Oral Oncology (2004) 40 6–12

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ORAL ONCOLOGY

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Effects of snuff extract on epithelial growth and differentiation in vitro

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Received 6 March 2003; accepted 13 May 2003

KEYWORDS
Cell culture;
Epithelial differentiation;
Keratinocytes;
Ki-67;
Smokeless tobacco;
Snuff

Summary Snuff is a locally irritative agent causing hyperkeratinization and hyperplasia of the oral epithelium. The aim of the study was to investigate the effects of snuff on epithelial cell growth and differentiation in vitro. Three-dimensional HaCaT cell cultures were grown for 6, 12, 14, and 18 days in the presence of 1% snuff extract. Ki-67, p53 and cytokeratins (Cks) 5, 13, 10, 19, 18, involucrin and filaggrin were studied by means of immunohistochemistry. Ki-67 indices were assessed, and the results analyzed statistically. Marked morphologic changes were seen with advanced culture time in the snuff group, probably as a result of increased toxic effects. Snuff exposure decreased the percentage of Ki-67 positive cells on days 6, 12, and 14, suggesting that snuff does not stimulate proliferation activity in this in vitro model. Cornification-related Ck 10 decreased after snuff exposure, indicating disturbances in the epithelial differentiation process.

Introduction

Snuff has carcinogenic potential with about 30 carcinogenic chemicals identified.[1] The most important carcinogens include tobacco-specific N-nitrosamines: N′-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.[2] Snuff-induced mucosal lesions typically show increased epithelial keratinization and thickness.[3,4] Dysplastic changes seem to be rare,[5–7] and the lesions usually regress or resolve rapidly after discontinuation of snuff use.[8]

Earlier epidemiologic studies have shown evidence for increased oral cancer risk[9] but, controversially, more recent surveys have not found any such correlation in the users of the Scandinavian type of moist snuff.[10,11] The divergent study results may relate to differences in the types of

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doi:10.1016/S1368-8375(03)00109-X

smokeless tobacco and their chemical compositions (product origin, manufacturing process, storage) or usage patterns. Concomitant or past cigarette smoking may also influence results.

Oral mucosal development is regulated by epithelial—mesenchymal interactions, accompanied by changes in growth and differentiation related molecules.[12] Three-dimensional epithelial cell culture is a simplified in vitro model mimicking these molecular events. The aims of our study were to investigate the effects of snuff extract on epithelial cell growth and differentiation.

Materials and methods

Keratinocytes and normal fibroblasts

HaCaT cells (spontaneously immortalized skin keratinocyte cell line) were maintained in Dulbecco's modified Eagles medium (Gibco BRL, Paisley, UK) supplemented with 1% non-essential amino acids (Gibco BRL), 2 mM L-glutamine (Gibco BRL), 10% fetal bovine serum (Gibco BRL, Life Technologies, Germany), 100 μg/ml streptomycin, and 100 IU/ml penicillin (Gibco BRL).

To establish fibroblast cultures, biopsy samples from normal buccal mucosa were placed in D-MEM containing 200 μg/ml streptomycin and 200 IU/ml penicillin (Gibco BRL). The lamina propria was separated from the epithelium and minced into small 1 mm^3 fragments, and explant cultures were started. The fibroblasts were grown in supplemented D-MEM and were from passages 1—5.

Cells were maintained in an incubator (90% relative humidity, 5% CO_2) at 37 °C and detached for experiments with 0.25% trypsin (Gibco BRL) containing 0.02% EDTA (Titriplex®III, Merck, Darmstadt, Germany).

Preparation of three-dimensional epithelial cell cultures

Eight ml of Cellon® (Cellon S.A., Strassen, Luxembourg) collagen solution was mixed with 1 ml of 10×D-MEM and 1 ml 0.01 M NaOH. pH was adjusted with 0.2 ml 0.1 M HCl to 7.4±0.2. Fibroblasts were suspended in the collagen solution to a density of 300 000 cells per ml. This mixture (0.5 ml) was placed in 12 mm cell culture inserts (Transwell, Costar, Cambridge, MA, USA) and allowed to solidify in the incubator for 1 h before medium was added.

Collagen gels were grown with FAD medium: D-MEM with 25% Ham-F12 medium (Gibco BRL), 10% fetal bovine serum, 4 mM L-glutamine (Gibco BRL), 5 μg/ml insulin (Sigma, St. Louis, MO, USA), 0.18 mM adenine (Sigma), 0.1 nM cholera toxin (Sigma), and 5 ng/ml epidermal growth factor (Boehringer Mannheim, Mannheim, Germany), and collagenase inhibitor hydrocortisone 0.4 μg/ml (Sigma).

After 5 days of culture, HaCaT cells were added at a density of 100 000 cells (suspended in 0.5 ml FAD medium) to the collagen gels. Cocultures were grown submerged for 3 days. After the cells had reached confluence, the cultures were cut out from the inserts and placed in six-well plates (Costar Corporation, Cambridge, MA, USA) on a stainless steel grid at the air—liquid interface. The FAD medium was replaced every second day.

Administration of snuff extract

Snuff extract was prepared from a commercial moist snuff brand (Ettan®, Gothia Snus, Sweden). One gram of moist snuff (containing 8 mg/g nicotine) was mixed with 10 ml of FAD medium and incubated for 2 h at 37 °C. It was centrifuged at 4000 rpm twice for 10 min and sterilized by filtration (0.2-μm filter). The 10% w/v solution (nicotine content 6 mg/ml) obtained was diluted to a 1% concentration. Snuff extract was administered after lifting to the air—liquid interface. The control and experiment cultures with at least three parallel experiments were grown for 6, 12, 14 and 18 days.

Fixation and preparation of tissue culture samples

The samples were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were cut at 5-μm intervals onto organosilane pretreated slides (Sigma Chemical Co., St. Louis, USA) for hematoxylin and eosin and immunostaining.

Immunohistochemistry (IHC)

Table 1 shows the monoclonal antibodies (Mabs) used. Immunostaining was done using the avidin—biotin complex technique with the Dako ChemMate Detection Kit, peroxidase/DAB-rabbit/mouse (Dako, Glostrup, Denmark) and an appliance for immunohistochemical analysis (Dako ChemMate TM 500, BioTek Solutions, USA). Counterstaining was done using Mayer's hematoxylin.

Analysis of sections

Cell proliferation was quantified by counting the percentage of Ki-67 positive nuclei in three randomly selected fields of each specimen from color

Table 1 Antibodies used In the immunohistochemical analyses

Antibody	Manufacturer	Clone	Dilution	Pretreatment	Positive control samples	Specificity
Ki-67	Immunotech, Marseille, France	MIB-1	1:50	No pretreatment	Skin	G1, S, G2 and M phase in proliferating cells
p53	Dako, Glostrup, Denmark	DO 7	1:1[a]	Microwave	Breast Cancer	Wild type and mutant p53 protein
Ck 18	Dako, Glostrup, Denmark	DC 10	1:20	Microwave + prot.K	Prostate	Simple glandular and ductal epithelia
Ck 19	Dako, Glostrup, Denmark	RCK 108	1:100	Microwave + prot.K	Breast	Glandular and stratified (basal compartment) epithelia
Ck 5	Monosan, Sanbio	C-50	1:10	Microwave + prot.K	Skin	Basal cells
Ck 13	Cymbus Biotechnology	KS13.1	1:50	Microwave + prot.K	Fibrous hyperplasia	Stratified squamous epithelia
Ck 10	Dako, Glostrup, Denmark	DE-K10	1:10	Microwave	Skin	Keratinizing stratified epithelia
Filaggrin	Argene, Varlines, France	AHF3	1:100	Microwave	Skin	Stratified epithelia; synthesized in granular cells
lnvolucrin	Novocastra, Newcastle upon Tyne, UK	SY5	1:100	Microwave	Skin	Stratified epithelia; precursor of cornified envelopes

[a] Ready-to-use reagent.

prints (UPC 1010, Sony Co., Tokyo, Japan) taken with a digital camera (SSC-DC38P, Sony Co) with a 25×objective of a Leitz Laborlux D microscope. The results are expressed as a Ki-67 index, which equals the percentage of positive cell nuclei in relation to all nuclei in the field. Staining for epithelial differentiation markers, Cks 18, 19, 5, 13, 10, involucrin, and filaggrin was scored as negative (0), staining of the lower one third (1), two thirds (2), through all cell layers (3), and the uppermost layer (4) of the epithelium.

Statistical methods

Ki-67 indices were compared by *t*-tests at days 6, 12—14, and 18 (SAS-system, version 8.2).

Results

Histology of three-dimensional epithelial cell cultures

Control cultures

The control cultures grown for 6, 12, 14, and 18 days showed stratification and features of epithelial dysplasia (Fig. 1). At days 6, 12, and 14, its thickness was 10—12 cell layers and at day 18 up to 16 cell layers. At day 6, no organization into epithelial layers was visible. With increasing culture time (12 and 14 days), the basal cell layer exhibited a slightly cuboidal morphology. At day 18, the basal cells had become smaller and less clearly distinguishable. At days 12, 14, and 18, a thin layer of flattened cells was seen in the uppermost layer, suggestive of keratinization.

Snuff exposed cultures

When the samples had been exposed to 1% snuff extract for 6 days (Fig. 1), the epithelium showed dysplastic changes in the same way as the controls did. Epithelial thickness varied from 5 to 12 cell layers. Intercellular dyskeratosis and cellular vacuolization were seen in the intermediate cell layers, indicating degeneration.

After 12 and 14 days of culture, the average thickness of the epithelium remained at 10 cell layers, but further changes were seen in morphology. The basal cell layer was no more visible, and the degenerative changes had progressed. Apoptotic cells with nuclear fragmentation and other nuclear anomalies were detected.

At day 18, the epithelium had reached considerable thickness but was severely degenerated. Necrotic cells showing an aberrant nucleus or no nucleus at all were abundant.

The matrix component

The matrix component, consisting of collagen and fibroblasts, varied in thickness in control cultures at days 6, 12, and 14. It was slightly thicker in cultures exposed to snuff extract for 6 days. At days 12 and 14, the matrix compartment was generally thin. However, intraexperimental variations

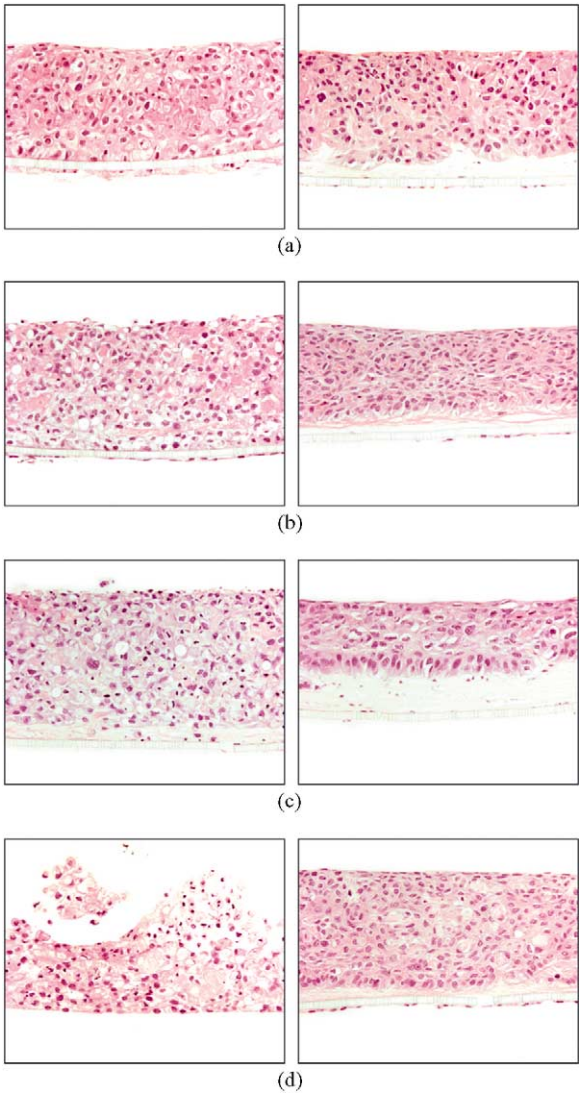

Figure 1 Morphology of snuff-exposed and control three-dimensional cell cultures (HE counter staining). Cultures grown for 6 days (a), 12 days (b), 14 days (c), and 18 days (d). Snuff-exposed cultures left column, control cultures right column. Original magnification 250×.

occurred. At day 18, the matrix was not distinguishable in either group.

Immunohistochemical staining of cultured specimens

Cell proliferation activity as detected by Ki-67 staining

Table 2 shows the Ki-67 indices. All cell layers of control and snuff-exposed epithelia showed Ki-67-immunoreactive nuclei at day 6. The proportion of proliferating cells in the snuff-exposed group had decreased to a level lower than that of the controls (Fig. 2). Similarly, at days 12 and 14, proliferation

Table 2 Mean Ki-67 indices in snuff-exposed and control three-dimensional cell cultures

Days of culture	Snuff-exposed (mean, ±S.D. error)	Control (mean, ±S.D. error)	*t* value	*P* value
6	42.66 (0.037)	73.79 (0.041)	5.52	0.0006[a]
12—14	15.30 (0.323)	26.13 (0.053)	1.79	0.0939
18	17.50 (0.018)	25.28 (0.050)	1.15	0.2957

[a] Statistically significant.

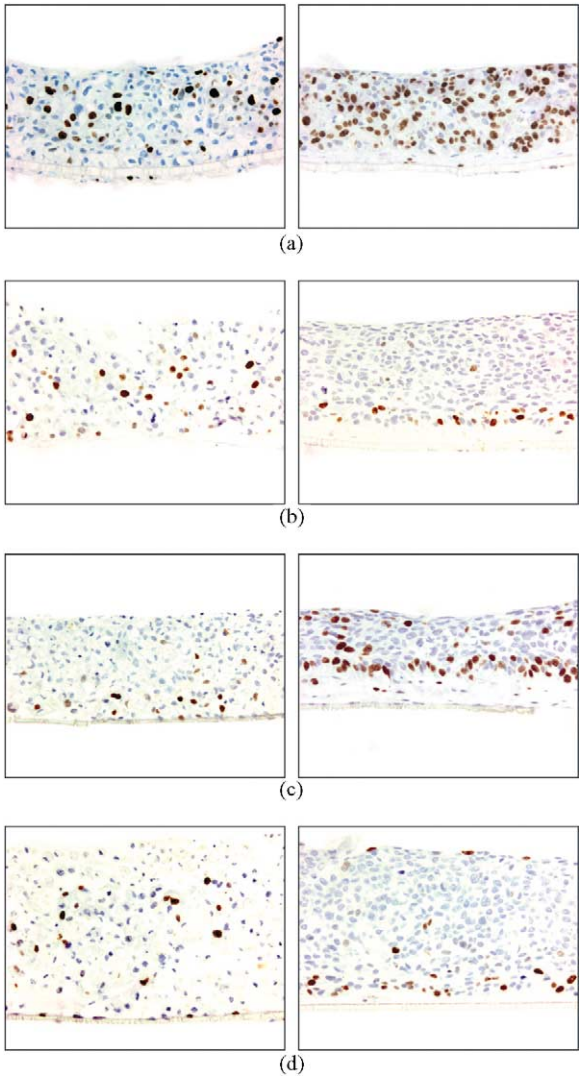

Figure 2 Ki-67 expression in snuff-exposed and control three-dimensional epithelial cell cultures. Cultures grown for 6 days (a), 12 days (b), 14 days (c), and 18 days (d). Snuff-exposed cultures left column, control cultures right column. Original magnification 250×.

activity was reduced, but the difference was not statistically significant. At this time point, the location of positive nuclei varied. In the controls, Ki-67-stained nuclei were confined to the basal and

a few suprabasal layers, whereas in the snuff group positive nuclei were scattered throughout the epithelial thickness. At day 18, the overall morphology of the cells was severely affected, and, consequently, Ki-67 positivity was only detected in cells with the most intact morphology.

Epithelial differentiation markers

Markers for simple epithelial cells, Cks 18 and 19, were detected throughout snuff-exposed and control epithelia (Table 3). Staining intensity for Ck 18 decreased slightly with advanced culture time.

Mabs to basal-cell-related Ck 5 and stratification-related Ck 13 stained weakly groups of cells in control tissues and snuff-exposed epithelia. At days 12 and 14, a slight decrease in Ck 5 staining intensity was seen below the level of the controls. At day 18, staining of both Mabs was only detected on the cell membrane in snuff-treated samples.

Mab to cornification-related Ck 10 stained strongly the upper two thirds of epithelium in all control cultures. In snuff-exposed groups, staining was notably weaker and confined to groups of scattered cells. Some snuff samples were completely negative (Fig. 3).

Immunoreactivity to involucrin, another marker of cornification-related differentiation, was detected in all cell layers of studied samples. At days 12 and 14, the amount of positively stained cells was higher in the snuff-treated group than in the controls. A late-stage cornification-related differentiation marker, filaggrin, was negative in all cultures at days 6, 12, and 14. A small area of filaggrin immunoreactive cells was detected in one 18-day control culture.

Cell-cycle regulators

As expected, all cells in the control cultures showed strong nuclear positivity for p53, owing to the heterozygous mutation of p53 in exon 5 (C > T mutation in codon 179) and exon 8 (CC > TT mutation in codons 281 and 282), which the HaCaT cell line carries, leading to overexpression of p53.[30] Several cells became p53-negative after exposure to snuff.

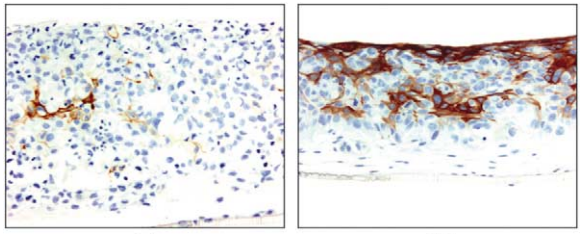

Figure 3 Ck 10 staining in snuff-exposed (a) and control (b) three-dimensional epithelial cell cultures grown for 6 days. Original magnification 250×.

Discussion

The three-dimensional cell culture used in this study, allowing cell—cell and cell—matrix interactions, is a suitable model for the study of pathogenesis by exogenous agents. A skin epithelium derived HaCaT cell line was used, as it is well-characterized and known to have good proliferation properties combined with differentiation capacity.[13] However, it reconstitutes an epithelium with a dysplastic phenotype in coculture,[14] but normal cells grow slowly and undergo senescence, limiting reproducibility.

Snuff extract was added when the co-culture was lifted to the air—liquid interface and the epithelial cells started to stratify. This setting shares similarities with wound healing, whereas a fully-developed epithelium cultured with serum-free media may bear greater resemblance to a mature epithe-

Table 3 Immunolocation of epithelial differentiation markers in snuff-exposed and control cultures (staining intensity in parentheses)

Antibody	6 days		12 days		14 days		18 days	
Group:	Snuff	Control	Snuff	Control	Snuff	Control	Snuff	Control
Ck 18	3 (++)	3 (++)	3 (++)	3 (++)	3 (++)	3 (++)	3 (++)	3 (++)
Ck 19	3 (++)	3 (++)	3 (++)	3 (+++)	3 (++)	3 (++)	3 (++)	3 (++)
Ck 5	0 or 3 (−,+)	3 (+)	3 (+)	3 (++)	3 (++)	3 (++)	3 (++)	3 (++)
Ck 13	3 (+)	3 (+)	3 (+)	3 (+)	3 (+)	3 (+)	3 (+)	3 (+)
Ck 10	0 or 4 (++)	3 or 4 (+++)	0 or 4 (+)	4 (+++)	0 or 4 (++)	4 (+++)	0 (−)	4 (+++)
Filaggrin	0 (−)	0 (−)	0 (−)	0 (−)	0 (−)	0 (−)	0 (−)	0 (+)
Involucrin	3 (+++)	0 or 3 (++)	0 or 3 (++)	3 (+++)	3 (+++)	3 (++)	3 (++)	3 (++)

−=no straining, +=mild staining intensity, ++=moderate, +++=strong. 0=negative sample, 1=staining of lower 1/3 of epithelium, 2=lower 2/3, 3=whole epithelium, 4=uppermost layer.

lium in vivo. Our morphologic finding of dysplastic cultured epithelium was consistent with the expression of epithelial differentiation markers. The detection of Ck 19 in all epithelial cell layers shows that the epithelium produced was simple and premature.

The results of the present study show that long-term snuff exposure, more than 12 days in culture, resulted in severe cellular damage and impaired cellular adhesion. In a similar experiment, Wang and coworkers found destructive morphologic changes after only 2 days of culture.[15] The differences in tolerance may be due to differences in the biological properties and metabolism of tobacco components in the cell lines used. Normal cells appear to be more vulnerable to snuff than their genetically altered counterparts.[16] Epidermal keratinocytes appear more sensitive to nicotine toxicity than mucosal cells.[17] Cell death may result from a specific signaling pathway leading to apoptosis or by events leading to necrosis. Fox et al.[18] have shown that cell death following long-term snuff exposure in vitro is not a result of apoptosis but related to epithelial–mesenchymal interactions resulting in the loss of cell adhesion.[18]

In the present study, we found increased cellular vacuolization in epithelial cells with increased culture time. Similar vacuolization has been reported to reversibly occur in HaCaT keratinocytes following nicotine exposure at high concentrations (>200 μl/ml).[19] It has been speculated that these vacuoles contain nicotine and may protect the cell from the toxic effects of free nicotine.[19] Others have suggested that this vacuolization results from tissue damage caused by the high pH of Scandinavian snuff.[20] We speculate that the vacuolizations seen in the present study may represent degenerative changes in response to the chemical components of snuff. The present study also showed an increase in nuclear aberrations with advanced culture time. Nuclear abnormalities have been reported in smears taken from the buccal mucosa of snuff users.[21] This has been thought to result from the toxic effects of snuff.[22]

In the present study, cell proliferation as detected by Ki-67 staining was not increased in the snuff-treated group compared to controls. Previous studies using cultured cells have shown that cell proliferation is related to the concentrations of snuff extract and nicotine. Low concentrations seem to increase cell proliferation whereas high concentrations, probably owing to their toxic effects, either decrease or have no effect on cell proliferation.[15,19] Similarly, in the study of Murrah and coworkers, high concentrations of smokeless tobacco extract (2%) exposure did not increase cell proliferation.[23] However, when the authors exposed the cells to purified tobacco carcinogens, proliferation activity was markedly increased.[23]

The results of the present study support the previous finding that high concentrations of snuff extract may have toxic effects that increase with prolonged exposure. Our recent in vivo study is in agreement with the present study showing that snuff extract did not stimulate cell proliferation.[24] In contrast, Wedenberg and coworkers detected increased Ki-67 protein expression in lesions from heavy snuff users.[25]

Snuff-induced lesions are histologically characterized by hyperkeratosis and hyperplasia. They are known to express Cks of cornified epithelia. Keratinization may be induced by snuff ingredients, including alkaline-buffering salts (such as calciumcarbonate) or nicotine.[19,26] Luomanen and coworkers detected Cks 1, 9, 10, and 11 in snuff users' lesions by immunohistochemistry.[27] Similarly, in vitro studies have shown increased Ck 1 and/or 10 expression in nicotine-treated oral keratinocytes.[17,26] and HaCaT cells.[19] The present study showed a decrease in terminal differentiation markers Ck 10 and filaggrin. The conflicting result may be explained by the relatively high concentration of the snuff extract used. Nicotine concentrations of more than 100 μg/ml have been reported to lead to disarrangement of Ck 1/10 filaments and to a decrease in their expression.[19]

p53 has been found to be expressed in snuff dippers' lesions varying from a low[28] to a high proportion of cells.[25,29] Upregulation may be explained as normal protective p53 production or alternatively p53 mutation. This study did not allow direct investigation of effects on p53, as the cell line used carries a heterozygous mutation of the gene.[30] The rate of cells expressing p53 diminished with increasing culture time in snuff extract (data not shown). The explanation for this could be that mutated p53 may remain functionally active or the reduction in protein expression simply reflects the overall decrease in protein synthesis as a result of toxic effects.

To conclude, the results of the present study show that snuff extract causes morphologic changes and that long-term snuff exposure does not increase epithelial cell proliferation activity but causes disturbances in the differentiation process.

Acknowledgements

The technical assistance of Ms. Sari Mäki and Ms. Marja-Riitta Uola is gratefully acknowledged. The study was supported by grants from the Academy of Finland, the Finnish Dental Society Apollonia and the Cancer Society of Finland.

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