased the incidence of bladder carcinomas, suggesting an important association of the instilled EGF with the transplanted bladder mucosa. Here, we indicated that locally administered EGF enhanced tumour formation in the hamster cheek pouch and forestomach. Together, it is likely that EGF could act from the luminal side if the normal architecture of the mucosa were impaired by wounding or neoplastic changes.

The results of this study suggest that salivary EGF acts as a stimulator of oral and gastric tumour formation. Alternatively, these results may indicate the action of exogenous EGF applied to the mucosa. Since the effect of EGF on wound healing has been established [3–6], local or oral EGF administration could be of clinical value for the treatment of lesions such as recurrent oral aphthae and chronic gastroduodenal ulcers. However, the potential effect of EGF on oral and gastric tumour formation should be taken into consideration when clinical trials of this agent are conducted.

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Acknowledgement—This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (04807146).