

The enhancing effect of excess retinol palmitate on induction of odontogenic tumors and inhibitory effect on squamous cell carcinoma of the gingiva in hamsters treated with N-methylnitrosourea

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Summary. The influence of excess retinol palmitate on induction of tumors in the oral region was examined histopathologically. Sixty-three weanling Syrian golden hamsters were divided into five groups and received either 0.2% N-methylnitrosourea (MNU) (1mg/100g body weight) or retinol palmitate (RP) (25,000 IU/100g body weight) twice a week for 16 weeks, singly or in combination. Animals received RP intraperitoneally or intragastrically and then, 6 hours later, the animals received intragastric administration of MNU. To accelerate the cell activity of the incisal tooth buds, intentional disocclusion of the left upper and lower incisor of all hamsters was carried out by repeated cutting with cooled diamond disks to a level just above the inter-dental papilla twice a week for 12 weeks. The right incisors were left in occlusion. In all animals exposed to RP+MNU, while the induction of squamous cell carcinomas of the gingiva and forestomach were prevented, the notable findings were a significantly increased incidence of odontogenic tumors in cut incisal regions of the animals with intragastric administration of RP+MNU and an induction of maxillary neurogenic tumors. The incidence of MNU-induced disturbances in odontogenesis in the incisors was reduced but marked disturbances were increased. RP seemed to have opposite effects of prevention and enhancement for development of neoplastic changes in the oral region.

Key words: Retinol palmitate, Odontogenic tumor, Squamous cell carcinoma of gingiva, Neurogenic tumor, N-methylnitrosourea

Introduction

Vitamin A is necessary for normal differentiation and maintenance of physiological functions of epithelial tissues. Vitamin A and its synthetic analogs (retinoids) are potent agents for control of cell differentiation in many epithelial tissues. Exogenous vitamin A has also been shown to have a profound *in vivo* effect on the developing dentition of rodents (Biggerstaff et al., 1971; Germain and Berry, 1973; Abbott and Pratt, 1988; Hurmerinta et al., 1980). Several studies have suggested the inhibitory effects of vitamin A on tumor development, although they have been somewhat contradictory. Some studies report a vitamin A-influenced inhibition of chemically-induced tumors (Bollag, 1972; Thompson et al., 1979; Shklar et al., 1980), other studies report a promoting influence of this substance (Levij et al., 1969; Polliack and Levij, 1969; Polliack and Sasson, 1972; Smith et al., 1975). Cavalari et al. (1971) has shown that vitamin A has an enhancing effect on DMBA-induced tumorigenesis. Natural retinoids, fed at high dietary levels, have some ability to prevent chemical carcinogenesis in epithelial tissues of the bronchi, trachea, stomach, uterus, bladder, breast, and skin of experimental animals (Sporn et al., 1976). There is considerable evidence for a positive role for vitamin A or some of its synthetic analogs (retinoids) in the prevention or control of cancer of tissues that depend on vitamin A activity for maintenance of normal differentiation and integrity (Berry, 1997; Newberne and Suphakarn, 1977). The aim of our study was to evaluate the effects of excess retinol palmitate (RP) on N-methylnitrosourea (MNU) tumorigenesis in the incisal tooth bud, gingiva and other regions preceding application of a tumor-inducing carcinogen.

Materials and methods

Sixty-three 15-day-old Syrian golden hamsters were

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divided into four groups; Group 1: 20 animals treated with MNU after intragastric administration of RP; Group 2: 20 treated with MNU after intraperitoneal administration of RP; Group 3: 18 treated only with MNU; Group 4: 5 treated only with RP intragastrically; Group 5: five only RP-treated intraperitoneally. To accelerate the cell activity of the incisal tooth buds, intentional disocclusion of the left upper and lower incisor of all hamsters was carried out by repeated cutting with cooled diamond disks to a level just above the inter-dental papilla twice a week for 12 weeks. The right incisors were left in occlusion. Simultaneously, with repeated cutting, animals (Groups 1 and 2) received RP (Sigma Chemical Co., St. Louis, USA) intraperitoneally or intragastrically (25,000 IU/100g body weight) and then, 6 hours later, the animals received intragastric administration of fresh MNU (0.2% fresh solution, 1 mg/100g body weight) (Nakarai Chemicals Ltd., Kyoto, Japan) twice a week for 16 weeks. Animals were maintained under humane conditions. They received appropriate anaesthetics and care to minimize pain and discomfort during operative and postoperative procedures. Serum vitamin A levels were measured on the third day after administration of RP at twelve weeks in the experimental period. The retinol estimates were performed by high-pressure liquid chromatography. At three weeks after the last administration of MNU, all animals were killed. The jaws and forestomach were fixed in 10% neutral-buffered formalin. Soft X-rays of the jaws were taken before decalcification in 10% ethylenediamine tetraacetic acid at pH 7.4. The specimens were embedded in paraffin wax or water-miscible methacrylate resin and semiseriably or serially sectioned at 4 μ m and were stained with hematoxylin-eosin and Azan Mallory stain. A comparative study of the incidence of odontogenic tumors, disturbances in odontogenesis of the incisors, gingival squamous cell carcinoma and other tumors in MNU-treated or RP plus MNU-treated hamsters was made histologically.

Results

Serum levels of retinoid after two months of continuous application were as follows (n=10): intact animals, 372 \pm 40ng/ml; Group 1 (RP(ig)+MNU), 677 \pm 127 ng/ml; Group 2 (RP(ip)+MNU), 612 \pm 146 ng/ml; and Group 3 (MNU), 317 \pm 43 ng/ml. Serum levels of retinoid in groups 1 and 2 (RP+MNU) were significantly higher than those in group 3 (MNU) (Student's t-test $p < 0.0001$). Group 3 showed a significantly lower serum level of retinoid than that in intact animals ($p < 0.01$). There was a significantly greater incidence of odontogenic tumors in Group 1 animals than in Group 3 animals. The incidence of squamous cell carcinomas (SCC) of the gingiva and forestomach in the RP plus MNU-treated animals was significantly lower than in the MNU-treated animals (Table 1). Hypoplastic changes of the incisors were more prominent and much more frequent in the cut mandibular incisors than in the uncut ones, while the maxillary incisors showed very slight hypoplastic changes. The incidences of disturbances in odontogenesis in Groups 1 and 2 were lower than in Group 3, though the hypoplastic changes were more prominent in a few animals of Groups 1 and 2 than those in the animals of Group 3.

Group 1 showed severe disturbances in odontogenesis occurring on the mandibular cut incisors. Occasionally, complete loss of enamel formation was seen (Fig. 1). The atrophied "U"-shaped parts of the tooth buds were transformed to Hertwig's root sheath (RS)-like features which irregularly induced dentin. The epithelial cell nests (Fig. 2) migrated from the transformed "U"-shaped part and aberrant RS. Keratinization in the epithelial cell nests was not frequent. Sometimes the alveoli were filled with irregular dentin masses (Fig. 3). Numerous, variously-sized neoplastic atypical odontogenic epithelial cell nests with partly inductive dentin formation migrated into the mandibular cut incisal region (Fig. 4). Sometimes

Table 1. Incidence of neoplastic changes and disturbances in odontogenesis of hamsters

	NUMBER OF HAMSTERS	INCISORS: DISTURBANCES IN ODONTOGENESIS: MANDIBLE	INCISORS: ODONTOGENIC EPITHELIUM: NEOPLASTIC PROLIFERATION		GIGIVAL EPITHELIUM: SQUAMOUS CELL CARCINOMA WITH INVASIVE GROWTH	MAXILLARY BONE: NEUROFIBROMA-LIKE TUMOR	FORESTOMACH: SQUAMOUS CELL CARCINOMA WITH INVASIVE GROWTH
			Mandible	Maxilla			
			Group 1: RP (ig)+MNU	20			
Group 2: RP(ip)+MNU	20	13	2 ^{3*}	0	3 ^c	1	5 ^d
Group 3: MNU	18	18 ^a	14 ^{4*} , b (2)	0	9 ^c	0	17 ^d
Group 4: RP(ig)	5	0	0	0	0	0	0
Group 5: RP(ip)	5	0	0	0	0	0	0

ig: intragastric administration; ip: intraperitoneal administration; 1*: three odontoameloblastoma-like tumors and two odontomas; 2*: odontomas; 3*: odontoameloblastoma-like tumors and odontoma; 4*: squamous odontogenic tumor-like tumor; a, b, c and d: significant differences are found between RP+MNU groups and MNU group. Values for p are derived from chi-square test: a and c, $p < 0.01$; b and d, $p < 0.05$; (); keratocysts

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odontogenic tumors which extensively destroyed bone were found in the mandibular cut incisors (Fig. 5a). These showed odontoameloblastoma-like features histologically. The tumor masses or strands were bounded by a layer of columnar cells or cuboidal cells and showed stellate reticulum-like features, keratinization and small cystic space formation in the central parts of some nests. Scattered mitotic figures were also seen. Moreover, inductive dysplastic dentin and tooth-like structures with numerous odontoblasts were seen in contact with the tumorous epithelial component (Fig. 5b,c). A large odontogenic keratocyst-like feature was seen in the mandibular cut incisal region (Fig. 6). In three animals, odontoma-like tumor masses were seen in the the maxillary cut incisal region (Fig. 7a,b). Histologically, the tumors were composed of a large

mass of dysplastic dentin and there was proliferation of the odontogenic epithelial component. In two animals, neurogenic tumor-like fibrous tumors were seen in the molar region of the maxillary bone. In Group 2, disturbances in odontogenesis and atypical migration of the odontogenic epithelial cell nests were coincidentally observed in the mandibular cut incisors. These changes of the incisors were almost the same as those seen in Group 1. Occasionally, the odontogenic epithelial nests showed keratinization. Two odontogenic tumors were found in the mandibular cut incisors. Histologically, the tumors were composed of numerous odontogenic epithelial cell nests proliferating in sheets or strands with partly irregular dentin formation. In one animal, large neurogenic tumors with extensive destruction of bone were seen in the molar region of the maxillary bone (Fig.

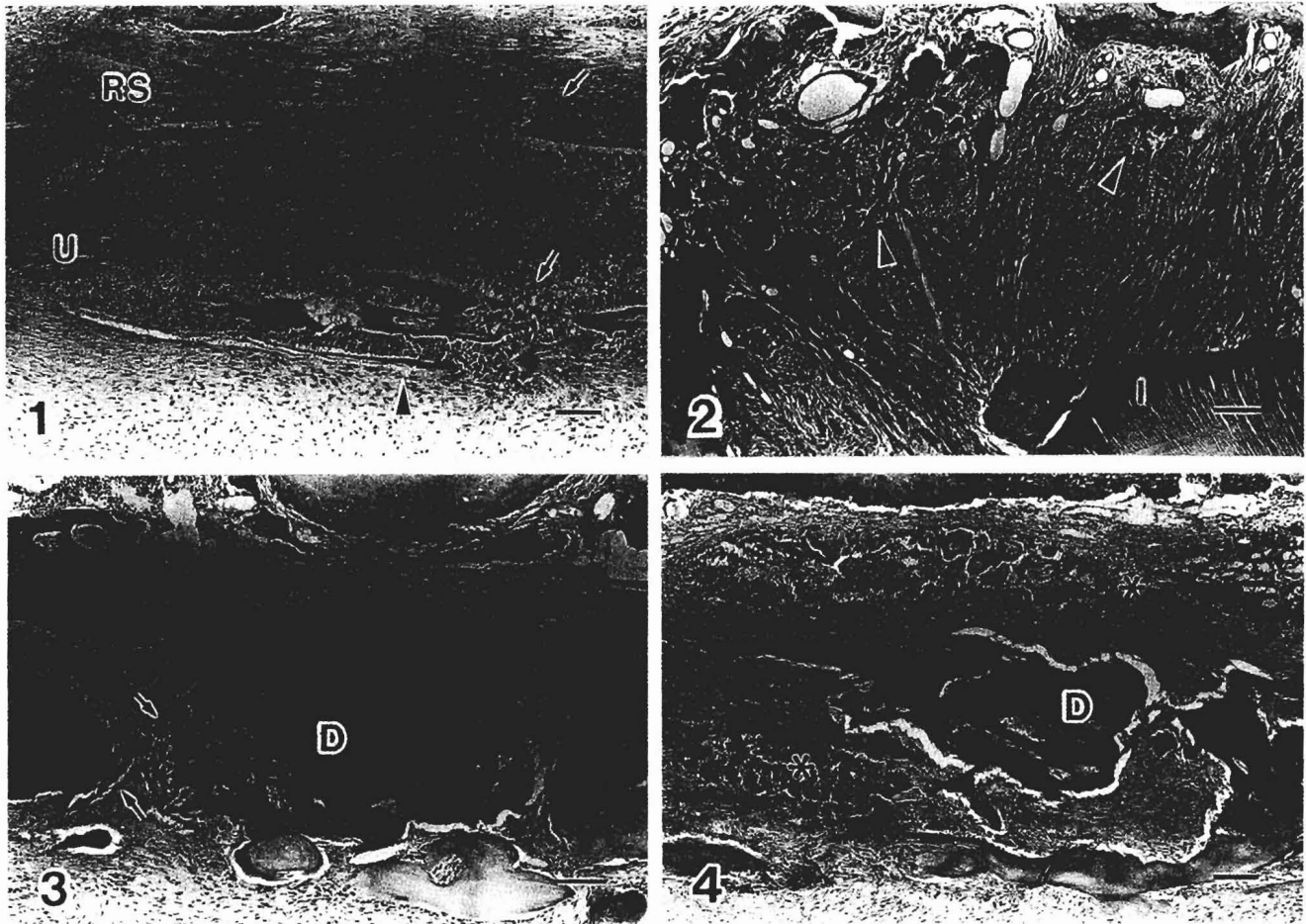


Fig. 1. Severe disturbances in odontogenesis of the mandibular cut incisors. Irregularly formed dentin (arrows) and complete loss of enamel formation (arrow-heads). Hertwig's epithelial root sheath (RS). "U"-shaped part (U). HE. x 65, Bar: 95 μ m.

Fig. 2. Numerous migrating odontogenic epithelial cell nests (arrow-heads) in the lingual periodontal ligament. Cross section of incisor (I). HE. x 100, Bar: 60 μ m.

Fig. 3. The alveolus are filled with irregular dentin-like masses (D) with epithelial components (arrow-heads) at the periphery. HE. x 65, Bar: 95 μ m.

Fig. 4. Odontogenic epithelial cell nests (*) atypically proliferating in sheets with partly irregular dentin formation (D). HE. x 65, Bar: 95 μ m.

8a,b). Group 3 showed hypoplastic changes of dentin and enamel in 14 animals, 10 of which had complete loss of amelogenesis in the mandibular cut incisors. Numerous migrating odontogenic epithelial cell nests in the cut incisal region of the mandible showed a tendency to marked keratinization and occasionally, small odontogenic keratocyst-like features and odontogenic tumors were found, which were almost the same histological changes as seen in a previous report.

In Group 1, one animal developed SCC with slight invasive growth in the molar region of the mandible (Fig. 9a). In Group 2, three animals developed SCC with invasion into the alveolar bone in the molar region (Fig. 9b), two of them in the mandible and another in the maxilla. In Group 3, many animals developed well-differentiated SCC with extensive invasion into the bone marrow (Fig. 9c). Groups 4 and 5 showed neither remarkable neoplastic changes nor perceptible signs of vitamin A toxicity.

Discussion

In the present study, the disturbance in odontogenesis ranged in extent from loss of pigment of the enamel surface and enamel hypoplasia to loss of enamel and irregular hypoplastic dentin formation. The histological findings of disturbances in odontogenesis in the incisors of the animals with RP plus MNU application were similar to those seen in MNU-treated animals (Kohgo, 1972; Edwards, 1978), but hypoplastic changes of the incisors were more prominent in RP plus MNU-treated animals than in MNU-treated animals. Vitamin A prevents tooth morphogenesis and odontoblast differentiation in the early bell stage; however, it does not prevent later events such as the secretion of predentine or the polarization of ameloblasts (Hurmerinta et al., 1980). With MNU administration alone the odontogenic epithelium of the tooth bud and numerous migrating odontogenic epithelial nests showed

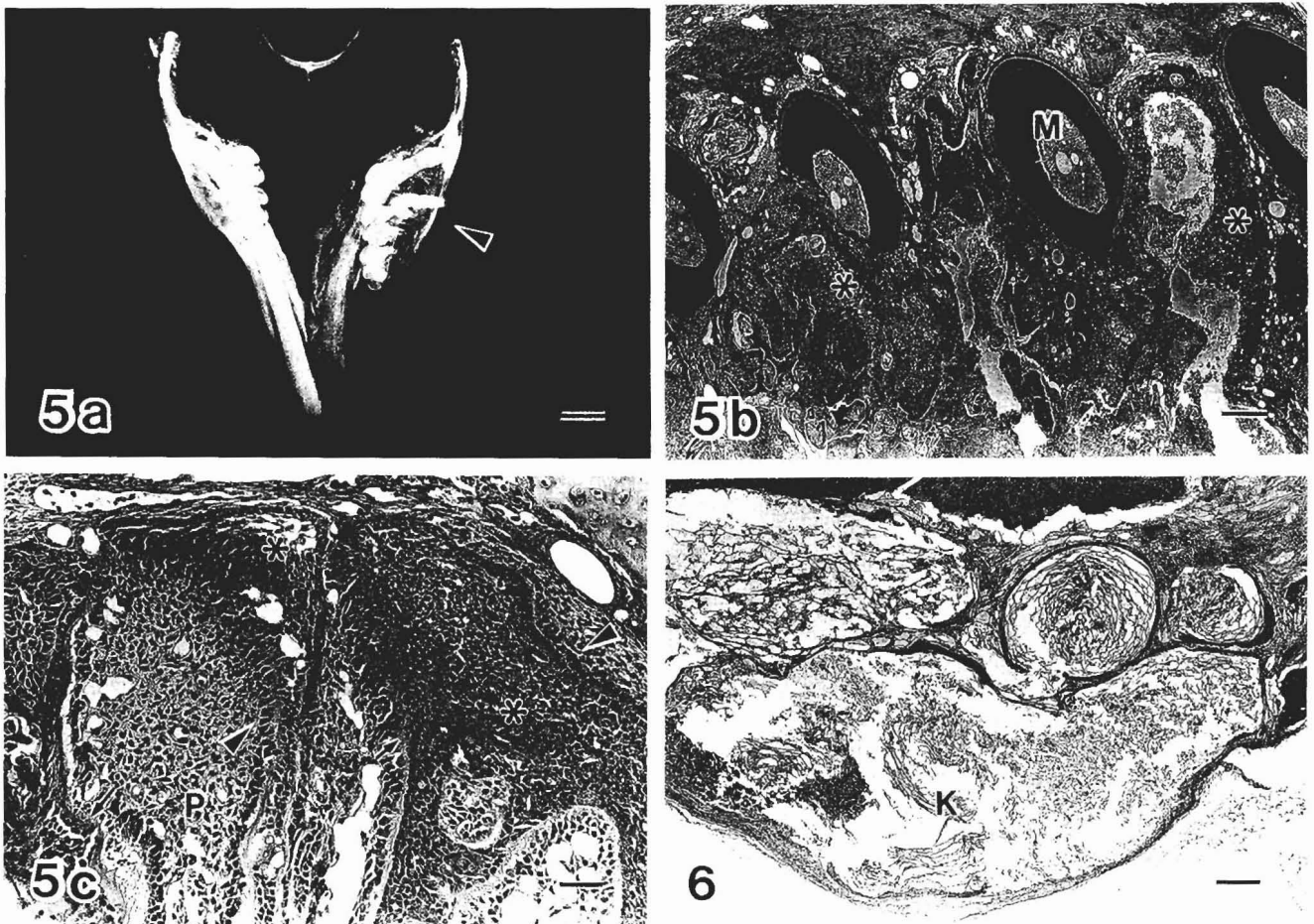


Fig. 5. a. Radiograph of mandible extensively destroyed by odontogenic tumor (arrow-head). x 2.5, Bar: 2.4mm. b and c. Atypical proliferation of odontogenic epithelium (*) showing ontoameloblastoma-like features with inductive dysplastic dentin formation (arrows) and numerous odontoblasts (arrow-heads) in pulp-like mesenchymal tissues (P). HE. b, x 25, Bar: 240 μ m; c, x130, Bar: 45 μ m.

Fig. 6. Odontogenic keratocyst-like features (K) in the mandible. HE. x 25, Bar: 240 μ m.

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squamous metaplasia. Consequently, the tooth bud seemed to lose its enamel-productive potential and to have disturbed dentin inductive potential. Furthermore, the migrating epithelial nests showed a tendency for degeneration with keratinization. However, in RP plus MNU administration, the migrating odontogenic epithelial nests exhibited reduced squamous metaplasia with keratinization and atypically proliferated; therefore, odontogenic tumors seemed to originate from the epithelial nests. The different incidences of the induction of odontogenic tumors between Groups 1 and 2 seemed to reflect the difference in the serum vitamin A levels. So far, there has been no information concerning enhancing the effect of RP on odontogenic tumors or allied lesions induced experimentally. β -Carotene application does not result in the enhancing of tumorigenesis of odontogenic tumors in MNU-treated hamsters (Kohgo et al., 1996). The inhibitory effects of RP on keratinization of the epithelium may be due to its

regulatory effect on epithelial tissues, ensuring their differentiation and growth. It is also possible that retinoic acid is the active metabolite of retinol (Bollag, 1972). Fuchs and Green (1981) reported that vitamin A contained in cultured epidermal and conjunctival keratinocytes prevented the synthesis of the 67 kd keratin characteristic of terminally differentiating epidermis and that it stimulated the synthesis of 40 and 52 kd keratins.

MNU has been used in an attempt to induce odontogenic tumors in the hamster (Herrold, 1968; Kohgo, 1972; Edwards, 1978). However, only a low yield was observed. The odontogenic epithelium is dependent on a specific interaction with underlying mesenchyme for fulfillment of differentiation. In the present study, the migrating odontogenic epithelial nests in the incisal region seemed to demonstrate interaction with the mesenchyme; therefore, several odontoameloblastoma-like tumors seemed to be induced. As for the

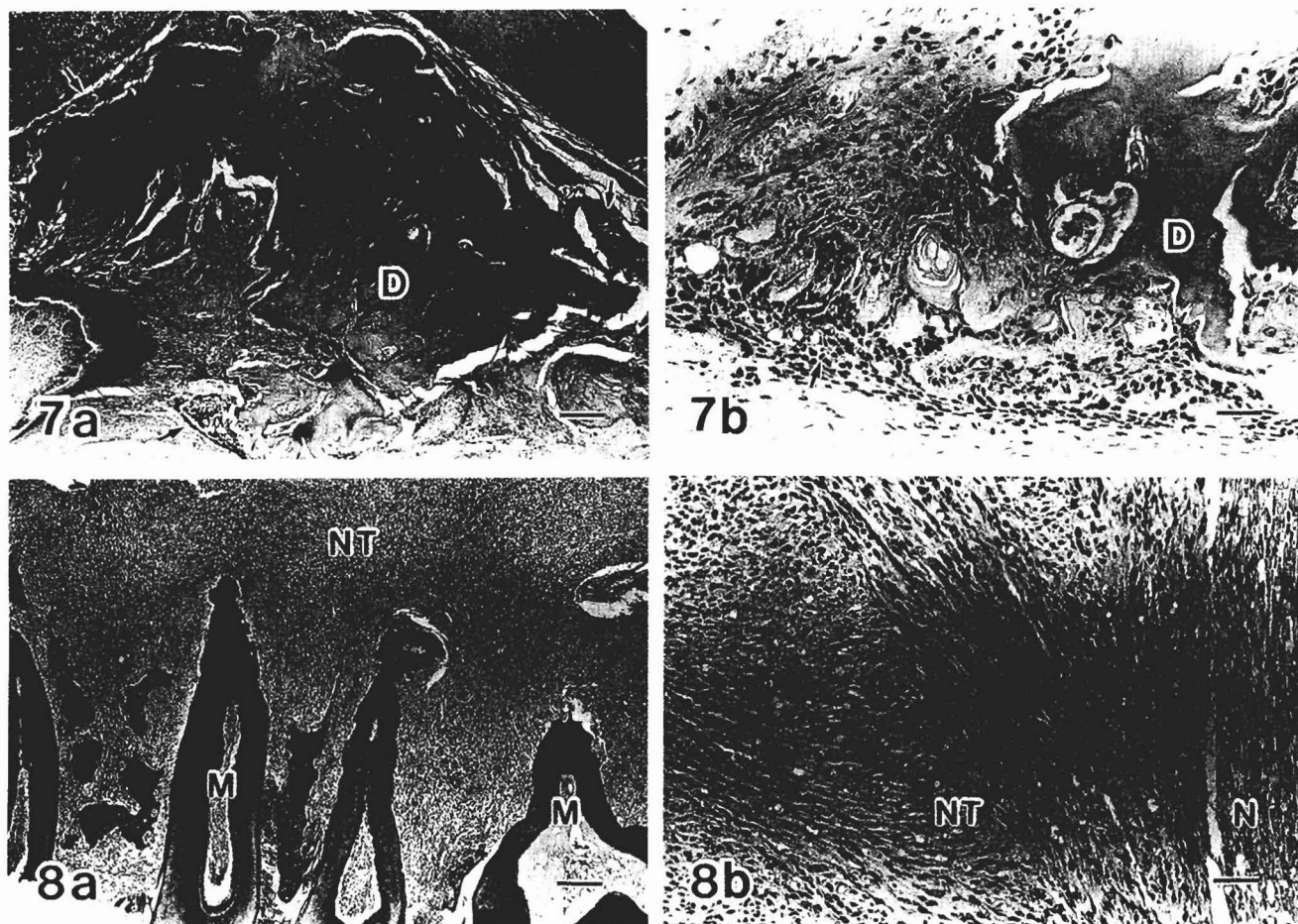


Fig. 7a,b. Odontoma-like tumor in the maxillary incisal region, composed of a large mass of dysplastic dentin (D) and proliferation of epithelial component (arrow-heads) at the peripheral area. HE. a, x 25, Bar: 240 μ m; b, x 200, Bar: 30 μ m.

Fig. 8a,b. Neurogenic tumor-like fibrous tumors (NT) with extensive destruction of the maxilla. Molar (M). Alveolar bone of maxilla (arrowheads). Nerve (N). Azan Mallory. a, x 25, Bar: 240 μ m; b, x 130, Bar: 45 μ m.

effects of RP, the odontogenic epithelial nests seemed to be deterred from squamous metaplasia and sometimes the epithelial nests seemed to gain a natural potential for induction of odontoblasts via epithelial-mesenchymal interaction. Localized vitamin A-induced proteolytic activity may play a role in the normal process of mesenchymal tissue remodeling, especially during embryonal development (Lotan, 1980). Vitamin A has been shown to increase collagen synthesis in some tissues (Chung and Houck, 1964; Strickland and Mahdavi, 1978) and pre dentine is secreted in the presence of vitamin A (Rouch et al., 1974). Since the tooth bud of the hamster incisor is always at the stage of embryonal development, rather a lot of dentin and cementum-like tissue seemed to be induced in the incisal region in the present experiment using retinol. Several *in vivo* studies have implied that under certain conditions retinoid might enhance tumor development (Levij et al., 1969; Polliack and Levij, 1969). Retinol would thus exert its prophylactic effect on tumor induction only via its transformation to retinoic acid (Bollag, 1972). While both retinoids and tumor promoters exert pleiotypic effects on cells *in vitro* and *in vivo*, this does not necessarily imply that retinoids possess tumor-promoting activity (Lotan, 1980). On the other hand, Kandarkar and Sirsat (1983) reported that higher doses of vitamin A palmitate, while delaying the effect of DMBA as a carcinogen, do not prevent tumor induction, and that application of vitamin A palmitate after carcinogen application does not inhibit tumor induction either. Since cell generation time in neuroepithelial cells

in animals with hypervitaminosis A is prolonged (Langman and Welch, 1966, 1967), in the present study, RP might have participated somewhat in the tumorigenesis of neurofibroma-like tumors. However, further information and research are needed to confirm this. In the application of RP plus MNU, keratinization and neoplastic invasive growth of the epithelium of the gingiva and forestomach were seen at a lower rate and to a lesser degree than in the MNU-treated animals (present study and Kohgo et al., 1990) and β -carotene plus MNU-treated animals (Kohgo et al., 1996), which seemed to suggest that RP has a definite effect of control on the progression of MNU-induced carcinogenesis. The different incidences of the tumors in Group 1 and Group 2 seemed to depend upon the different serum vitamin A levels due to methods of RP application. Vitamin A and the synthetic retinoids may inhibit proliferation through a tendency to induce maturation of epithelia with secondary suppression of proliferation (Boone et al., 1990). Retinoids may prevent the carcinogen-mediated development of premalignant and malignant cells by redirecting the progeny of dividing cells toward normal pathways of differentiation (Bollag and Holdener, 1992). Thus, the present results may imply that RP plays important roles in both enhancing and preventing tumorigenesis of the oral region. The results suggest that this method of application of RP plus MNU to hamsters may represent a useful model for induction of odontogenic tumors.

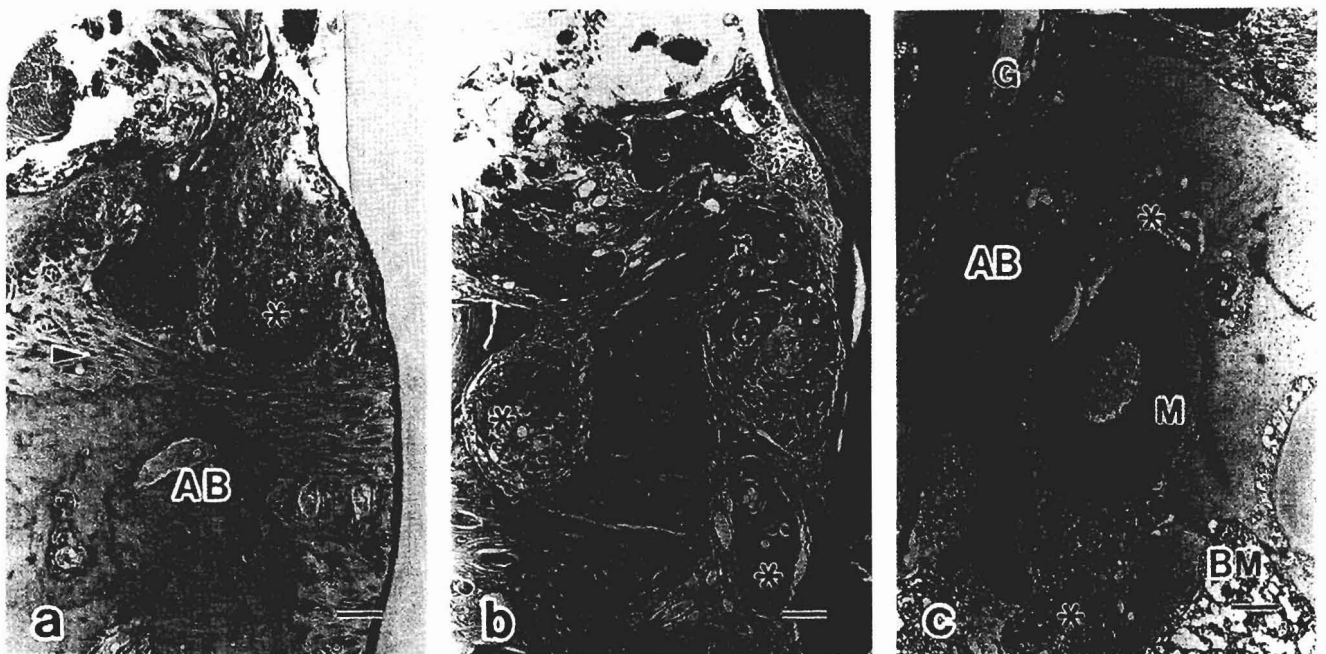


Fig. 9. Well-differentiated squamous cell carcinoma of the gingiva. Downward proliferation of neoplastic epithelium (*). **a.** Group 1. **b.** Group 2. **c.** Group 3. Gingiva (G). Alveolar bone of mandible (AB). Transseptal ligament (arrow-head). Molar (M). Bone marrow (BM). HE. **a,** x 33, Bar: 180 μ m; **b,** x 100, Bar: 60 μ m; **c,** x 10, Bar: 60 μ m.

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