Immunohistochemical and Ultrastructural Studies of Experimental Epithelial Dysplasia Induced by Excisional Wounding and Trp-P-2 Application in Hamster Tongues

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Fujita K, Kameyama Y, Maeda H, Tanaka H, Konishi S, Kato H and Sato E: Immunohistochemical and ultrastructural studies of experimental epithelial dysplasia induced by excisional wounding and Trp-P-2 application in hamster tongues. *Oral Med Pathol* 1997; 2:75-80 ISSN 1342-0984.

The present study was carried out to investigate histologically, immunohistochemically and ultrastructurally the effects of Trp-P-2 applications together with excisional wounding of hamster tongues. Histologically the animals which received Trp-P-2 treatment in combination with excisional wounding developed epithelial dysplasias in their tongues. Immunohistochemically the cells of these epithelial dysplasias stained positive for papillomavirus genus-specific antigen. Furthermore, ultrastructurally these dysplastic cells exhibited the presence of virus-like particles within their nuclei. In the present study, it seemed obvious that epithelial dysplasia was induced by excisional wounding and Trp-P-2 treatment, but the relationship between the appearance of epithelial dysplasia and the presence of virus-like particles was not clear.

Key words: hamster tongue, excisional wounding, Trp-P-2 application, epithelial dysplasia, virus-like particles

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Introduction

Dietary factors play an important role in the occurrence of human cancer (1). Many mutagenic heterocyclic amines have been isolated from pyrolysates of amino acids and protein, from boiled fish and meat and from beef extract (2) and shown to be carcinogenic in animals (3). Trp-P-2 (3-amino-1-methyl-5H-pyrido[4,3-b]indole) is one of these heterocyclic amines. In a previous histological investigation (4), we have reported that Trp-P-2 application in combination with excisional wounding could produce epithelial dysplasia in the tongue of hamsters. Furthermore, excisional wounding was assumed to act as a promotional stimulus in inducing the appearance of epithelial dysplasia in hamster tongues initiated with Trp-P-2. The present study was carried out to investigate histologically and ultrastructurally the effects of Trp-P-2 treatment together with excisional wounding on the tongues of hamsters. In addition, the changes in the tongues were examined immunohistochemically for papillomavirus genus-specific antigen with the use of antisera to bovine papillomavirus type I.

Materials and Methods

Thirty Golden hamsters, 3 weeks old, were divided equally into 6 groups (Groups A, B, C, a, b and c). In all 6 groups, the tip (2mm) of the tongue was excised with scissors. Then, in Groups A and a, the wound was painted three times weekly with DMSO (dimethyl sulfoxide, Yoneyama Yakuhin Kogyo Co., Ltd.) for 8 weeks. In Groups B, C, b and c, the wound was painted three times weekly with a 0.5% solution of Trp-P-2 in DMSO for 8 weeks. At the end of 8 weeks, in all 6 groups, the tip (2mm) of the tongue was again excised with scissors. Subsequently, in Groups A, B, a and b, the wound was painted daily with DMSO until the animals were killed. In Groups C and c, the wound was painted daily with Trp-P-2 until the animals were killed. All animals of the 6 groups were killed 16 days after the second excision and their tongues were removed. For histological and immunohistochemical study, the tongues of 5 animals from Groups A, B and C were fixed in 10% neutral buffered formalin. Four μ m serial sagittal sections were prepared, stained with hematoxylin and eosin, and subjected to a peroxidase-antiperoxidase immunohisto-

chemical method for detection of papillomavirus genus-specific antigen (DAKO, Kit K541, Denmark). For ultrastructural study, the tongues of animals in Groups a, b and c were cut into small pieces of less than 1 mm and fixed for 2 hours in a mixed solution of 2% paraformaldehyde and 2.5% glutaraldehyde at pH 7.4. After a buffer rinse, all pieces were postfixed for 2 hours in 1% buffered osmium tetroxide. Tissues were dehydrated through a graded series of ethanol and embedded in Epon 812. The embedded blocks were cut into about 1 μ m-thick semi-sections, which were then stained with toluidine blue and examined with a light microscope. Thin sections were stained with both uranyl acetate and lead citrate and examined with an electron microscope. Ultrastructural observations were performed mainly on the cells of the basal, the prickle-cell, the granular and the keratinized layer of the tongue.

The 6 groups of animals and their treatment are summarized in Fig. 1.

Fig. 1: Treatment for 6 groups

T : application of Trp-P-2 S : sacrifice
D : application of DMSO (3T/W) : three times per week
E : excision of tongue

Fig. 2: Group A. The wound of the tongue was completely covered by hyperkeratinized epithelium. The basement membrane was seen clearly. HE $\times 25$

Results

Histological and immunohistochemical findings

In Group A, the wounds of the tongues in all 5 animals were completely covered by hyperkeratinized epithelium (Fig. 2). The basement membranes were seen clearly. The underlying connective tissues displayed a slight degree of inflammatory cell infiltration. The epithelial cells of the tongues in all animals were negative for papillomavirus genus-specific antigens.

In Group B, the wounds of the tongue in 4 of 5 animals showed moderate epithelial dysplasia (Fig. 3). Most of

Fig. 3: Group B. The wound of the tongue showed moderate epithelial dysplasia. The basement membrane was irregular or indistinct. HE $\times 25$

the basement membranes were irregular or indistinct. The underlying connective tissues showed a slight degree of inflammatory cell infiltration. The remaining single animal displayed a wound similar to those in the animals of Group A. The nuclei of dysplastic swollen epithelial cells which were located in the superficial layers of the epithelial dysplasia of the tongues stained positive for papillomavirus genus-specific antigens (Fig. 4). The epithelial cells of the tongue in the remaining single animal stained negative.

Fig. 4: Group B. The nuclei of dysplastic swollen epithelial cells in the superficial layers of the epithelial dysplasia stained positive for papillomavirus genus-specific antigens. PAP, counterstained with hematoxylin $\times 50$

Fig. 5: Group C. The wound of the tongue showed more marked epithelial dysplasia. The basement membrane was similar to that in the animals of Group B. HE×25

In Group C, the wounds of the tongues in all 5 animals showed more severe epithelial dysplasia than those in the animals of Group B (Fig. 5). The basement membrane and the connective tissue were similar to those in the animals of Group B. The nuclei of dysplastic swollen epithelial cells in the superficial layers of epithelial dysplasia also showed positive staining for papillomavirus genus-specific antigens similar to those in the 4 animals of Group B.

The number of animals with epithelial dysplasia in each group is summarized in Table 1.

Table 1: The number of animals with epithelial dysplasia in each group

Group A	0/5	Group a	0/5
Group B	4/5	Group b	3/5
Group C	5/5	Group c	5/5

Fig. 6: Group a. The nucleus of the prickle cell was slightly flattened and contained a moderate number of free ribosomes, rough surfaced endoplasmic reticulum and mitochondria. The bundles of tonofilaments in the cytoplasm surrounded the round or oval nucleus. ×5,000

Ultrastructural findings

In Group a, in the semi-sections, none of the 5 animals showed any epithelial dysplasia in the tongue. In the tongues of these animals, the cells of the prickle-cell layer were slightly flattened and contained a moderate number of free ribosomes, rough surfaced endoplasmic reticulum and mitochondria (Fig. 6). The bundles of tonofilaments in the cytoplasm surrounded the round or oval nucleus or were oriented towards the desmosomes (Fig. 6). The cells of the granular layer were moderately flattened and contained various amounts of keratohyaline granules. The cells of the keratinized layer were very flattened and had no nucleus.

In Group b, in the semi-sections, 2 of the 5 animals did not show any dysplasia in the tongue, but the remaining 3 animals exhibited moderate epithelial dysplasia. Ultrastructural findings of the former were similar to

Fig. 7: Group b. The nucleus of the prickle cell was indented and the nucleolus was enlarged. The intranuclear virus-like particles were observed within the nucleus. The bundles of tonofilaments increased in number and surrounded the nucleus or showed a swirl-like arrangement. ×5,000

Fig. 8: Group b. High power magnification of virus-like particles in the prickle cell in Fig. 8. The size was about 40 nm in diameter. ×50,000

those of Group a. In the tongues of the latter, most of the cells of the prickle-cell layer were irregular in shape and their intercellular spaces were enlarged. The nucleus

was round or oval and sometimes indented. The nucleolus was enlarged. In some cells, intranuclear virus-like particles (about 40 nm in diameter) were observed within the nucleus (Figs. 7, 8). These intranuclear virus-like particles showed a crystalline array arrangement. The number of free ribosomes, rough surfaced endoplasmic reticulum and mitochondria increased compared with those in the prickle cells in Group a. The bundles of tonofilaments also increased in number and surrounded the nucleus or showed a swirl-like arrangement (Fig. 7). The number of desmosomes was fewer than those in the prickle cells in Group a. Most of the cells in the granular layer were flattened and sometimes the nucleus of these cells also contained the intranuclear virus-like particles (about 40 nm in diameter) showing a crystalline

Fig. 9: Group b. The cell of the keratinized cell had a nucleus with the intranuclear virus-like particles, exhibiting a crystalline array arrangement. $\times 5,000$

Fig. 10: Group b. High power magnification of virus-like particles in the keratinized cell in Fig. 9. The size was about 40 nm in diameter. $\times 50,000$

array arrangement. Both bundles of tonofilaments and free ribosomes surrounded the nucleus. Various amounts of keratohyaline granules were observed. The cells of the keratinized layer usually lacked a nucleus, but occasionally had a nucleus with intranuclear virus-like particles (about 40 nm in diameter) which exhibited a crystalline array arrangement (Figs. 9,10)

Table 2: The number of animals with virus-like particles within the epithelial layers in each group

	Basal Cell	Prickle Cell	Granular Cell	Keratinized Cell
Group a	0/5	0/5	0/5	0/5
Group b	0/5	3/5	3/5	3/5
Group c	0/5	4/5	5/5	5/5

In Group c, in the semi-sections, all 5 animals showed epithelial dysplasia in the tongue. In these tongues, the changes in the cells of the prickle-cell, the granular and the keratinized layer were almost similar to those in the cells of each corresponding cell layer in Group b.

The number of animals with virus-like particles within the epithelial layers in each group is summarized in Table 2.

Discussion

Many mutagenic heterocyclic amines have been demonstrated to be carcinogenic in animals as mentioned earlier (2). Trp-P-2 was the first one to be isolated and identified. It was reported that, after Trp-P-2 was applied to the skin of mice twice weekly for 5 weeks followed by application of TPA (12-O-tetradecanoylphorbol-13-acetate) twice weekly for 47 weeks, skin squamous cell papilloma and carcinoma developed in these animals (5). It was also demonstrated that papillomas were produced in the mice after Trp-P-2 was topically applied twice weekly for 5 weeks on the skin followed by TPA administration for 47 weeks (6). With regard to oral carcinogenesis, we have reported that epithelial dysplasias could be produced in the hamster tongue in about 10 weeks using double excisional wounding and application of Trp-P-2 (4). In the present study using the same method, it was possible to produce epithelial dysplasia by Trp-P-2 treatment in combination with excisional wounding in the hamster tongues of Groups B and C, and these results were almost the same as those of our previous study (4). The present study likewise seemed to confirm the fact that excisional wounding acted as a promotional stimulus in inducing the appearance of epithelial dysplasia in hamster tongue initiated with Trp-P-2.

Previous studies (7, 8) demonstrated that the oral lesion, namely lingual epithelial dysplasia of hamsters was induced by both trauma and chemical carcinogen DMBA treatment. It was also found ultrastructurally that these cells contained virus-like particles resembling papillomavirus in the nuclei (8). Furthermore, these cells showed the presence of papillomavirus antigens within the dysplastic cells (9). Recently, a novel papillomavirus was identified in these lesions of the hamster tongue (10), suggesting there might be some relationship between the appearance of the dysplastic changes and the presence of papillomavirus. In the present study, the presence of papillomavirus was also recognized by

immunohistochemical staining for papillomavirus genus-specific antigen in the cells of the epithelial dysplasia of the tongue induced by Trp-P-2 treatment with traumas in the animals of Groups B and C. The reaction products were localized within the nuclei of epithelial cells, particularly in the superficial layers of the epithelial dysplasia. These positive cells were swollen cells showing a ballooning or vacuolated cytoplasm, termed koilocytes (11). In the lingual epithelium in the animals of Group A, neither koilocytes nor papillomavirus antigen positive cells were observed.

Various types of human papillomaviruses are known to cause benign epithelial tumors of the mucosa, and some of them such as types 16 and 18 are considered to be closely related to the development of cancer (11, 12, 13), although it is difficult, as suggested by many investigators (11, 13), to prove a direct involvement of potentially carcinogenic viruses in oral cancer development. Human papillomavirus is reported to be detected in human oral lesions such as papilloma (14), focal fibrous hyperplasia (15), oral wart (16), oral condyloma acuminatum (17), lichen planus (18), leukoplakia (18, 19) and carcinoma (18, 20). Human papillomaviruses were usually found in the koilocytes of the lesions of the oral mucosa in humans (11, 18).

Ultrastructurally, in the animals of Groups b and c in the present study, the intranuclear virus-like particles (about 40nm in diameter) were identified within the nuclei of prickle, granular and keratinized cells of epithelial dysplasia in the tongue. The presence of these virus-like particles probably corresponded to the presence of papillomavirus antigen in the cells of epithelial dysplasia revealed by immunohistochemistry in the present study. These virus-like particles were probably the same type of papillomavirus which were identified in the dysplastic lesion of the hamster tongue produced by DMBA treatment and trauma (10). The virus-like particles were not observed in the epithelial cells of the tongue in the animals of Group a in which no animals showed epithelial dysplasia. To date, there has been no study demonstrating ultrastructurally the presence of the intranuclear virus-like particles in the chemically induced oral cancerous or precancerous lesions in the animals. However, intranuclear virus-like particles were observed in focal epithelial hyperplasia in rabbits and chimpanzees (21-23). In the human oral lesions such as papilloma (24, 25), focal epithelial hyperplasia (26-29), oral verruca vulgaris (30), oral condyloma acuminatum (31) and oral warts (32), the intranuclear virus-like particles were also found ultrastructurally within the cells of the prickle cell to the parakeratinized cell layer. To our knowledge, there has been no ultrastructural study describing the presence of virus-like particles in the normal oral mucosa in humans or animals, although human papillomavirus DNA was detected in the normal oral mucosa in humans (20, 33). It was difficult to interpret the significance of the presence of both the papillo-

mavirus antigen and the virus-like particles in the cells of carcinogen-induced epithelial dysplasia in the tongues of hamsters, but it seems to be clear from the results of the present study that the development of epithelial dysplasia in the tongue of animals was always associated with the manifestation of papillomavirus antigen and virus-like particles. The relationship between the appearance of epithelial dysplasia and the presence of virus-like particles remains to be elucidated.

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(A part of this article was reported in *Aichi-Gakuin Dent Sci* 1989; **27**: 227-76, in Japanese)

(Accepted for publication November 4, 1997)