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Apoptosis in Normal Epithelium, Premalignant and Malignant Lesions of the Oropharynx and Oral Cavity: a Preliminary Study

M.A. Birchall, C.M. Winterford, D.J. Allan and B.V. Harmon

To explore the involvement of apoptosis in the development of oral and oropharyngeal squamous cell carcinoma (SCC) *in vivo*, biopsies were taken from patients with macroscopically normal ($n=6$), leukoplakic ($n=12$) or malignant ($n=8$) mucosa. Leukoplakic lesions were divided histologically into dysplasia ($n=5$) or carcinoma *in situ* (CIS: $n=7$). Material was prepared for light and electron microscopy. The apoptotic index (AI), vertical cell position of apoptoses (cp), mitotic index (MI) and AI:MI ratio were calculated for each patient. AI increased from 0.12% ± 0.07 S.E.M. (normal) to 0.58 ± 0.13 (CIS) but fell to 0.14 ± 0.14 in SCC. Apoptoses were suprabasal in normals, but generalised in CIS. MI increased from normal (0.20 ± 0.06) to SCC (0.32 ± 0.09), and AI:MI was at its maximum in CIS (1.57; SCC: 0.44). The results suggest that a change in apoptosis accompanies the onset of invasion in a premalignant lesion of the human oral cavity and oropharynx.

Keywords: apoptosis, oral cavity, oropharynx, normal epithelium, carcinoma *in situ*, squamous cell carcinoma, human

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INTRODUCTION

Apoptosis, or programmed cell death, is a distinct cellular event which has been identified under a wide range of physiological and pathological circumstances [1, 2]. It may be induced by a variety of stimuli, and is thought to play an important part in carcinogenesis [3]. Unfortunately, little is known of the exact nature of change in apoptosis during the progression from normal to malignant tissue, and much of the available data is contradictory. Thus, in rat liver, apoptosis appears to increase in premalignant lesions compared to normal [4], whilst in the human breast, premalignant changes are accompanied by a decrease in observed apoptosis relative to mitosis [5]. Hence, there is a need to study the process in more detail.

The mucosa of the oral cavity and oropharynx is a common site for the development of dysplastic change, usually after exposure to cigarette smoke and alcohol, and this may in turn progress to squamous cell carcinoma [6]. Since they are easily observed and accessible, the mouth and pharynx provide an ideal site for the study of carcinogenesis *in vivo*.

The aim of the present study was to identify and characterise apoptosis in normal, dysplastic and carcinomatous epithelia of the human oral cavity and oropharynx and to provide preliminary data for further study.

MATERIALS AND METHODS

Twenty-seven biopsies were obtained prospectively from the mouth and oropharynx (soft palate) of patients presenting to the Ear, Nose and Throat (ENT) Clinic, Royal Brisbane Hospital, and Head and Neck Clinic, Queensland Radium Institute over a 3 month period. Of these, eight presented with lesions that proved to be invasive squamous cell carcinoma, whilst 12 had leukoplakic or erythroplakic lesions that proved to contain varying degrees of epithelial dysplasia. In addition, control tissue was obtained from the anterior faucial pillar during tonsillectomy for recurrent tonsillitis (3 cases) and from the inferior surface of the soft palate during uvulopharyngoplatoplasty for simple snoring (3 cases). One case was biopsied but not included as it showed scar tissue from a previous intraoral procedure.

The study was performed with the approval of the Research and Ethics Committee of the Royal Brisbane Hospital (Protocol 94/03), and fully informed consent was obtained in each case.

Correspondence to M.A. Birchall.
M.A. Birchall is at the Department of ENT Surgery, Royal Brisbane Hospital, Herston, Queensland 4006; C.M. Winterford is at the Department of Pathology, University of Queensland Medical School, Herston, Queensland 4006; and D.J. Allan and B.V. Harmon are at the School of Life Sciences, Queensland University of Technology, GPO Box 2434, Brisbane, Queensland 4001, Australia.
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HISTOLOGICAL EXAMINATION

After atraumatic sampling, biopsies were fixed in 4% formaldehyde in 0.9% saline for a minimum of 1 h. Paraffin sections, 4 μm thick, were prepared and stained with haematoxylin and eosin (H&E) for light microscopy. Care was taken to ensure that sections were as vertical as possible relative to the surface. At least two sections were cut in each case to reduce sampling error. In two cases, after fixing with a glutaraldehyde/paraformaldehyde solution, semithin (1 μm) sections were cut and stained with Toluidine Blue to select areas for electron microscopy. This was performed with a JEOL JEM-1200 EX II, whilst light microscopy was performed using an Olympus BH-2 light microscope.

Histological assessment of the tissue was performed blind by an experienced histopathologist (Professor J. Searle). The tissue was graded as normal, dysplastic (mild to moderate dysplasia), carcinoma *in situ* (CIS) or squamous cell carcinoma (SCC). Counting was indertaken on the H&E sections with oil immersion at 100 × magnification, using a 10 × 10 cell graticule. Sections were examined by one observer (MAB) who was blind to the histological diagnosis, and a minimum of 1000 cells were counted per slide. The numbers of apoptoses and mitoses, expressed as a percentage of observed cells (apoptotic index, AI; mitotic index, MI) were recorded, along with vertical cell position (cp) of observed apoptotic bodies or cells. The AI:MI ratio was calculated. Data were analysed using Kruskal–Wallis non-parametric analysis of variance.

RESULTS

Demography

Demographic characteristics of the four histological groups are given in Table 1. The average age was younger (56.0 years, range 18–76 years) in the normal group compared to the other groups and one third of the patients in this group were non-smokers. However, the other categories contained patients that were highly comparable in terms of age, sex and smoking habit. The sites of biopsy were floor of the mouth, tongue, anterior pillar/retromolar region, soft and hard palate.

Morphology

Apoptotic cells and, more frequently, apoptotic bodies were observed in all sections examined. The commonest appearance was a group of hyperdense bodies contained within migrating epithelial cells (Fig. 1). However, occasionally, typical early changes, such as margination of chromatin and condensation of cytoplasm, were also noted (Fig. 2). Mitotic figures were readily identifiable and were morphologically distinct from the appearance of apoptoses (Fig. 1). Electron microscopy confirmed the typical changes of apoptosis within SCC and dysplastic epithelium: besides phagocytosed apoptotic bodies containing recognisable nuclear fragments, it was possible to see apoptotic cells whose nuclei showed margination of chromatin, with relative preservation of the organelles within the condensed cytoplasm (Fig. 3). Apoptotic neutrophils were also observed within the stroma of SCC (Fig. 4).

Table 1. Demography of patients in each of the four study groups

	Number	Average age (range)	Females	Non-smokers
Normal	6	56 (18–76)	0	2
Dysplasia	5	70 (61–79)	3	1
Carcinoma *in situ*	7	63 (62–70)	3	0
Squamous cell carcinoma	8	62 (59–74)	2	1

Fig. 1. Severely dysplastic mucosa with apoptotic bodies (A) at cell position 6. Clearly distinct mitoses (M) are also seen (× 540, bar = 50 μm).

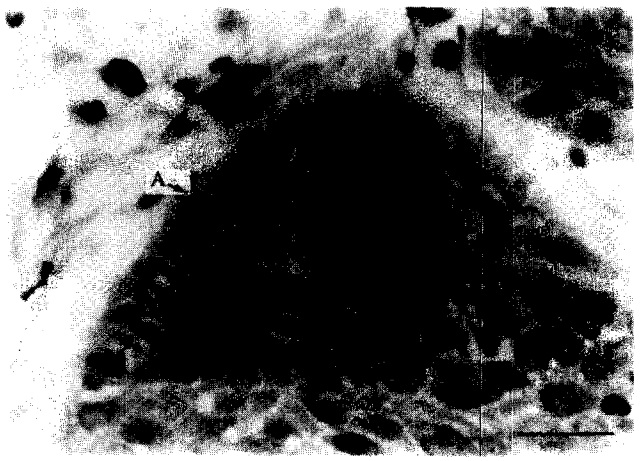

Fig. 2. Mild dysplasia: basal apoptosis with characteristic margination of chromatin is visible (A) (× 1350, bar = 20 μm).

Cell position

The depth of the epithelium increased with the degree of dysplasia, from 7 nucleate cells thickness to over 20 in CIS. Apoptoses were found in modal cell positions 2 and 3 in normal tissue and mild to moderate dysplasia, respectively. However, in CIS, apoptoses became distributed throughout the epithelium. Clearly, it was not possible to assess the vertical position of cells within invasive SCC.

Apoptotic and mitotic indices

Results for the various indices are shown in Table 2 and Fig. 5. AI increased from normal (0.12% total cells, S.E.M. 0.7) through dysplastic epithelia to reach a maximum in CIS (0.58

Fig. 3. Electron micrograph from a section of carcinoma of the tonsil. Phagocytosed apoptotic bodies in different stages of degradation are seen. Margination of chromatin is clearly visible in the central body (× 12 000, bar = 3 μm).

Fig. 4. Electron micrograph showing an apoptotic neutrophil in the stroma of a tongue carcinoma (× 12 000, bar = 3 μm).

Table 2. Apoptotic index (AI), mitotic index (MI) and AI/MI ratio for the four groups of patients (mean and S.E.M.). The modal cell position (cp) of apoptoses/apoptotic bodies is shown for the first three groups

	Normal	Dysplasia	Carcinoma *in situ*	Squamous cell carcinoma
A.I.	0.12 ± 0.07	0.28 ± 0.10	0.58 ± 0.13	0.14 ± 0.14
M.I.	0.20 ± 0.06	0.26 ± 0.09	0.37 ± 0.10	0.32 ± 0.09
A.I./M.I.	0.60	1.08	1.57	0.44
c.p.	2	3	5	—

S.E.M. 0.13). However, it fell to "normal" levels in overt carcinoma (0.14 S.E.M. 0.14). In contrast, MI rose steadily and reached similar levels in both CIS (0.37 S.E.M. 0.10) and SCC (0.32 S.E.M. 0.9). Accordingly, the AI:MI ratio reached a maximum in CIS (1.57) and a minimum in SCC (0.44). The fall in AI:MI between CIS and SCC was significant at the 5% level ($P = 0.041$), but all other differences failed to reach statistical significance.

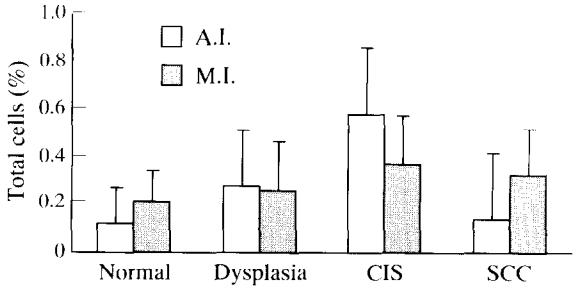

Fig. 5. Bar chart of apoptotic index (AI) and mitotic index (MI) in normal and pathological oral and oropharyngeal epithelia, expressed as a percentage of the total number of cells. Error bars = 95% confidence intervals.

DISCUSSION

This study shows that apoptosis is readily observed in biopsies of the human oral and oropharyngeal mucosa. Electron microscopy findings confirmed the light microscopic appearances as being apoptotic. The position of apoptotic bodies was localised to the deepest layers in normal and dysplastic epithelia, but became generalised in CIS and SCC. The apoptotic index increased progressively from normal to CIS, but fell again in SCC, and this was reflected by an increase in the AI:MI ratio, followed by a marked drop in SCC.

The numbers of patients in this report were small. Whilst the three abnormal categories were similar in terms of age, sex and smoking habit, the fact that the normal group were younger and contained more non-smokers may have introduced error. In particular, it is known that nicotine and its metabolites inhibit apoptosis in various tissues, including pharyngeal carcinoma [7]. If this was true in the present setting, then the real difference between normal and abnormal oral epithelia is likely to be even greater than described. The effect of age and sex on apoptosis is unknown. However, there are hormonal differences with age and sex, and the inductive effect of steroids on apoptotic cells *in vitro* is well described [8].

Likewise, the differences between the various sites of biopsy exert an unknown effect, which should be explored by a larger study of normal tissue. Certainly, SCC tends to behave in different ways depending on the subsite [9] and the biological difference this implies may extend to changes in apoptosis.

The light and electron microscopic appearances of apoptosis have been described previously [2] and all the typical features were visible in the present study. It was interesting that some of the neutrophils associated with SCC were also observed to be apoptotic, since it is recognised that an immune suppressive factor released by oesophageal carcinoma cells induces apoptosis in lymphoid cells [10]. This may represent an important aspect of the invasiveness of these tumours.

The observation that apoptosis occurs in the suprabasal layers under normal and early dysplastic conditions is consistent with the models proposed for skin and intestine [11]. In these models, epithelial cells of second and later generations are destined to undergo either apoptosis or terminal differentiation, with eventual desquamation of the latter and phagocytosis of the former. The degree of apoptosis, it is proposed, depends on the presence of various survival

factors as well as the effects of topical factors and intrinsic "suicide" mechanisms for disordered cells. The fact that apoptosis becomes more diffuse in CIS and SCC is not surprising and has been recognised in other carcinomas [12].

Values for MI were very much as observed in other normal and abnormal tissues [4, 5, 13]. MI increased steadily as one progressed from normal to malignant tissue. This is a well-known observation, which has been used to help diagnose malignancy in some situations [13]. However, in the present context, this rise served to highlight the drop in apoptosis in SCC compared to CIS.

The values of AI in normal tissue found in the present study are consistent with figures from other parts of the body, such as the breast [5]. The finding that apoptosis increased with increasing degree of dysplasia has not been previously reported for head and neck mucosa. The data of Columbano *et al.* from rat liver, although also descriptive, suggests that apoptosis increases in premalignant nodules, which is consistent with our results [4]. Although Allan *et al.* [5] found that AI fell significantly in fibrocystic disease of the breast, the association of this condition with the development of breast cancer is much weaker than that between mucosal dysplasia and SCC [6]. Teleologically, it might be argued that the epithelium is attempting to remove cells with damaged genomes by the mechanism of apoptosis. This may be intrinsic "suicide" or be induced by adjacent normal or infiltrating inflammatory cells. Alternatively, the topical factors responsible for dysplastic change may themselves be inducing increased levels of apoptosis, which may, therefore, simply be an association.

The drop in apoptosis between CIS and invasive squamous cell carcinoma is a striking and novel observation. Taken in isolation, this would suggest that a change in the control of apoptosis is a critical step in the development of invasive change in a premalignant tissue. This difference becomes even more marked when the AI:MI ratio is calculated, since MI is similar in CIS and SCC: AI:MI actually drops below normal levels in SCC. However, AI:MI values must be viewed with caution, as cells undergoing apoptosis are not necessarily from the same population as those undergoing mitosis.

The distinction between CIS and SCC, from the clinical point of view, is very important, since the latter necessitates radical therapy, whilst the former is usually kept under observation, and commonly reverses with the removal of luminal exposure to cigarette smoke and alcohol [6]. Hence, the fall in AI between CIS and SCC requires detailed scrutiny.

In conclusion, apoptosis is easily recognised in light and electron microscopic preparations of normal, premalignant and malignant epithelia of the human oral cavity and oropharynx. It appears to occur in the suprabasal layers under resting conditions, but becomes generalised in advanced dysplasia. Apoptotic indices increase from normal, through dysplastic epithelia to carcinoma *in situ*. However, whilst mitotic indices are even higher in invasive carcinoma, apoptosis appears to fall markedly. Hence, inhibition of apoptosis may be an important part of the final step from premalignant epithelium to malignancy *in vivo*. These findings should be further explored in larger studies.

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