Snuff tumorigenesis: effects of long-term snuff administration after initiation with 4-nitroquinoline-N-oxide and herpes simplex virus type 1

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The tumor promoting effects of snuff was studied in Lewis rats initiated with 4-nitroquinoline-N-oxide (4-NQO) and Sprague Dawley rats repeatedly inoculated with herpes simplex virus type 1 (HSV-1). The test substances were administered in a surgically created canal in the lower lips of the rats. There were 15 rats in each test group and 10 rats in the control group. In the groups treated with 4-NQO and 4-NQO + snuff, 8 and 12 tumors (5 and 9 malignant) were found, respectively. In the group subjected to HSV-1 only, 3 tumors were found (2 malignant), in the group subjected to snuff only, 4 tumors were found (3 malignant) and in the group subjected to the combination of HSV-1 and snuff, 13 tumors were found (7 malignant). In the control group only one malignancy was found. The study did not show any promoting effects of snuff in the oral cavity after initiation with 4-NQO. Neither was there any increase in the number of oral tumors in rats treated with HSV-1 and snuff. However, there was a marked increase in the number of malignant tumors outside the oral cavity in the group treated with HSV-1 and snuff, underlining the importance of interactions between these two agents in the development of malignant lesions.

Key words: herpes simplex; smokeless tobacco; snuff; tumorigenesis.

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Earlier studies have demonstrated that snuff dipping affects both general and oral health (1–3). Snuff dipping results in characteristic intraoral mucosal lesions, both in rats and humans. In rats these lesions have been shown to be reversible after discontinuation of snuff (4).

The most serious complication associated with snuff dipping is the markedly increased risk of developing oral cancer after long exposure. Thus, WINN *et al.* (5) demonstrated that snuff dipping is strongly associated with an increased risk of developing oral squamous cell carcinoma. In contrast to the human situation sufficient evidence to support carcinogenicity of snuff in experimental animal is lacking (6). To some extent this has earlier been related to the lack of a pertinent animal model. Such a model (7) has been developed, allowing life-long administration of snuff in a surgically created canal in the lower lip of the rat. Simulated snuff-dipping using this model have resulted in the development of oral tumors (8–10).

The development of cancer can be regarded as a two stage process, initiation and promotion (11). It has earlier been shown that 4-nitroquinoline-N-oxide (4-NQO) is a potent intraoral carcinogen. Thus 100% of animals painted in the hard palate for 6–8 months with 4-NQO developed carcinomas at the site of application (12). There is evidence supporting that a limited number of applications of 4-NQO is sufficient to initiate intraoral squamous cell carcinomas (13).

A large number of reports have associated herpes simplex virus type one (HSV-1) with leukoplakias, epithelial dysplasias and oral as well as cancers of the head and neck region (14, 15). HSV-1 is a ubiquitous human oral pathogen and approximately 70% of the adult population in the USA and western Europe is seropositive for HSV-1. Several previous reports have demonstrated that HSV-1 is capable of transforming cells in vitro (16, 17). A prerequisite for HSV to cause cell trans-

Table 1. Experimental protocol.

Group	Treatment		
I	Surgery	HSV-1	
II	Surgery		Snuff
III	Surgery	HSV-1	Snuff
IV	Surgery	4-NQO	
V	Surgery	4-NQO	Snuff
VI	Surgery		

formation is that virus induced cell lysis is prevented. In an earlier study we have demonstrated that snuff is capable of preventing HSV induced cell lysis (18), which may be one mechanism in snuff/HSV-1 tumorigenesis. Earlier studies have also shown that repeated infection with HSV-1 and simulated snuff dipping in rats and hamsters have resulted in induction of oral squamous cell carcinomas (10, 19).

The aim of the present study was to evaluate if snuff functions as a tumor promoter in rats initiated with 4-NQO or HSV-1. Using the earlier described model, rats were initiated with 4-NQO or inoculated with HSV-1 followed by life-long simulated snuff dipping in order to assess the tumor promoting effects.

Material and methods

Animals

Male, inbred Lewis rats (Groups IV & V) and Sprague Dawley rats (Groups I, II, III and, VI), purchased from Anticimex AB, Stockholm, were used. The rats were 3-months old with an avarage weight of 400 g when the experiment started. Three to four rats were kept in macrolon cages, (NO 3, Jacoby, Stockholm) with hardwood bedding. The animals were fed a standard pelleted diet (Astra Ewos AB, Södertälje), and tap water *ad libitum*. Temperature was kept constant between 21–23°C and the relative humidity 40%. Light followed diurnal rhythm and was never less than 8 h. The rats underwent a surgical procedure, described earlier, to create a canal in the lower lip which functioned as a reservoir for the test substances (7). The operation was followed by a 10-day healing phase, whereupon the experimental treatment began.

Snuff

A commercially available Swedish brand was used in the study. Analyses of the tobacco specific N-nitrosamines (TSNA) in this brand has been performed earlier. The average concentration of TSNA is: N-nitrosonornicotin 33000 µg/kg, 4-(methylnitrosamino)-1(-3pyridyl)-1-butanon 4600 µg/kg, N-nitrosoanatabin 40000 µg/kg, and N-nitrosoanabasin 1900 µg/kg respectively. The snuff was applied in the test canal, 8 AM and 4:30 PM 5 days weekly by means of a plastic syringe. The test canal was completely filled which on the average meant the position of 200 mg of snuff. The average exposition time was 12 h per day (7).

4-nitroquinoline-N-oxide (4-NQO)

4-NQO was used as an initiator and purchased from Fluka Ag Buchs S.G. Switzerland. The chemical was dissolved in propyleneglycol to a concentration of 0.5%.

Virus

Herpes simplex virus, type 1, strain F (HSV-1) was used in this study. Virus infectivity was assayed by plaque titration on green monley kidney cells (GMK-AH1) and expressed as plaque forming units (pfu) per ml.

Experimental design

Eighty-five rats were divided into six groups. All rats were operated on as described above and the experimental treatment began after a healing phase of 10 days. The experimental design is summarized in Table 1.

Group I – comprising 15 Sprague-Dawley rats, received 0.5 ml of HSV-1 suspension, 2.5×10^7 pfu/ml, absorbed in a cotton swab. The swab was placed in the test canal in the lower lip after scarification with a 26 gauge needle. This was repeated once monthly until the animals were killed at the end of the experiment, thus mimicking a recurrent HSV-infection (10).

Group II – comprising 15 Sprague-Dawley rats received snuff five days weekly, as described above until killed.

Group III – comprising 15 Sprague-Dawley rats, was treated with HSV-1 identically to Group I, but the treatment also included snuff administration identical to Group II, except for 1 day monthly which was occupied by the virus inoculation.

Group IV – comprising 15 Lewis rats received 4-NQO once weekly during five consecutive weeks. 0.05 ml of the solution (0.25 mg 4-NQO) was absorbed in a cotton swab. During each application, the swab was put in the test canal 24 h and then removed.

Group V – comprising 15 Lewis rats, was treated with 4-NQO identical to Group IV for 5 weeks followed by snuff administration 5 days weekly, identically to Groups II and III.

Group VI – comprising 10 Sprague-Dawley rats served as a control group and was treated with propyleneglycol on a cotton swab once weekly during 5 wk.

Two rats in Group I died from encephalitis after 21 and 27 wk, respectively and were excluded from the study. Another 11 rats were found dead and suffered from pronounced autolysis and were therefore excluded from the experiment. The remaining rats constitute the effective number of experimental animals shown in Tables 2–4.

All animals were killed when moribund after 16–30 months by injection of phenobarbital followed by exsanguination until cardiac arrest.

Histopathologic methods

Following gross exterior inspection all animals underwent necropsy for the recording of tumors. Specimens from gingival sulcus, test canal, lower lip, lungs, heart, liver, forestomach, urinary bladder, spleen, brain and other grossly abnormal tissues were taken for light microscopy examination. Tissue specimens were fixed in 4% neutral buffered formalin solution, embedded in paraffin, sectioned and stained by routine methods, hematoxylin – eosin and according to Weigert van Gieson. The specimens were coded and evaluated independently by two of the authors (P-A L. and SL J). The preneoplastic and reactive changes of the oral cavity were classified in accordance with the criteria given in "Definitons of leukoplakia and related lesions: an aid to studies of oral precancer" (20). Rat nephrosis was in three grades,

Table 2. Mean survival time in the different groups.

Group	*n*	Wk	Range
I	12	85	54–102
II	13	86	70–94
III	15	89	76–102
IV	12	116	82–131
V	12	115	95–123
VI	8	90	61–102

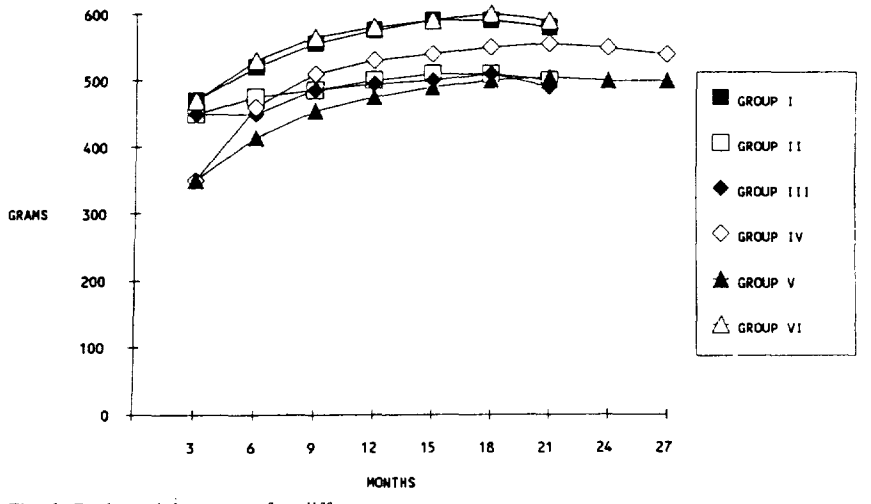

Fig. 1. Body weight curves for different groups.

as 1 (+), 2 (++), and 3 (+++) corresponding to mild, moderate, and severe.

Statistical methods

Statistical significance of differences in tumor incidence between the groups was calculated with Fisher's exact test. $P < 0.05$ was regarded statistically significant.

Results

General health effects

The mean survival time is given in Table 2. The Lewis rats (Groups IV and V) survived 6 months longer than the Sprague-Dawley rats. The mean body weight of the rats during the experiment is presented in Fig. 1. The weight of the Lewis rats at the start of the experiment was approximately 100 g less than the weight of the Sprague-Dawley rats. This difference diminished slightly but persisted throughout the experiment. Groups II, III, and V (snuff treated) had a slower weight gain than Groups I, IV, and VI. The difference in weight between snuff treated and not snuff treated groups was also statistically significant at 20 months age. Brain specimens from the two rats in Group I who died after 21 and 27 wk showed acute encephalitis but brain specimens from all other animals were normal by histologic examination.

Fig. 2. Well-differentiated keratinizing squamous cell carcinoma of lip in Group III rat. H-E, ×96.

Tumor incidence

The incidence, location, and histologic type of tumors is given in Table 3. One squamous cell carcinoma of the lower lip was found in Group I and two in Group IV (Fig. 2). They were all growing in close connection to the entrance of, but not in, the test canal. Squamous cell carcinomas of the oral cavity, located in the crevicular epithelium close to the orifice of the lip canal were found in two rats of Group V and in one rat of Groups II and IV, respectively (Fig. 3). Squamous cell carcinomas of the ear duct were found in one rat in Groups I and III, respectively. One nasal squamous cell carcinoma was found in a rat of Group II and one forestomach tumor was found in a Group V rat. None of those squamous cell carcinomas had metastasized and no animals had more than one malignant tumor. The incidence of squamous cell carcinomas of the head and neck region did not significantly differ between the different groups. However, the total number of tumors was significantly higher in Group III as compared to Groups I, II and VI (Table 3), ($P < 0.05$). The difference between Groups IV and V in tumor incidence was not statistically significant.

Fig. 3. Poorly differentiated squamous cell carcinoma of oral cavity in Group I rat. H-E, ×130.

Fig. 4. Spindle cell sarcoma of stomach in Group III rat. H-E, ×196.

Table 3. Incidence, location, and histologic type of tumors in the different groups (numbers of rats affected).

Group number	I	II	III	IV	V	VI
Effective number of rats	12	13	15	12	12	8
Type of lesion and location						
Squamous cell carcinoma						
Ear duct	1		1			
Lip	1			2		
Oral cavity		1		1	2	
Nose		1				
Forestomach					1	
Squamous cell papilloma						
Forestomach				1		
Lip					1	
Adenocarcinoma						
Breast			2			
Colon		1				
Hepatoma						
Liver					2	
Pheochromocytoma						
Adrenal gland			1	1	3	
Carcinoid						
Lung				1		
Sarcoma						
Stomach			1			
Salivary gland			1			
Scrotum			1			
Leukemia					1	1
Adenoma						
Breast	1		2	1		
Adrenal cortex			2			
Cavernous hemangioma						
Gingival mucosa			1	1	2	
Fibrous histiocytoma						
Breast			1			
Desmoplastic fibroma						
Skin		1				
Total	3	4	13	8	12	1
Number of tumor bearing animals	3	3	8	5	7	1
Number of malignant tumors	2	3	7	5	9	1

Preneoplastic lesions and reactive changes

Dysplasia of squamous epithelium of the lip was most commonly seen in Group V, (Table 4).

Hyperplastic lesions were most prevalent in the rats of Group III. Hyperplastic lesions of the labial epithelium was not only confined to the test canal. One striking observation was the presence of foreign body giant cell granulomas in the connective tissue of the lips which were significantly more common in Group V rats (Table 4). These granulomas were not only more prevalent, but their size was also significantly larger in Group V than in the other groups.

The kidneys were enlarged with variable degrees of rat nephrosis in most of the animals. The average grade of rat nephrosis in the different groups is shown in Table 5. The results clearly show that the Lewis rats were not affected by severe rat nephrosis but mostly by mild forms.

Discussion

There were no significant differences in survival time between rats treated with snuff or virus alone, compared to rats treated with a combination of snuff and HSV-1 (Table 2). Neither was there significant difference in survival between 4-NQO treated rats and rats treated with 4-NQO + snuff. The 6-month longer survival time of the Lewis rats as compared to the Sprague-Dawley rats may be explained by the fact that these rats had much less pronounced rat rephrosis than the Sprague-Dawley rats (Table 5). The rat-nephrosis is related to a high diet protien intake, and is known to be one of the most important factors causing early health detoriation and death in ageing laboratory rats, especially male Sprague-Dawley rats (21). It is unlikely that treatment differences could account for the longer life span of the Lewis rats.

The weight gain followed the same course for both Lewis and Sprague – Dawley rats during the experiment, but Groups II, III, and V had a slower weight gain than Groups I, IV, and VI (Fig. 1). There are several possible explanations to the slower weight gain of the snuff treated groups. One could be that the snuff exposure resulted in general toxic effects. Another is an increased metabolic activity induced by nicotin. A third possible explanation is the fact that snuff administration resulted in chronic inflammaton of the lip region with soreness and pain which might have prevented maximum food intake.

There were no significant differences

Table 4. Incidence of reactive and preneoplastic lesions in the different groups (numbers of rats affected).

Group number	I	II	III	IV	V	VI
Effective number of rats	12	13	15	12	12	8
Type of lesion and location						
Giant cell granulomas						
Lip (small)	1	2	4			1
(large)	1	3	1	2	10	1
Hyperplasia						
Lip (minor)	4	8	12	5	5	1
(severe)	1	3	2		3	
Crevicular epith. (minor)	5	5	8	4	3	1
(severe)		1	2	1	3	
Forestomach (minor)		1	3	4		
(Severe)		1	2		1	
Dysplasia						
Lip					2	
Crevicular epithelium			2	1	2	
Forestomach					1	

Table 5. Incidence and severity of rat nephrosis in different groups.

Group number	I	II	III	IV	V	VI
Effective number of rats	12	13	15	12	12	8
No nephrosis	1	1	1	5	1	
Grade 1 (+)	3	5	6	7	10	
Grade 2 (++)	2	3	4		1	5
Grade 3 (+++)	6	4	4			3
Average of grade of severity of nephrosis	2.1	1.8	1.7	0.5	1.0	2.7

in the incidence of tumors in specific organs or of specific histologic types between the different groups but there was a marked and statistically significant difference between the total number of tumors in Group III versus Groups I, II, and VI. The difference in total tumor incidence between Groups IV and V was not statistically significant. Although there are no earlier reports on differences in spontaneous or induced tumor incidence between Lewis and Sprague-Dawley rats, we regard it important to interpret the tumor incidence in Groups IV and V separately. The reason for using Lewis rats in the 4-NQO treated groups was the intention to explant the tumor cells into cultivating medium in order to examine for chromosomal abberations, for which inbred animals is a prerequisite. However, due to technical problems no cell growth was obtained.

The highest tumor incidence was found in Group III which had been subjected to both virus and snuff exposure. The results support that the combination of HSV-1 and snuff has tumorigenic properties and they are consistent with our earlier results and those of other investigators (9, 19). However, oral squamous cell carcinomas were not induced in this study after exposure to HSV-1 and snuff. This may be explained by differences in methods of infecting the rats. The absorbtion of the virus suspension in a cotton pellet before application in the test canal instead of direct application on the mucous membrane might explain the induction of fewer oral squamous cell carcinomas in this study. The intention when inoculating virus in the test canal was to study a possible local carcinogenic effect of the combination HSV-1 and snuff but our data do not support the presence of such local effects when using a cotton pellet as a vehicle for the virus suspension.

There may be several mechanisms to account for the higher tumor incidence in the group subjected to the combination of HSV-1 and snuff. One factor is the fact that chemicals in snuff inhibit the cytolytic properties of HSV-1 (18) which enables HSV-1 to express its possible carcinogenic properties. Other investigators have also shown that uv-inactivated HSV-1 may transform cells *in vitro* and that these cells when injected into the homologus host formed tumors which metastasize (17). Another possiblity is that some of the 2500 chemical substances in snuff (22) may act as initiators and chronic HSV-1 infection functions as a promoter, by inhibiting DNA repair by disruption of host DNA (23). Important chemicals in snuff are the tobacco specific N-nitrosamines (TSNA) which are present in mg/kg concentrations (22,24). Administration of these compounds result in tumors locally at the site of application as well as organ-specific tumors in nasal cavity, esophagus, lungs, and liver (24). The rats subjected to repeated HSV-1 infection exhibited signs of a generalized infection. The autopsies also confirmed this clinical observation in the two rats of Group I who died from encephalitis. It is possible that such a generalized HSV infection makes eucaryotic cells more susceptible to the carcinogens present in snuff, which may explain why the rats developed tumors distant from the site of HSV-1 inoculation and snuff application. A generalized HSV infection might also supress the immune response and if so facilitate tumor development. Another mechanistic possibility is that HSV-1 may, although it seems doubtful, even in its infectious state induce cancer and that snuff acts as a tumor promoter. The possibility that snuff acts only as a tumor promoter may be supported by the fact that the hyperplastic epithelial lesions induced by snuff are reversible (4). In the present investigation no significant increase in tumor incidence was found in group V (4-NQO + snuff) compared to group IV (4-NQO only). Thus we were not able to show that snuff functions as a tumor promoter in rats initiated with 4-NQO in the lip. Neither have we been able to promote cancer development with snuff in rats initiated with 4-NQO is the hard palate (Johansson *et al.*, to be published). Even though snuff appeared as a general tumor promoter in combination with HSV-1, it did not exert any specific promoting effects on the oral cavity. The reason for this is uncertain, but possibly polyphenols in snuff could function as local inhibitors of cancer development, as suggested earlier in a dose-response study in which rats, using the same experimental model were exposed to TSNA-enriched snuff. No correlation could be found between the TSNA content and tumor development which was ascribed to the presence of an inhibitor in the snuff (25, 8).

Of 13 rats who were exposed to HSV-1 and snuff, 6 developed malignant tumors compared with only 1 leukemia in the control group of 8 rats. No significant increase in the number of malignant tumors was found in the groups subjected to HSV-1 or snuff only. This has further underlined the hypothesis that HSV-1 and snuff interact in the development of malignant lesions. The relevant mechanisms are still unknown but further studies in this field are now conveyed.

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