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Strain Difference of Susceptibility to 4-Nitroquinoline 1-Oxide-induced Tongue Carcinoma in Rats

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Strain difference of susceptibility to 4-nitroquinoline 1-oxide (4NQO)-induced squamous cell carcinomas of the tongue among Dark-Agouti, Long-Evans, Sprague-Dawley, ACI/Ms, Fischer 344, Donryu and Wistar/Furth rats was surveyed by evaluating the survival times, incidences and sizes of developed tumors as markers of susceptibility. Administration of 4NQO dissolved in drinking water induced squamous cell carcinomas in various sites of the upper digestive tract mucosa of all the experimental male and female rats of the seven strains. Regarding the mean survival times, Wistar/Furth rats survived much longer than any other strain of rats, and Dark-Agouti showed the shortest survival. The incidence of large, mass-type carcinomas of the tongue of Dark-Agouti rats was higher than in any other strain of rats, while that of Wistar/Furth rats was the lowest. Subsequently the mitotic activity and bromodeoxyuridine incorporation in the tongue epithelium of Dark-Agouti and Wistar/Furth rats were estimated after a short-term administration of 4NQO. There was a pronounced difference between the two strains of rats, because the proliferative responses of the tongue epithelium of Dark-Agouti rats to the 4NQO stimulation were much higher than those of Wistar/Furth rats. These results indicated that there are marked differences in the susceptibility to 4NQO-induced tongue carcinoma among the seven strains of rats, and that Dark-Agouti and Wistar/Furth rats could be useful as models of highly and poorly susceptible strains, respectively, for further genetic analysis.

Key words: Strain difference — 4NQO-induced tongue carcinoma — Rat

4-Nitroquinoline 1-oxide (4NQO) exhibits potent and pleiotrophic carcinogenic effects on various species of animals.[1,2] Several experiments have shown that administration of 4NQO dissolved in drinking water induces a high incidence of squamous cell carcinoma in the upper digestive tract, including the tongue.[3,4] This methodology, naturally applying the carcinogen by dissolving it in drinking water, has the advantage of involving minimal stress to the animals. Further, it was reported that this model of squamous cell carcinoma is very convenient.[4] Thus, we adopted this methodology to induce carcinogenesis of rat tongue.

This study was designed to examine the differences of susceptibility to 4NQO-induced tongue carcinoma among seven strains of rats. The purpose of the study was to identify strain differences which could guide further analysis of the genetic background controlling rat tongue carcinogenesis. We therefore evaluated the survival times of rats, and the incidences and sizes of carcinomas induced on the rat tongue. Then we measured the mitotic activity of epithelial cells of the tongue and the rate of bromodeoxyuridine (BrdU) incorporation into them after short-term administration of this carcinogen to estimate whether proliferative responses reflect the susceptibility or not.

MATERIALS AND METHODS

Rats Seven strains of rats were used in this study, as follows. 1) Inbred Dark-Agouti (DA/Slc) rats were purchased from Shizuoka Laboratory Animal Center, Hamamatsu. 2) Inbred Fischer 344 (F344/DuCrj) rats were purchased from Charles River Japan, Inc., Atsugi. 3) Long-Evans rats were introduced into the laboratory of Saitama Cancer Center, Saitama, in 1969 from a closed colony maintained in the Ben May Laboratory for Cancer Research, University of Chicago, Chicago. A mutant with pink-eyed dilution (p/p) was found in 1970 in the Long-Evans colony, and the mutation (Long-Evans/stm) was fixed by selective mating. Thereafter, they were maintained by sister-brother mating and used at their 44th or 45th generation. 4) Wistar/Furth rats were introduced in 1976 from Hiroshima University, Hiroshima. They were at the 29th or 30th generation of sister-brother mating in our laboratory. 5) Donryu rats were derived from a breeding pair obtained in 1976 from Osaka University, Osaka. They were at the 30th to 32nd

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generations of sister-brother mating in our laboratory. 6) ACI/Ms rats were progenies of an inbred breeding pair obtained in 1970 from the National Institute of Genetics, Mishima. They were used at the 38th to 40th generations of inbreeding in our laboratory. 7) Sprague-Dawley rats (SD/Jcl) were outbred rats purchased from Japan Clea Co., Tokyo. None of these rat developed a spontaneous tumor before 12 months of age.

4NQO administration 4NQO (Nakalai Tesque, Kyoto) was dissolved in 0.5% ethanol solution at a concentration of 200 mg/liter to prepare a refrigerated stock solution, which was diluted with distilled water just before use to obtain a concentration of 0.001%. All the rats were allowed access to 4NQO-containing drinking water *ad libitum* from 5 p.m. to 9 a.m., but no water was given thereafter. The rats were inspected twice a day and weighed once a week. The volume of water consumed was measured daily to estimate the amount of 4NQO taken. The amount was approximately 70 mg/100 g body weight/day in all cases, without significant sex, strain or age difference.

Experiment 1: Sex and strain differences in 4NQO-induced carcinogenesis in rat tongue Administration of 4NQO to the experimental male and female rats of the seven strains was started at the age of six weeks, and lasted until death. All the rats were killed when they became moribund, and full autopsy including histopathological examination of various sites of the oral mucosa, pharynx, esophagus, stomach, small and large intestines, liver, pancreas, larynx, trachea, lung, heart, spleen, kidney, urinary bladder, adrenal, pituitary gland, brain, bone marrow and lymph nodes was carried out.

The mean survival time of each group of rats was evaluated and analyzed statistically. The autopsy findings were recorded individually, and incidence of mass-type carcinomas of the tongue was compared among the seven strains of rats and between both sexes of each strain. In this paper, we use the term 'mass-type carcinoma' to describe a carcinoma more than 7 mm in the longest diameter. Carcinomas of this size showed macroscopically as well as histopathologically an apparent infiltrative growth into the muscular layer.

Experiment 2: Mitosis index Six-week-old male rats of Dark-Agouti and Wistar/Furth strains were supplied with drinking water containing 0.001% 4NQO for four weeks. After that, they were treated i.p. with colchicine (0.5 mg/100 g body weight) and were killed at 4:30 p.m., 3.5 h later, under ethyl ether anesthesia. Following decapitation, the tongue with both mandibles was soaked carefully in 10% buffered formalin. Serial paraffin sections (6 μm in thickness) parallel to the sagittal direction were prepared from the vallecula epiglottica to the anterior border of the mouth floor. The sections were stained with hematoxylin and eosin.

Out of 100 sections from each specimen, every tenth section was projected at 200 times magnification. The number of arrested metaphases in the surface epithelium was counted and used for the calculation of the mitosis index. The length of the outer surface of the epithelium within the delineated area was measured on each drawing

Table I. Survival Time of Seven Strains of Rats Treated with 4NQO

Strain	Sex	N	Survival time (days)	Mean (days)	M+F, Mean (days)	SD
Dark-Agouti	M	16	126–217	167.44	171.10	29.59
	F	15	120–226	175.00		
Long-Evans	M	12	129-234	176.58	182.00	38.80
	F	10	126–257	188.50		
Sprague-Dawley	M	12	142–235	174.92	186.21	31.21
	F	12	151–241	197.50		
ACI/Ms	M	38	141–270	186.14	189.44	27.42
	F	13	130–226	196.54		
Fischer 344	M	12	147–217	193.08	199.54	30.13
	F	12	154–266	206.00		
Donryu	M	11	130–243	195.55	205.39	31.02
	F	12	190–257	214.42		
Wistar/Furth	M	14	213–290	236.50	238.24	23.55
	F	15	182–290	239.87		

Scheffe's S test: * $P<0.01$, ** $P<0.05$.

with a curvometer. The values were used for calculating the mitosis index, which is the mean number of metaphases per 1 mm of the outer epithelial surface of each animal. This method for evaluation of the mitosis index was modified from the method of Karring and Löe.[5]

Experiment 3: BrdU-labeling index Six-week-old male rats of Dark-Agouti and Wistar/Furth strains were supplied with drinking water containing 0.001% 4NQO for four weeks. One hour before being killed at 4:30 p.m., all the rats were treated i.p. with BrdU (5 mg/1 ml saline/100 g body weight). The whole tongue with both mandibles was removed carefully, and fixed in cold 70% ethanol for about 12 h. The tongue blocks were cut parallel to the sagittal direction and then dehydrated and embedded in paraffin. The paraffin sections were prepared as in Experiment 2, and avidin-biotin complex immunostaining (Vectastain ABC kit, Vector) was done with anti-BrdU antibody (Beckton-Deckinson; monoclonal, dilution 1/500) for demonstration of BrdU incorporation into the cellular nuclei.

For assessment of the proliferative characteristics of the tongue epithelium, the number of immunohistochemically positive cells was counted. The longitudinal sections of the tongue extending from the vallecula epiglottica to the anterior border of the mouth floor were analyzed. For the scoring of each 1 mm length value, the number of nuclei of epithelial cells labeled with BrdU was estimated according to the method described above.[5]

Dark-Agouti		Tongue	Floor of mouth	Mandibular gingiva	Buccal mucosa	Maxillary gingiva	Hard palate	Pharynx	Larynx	Trachea	Esophagus	Forestomach	Others	Survival Time (days)
Male-	1	●*	○			○	○	○*			○		Lip	217
	2	●*	○	●		○	○					○		136
	3	●*		●		○	○						Pn	190
	4	●*		●			○	○						196
	5	●*	●		○		○							189
	6	●				○	○	○						206
	7	●*				○	○	○*						179
	8	●				○	○							130
	9	●*		○	●	○	○						Pn	171
	10	●												126
	11	○*		●			○							158
	12	○*				●	○	○*			○			189
	13	●*					○							127
	14	●		○		○	○							153
	15	●				○	○	○						154
	16	●*		○		○	○							158
Female-	1	○*		●			○							154
	2	●*					●	○						181
	3	●				○	●							200
	4	●*				○	○	○						120
	5	●						○					Pn	158
	6	●*			●	○	○	○						190
	7	●*				○	○	○*					Lip	226
	8	●*				○	○							175
	9	●*				○	○	○*					Lip	226
	10	●*					○	○*					Lip	212
	11	●*	○	○			○	○				○		137
	12	●*					○							154
	13	●*		○		○	○							154
	14	●		○		○*	○	○					Pn	169
	15	●*				○*	○	○					Pn	169

Fig. 1. Summary of autopsy findings of Dark-Agouti rats. Note that every rat had multiple carcinomas in the various sites of the upper digestive tract and most rats had one or more mass-type carcinoma(s) in the tongue. ●; Mass-type carcinoma measuring more than 7 mm in its longest diameter. ●*; Multiple carcinomas with one or more mass-type carcinoma(s). ○; Carcinoma measuring less than 7 mm in its longest diameter. ○*; Multiple carcinomas without a mass-type carcinoma. Lip; Carcinoma of the lip. Pn; Pneumonia.

RESULTS

Experiment 1: Sex and strain differences in 4NQO-induced carcinogenesis in rat tongue Administration of 4NQO p.o. by our protocol was highly carcinogenic in all seven strains of rats. However, there was a remarkable difference among strains in the survival times (Table I). The mean survival time was the shortest in Dark-Agouti rats, increasing in the order of Long-Evans, Sprague-Dawley, ACI/Ms, Fischer 344, Donryu, and being longest in Wistar/Furth rats. The differences in mean survival times of the examined seven strains of rats were found to be statistically significant by Scheffe's S test. The survival times were slightly shorter in males than in females in all seven strains, but there was no statistically significant difference between the males and females in any strain.

Multiple, various-sized squamous cell carcinomas were observed macro- and microscopically in all the experimental rats. Almost all of the carcinomas were of well differentiated keratinizing type. No significant sex or strain difference was observed in the incidence of overall squamous cell carcinomas in the upper digestive tract mucosa, from the lips to the forestomach. The autopsy findings of Dark-Agouti rats and Wistar/Furth rats, having the longest and the shortest mean survival times, respectively, are summarized in Figs. 1 and 2.

The predominant and characteristic autopsy finding was that all the experimental rats of Dark-Agouti, Sprague-Dawley, ACI/Ms, Fischer 344 and Donryu strains had one or multiple squamous cell carcinoma(s) in the tongue (incidence: 100%), and they showed a high incidence of mass-type carcinoma of the tongue, in the order of Dark-Agouti (28/31, 90%) (Fig. 3), Fischer 344 (20/24, 83%), ACI/Ms (33/41, 80%), Sprague-Dawley (15/24, 63%), and Donryu (14/23, 61%) rats. In Long-Evans rats, the incidence of tongue carcinoma was also high (100%), but the incidence of mass-type carcinoma was rather low (10/22, 45%). Peculiarly, Wistar/Furth rats had a rather high incidence of tongue carcinoma (22/29, 76%), but only seven of them had

Wistar/Furth		Tongue	Floor of mouth	Mandibular gingiva	Buccal mucosa	Maxillary gingiva	Hard palate	Pharynx	Larynx	Trachea	Esophagus	Forestomach	Others	Survival Time (days)
Male-	1	○*		●		○	○	○						232
	2	○		●			○	○*					Pn	246
	3	●*		○		●	○	○	○	○	○		Pn	290
	4		○	●		○	○	○*						217
	5	○		●			○	○*	○				Pn	230
	6						○	○*	○*	○				222
	7	○				○	○	○	○*	○*			Pn	262
	8						○	○	○				Pn	213
	9		●				○	○*	○				Lymph node metastasis	231
	10	○				○	○	○	○				Pn	217
	11	●*		●			○		○	○			Lip	246
	12	○	○*	●	○	○	○	○	○				Pn	230
	13	●					○	○	○	○	○			246
	14	○				○	○	○*	○*	○			Pn	229
Female-	1	○			○	○	○	○*	○*	○*			Pn	273
	2	○				○	○	○	○*	○	○		Pn	262
	3	○		○		○	○	○	○	○*		○	Pn	246
	4	●				○	○	○*						258
	5	●		●			○	○						217
	6			●		○	○	○	○		○			230
	7	○	○	●			○		○					246
	8	○		○		○	○	○*	○	○		○	Pn	256
	9	●		●	○	○	○	○*	○					230
	10	●				○	○		○	○*			Pn	215
	11	○*				●	○	○*	○					239
	12	○		○		○	○	○	○*	○			Pn	290
	13			●			○	○*						182
	14					●	○	○	○				Pn	208
	15	○				●	○	○*	○*				Pn	246

Fig. 2. Summary of autopsy findings of Wistar/Furth rats. Note that every rat had multiple carcinomas in various sites of the upper digestive tract and rather few rats had mass-type tongue carcinoma(s). Symbols; See the legend to Fig. 1.

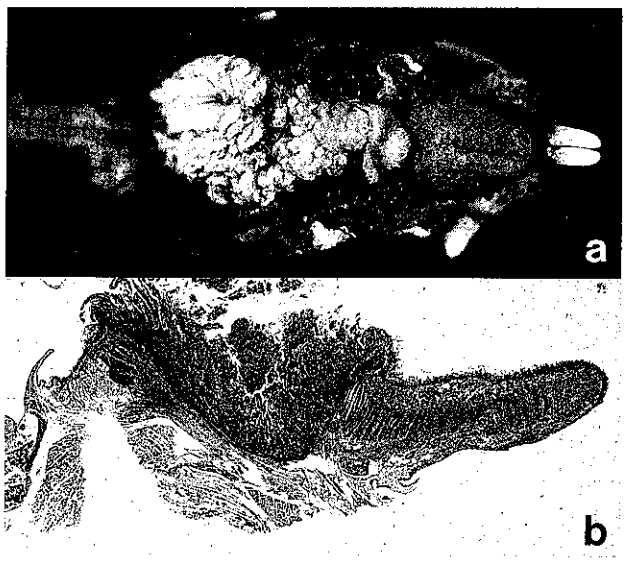

Fig. 3. a: A large, mass-type carcinoma of the posterior half of the dorsal aspect of the tongue of a Dark-Agouti rat (No. male-10) on the 126th experimental day. b: Histological section of the same material as shown in Fig. 3a. Note the carcinoma invading the tongue muscles.

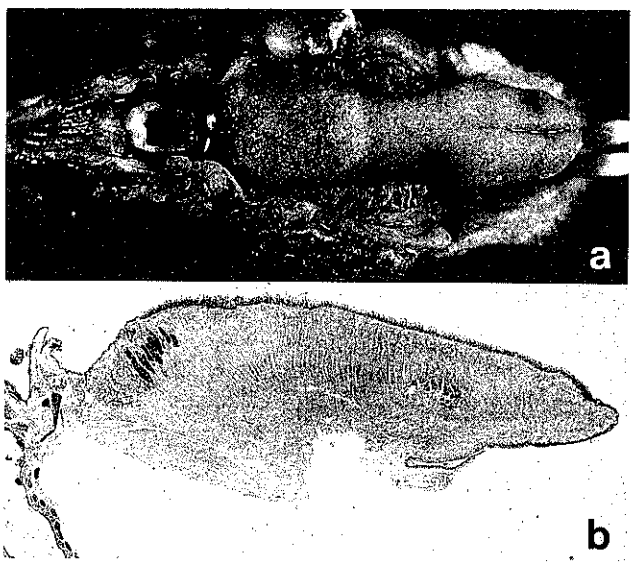

Fig. 4. a: No visible carcinoma in the dorsal aspect of the tongue of a Wistar/Furth rat (No. male-8) on the 213th day. b: Hitstological section of the same material as shown in Fig. 4a. The surface epithelium is diffusely hyperplastic without a carcinoma.

mass-type carcinoma(s) of the tongue (7/29, 24%). There was no visible carcinoma in the tongue of seven out of the 29 rats examined (Fig. 4). The chi square-test disclosed that there were statistical significant differences in the incidences of mass-type carcinoma of the tongue between Wistar/Furth rats and each of the other strains except for Long-Evans rats (Table II).

Metastatic lesion was found in a cervical lymph node of a Wistar/Furth rat (Fig. 2), but distant organ metastasis was not detected in any of the examined animals.

Experiment 2 and Experiment 3: Proliferative response of tongue mucosa during carcinogenesis induced by 4NQO in highly susceptible Dark-Agouti rats and poorly susceptible Wistar/Furth rats There was no significant difference in the numbers, position or ranges of distribution of cells showing arrested metaphases, or cells labeled with BrdU in control Dark-Agouti and control Wistar/Furth rats as shown in Figs. 5a and 6a. However, after treatment with 4NQO for four weeks, the mean number of metaphases and the mean number of labeled cells in both strains were significantly larger than those in each of the control groups (Figs. 5b and 6b). In Dark-Agouti rats, the mean numbers of metaphases and BrdU-labeled cells were significantly larger than those in Wistar/Furth rats (Figs. 7). In Experiments 2 and 3, no dysplastic change was apparent in the surface epithelium of the tongue in any of the rats examined.

DISCUSSION

Experiment 1 of this study was conducted in rat models for tongue carcinoma to generate information bearing on strain differences in response to 4NQO administration. The results or Experiment 1 could be summarized as follows: 1) 4NQO administration produced squamous cell carcinomas in the upper digestive tract mucosa of all the experimental rats, 2) the mean survival time of Dark-Agouti rats was the shortest among the seven strains of rats and they showed multiple mass-type carcinomas in the tongue, and 3) the mean survival time of Wistar/Furth rats was the longest among the seven strains of rats, and they had a much lower incidence of mass-type tongue carcinomas.

Regarding the autopsy findings of Dark-Agouti rats, the survival time seemed to be closely associated with the growth of tongue carcinoma and subsequent general emaciation, while aspiration pneumonia and/or respiratory disturbances due to the development of pharyngolaryngeal carcinomas seemed to be mainly related to the cause of death of Wistar/Furth rats. In Wistar/Furth rats survival time was much longer than in any other strain of rats, and this lengthening of the survival time seemed to allow the development of carcinomas, although they were not so large, in the pharyngolaryngeal

Table II. Comparisons of the Number of Rats with Mass-type Carcinoma(s) of the Tongue

Strain	Sex	No. of rats (total)	No. of rats with carcinoma(s) of the various sites of the upper digestive tract (total : incidence)	No. of rats with carcinoma(s) of the tongue (total : incidence)	No. of rat with mass-type carcinoma(s) of the tongue (total : incidence)
Dark-Agouti	M	16	16	16	14
	F	15 (31)	15 (31:100%)	15 (31:100%)	14 (28:90%)
Long-Evans/Stm	M	12	12	12	6
	F	10 (22)	10 (22:100%)	10 (22:100%)	4 (10:45%)
Sprague-Dawley	M	12	12	12	7
	F	12 (24)	12 (24:100%)	12 (24:100%)	8 (15:63%)
ACI/Ms	M	28	28	28	23
	F	13 (41)	13 (41:100%)	13 (41:100%)	10 (33:80%)
Fischer 344	M	12	12	12	10
	F	12 (24)	12 (24:100%)	12 (24:100%)	10 (20:83%)
Donryu	M	11	11	11	7
	F	12 (23)	12 (23:100%)	12 (23:100%)	7 (14:61%)
Wistar/Furth	M	14	14	10	3
	F	15 (29)	15 (29:100%)	12 (22:76%)	4 (7:24%)

Chi square-test: * $P<0.001$, ** $P<0.01$.

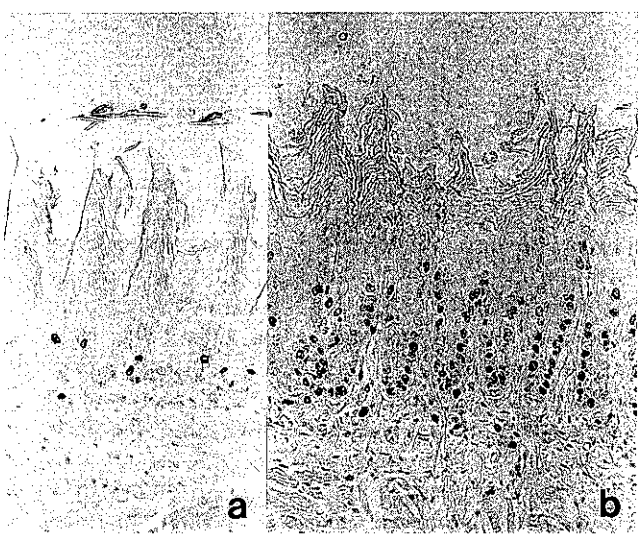

Fig. 5. a: BrdU incorporation in the nuclei of the epithelial cells of the tongue of a control Dark-Agouti rat without 4NQO treatment. Immunostaining for BrdU using monoclonal antibody (mouse IgG). b: BrdU incorporation in the nuclei of the epithelial cells of the tongue of a Dark-Agouti rat treated with 4NQO for four weeks.

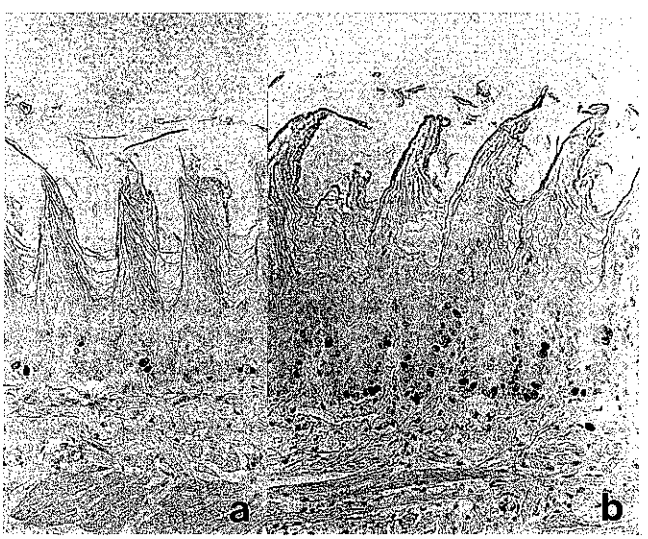

Fig. 6. a: BrdU incorporation in the nuclei of the epithelial cells of the tongue of a control Wistar/Furth rat without 4NQO treatment. b: BrdU incorporation in the nuclei of the epithelial cells of the tongue of a Wistar/Furth rat treated with 4NQO for four weeks. Note that the incorporation is less marked than that of the Dark-Agouti rat shown in Fig. 5b.

regions. Mass-type carcinomas with a longest diameter measuring more than 7 mm, which were seen in most Dark-Agouti rats and a few Wistar/Furth rats, showed a characteristic infiltrative growth into the muscular layer, suggesting a rapid, aggressive proliferation. Accordingly, it is reasonable to consider that Dark-Agouti rats had

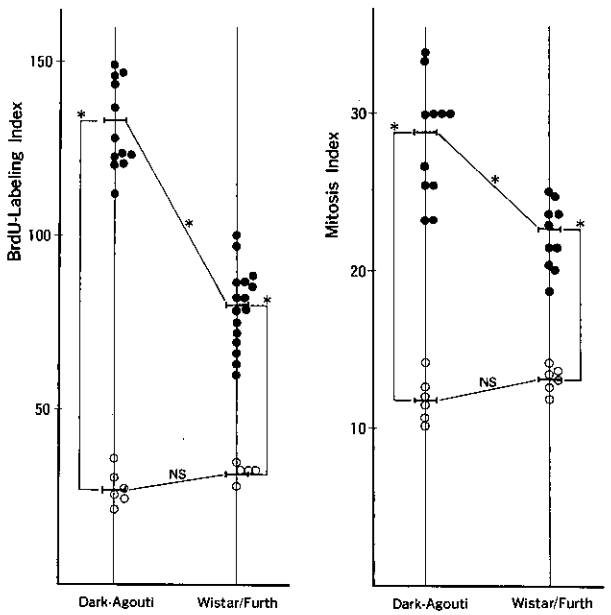

Fig. 7. BrdU-labeling index (left) and mitosis index (right) of the surface epithelium along the saggital direction near the midline of the tongues of Dark-Agouti and Wistar/Furth rats with and without 4NQO treatment for four weeks. The indexes are expressed in terms of the number of epithelial cells in 1 mm length of the tongue surface. Both mean indexes are different with statistical significance between the two treated strains and also between the treated rats and nontreated rats of each strain. ●; A rat treated with 4NQO for four weeks. ○; A control rat without 4NQO treatment. *; *t* test, significant ($P<0.01$). NS; *t* test, not significant.

the highest susceptibility to 4NQO-induced tongue carcinogenesis while Wistar/Furth rats had the lowest.

Carcinogenesis is considered to be related to the cell proliferation rate. Some investigators[6-8] have reported that the proliferative responses of the target cells of experimental animals to some chemical carcinogens were higher in susceptible strains than in nonsusceptible strains, suggesting that susceptibility is controlled by the genetically determined indigenous proliferative characteristics of the organs and tissues. Therefore, we conducted the additional Experiments 2 and 3 reported herein. Experiments 2 and 3 disclosed that there was a pronounced difference between Dark-Agouti and Wistar/Furth rats in the mitosis and BrdU-labeling indexes of the tongue epithelium after short-term (for four weeks) administration of 4NQO. The results of Experiments 2 and 3 are thus consistent with the results of Experiment 1.

With respect to 4NQO-induced tongue carcinogenesis, host genetic control can be assumed in, for example, metabolic conversion of 4NQO to 4-hydroxyaminoquinoline 1-oxide (4HAQO),[9-11] which is believed to be a proximate derivative of the potent carcinogen 4NQO, reaction of 4HAQO with DNA to yield several kinds of adducts,[12,13] excision of these 4HAQO-DNA adducts with repair of injured DNA strands,[13-15] access of 4NQO and/or 4HAQO to the target tongue epithelium, anatomical-physiological characteristics of the oral cavity and tongue, immunological responses to transformed cells, endocrinological influences, etc.

At the present time, however, the question of what is the principal genetic factor influencing the carcinogenic mechanism of 4NQO is not precisely resolved. To clarify the genetic factors involved in the susceptibility to 4NQO-induced tongue carcinogenesis, we should conduct a number of cross and backcross experiments involving highly susceptible Dark-Agouti and poorly susceptible Wistar/Furth rats. Observation of the incidences of tumors in the parental strains, crosses and backcrosses will allow us to draw some conclusions about the inheritance of tumor susceptibility and to propose a tentative genetic model in which tongue carcinoma susceptibility is determined by certain genes.[16-19]

Battisto *et al.*[20] reported that Dark-Agouti rats were highly susceptible to adjuvant arthritis and this susceptibility was controlled by an autosomal dominant gene. We have no evidence suggesting an interrelationship between the high susceptibilities to both adjuvant arthritis and 4NQO-induced tongue carcinoma in Dark-Agouti rats, and indeed the gene controlling the susceptibility to adjuvant arthritis was presumed not to control directly the susceptibility to 4NQO-induced tongue carcinoma, because Fischer 344 rats, which are highly susceptible to 4NQO-induced tongue carcinoma like Dark-Agouti rats, as seen in the present study, were highly resistant to adjuvant arthritis in the survey of Battisto *et al.*[20]

In the present study, no statistically significant difference was detected in the mean survival times or the incidences of mass-type carcinoma of the tongue between the male and female rats of each strain, though the mean survival times of the males were a little shorter than those of the females of each strain.

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