Original Article

Effects of Sex Hormones on Rat Tongue Carcinoma Induced by 4-Nitroquinoline 1-Oxide (4NQO)

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Abstract

We investigated the regulatory effects of sex hormones on tongue carcinoma initiated by orally administration 4-nitroquinoline 1-oxide (4NQO) to rats. Animals of either sex were classified into three groups. The male rats in each group received an estrogen administration (Me), orchiectomy (Mor), or both treatments (Me/or) while the female rats also received testosterone administration (Ft), ovariectomy (Fov), or both treatments (Ft/ov).

The differences in the carcinogenic progress among these groups were examined by macroscopic and microscopic observation of tongue tissues. The incidence of cancer in the tongue tissue was 100% in the group reinforced with testosterone (testosterone + group) (Ft, Ft/ov, Me) but only 56.0% in the group not reinforced with testosterone (testosterone – group) (Fov, Mor). These findings suggest that sex hormones play a role in the onset of 4NQO-induced tongue carcinoma.

Key words: 4NQO—Tongue carcinoma—Rat—Sex hormone—Carcinogenesis

Introduction

The chemical compound, 4-nitroquinoline 1-oxide (4NQO), is carcinogenic. When administered to animals, it is metabolized by DT-diaphorase into the active carcinogen quinoline 4-hydroxyaminoquinoline 1-oxide (4HAQO)¹³⁾. This derivative produces fluorescent compounds that covalently bind to DNA and induce mutations and/or carcinogenesis in affected cells^{16,17)}. Ohne *et al.* in our labo-

ratory selectively induced tongue carcinoma in rats by oral administration of 4NQO^{10,11)} dissolved in drinking water. The developmental process of, and pathological findings on 4NQO-induced carcinomas bear similar clinical features to those of tongue carcinoma. Therefore, this experimental model may offer a method of studying human oral carcinoma. Many studies have used 4NQO-induced tongue carcinoma models, both histological and genetic^{6,15)}. Oral administration of 0.005%

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4NQO in drinking water for 6 months resulted in squamous cell carcinoma of the tongue in male rats. However, this type of carcinoma rarely developed in female rats, except for the occasional occurrence after 8 months⁵⁾.

In the present study, we examined the effect and role of gonadectomy and/or sex hormones on the development of 4NQO-induced tongue carcinoma in rats.

Materials and Methods

This study was performed according to the institutional guidelines and principles of the Animal Care Unit of Tokyo Dental College.

1. Experimental animals and rearing methods

Male and female Sprague Dawley (SD) rats weighting about 200 g each were purchased from Sankyo Laboratory Service, Tokyo. Animals were allowed *ad libitum* access to commercially available diet (Oriental Co., Tokyo) and water containing 0.005% 4NQO.

2. Experimental protocol

1) Administration of 4NQO

4NQO (1.0 g; Nakarai Chemical Co., Kyoto) was dissolved in 5% ethanol-containing water to make a 0.02% stock solution. This solution was diluted with tap water to a concentration of 4NQO at 0.005% and then orally administered to the rats from brown, light shielded polyvinyl bottles that were checked and refilled with freshly prepared 4NQO solution once a week.

2) Experimental agents

Testosterone propionate ("Testinon" Mochida Pharmaceuticals Co., Ltd., Tokyo) and estradiol benzoate ("Pelanin Depot" Mochida Pharmaceuticals Co., Ltd.) were administered to the rats as described below.

3) Female groups

The female rats were classified into an Ft (testosterone), an Ft/ov (ovariectomy and testosterone) and an Fov (ovariectomy) group (Table 1), containing 9, 11, and 12 animals, respectively. Two weeks after ovariectomy,

testosterone (10 mg/kg) was intraperitoneally injected into the rats of the Ft and Ft/ov groups twice a week for 6 months as described by Deguchi *et al.*³⁾.

4) Male groups

The male rats were classified into an Me (estrogen), an Me/or (orchietomy and estrogen), and an Mor (orchietomy) group (Table 1), each containing 9 animals. Two weeks after orchietomy, estrogen (5 mg/kg) was intraperitoneally injected into the rats of the Me and Me/or groups once a week for 6 months as described by Deguchi *et al.*³⁾.

3. Observation methods

Macroscopic changes in the tongue of each animal were examined and photographed once a week for 6 months under anesthesia induced by diethyl ether (pentobarbital®) inhalation. At 8 months after starting 4NQO administration, the rats were euthanized with an injection of 0.5 ml/kg of 1.0% pentobarbital into the heart. Excised tongues were immediately immersed and fixed in 10% neutral buffered formalin, thinly sliced in the sagittal direction, embedded in paraffin, sectioned at 4 μ m, and stained with hematoxylin and eosin for histopathological examination. Major organs were also examined at autopsy.

4. Comparisons of groups

The Mann-Whitney test compared various parameters among the experimental groups using a Windows version of the statistical software, SPSS (SPSS Ltd., Tokyo, Japan).

Table 1 Grouping of experimental animals

Sex	Group	Experimental Agent
Female	A	Testosterone
	B	Testosterone + Ovariectomy
	C	Ovariectomy
Male	A	Estrogen
	B	Estrogen + Orchietomy
	C	Orchietomy

Results

1. Survival rates

The survival rates over the 6-month experimental period were 55.5% (5/9), in the Ft group, 72.7% (8/11) in the Ft/ov group, 91.6% (11/12) in the Fov group, 77.7% (7/9) in the Me group, 66.6% (6/9) in the Me/or group and 88.8% (8/9) in the Mor group.

2. Macroscopic observation

Table 2 shows the results of macroscopic observations. Lesions of the tongue became apparent in all groups by the 20th experimental week. The macroscopic changes were classified into Ulcer type (Fig. 1), Massive type (Fig. 2), Papillary type (Fig. 3), Leukoplakia

type (Fig. 4), and Erosion type (Fig. 5).

All of the Ft groups developed Ulcer- or Papillary-type lesions. All of the Ft/ov groups developed Papillary-, Mass-, or Leukoplakia-type lesions. In contrast, Ulcer- or Papillary- and Erosion-type lesions were discovered in only 45.5% (5/11) and 54.5% (6/11), respectively, of the Fov group.

Papillary- or Mass-type lesions were developed in 100% (7/7) of the Me group. Papillary- or Leukoplakia-type lesions were evident in 100% (6/6) of the Me/or group. Papillary- and Mass-type lesions were developed in 75.0% (6/8) and Erosive-type lesions in 25.0% (2/8) of the Mor group. No abnormalities were evident in other major organs.

Table 2 Results of macroscopic appearance

Sex	Group	Ulcer	Papillary	Mass	Leukoplakia	Erosion
Female	A	2	3	0	0	0
	B	0	6	1	1	0
	C	4	1	0	0	6
	total	6	10	1	1	6
Male	A	0	6	1	0	0
	B	0	4	0	2	0
	C	0	4	2	0	2
	total	0	14	3	2	2
Total		6	24	4	3	8

Fig. 1 Ulcer-type lesion on dorsum of the tongue

Fig. 2 Massive-type lesion on dorsum of the tongue

3. Histopathological observation

Table 3 shows the results of histopathological observations. The carcinoma were classified into 5 types: well-differentiated squamous cell

Fig. 3 Papillary-type lesion on dorsum of the tongue

Fig. 4 Leukoplakia-type lesion on dorsum of the tongue

Fig. 5 Erosion-type lesion on dorsum of the tongue

carcinoma with a strong tendency towards invasion (Fig. 6), early invasive squamous cell carcinoma (Fig. 7), carcinoma *in situ* (Fig. 8), and moderate or mild dysplasia (Fig. 9).

In the female groups, we confirmed well-differentiated type squamous cell carcinoma in all of the Ft group and in 25.0% (2/8) of the Ft/ov group. Early invasive type squamous cell carcinoma was confirmed in the remaining 75.0% (6/8) of the Ft/ov group. Squamous cell carcinoma and dysplasia were confirmed in 45.5% (5/11) and in 54.5% (6/11), respectively, of the Fov group.

In the male groups, we observed well-differentiated squamous cell carcinoma in 71.4% (5/7) and early invasive squamous cell carcinoma in the remaining 28.6% (2/7) of the Me group. Well-differentiated squamous cell carcinoma occurred at an incidence of 50.0% (2/4) in the Me/or group. We also

Table 3 Results of histopathological observation

	Group	Carcinoma			Dysplasia	
		invasive	early invasive	Ca <i>in situ</i>	moderate	mild
Female	A	5	0	0	0	0
	B	2	6	0	0	0
	C	5	0	0	4	2
Male	A	5	2	0	0	0
	B	2	1	1	2	0
	C	4	1	0	1	2

Fig. 6 Well differentiated-type squamous cell carcinoma with keratin pearls, infiltrating down into muscle

Fig. 7 Early invasive-type squamous cell carcinoma with keratin pearls formation

confirmed squamous cell carcinoma in 62.5% (5/8) and dysplasia in 37.5% (3/8) of the Mor group (Table 3).

4. Comparison of different groups

The lesions in both the female and male groups were macroscopically and histopathologically similar to those in the reported rat models of tongue carcinoma induced by 4NQO. The histopathological findings among the females, among the males, and between the female and male groups showed no significant difference.

In order to determine the influence of sex hormones in the carcinogenetic process, we compared the results of histopathological

observation by dividing each group into a Testosterone(+) subgroup (T+: Ft, Ft/ov, Me) and a Testosterone(-) subgroup (T-: Fov, Me/or, Mor). We further divided the Testosterone(-) subgroup into a Testosterone(-)/Estrogen(-):T(-)E(-) cohort and a Testosterone(-)/Estrogen(+):T(-)E(+) cohort. Carcinogenesis leading to squamous cell carcinoma in the T(+) subgroup was observed in 100% (20/20), and in the T(-) group in 56.0% (14/25), while the remaining 44.0% (11/25) of the samples showed dysplasia. Furthermore, dysplasia was found at an incidence of 47.4% (9/19) and squamous cell carcinoma at 52.6% (10/19) in the T(-)E(-) cohort, while in the T(-)E(+)

Fig. 8 Carcinoma *in situ* showing irregular rate process

Fig. 9 Severe dysplasia showing severe atypism of basal cells and increased number of mitotic figures

cohort, dysplasia was found in 33.3% (2/6), and squamous cell carcinoma in 66.7% (4/6).

A significant difference in carcinogenesis was observed between the T(+) and T(-) subgroups. (The Mann-Whitney test: $p < 0.05$) (Figs 10, 11)

Discussion

Ohne *et al.*¹⁰⁾ originally described 4NQO-induced rat tongue carcinoma, and then Katakura *et al.*⁵⁾ induced well-differentiated type squamous cell carcinoma in male S-D rats. Nevertheless, the influence of sex hormones in the carcinogenesis of 4NQO-induced

tongue carcinoma has not been studied in detail.

In this study, squamous cell carcinoma developed in all rats that were given testosterone regardless of sex. On the other hand, estrogen elicited no significant difference in rate of carcinogenesis. Testosterone and/or ovariectomy increased the rate of carcinogenesis, whereas estrogen and/or orchiectomy elicited a decrease. These findings indicate that the induction of squamous cell carcinoma in the tongue was influenced by testosterone and gonadectomy and/or the opposite sex hormone.

Squamous cell carcinoma was found to develop specifically in the swollen regions of

Fig. 10 Analyzed with respect to the presence of testosterone
 $p < 0.05$ (compared with testosterone (+))

Fig. 11 Analyzed with respect to the presence of estrogen

the tongue when the rats were allowed free access to drinking water containing 4NQO. This carcinogen is metabolized by 4NQO reductase to 4HAQO, which then initiates carcinogenesis by blocking the transforming activity of DNA^{1,4)}. However, whether this enzyme is present in the tongue remains unclear. Mochizuki⁸⁾ found a high concentration of 4NQO in the posterior surface of the filiform papillae where the tongue was swollen and concluded that since tongue carcinoma specifically developed in this area, local metabolism of 4NQO facilitated carcinogenesis. However the relationship between sex hormones and localization of 4HAQO or existence of 4HAQO in oral tissue remains to be clarified.

Toyokuni *et al.*¹⁸⁾ investigated the relationship between a carcinogen and experimentally-induced renal cell carcinoma (without sex differentiation). They administered ferric nitriloacetate (Fe-NTA) to ddY mice to inves-

tigate the relationship between sex hormones and gonadectomy. They classified mice into groups in a manner similar to those in the present study and found that the rate of carcinogenesis was significantly higher in mice that received testosterone. The results of their study suggested that sex hormones affected the lipid peroxidation of Fe-NTA. Deguchi *et al.*³⁾ conducted a similar study and Okada *et al.*¹⁴⁾ found a sex difference in the lipid peroxidation of Fe-NTA in the kidney of mice, and concluded that the sensitivity to Fe-NTA was localized.

It is reported that sex steroids such as androgen, estrogen, or progesterone and cortisol bind to cell-receptors in the gingival tissue and parotid or minor salivary glands^{2,7)}.

Testosterone is metabolized in human males with healthy gingiva, and in both human males and females with inflamed gingiva^{9,12,19)}. However, it is uncertain whether the receptor is in the tongue.

Even if there is a testosterone's receptor in rat tongue, this does not indicate that it is directly involved in 4NQO-induced carcinogenesis. The influence of testosterone on 4NQO-induced carcinogenesis, merits further investigation. The result of this study, suggest a role for sex hormones in 4NQO-induced carcinogenesis.

Conclusions

1. Macroscopic findings on tongue lesions revealed no significant differences among the six groups of rats given with 4NQO and treated with either sex hormones or gonadectomy.
2. Squamous cell carcinoma developed in the tongue of all rats given testosterone, and the rate of carcinogenesis significantly differed between the rats with and without testosterone ($p < 0.05$).
3. The results suggest that testosterone plays a role in 4NQO-induced carcinogenesis in rat tongue carcinoma.

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