Pergamon

Oral Oncology, Vol. 33, No. 6, pp. 419–425, 1997

Printed in Great Britain
1368-8375/97 $17.00+0.00

PII: S0964-1955(97)00033-X

Apoptosis, Mitosis, PCNA and bcl-2 in Normal, Leukoplakic and Malignant Epithelia of the Human Oral Cavity: Prospective, *In Vivo* Study

M. A. Birchall,[1] E. Schock,[2] B. V. Harmon[3] and G. Gobé[2]

[1]Department of ENT Surgery, Royal Brisbane Hospital; [2]Department of Pathology, University of Queensland Medical School; and [3]School of Life Science, Queensland University of Technology, Brisbane, Australia

Disordered balance between proliferation and apoptosis may contribute to carcinogenesis. Thirty-two oral biopsies were collected prospectively: 10 normal (N), 10 leukoplakia (dysplasia, D = 5; hyperplasia, H = 5) and 12 squamous cell carcinoma (C: 11). Distant normal tissue was also collected (HN, DN, CN). Based on counts of 1000 cells/slide, mitotic (MI), apoptotic (AI) and proliferating cell nuclear antigen (PCNA: PI) indices were calculated and bcl-2 expression recorded. AI correlated with MI ($P < 0.001$), but not PI or bcl-2 expression. PCNA was higher in H and HN than other groups ($P < 0.0001$). bcl-2 was reduced in C and CN ($P < 0.001$). Peak mitosis shifted basally in dysplasia, whilst peak apoptosis remained unaltered. These data confirm topographical alterations in proliferation relative to apoptosis in dysplasia of the oral cavity. Reduced bcl-2 in carcinoma and related 'normal' epithelium was unexpected, and may contribute to the high incidence of metachronous carcinomas in these patients. © **1997 Elsevier Science Ltd. All rights reserved.**

Key words: apoptosis, mitosis, bcl-2, PCNA, hyperplasia, dysplasia, carcinoma

Oral Oncology, Vol. 33, No. 6, pp. 419–425, 1997

INTRODUCTION

We have previously demonstrated that absolute levels of apoptosis in normal, premalignant and malignant epithelium of the oral cavity and oropharynx appear relatively static compared to the increase in mitotic activity. However, apoptosis occurs in a more superficial position in dysplasia than in normal epithelium. We have postulated that this separation from the presumptive stem cell layer represents a reduction in the 'efficiency' of the apoptotic 'house-keeping' mechanism in these lesions, and may be fundamental to their malignant potential [1].

The bcl-2 gene product is thought to play an important role in cellular decision-making [2, 3]. Overexpression of bcl-2 in B-cells leads to prolonged survival and subsequent malignancy [4]. Recently, bcl-2 has been shown to exhibit reciprocity with p53 in the development of colorectal adenocarcinoma [5], with bcl-2 expression being apparently suppressed in malignancy compared to premalignant adenomas. Although a serial change similar to that for bowel, from normal, through progressive degrees of dysplasia, to squamous cell carcinoma, is believed to occur in the head and neck, bcl-2 expression has been little studied. In a small study of nasopharyngeal carcinoma, bcl-2 positive cells were observed in respiratory-type epithelium, but not in squamous epithelium, adjacent to carcinomas [6]. However, no studies have been reported on bcl-2 expression in stratified squamous epithelium, despite the fact that this is commonly involved in premalignant and malignant change.

If the normal response to genetic mutation in squamous stem cells is that of apoptosis, and this response, as our earlier findings suggest [1, 7], is altered during the development of malignancy, then one might expect that bcl-2 gene expression might also be altered. We therefore hypothesised that bcl-2 expression would increase in dysplasia and malignant lesions compared to normal epithelium and non-premalignant oral lesions (hyperplasia). Further, we hypothesised that the increase in expression in dysplasia would be confined to the basal layer and would be proportional to the amount of observed apoptosis.

Proliferating cell nuclear antigen (PCNA) is a 36kD non-histone nuclear protein that is auxiliary to DNA-polymerase delta and necessary for replication [8, 9]. It has previously been used as a marker of cell proliferation, but more recently has been shown to represent both induction by growth factors and excision repair processes [10, 11]. In a previous study of the oral cavity, PCNA counts were suggested to increase with

Correspondence to M. A. Birchall at University Department of Otolaryngology, Head and Neck Surgery, Medical School Building, Southmead Hospital, Bristol BS10 5NB, U.K.
Received 21 Jan. 1997; provisionally accepted 11 Feb. 1997; revised manuscript received 21 Apr. 1997.

increasing dysplasia and grade of carcinoma, although no statistics were applied to support the conclusions and there was no indication of delay times prior to fixation [12]. Although changes in proliferative activity are known to occur in both hyperplasia and dysplasia, these have not been related to excision repair *in vivo*. One way of accomplishing this in theory would be to perform counts of both PCNA expression and mitotic figures simultaneously. We hypothesised that PCNA expression would be greater in hyperplasia, a condition rarely associated with malignancy, than in dysplasia, where DNA repair mechanisms may be defective. We further hypothesised that PCNA positive nuclei would be numerically and topographically distinct from observed mitoses.

In the present study we (1) compare the relationships of apoptosis, mitosis, bcl-2 and PCNA in prospectively collected biopsies of the oral cavity and oropharynx; (2) relate the expression of these markers to four different histologies: normal epithelium, hyperplasia, dysplasia and invasive squamous cell carcinoma; (3) compare topographical distribution of apoptosis, mitosis, bcl-2 and PCNA positive cells.

MATERIALS AND METHODS

Tissue was collected from patients under the ethical guidelines of the Royal Brisbane Hospital (ref.: 94/03). All tissue used in this study was from patients who were consulted and consented to its use.

Tissue from consecutive patients presenting to the Otolaryngology and Plastic Surgery Departments of the Royal Brisbane Hospital with either leukoplakia, erythroplakia or frank carcinoma of the oral cavity and oropharynx, as determined by two experienced clinicians, was collected over an 8 month study period in 1994 (9 with leukoplakia, 1 with erythroplakia and 12 with clinical carcinoma). Biopsies (at least 8 mm^2) were taken from the edge of the visible lesions. Internal control tissue was harvested from normal epithelium at least 10 mm from the area of concern.

External control tissue was taken from the anterior fauceal pillar of 7 patients undergoing tonsillectomy for recurrent sore throats and the soft palate of 3 patients undergoing surgery for simple snoring. These control patients had no clinical abnormality of the oral or oropharyngeal mucosa and were selected to match the other groups for age and sex, as far as possible.

Demographic details comprising age, sex, smoking and alcohol habits, and the anatomical subsite of the biopsy were recorded for all patients. Alcohol intake was classified as zero/occasional, 20 or less units a week or more than 20 units a week. Similarly, cigarette consumption was classified as zero/ex-smoker, 20 or less cigarettes a day or more than 20 cigarettes a day [13]. Subsite was classified as oropharynx, lateral tongue or other oral site.

Histological examination

The specimens were immediately placed in formalin and paraffin sections prepared within 24 h. Four micrometre sections were stained with haematoxylin and eosin (H&E) for light microscopy. Four cases (1 from each group) had their biopsies divided in two at the time of surgery. One of these was processed for light microscopy and one fixed with a glutaraldehyde/paraformaldehyde solution embedded in epoxy resin, prepared as 1 μm sections and stained with Toluidine Blue to select areas for electron microscopy. This was performed with a JEOL JEM-1200 EXII, whilst light microscopy was performed using an Olympus BH-2 light microscope. Histological diagnosis was made by a single, experienced histopathologist with special expertise in head and neck pathology, who was blinded to the clinical history.

Counting of H&E slides was performed by one observer (MB) using 100 × magnification, oil immersion and a 10 × 10 cell graticule centred on the basement membrane. A minimum of 1000 cells per slide was counted. For carcinomas, counts were performed at the advancing edge, away from areas of necrosis. Apoptosis and mitosis were recognised using morphological characteristics as previously described [7], which were then confirmed ultrastructurally. The numbers of apoptoses and mitoses, expressed as percentage of observed cells (apoptotic index: AI; mitotic index: MI), were recorded, along with the vertical cell position (cp) of observed apoptotic events (bodies or cells). The AI:MI ratio was calculated. Cell position was assessed relative to the basal layer (cp = 1) via the shortest straight line. Alternative *in situ* staining methods for apoptosis have been trialled in our laboratory and by others [14], but have been assessed as unsuitable in the present model due to discrepancies between 'apoptoses' demonstrated and accepted ultrastructural and other features.

Immunohistochemistry

For immunostaining, standardised and controlled protocols were followed. Staining for PCNA expression used a monoclonal rabbit anti-human PCNA antibody. An indirect labelled streptavidin peroxidase biotin method was used with diaminobenzidine (DAB) as the substrate [9]. Light counterstaining was performed with Mayer's haematoxylin. Positive and negative controls (tonsillar tissue with lymphoid aggregates) were run with each immunohistochemical labelling procedure. A minimum of 1000 cells per slide was counted and the PCNA index (PI) calculated as a percentage of all cells. Only cells with a distinct brown staining confined to the nucleus were considered positive. For each positive cell, vertical cell position was plotted, as for apoptosis and mitosis.

Staining for bcl-2 expression used a monoclonal mouse anti-human bcl-2 antibody (Dako). An indirect labelled streptavidin peroxidase biotin method was used with diaminobenzidine (DAB) as the substrate. Positive and negative external controls (tonsil) were run with each immunohistochemical labelling procedure. In addition, subepithelial lymphocytes, where present, were used as internal positive controls. Sections were scored as positive or negative, and the distribution noted.

Statistical analysis

This was performed using the Statgraphics software package (V.2.6 Statistical Graphics Corp, U.S.). To compare demographic characteristics, including age, paired *t*-tests and chi-squared tests were performed as appropriate. Exploratory data analysis included distribution testing (Kolmogorov–Smirnov), and logarithmic transformation was performed where appropriate to allow the use of parametric tests. A backward stepwise linear regression model was fitted to the data [15], with AI, bcl-2 expression and histology as dependants in turn. Independent variables were age, sex, drinking and smoking habits, anatomical subsite, MI, PI, and bcl-2 expression.

For each group, mean score at each cell position (cp) was plotted as a function of vertical position, and the resultant plot smoothed over three cell positions [16]. The cell position corresponding to the peak of each curve was noted. Multifactorial analysis of variance was used to examine differences in cell distribution curves between different histological groups. A chi-squared test was used to explore differences in bcl-2 expression between groups. Significance at the 5% level was accepted.

RESULTS

Histology

Of the leukoplakic lesions, four demonstrated epithelial dysplasia (one mild, three moderate) and five hyperplasia by conventional criteria [17]. The erythroplakia proved to be severe dysplasia. All of the carcinomas were squamous cell, of which five were well differentiated, five moderately differentiated, and two poorly differentiated. All of the internal controls appeared normal microscopically. The specimens were grouped as normal (N), hyperplasia (H), hyperplasia normal (HN), dysplasia (D), dysplasia normal (DN), carcinoma (C) and carcinoma normal (CN). There were no significant differences in age, sex, alcohol or cigarette consumption between the groups (Table 1). The characteristic morphological appearances of mitosis and apoptosis were easily recognised by high power light microscopy and were confirmed by electron microscopy (Fig. 1). Both apoptotic cells and apoptotic bodies were identified and recorded.

Table 1. *Demographic details of the four subgroups*

		Normal	Hyperplasia	Dysplasia	SCC
Number		10	5	5	12
Subsite	Lateral tongue	5	3	2	1
	Floor of mouth	1	1	0	4
	Oropharynx	4	1	4	7
Smokers		4	2	1	5
Male		8	0	5	8

SCC, squamous cell carcinoma.

Demography

All patients in this study were Caucasian. The sex and age of the patient, cigarette or alcohol consumption, and anatomical subsite were not significantly related to AI, MI, PI or bcl-2 expression.

Indices

MI increased in D and C (N median 1.8, Q1–Q3 0.97–0.76; D 3.2, 2.8–6.4; C 4.7, 1.5–2.7: $P < 0.05$, Figs 2 and 3). AI was highly correlated with MI ($P < 0.001$, Figs 2 and 4), but not correlated with PCNA or bcl-2 expression.

PI was significantly higher in H and HN than all other groups (N mean 0.65, 95% CI 0.32–0.96; H 1.71, 1.39-2.03; HN 1.2, 0.9–1.5: $P < 0.0001$, Fig. 5). On regression, histology was the best predictor of PI, accounting for 17% of all variation.

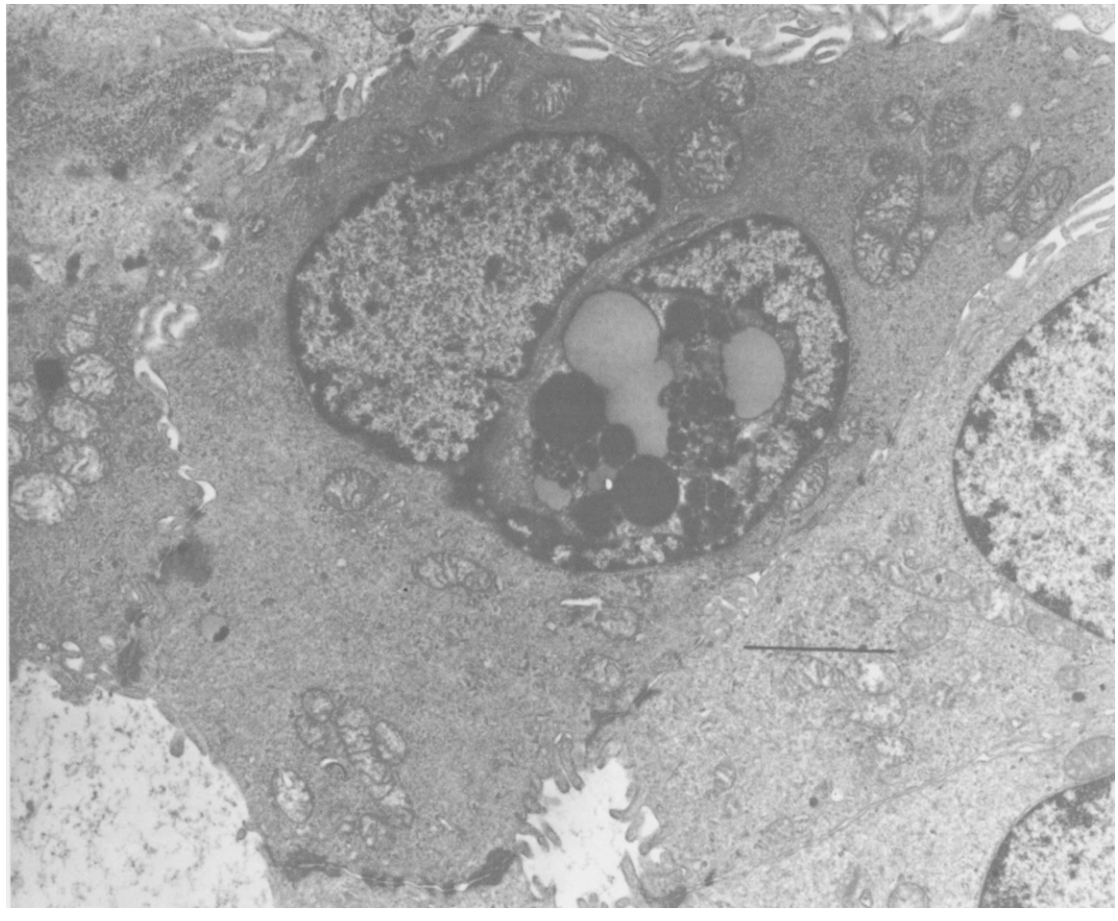

Fig. 1. Peri-nuclear apoptotic bodies in a section of squamous cell carcinoma of tonsil (E.M. × 18,000: bar = 2 μm). Typical preservation of organelles is demonstrated.

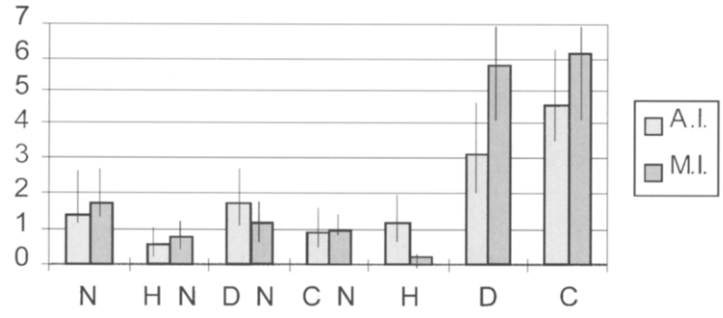

Fig. 2. Apoptotic (AI) and mitotic (MI) indices (% total cells: vertical axis) by histological diagnosis. N = normal, HN = normal tissue from hyperplasia patients, DN = normal tissue from dysplasia patients, CN = normal tissue from carcinoma patients, H = hyperplasia, D = dysplasia, C = squamous cell carcinoma. Means and 95% confidence intervals.

bcl-2 expression was significantly reduced in both C and CN when compared to normal epithelia (pooled normal 30% positive, C/CN 7% positive: $P < 0.001$ χ^2_3, Fig. 6).

Cell position

Peak apoptosis was in the basal layer in all epithelia examined ($P < 0.001$). Peak mitosis was suprabasal in normal (cp 2; $P < 0.01$) and hyperplasia (cp 3; $P < 0.05$), with a broader curve in hyperplasia (Fig. 3). Peak mitoses were basal in dysplasia ($P < 0.001$). Peak PCNA staining was observed in the basal layer for all epithelia, except normal epithelium from patients with squamous cell carcinoma (cp 2). In those cases where bcl-2 expression was observed, stained cells were exclusively in the basal layer, except for 1 case of dysplasia, where some expression in cell positions 2 to 4 was observed.

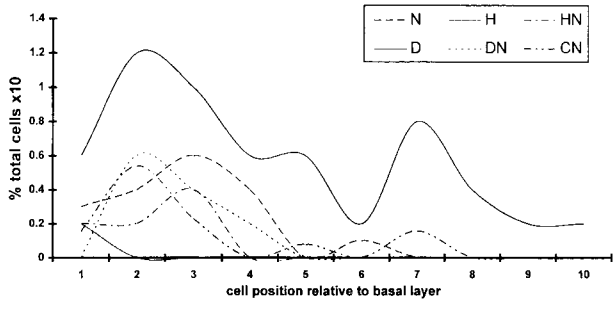

Fig. 3. Percentage of cells at each vertical cell position (counted along a perpendicular from the basement membrane: vertical axis) exhibiting mitosis, in normal epithelium (N), hyperplasia (H) and dysplasia (D). Curves smoothed over three cell positions. HN = normal tissue from hyperplasia patients, DN = normal tissue from dysplasia patients, CN = normal tissue from carcinoma patients.

DISCUSSION

This is the first study to relate cell death, proliferation and repair markers in the oral cavity and oropharynx in humans *in vivo*. It is also the first description, to our knowledge, of bcl-2 expression in the complete sequence from normal to malignant epithelium in the head and neck. Significant differences were detected between the various groups for all these parameters. Numbers in the present study are small, with only five specimens in four of the groups studied (H, HN, D, DN). Thus, although the main findings appear clear-cut, interpretation should be cautious.

Large studies of leukoplakia have shown a heavy preponderance of hyperplasia compared to dysplasia [18]. In the current study, the yield of dysplasia approached 50%. This probably reflects the bias in referral to head and neck surgery departments, in that there tends to be an increased index of suspicion on the part of the referring doctor. Due to the small numbers in this study, grade of dysplasia and degree of differentiation/inflammation in the carcinoma specimens could not be adequately accounted for. This is offset to some degree by the fact that nearly all the carcinomas were moderately differentiated.

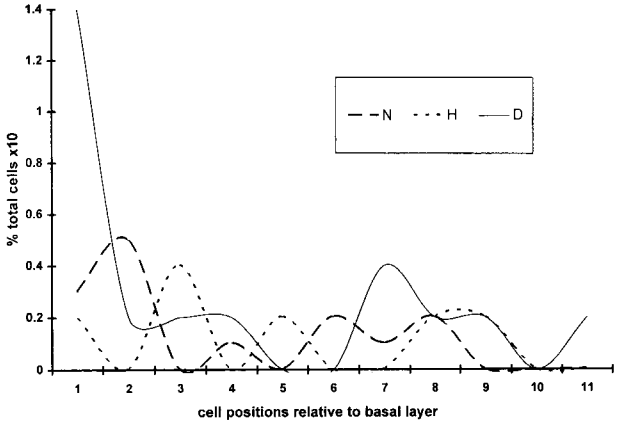

Fig. 4. Percentage of cells at each vertical cell position (counted along a perpendicular from the basement membrane: vertical axis) exhibiting apoptosis in normal epithelium (N), hyperplasia (H) and dysplasia (D). Curves smoothed over three cell positions.

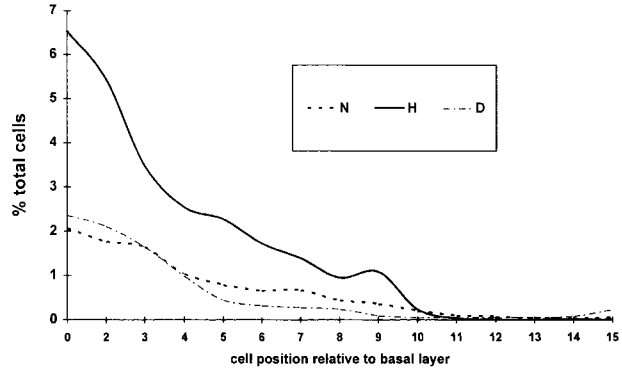

Fig. 5. Percentage of cells at each vertical cell position (counted along a perpendicular from the basement membrane: vertical axis) exhibiting PCNA positivity, in normal epithelium (N), hyperplasia (H) and dysplasia (D). Curves smoothed over three cell positions.

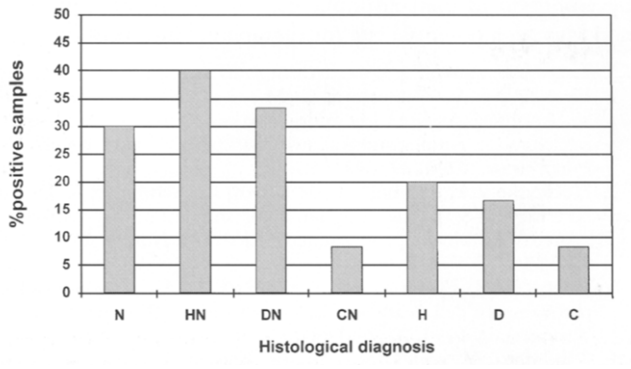

Fig. 6. bcl-2 expression in different histological groups. Expression was significantly reduced ($P < 0.05$) in squamous cell carcinoma and normal epithelium from patients with carcinoma, compared to all other groups. N = normal, HN = normal tissue from hyperplasia patients, DN = normal tissue from dysplasia patients, CN = normal tissue from carcinoma patients, H = hyperplasia, D = dysplasia, C = squamous cell carcinoma.

The thickness of the epithelium, and its malignant potential, is affected by anatomical subsite [19]. In the present study, although pathological groups were well matched, the normal group did not contain any specimens from the floor of mouth or tongue, areas known for high malignant potential. Future studies should include biopsies from these areas in normal individuals. Additionally, it may not be fair to assume that tissue as close as 1 cm from a squamous carcinoma indeed represents normal epithelium from those individuals: 'field change' can often spread this far and further. However, in a prospective study of this kind, it is unlikely that more distant material may be easily harvested without undue morbidity. Despite these limitations, we still feel that the tissue sampled represents a better internal control than immediately adjacent tissue, as studied by other authors [7, 12]. Finally, it may not be reasonable to assume that patients with snoring and sore throats will have entirely normal oral and oropharyngeal mucosa, even in the absence of macroscopic changes.

We have previously used a mixture of prospectively collected and archival material for studies of apoptosis in the human upper aerodigestive tract. However, some retrospective material needed to be rejected due to poor specimen preservation, oblique plane of section or incomplete patient details. The present study was therefore entirely prospective. This also ensured complete consistency of specimen collection, fixation, sectioning and staining. Immunohistological techniques are particularly sensitive to changes in fixation and processing. Indeed, PCNA is the classic example of such variation, which accounts for some of the widely differing counts obtained in ostensibly similar tissues from different laboratories [11, 20]. These errors were minimised in the current study by prospective collection, rigorously standardised, protocols [20].

The significant increase in numbers of mitoses from normal through dysplasia to carcinoma is well documented [1, 17]. The change in peak mitosis from suprabasal to basal in dysplasia of the oral cavity confirms the results of a previous, retrospective study. It is suggested that this is an important kinetic change, possibly representing increased stem cell turnover.

We have previously demonstrated that the prevalence of apoptosis does not change quantitatively between normal, dysplastic and malignant epithelia in the mouth and oropharynx [1]. This finding, confirmed by the present study, is put into context by the apparent topographical changes in relative peaks for apoptosis and mitosis. Our earlier study suggested that peak apoptosis was found in the basal, presumed stem cell [21], layer in normal and mild–moderate dysplastic epithelium. This peak moved superficially (towards the right in the graphs: 'right shift') in severe dysplasia and carcinoma *in situ*, whilst peak mitosis moved to the basal layer (towards the left of the graphs: 'left shift'). Here, the absence of a right shift in peak apoptosis may be explained by the lack of severe dysplasia from the specimens examined, although the significant left shift in mitosis may still act to reduce the potential 'policing' efficiency of the apoptotic process.

Recent reviews have urged caution in the interpretation of a demonstration of PCNA immunoreactivity. PCNA expression may be due to cycling of cells, unscheduled DNA synthesis (excision repair), or induction by the presence of tumour cells or growth factors, such as transforming growth factor alpha [11, 20]. The present data are not unique in showing a dissociation between estimates of mitosis and PCNA-immunoreactivity [22]. However, the significant increase in hyperplasia and adjacent 'normal' tissue is a new finding. A simple interpretation is that there is increased turnover in these epithelia, exaggerated by the fact that, due to its long half-life [22], expression of PCNA can persist well beyond S-phase [20]. It is interesting, however, that the present study failed to demonstrate an increased expression in dysplastic epithelium.

The significant increase in PCNA expression seen in hyperplasia may reflect an increase in DNA excision repair in this tissue. This has been demonstrated after exposure to u.v. light in other systems *in vitro* and *in vivo* [10]. It is possible that this explains the relatively low malignant potential of hyperplasia compared to dysplasia. However, the fact that adjacent, macroscopically normal tissue also exhibited high levels of immunoreactivity to PCNA suggests a more diffuse mucosal phenomenon in these patients, possibly involving induction by raised local levels of growth factors [11]. This would merit further study.

Peak PCNA expression was observed in the basal layers. Above this, there was a much broader distribution of staining than that seen with either apoptosis or mitosis alone. Again, this may simply reflect the long half-life of PCNA, with cells proliferating in deeper layers retaining some protein as they migrate beyond the proliferating compartment. Alternatively, this may mean a more even distribution of the excision repair process than the mitotic process.

There have been few reports of bcl-2 gene expression in oral and oropharyngeal epithelium [23–25], and this is the first to include internal and external control tissue. Although similar cautions apply as with PCNA, meticulous fixation and preparation ensure a maximum of consistency of staining in the present study. As with other gene product immunohistochemistry such as p53, there arise problems with interpretation due to the difficulties in differentiating normal, induced and mutated gene expression. With the bcl-2 protein there is the additional consideration of different dimerisation patterns between members of the bcl-2 gene product family [26].

In all but one (non-malignant) sample, expression of bcl-2 was confined to the basal layer of the epithelium. This is consistent with previous observations for the oral cavity [23, 24], skin [21] and nasopharynx [6]. Lu found expression

uncommon, but consistently basal, in three samples of normal postnasal squamous stratified epithelium, contrasting with columnar epithelium where expression was common, although still basal. It has been suggested that bcl-2 expression may be used as a marker for stem cells in hierarchical epithelia [21]. If this is the case, then the lack of significant changes in staining patterns between the non-malignant epithelia would imply that no change in stem cell numbers is occurring in either hyperplasia or dysplasia.

In the present study, there was a lack of correlation between bcl-2 expression and apoptotic index. Whilst bcl-2 is known to be important in switching cells away from the apoptotic pathway, it is now recognised that it does not operate in isolation, but is likely to differ in effect depending on dimerisation patterns with other members of its burgeoning family, such as Bax and Bcl-cl [25, 26].

A pilot study [7] suggested that a fall in epithelial apoptosis might be crucial to the development of carcinoma in a previously dysplastic lesion. Subsequent larger studies [1], however, showed that apoptosis did not vary significantly with degree of dysplasia or with the onset of squamous cell carcinoma, although important topographical changes occurred. This was confirmed in the present study, despite significant increases in mitotic index in dysplasia and SCC. Thus, we predicted an increase in bcl-2 expression might occur to prevent apoptosis 'keeping pace' with proliferation in dysplastic and malignant lesions. That the reverse occurred was therefore surprising. One possibility is that this represents aberrant expression of the gene, with the production of an unrecognised product. More likely, however, is that bcl-2 expression needs to be interpreted in the context of levels of other components of the apoptosis-controlling soup, particularly products of the other members of the bcl-2 family and p53. In support of this, recent studies have demonstrated reciprocity of bcl-2 and bax expression in squamous cell carcinoma of the oral cavity [25], and of bcl-2 and p53 expression in carcinoma of the colon [5].

An important finding was the lack of bcl-2 expression in macroscopically and histologically normal epithelium taken more than 1 cm away from SCC. This is too remote for direct suppression by tumour products, and we suggest is more likely to represent a constitutive suppression in these patients. Since this group of patients is particularly prone to the development of multiple primary cancers of the upper aerodigestive tract [27], suppression of bcl-2 expression may be an important marker for such metachronous malignancy. This requires confirmation by the prospective evaluation of a larger cohort. If this hypothesis were confirmed, manipulation of the bcl-2 family of genes may represent a possible mode of prophylaxis against metachronous malignancy. In addition, levels of expression may prove a useful intermediate endpoint for preventive studies.

In summary, we have shown that, in squamous epithelia of the head and neck in humans *in vivo*, there are topographical alterations in mitosis relative to apoptosis in dysplasia of the oral cavity/oropharynx. PCNA expression is dissociated from numbers of observed mitoses, suggesting that an alternative process, such as excision repair. This may be an important factor in the relatively low rate of malignant change in hyperplasia relative to dysplasia. bcl-2 gene expression is generally confined to the basal layer in this system, and is suppressed in malignant and related 'normal' epithelium. The latter finding may be an important factor in the development of metachronous carcinomas in these patients, and may be a potential site for therapeutic intervention.

1. Birchall, M. A., Winterford, C. M., Gobé, G. and Harmon, B. V., Apoptosis and mitosis in oral and oropharyngeal epithelium; evidence for a topographical switch in premalignant lesion. *Cell Proliferation*, 1996, **29**, 447–456.
2. Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R. D. and Korsmeyer, S. J., Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature*, 1990, **348**, 334–336.
3. Jacobson, M. D., Burne, J. F. and Raff, M. C., Mechanisms of programmed cell death and Bcl-2 protection. *Biochemical Society Transactions*, 1994, **22**, 600–602.
4. Tsujimoto, Y. and Croce, C., Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. *Proceedings of the National Academy of Sciences USA*, 1986, **83**, 5214–5218.
5. Watson, A. J. M., Merrit, A. J., Jones, L. S., *et al.*, Evidence for reciprocity of bcl-2 and p53 expression in human colorectal adenomas and carcinomas. *British Journal of Cancer*, 1996, **74**, 889–895.
6. Lu, Q. L., Elia, G., Lucas, S. and Thomas, J. A., Bcl-2 proto-oncogene expression in Epstein–Barr associated nasopharyngeal carcinoma. *International Journal of Cancer*, 1993, **53**, 29–35.
7. Birchall, M. A., Winterford, C. M., Allan, D. J. and Harmon, B. V., Apoptosis in normal epithelium, premalignant and malignant lesions of the oropharynx and oral cavity: a preliminary study. *Oral Oncology, European Journal of Cancer*, 1995, **31B**, 380–383.
8. Bravo, R., Frank, R., Blundell, P. A. and McDonald-Bravo, H., Cyclin/PCNA is the auxiliary protein of DNA polymerase-delta. *Nature*, 1987, **326**, 515–520.
9. Hall, P. A., Levison, D. A., Woods, A. L., *et al.*, Proliferating cell nuclear antigen (PCNA) immunolocalisation in paraffin sections: an index of cellular proliferation with evidence of deregulated expression in some neoplasms. *Journal of Pathology*, 1990, **162**, 285–294.
10. Shivji, M. K. K., Kenny, M. K. and Wood, R. D., Proliferating cell nuclear antigen (PCNA) is required for DNA excision repair. *Cell*, 1992, **69**, 657–676.
11. Hall, P. A., Coates, P. J., Goodlad, R. A., Hart, I. R. and Lane, D. P., Proliferating cell nuclear antigen expression in non-cycling cells may be induced by growth factors *in vivo*. *British Journal of Cancer*, 1994, **70**, 244–247.
12. Steinbeck, R. G., Moege, J., Heselmeyer, K. M., *et al.*, DNA content and PCNA immunoreactivity in oral precancerous and cancerous lesions. *Oral Oncology, European Journal of Cancer*, 1994, **29B**, 279–284.
13. Field, J. K., Spandidos, D. A., Malliri, A., *et al.*, Elevated p53 expression correlates with a history of heavy smoking in squamous cell carcinoma of head and neck. *British Journal of Cancer*, 1991, **45**, 450–456.
14. Grasl-Knapp, B., Ruttkay-Nedecky, B., Kovdeska, H., Bukowska, K., Bursch, W. and Schulte-Hermann, R., *In situ* detection of fragmented DNA (TUNEL) assay fails to discriminate among apoptosis, necrosis and autolytic cell death: a cautionary note. *Hepatology*, 1995, **21**, 1465–1468.
15. Altman, D. G., *Practical Statistics for Medical Research*. Chapman & Hall, London, 1991.
16. Li, Y. Q., Fan, C. Y., O'Connor, P. J., Winton, D. J. and Potten, C. S., Target cells for the cytotoxic effects of carcinogens in the murine small bowel. *Carcinogenesis*, 1992, **13**, 361–368.
17. Michaels, L., *ENT Histopathology*. Springer Verlag, London, 1987.
18. Silverman, S., Gorsky, M. and Lozada, F., Oral leukoplakia and malignant transformation. A follow-up study of 257 patients. *Cancer*, 1984, **53**, 563–568.
19. Maran, A. G. D., Nature of head and neck cancer. In *Stell and Maran's Head and Neck Surgery*, eds A. G. D. Maran, M. Gaze and J. A. Wilson. Butterworth–Heinemann, Oxford, 1993.
20. McCormick, D. and Hall, P. A., The complexities of proliferating cell antigen. *Histopathology*, 1992, **21**, 591–594.
21. Polakowska, R. R. and Haake, A. R., Apoptosis: the skin from a new perspective. *Death and Differentiation*, 1994, **1**, 19–31.
22. Scott, R. J., Hall, P. A. and Hladane, J. S., A comparison of immunohistochemical markers of cell proliferating with experi-

mentally determined growth fraction. *Journal of Pathology*, 1991, **87**, 173–178.
23. Ravi, D., Nalinakumari, K. R., Rajaram, R. S., Nair, M. K. and Pillai, M. R., Expression of programmed cell death regulatory p53 and bcl-2 proteins in oral lesions. *Cancer Letters*, 1996, **105**, 139–146.
24. Dawson, C. W., Eliopoulos, A. G., Dawson, J. and Young, L. S., BHRF1, a viral homologue of the Bcl-2 oncogene, disturbs epithelial cell differentiation. *Oncogene*, 1995, **10**, 69–77.
25. Jordan, R. C. K., Catzavelos, G. C., Barrett, A. W. and Speight, P. M., Differential expression of bcl-2 and bax in squamous cell carcinoma of the oral cavity. *Oral Oncology, European Journal of Cancer*, 1996, **32B**, 394–400.
26. Farrow, S. N. and Brown, R., New members of the bcl-2 family and their protein partners. *Current Opinion in Genetics and Development*, 1996, **6**, 45–49.
27. Jones, A. S., Morar, P., Phillips, D. E., Field, J. K., Husband, D. and Helliwell, T. R., Second primary tumours in patients with head and neck squamous cell carcinoma. *Cancer*, 1995, **75**, 1343–1353.

Acknowledgements—We would like to thank Professors John Kerr, Stephen Prime and John Searle for their kind advice, Dr David Allan for inspiration and Drs Rob Hodge, Maurie Stevens, Julie Agnew and Del Hinkley, Consultant Surgeons, Royal Brisbane Hospital, whose patients contributed biopsies to this study. Mr Clay Winterford and Mr Mike Lucketts assisted with the preparation of specimens for electron microscopy. Mr Birchall was supported in part by a Nan Blofeld Fellowship, Institute of Laryngology and Otology, London and a Lionel Colledge Memorial Fellowship, the Royal College of Surgeons of England.