Characterization of the Nuclear Activity of Aging Acinar and Ductal Cells of Palatal Salivary Glands

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Purpose: To investigate age-related changes in proliferative markers in palatal salivary glands and to discuss the potential role of the age-related increase in the inflammatory infiltrate in these changes.

Material and methods: Twenty-four post-mortem samples were equally divided into young (12-21 yr), adult (32-45 yr), and old (72-97 yr) age groups. Thirty acinar and ductal nuclei from each sample were assessed for the number and area of AgNORs (nNOR and TVNOR, respectively). PCNA-, Ki-67- and p53-stained nuclei were recorded in 10 high power fields and expressed as a percentage from the total number of nuclei of each cell type. One-way ANOVA and Kruskal-Wallis one-way ANOVA statistical tests were used.

Results: Among AgNOR parameters, only nNOR of the acini decreased significantly with age ($p=0.013$); all other results were not statistically significant. A 3- and 7-fold increase in mean percent of PCNA acinar and ductal cells was found with age ($p=0.041$ and $p=0.0095$, respectively). Neither acinar nor ductal Ki-67 positive nuclei were found. Nuclear p53 staining was absent.

Conclusions: In the acinar cells, the pattern of changes of the examined markers infers an increase in their metabolic synthetic activity with age. In the ductal cells, the pattern of changes indicates that they are under a strong cellular defence stress response to a high load of DNA damage. It is suggested that the damage was induced by the adjacent inflammatory infiltrate and its associated products, which accumulate with aging in these glands.

Key words: palatal salivary glands, aging, inflammatory infiltrate, DNA damage, neoplasm

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INTRODUCTION

About 50% of the minor salivary gland tumours (both benign and malignant) occur in palatal salivary glands (PSG). Typically, patients diagnosed with these tumours are in their fourth to seventh decade of life (Auclair et al, 1991). Factors responsible for, and leading to predominance of tumours in this particular location have not been yet elucidated. Regarding the etiopathology of tumours in the major salivary glands, a first hint of an association between presence of inflammatory infiltrates (e.g. obstructive sialadenitis) and tumour development has been made (Regezi et al, 2003).

In a histomorphometric study on age-related changes of PSG, mean volume fraction (Vv) of the acini decreased significantly, whereas that of the ducts increased significantly with aging (Dayan et al, 2000). The mean Vv of all examined stromal components, inflammatory infiltrate, blood and lymphatic vessels, adipose tissue and connective tissue, demonstrated a significant age-related increase. Among all these stromal components, the mean Vv of the inflammatory infiltrate showed the highest increase, 1,471%. The described changes in PSG differ from those reported in the labial salivary glands (LSG) (Vered et al, 2000) especially in regard to changes in the inflammatory infiltrate

components. In PSG, the age-related increase in the inflammatory component was found to occur earlier, to continue throughout life, and to be more severe compared to LSG (Vered et al, 2003).

In view of the different histomorphometric age-related changes in PSG as compared to LSG, the aim of this study was to examine age-related changes in the proliferative potential of the parenchymal cells (acini and ducts) in PSG, and discuss the possible role that the age-related increase in the inflammatory infiltrate might have on them. Therefore, PSG were examined for AgNOR parameters and Ki-67, as previously described in respect to LSG (Dayan et al, 2002), as well as for PCNA and p53. PCNA serves as a marker of the proliferating compartment of normal tissues and as a means to evaluate the prognosis of various tumours (de Paula et al, 2000). p53 activation is expected to play a role in inducing cellular responses in respect to the age-related increase in DNA damage (Sharpless et al, 2002).

MATERIAL AND METHODS

Study Group

Histologic specimens were prepared from palatal salivary gland biopsies of the posterior area of the hard palate obtained at autopsy (Greenberg Institute of Forensic Pathology, Tel-Aviv) from 24 subjects who had died as a result of road accidents, gun shots, cardiac arrest, traumatic injuries, drowning and suicide. Insofar as could be determined, subjects were not affected by cytotoxic drugs, autoimmune diseases, lymphoma, leukaemia, tumours of the head and neck, or infectious diseases. Specimens were equally divided into three age groups: young (n=8, age range 12-21 yr, mean 17 yr); adult (n=8, age range 32-45 yr, mean 38 yr); and old (n=8, age range 72-97 yr, mean 84 yr).

AgNOR Staining and Histomorphometry

Sections, 3 μm thick, were mounted on silane-coated glass slides, stained with silver nitrate (Dayan et al, 2002) and improved by Yekeler's solution (Yekeler et al, 1995).

AgNOR staining was assessed in acinar and luminal intercalated ductal cells as marked at a low magnification. Acinar cells were easily recognized by their characteristic pyramid shape, clear cytoplasm and flattened, basally located nucleus. Ductal cells were cuboidal and possessed a round nucleus that occupied nearly the whole cell cytoplasm.

For each cell type, 30 nuclei were randomly selected from different areas of the glands, particularly where parenchyma was well conserved and both cell types were present. Histomorphometry was performed as previously described (Dayan et al, 2002).

The parameters examined and calculated for each nucleus were as following (Cabrini et al, 1992):

1. Number of profiles of AgNOR per nuclear profile, which yields information on the number of AgNORs per nucleus (nNOR)
2. AgNOR profile area per nucleus (μ^2), which yields information on the total AgNOR volume per nucleus (TVNOR).

Data are presented as the mean score (±SD) of each AgNOR parameter for each age group.

Ki-67, p53 and PCNA Immunohistochemical Staining

Sections, 3 μm thick, were mounted on positive-charged microscope slides (Optiplus™, Biogenex, San Ramon, CA, USA). After dewaxing in xylene, sections were dehydrated in ethanol, rinsed in distilled water, placed in 3% H_2O_2 for 10 min and rinsed in distilled water for 10 min. Sections were dipped in Nuclear Decloaker™ solution, pH=9.5, (Biocare Medical, Walnat Creek, CA, USA) in a steam pot and microwave-treated for 10 min for Ki-67 and p53 antigen retrieval procedure. For PCNA antigen retrieval procedure, slides were incubated in citrate buffer (pH=6) in a microwave oven at 92 ° for 10 min. Sections were cooled for 20 min at room temperature, rinsed in PBS for 10 min and then incubated at room temperature for 1 h with DO-7 primary antibody (1:100, Dako A/S, Denmark) for p53; MIB-1 (1:100, Dako A/S, Denmark) for Ki-67; and PC-10 (1:800, Zymed, San Francisco, CA, USA) for PCNA detection. For antibody detection, universal immune peroxidase polymer anti-mouse and rabbit Histofine^R (Multi) kit was used following the manufacturer's instructions (Nichirei, Tokyo, Japan). Sections were rinsed in PBS for 10 min, reacted with AEC substrate-chromagen kit (Zymed, San Francisco, CA, USA), rinsed in distilled water for 2 min and finally counterstained in Mayer's hematoxylin (Pioneer Research Chemicals, Colchester, UK) and covered with a non-aqueous GVA mounting medium (Zymed, San Francisco, CA, USA).

Ki-67, PCNA and p53 Staining Evaluation

Staining with the examined markers is expressed in the nuclei of cells from the proliferative compartment as a brown homogenous staining. Ki-67, PCNA and

Table 1 AgNOR-parameter scores in relation to age groups and cell types

Morphologic parameter (Biologic parameter) Cell types	Mean number of profiles of AgNOR (*nNOR*)			Mean AgNOR profile area per nucleus (μ^2) (*TVNOR*)		
	Young	Adult	Old	Young	Adult	Old
Acini	2.5+1.4[1,2]	2.13+1.1[1]	2.2+1.4[2]	1.2+0.9	1.2+1.0	1.3+1.0
Ducts	2.3+1.4	2.2+1.4	2.2+1.4	1.4+1.1	1.8+1.5	1.9+1.7

[1,2]p=0.013

p53 positively stained nuclei of each examined cell type were scored in 10 randomly chosen high power fields (x400 magnification) in each slide. Additionally, the number of unstained nuclei of each examined cell type in the same fields was recorded. The fraction of stained nuclei from the total number of nuclei (stained and unstained) was calculated for each slide. Results are presented as the mean percentage (fraction ×100) of stained nuclei for each cell type in each age group.

Statistical Analysis

Differences in the mean value of the AgNOR parameters, Ki-67, PCNA and p53 scores among age groups were statistically analyzed using one-way ANOVA and Kruskal-Wallis one-way ANOVA tests. Computations were carried out using the Statistical Package for the Social Sciences (SPSS, 6.1) software.

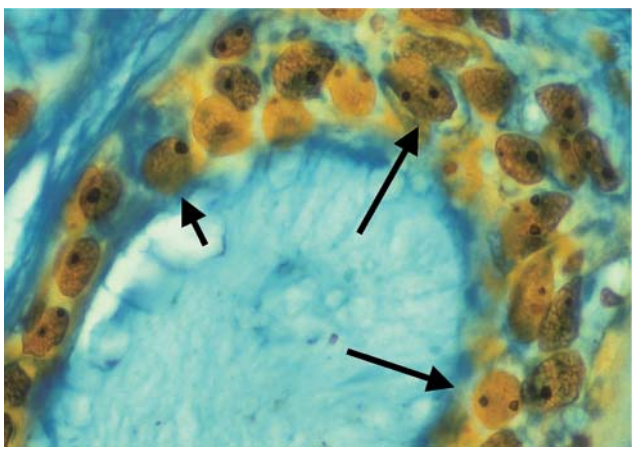

Fig. 1 Silver staining of nucleolar organizing regions in palatal ductal cells. Short arrow shows a nucleus with one positive dot and long arrows show nuclei with two positive stained dots (original magnification ×1000).

RESULTS

Age-related Changes in AgNOR Parameters (Table 1)

The nNOR of the acini showed a significant decrease in the adult and old groups as compared to the young group (p=0.013). No significant changes were found in the TVNOR with aging (p=0.05).

The nNOR and TVNOR scores of the ducts did not demonstrate significant changes with aging (p=0.05). Fig. 1 shows AgNOR-positive dots in ductal nuclei.

Age-related Changes in PCNA-positive Cells (Table 2)

The mean number of acinar cells showed a 3-fold increase from the young to the old age groups (p=0.041) and the mean number of the ductal cells showed a 7-fold increase between the same age groups (p=0.0095) (Fig. 2).

Ki-67 Evaluation

Since only isolated positively stained acinar and ductal cells were found in all examined slides, the proliferative index was calculated as 0%, and no statistical test was applied.

p53 Evaluation

No nuclear p53 staining of either acinar or ductal nuclei was found in any of the examined slides. However,

Table 2 Mean number of PCNA-positive acinar and ductal cells in relation to age groups

	Young	Adult	Old
Acini	9.0+4.5[1]	4.7+3.9	27.5+33.0[1]
Ducts	2.0+3.02[2]	5.1+4.1	14.7+12.7[2]

[1]p=0.041[2]p=0.0095

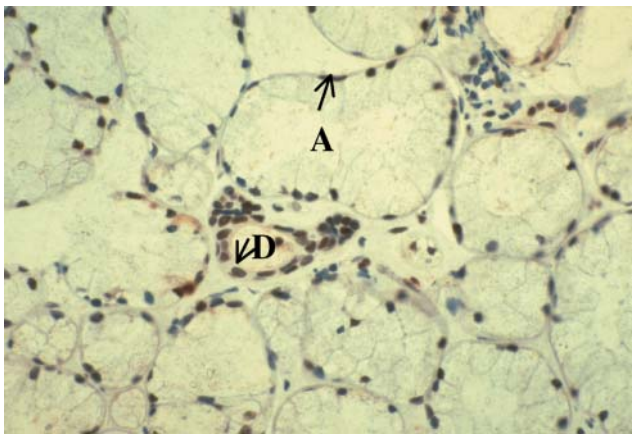

Fig. 2 Photomicrograph of PCNA positive stained nuclei of ductal (D) and acinar (A) cells (arrows) (original magnification ×200).

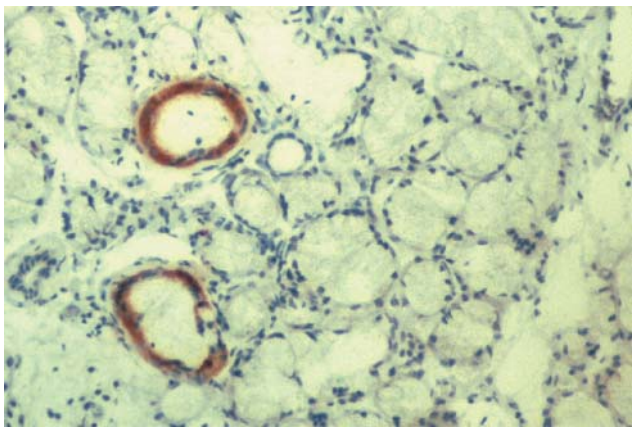

Fig. 3 Photomicrograph of p53 cytoplasmic positive staining in ductal cells (original magnification ×100).

cytoplasmic staining in several ducts was observed in four cases from the old group (Fig. 3).

DISCUSSION

The present study examined age-related changes in the nuclear activity of acinar and ductal cells of PSG with proliferative markers AgNOR, Ki-67 and PCNA, and cell cycle regulatory protein p53.

AgNOR and Ki-67 are considered markers of the proliferative activity of cells. However, there may be situations where their distribution may not be in agreement with each other, as found in a study on aging LSG (Dayan et al, 2002). In that study, age-related changes of AgNOR parameters (i.e., nNOR and TVNOR) were analyzed. It was concluded that a net age-related increase or decrease in the area of each AgNOR, accompanied by the absence of Ki-67 positivity, could reflect changes in the metabolic cell activity rather than changes in their proliferative capacity.

The significant age-related decrease in nNOR of the palatal acinar cells accompanied by no significant changes in the TVNOR and a null proliferative index, indicate that in the old group, these cells were implicated in an increased metabolic activity of protein synthesis with no proliferative activity. This could be explained by three different physiological phenomena. Firstly, part of the metabolic activity is probably invested in the synthesis of lysosomal enzymes necessary for undergoing atrophy (Harrison and Garrett, 1976), which is supported by the histomorphometric finding of about 50% reduction in the volume fraction of the acinar component with aging (Dayan et al, 2000). Secondly, the remaining functional acinar cells required to compensate for the lost secretory tissue probably consume another part of the metabolic activity. Thirdly, age-associated redifferentiation of acinar cells into ductal cells (Scott, 1980; Redman, 1995) is an additional phenomenon that might need excessive protein synthesis, mainly lysosomal enzymes for decomposition of the abundant cytoplasmic organelles characteristic of acinar cells, so that the acinar-derived ductal elements would possess only sparse cellular organelles.

In contrast to the acinar cells, ductal cells did not demonstrate any significant age-related changes either in their nNOR or TVNOR parameters, which infer no significant changes in their metabolic rate. The different behaviour pattern between the acinar and ductal cells probably derives from the impact that the chronic inflammatory infiltrate might have on the ductal epithelium located adjacent to it (Dayan et al, 2000; Vered et al, 2001). The presence of chronic inflammation is closely associated with the production of reactive oxygen species (ROS) and metabolites within adjacent cells, with an ultimate result of inducing DNA damage (Larsson et al, 2001). The DNA damage that ROS are capable of inducing includes activation of proto-oncogenes and/or impairment of tumour suppressor genes in neighbouring target cells (e.g. ductal cells), which ultimately may pave the way for the appearance of single tumour cells (Schwartsburd, 2003).

PCNA plays an important role in DNA damage repair in addition to its being an essential component of the DNA replication system (Prosperi, 1997). PCNA scores in the ductal cells in the old age group demonstrated a seven-fold increase as compared to the young group. In light of the fact that ductal cells had yielded negative

results in regard to Ki-67, it can be assumed that these cells, which are usually surrounded by a remarkable inflammatory infiltrate in the old age group, are primarily engaged in repairing inflammatory-associated DNA damage rather than in proliferative activity. This implies that the histomorphometric age-related increase in the volume fraction of the ducts (Dayan et al, 2000) does not represent a true ductal hyperplasia but rather edema and sialectasia. Further support for this is found in a study that examined ductal cells from sialadenitis-involved (Sjögren's syndrome) LSG (Larsson et al, 2001) and suggested that an immunoreactivity pattern of PCNA-positive and Ki-67-negative ductal cells reflects a strong cellular defence stress response to a high load of DNA damage.

In the present study, the acinar cells demonstrated a three-fold increase in their PCNA score from the young to the old group, which could also point to ROS-induced DNA damage repair activity. In the acinar cells, ROS can originate from either the adjacent inflammatory infiltrate or the intracellular amplified metabolic activity (Sharpless et al, 2002).

An important factor that mediates the generation of age-related cellular response to increased amounts of DNA damage is activation of the tumour-suppressor gene *p53*. In normally functioning cells, p53, which is the protein product of the *p53* gene, has a short half-life. Therefore, it is below the detection threshold of the immunostaining methods. However, in response to DNA damage, such as ROS production, p53 accumulates within cell nuclei and can be immunohistochemically detected. In the present study no nuclear p53 staining was found in any of the examined cell types, including the old age group, where DNA damage is high. Nevertheless, this does not imply that p53 mechanism is not initiated. Age-related cellular response regulation by *p53* can be achieved with only increased transactivation of *p53* dependent genes without excessive production of the p53 protein and subsequent immunohistochemical detection (Sharpless et al, 2002), which could be the case in the present study. The cytoplasmic staining of p53 in the cells of several ducts in the old group can be explained by the effect some mediators of inflammation have on the normal *p53* gene. As a result, they can induce conformational changes in the p53 protein product that correspond with its inactivation and aberrant redistribution from the cytoplasm to the nucleus (Moos et al, 2000). As a result, the role of the normal *p53* gene and its protein product as a guardian of genomic integrity could be impaired, predisposing to development of premalignant and malignant conditions. To establish this assumption in respect to the present findings, further studies are needed to determine the actual state of the *p53* gene and its downstream factors.

Associations between chronic inflammatory conditions and development of tumours at the precise site of the inflammation have been shown in several settings. Chronic inflammation has carcinogenic potential due to induction of DNA damage and mutations and is capable of generating a potentially pro-cancer microenvironment favourable for survival and growth of tumour cells (O'Byrne et al, 2001; Schwartsburd, 2003). As previously demonstrated, PSG are continuously exposed to a considerable chronic inflammation, a condition that might create a favourable background for development of tumours. As about half of the minor salivary gland tumours (both benign and malignant) are found in these glands, an association between these two facts is a possibility. However, further studies are needed to elucidate the particular factors that may contribute to this association.

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