Pergamon

Oral Oncol, Eur J Cancer, Vol. 32B, No. 3, pp. 169–175, 1996

Printed in Great Britain
0964–1955/96 $15.00 + 0.00

0964–1955(95)00086–0

Nuclear Morphometry in Experimental Oral Mucosal Carcinogenesis

A.M. Rich and P.C. Reade

School of Dental Science, Faculty of Medicine, Dentistry and Health Sciences, The University of Melbourne, 711 Elizabeth St., Melbourne 3000, Australia

The area, perimeter and diameter of basal cell nuclei of rat palatal epithelium were measured and the deviation of the basal cell nuclear profile from the form of a sphere was assessed after the application of the carcinogen 4-nitroquinoline-1-oxide (4NQO). After a 24-week treatment-free period, designed to eliminate the irritant effect of the carcinogen, the rats were killed, the palatal mucosa was recovered and processed and the nuclear histometry was assessed with image analysis techniques. The basal cell nuclear area increased as the epithelium became dysplastic and then decreased as carcinoma developed, but there were significant variations in this parameter in the control groups. Basal cell nuclei from moderately or severely dysplastic epithelium, and from epithelium adjacent to areas of invasive carcinoma, were significantly less regular in profile by comparison with control nuclei. Variations in nuclear profile, but not nuclear area, perimeter or diameter, might reflect fundamental nuclear alterations of significance during the process of carcinogenesis. Copyright © 1996 Elsevier Science Ltd

Keywords: nuclear shape, morphometry, oral cancer

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INTRODUCTION

Squamous cell carcinoma (SCC) of the oral mucosa may develop in precancerous lesions, such as leucoplakia and erythroplakia, or it may arise from tissue where there was no pre-existent, clinically apparent abnormality. Currently there is no certain means of determining which putative oral precancerous lesions will undergo malignant transformation. The degree of epithelial dysplasia in such lesions can be determined from microscopic examination of tissue, but this is a subjective assessment and lesions that are dysplastic do not necessarily progress to carcinoma [1, 2]. The inherent problems of subjective assessment can be minimised by using quantifiable features to compare pathological with normal tissue which will lead to a diagnosis based, as much as possible, on objective criteria.

It has been recognised for many years that the size of premalignant and malignant cells may vary from normal. As long ago as 1851, Lebert measured tumour cell nuclear diameter and noted a variation from normal [3]. Since then, many investigators have assessed cell size in various organs affected by a range of diseases. A number of different types and sites of epithelial malignancy have been studied in humans and in animals and varying morphometric techniques have been employed. The results have varied, but most authors report an increase in nuclear size as the disorder progresses from normal, to dysplasia, to neoplasia [4–6].

Cellular and nuclear pleomorphism are parameters in an index of oral epithelial dysplasia [7] and are constant features of oral SCC. These characteristics are usually assessed subjectively with conventional microscopy. Quantitative image analysis techniques provide a rigorous and reproducible means of determining nuclear shape or profile. This is expressed as the nuclear roundness or nuclear form factor and is unitless. Using these methods a distinction has been found between the nuclear form factor of oral mucosal lesions that subsequently underwent malignant transformation and those that did not progress [8, 9]. An association between the nuclear profile of tumour cells and prognosis has been established in prostatic carcinoma [10, 11] and colorectal carcinoma [12].

Since the behaviour of putative precancerous oral lesions in humans is unpredictable, and since temporal studies of carcinogenesis in humans are inappropriate, animal models of carcinogenesis have been developed. Wallenius and Lekholm [13] described an animal model of oral mucosal carcinogenesis whereby the thrice weekly application of 4-nitroquinoline-1-oxide (4NQO) to the palatal mucosa of rats, for approximately 6 months, led to the development of SCC. This model has proven to be reliable in the production of lesions which pass through various stages of dysplasia to frank neoplasia [14–16].

It was the purpose of this study to undertake a temporal

Correspondence to A.M. Rich.
Received 17 July 1995; provisionally accepted 9 Aug. 1995; revised manuscript received 14 Nov. 1995.

histometric assessment of the area (Na), perimeter (Np), maximum diameter (Ndmx) and nuclear profile or form factor (Nf) of rat palatal epithelial basal cell nuclei, with a delay following the application of low doses of 4NQO. In an attempt to avoid some of the difficulties associated with the interpretation of tissue changes after the administration of potent carcinogens, the model as described previously was modified such that relatively few applications of 4NQO were used and the tissues were harvested after a 24-week treatment-free observation period. Any non-specific changes related to the irritant effect of the solution which might have affected the epithelium would have resolved in this treatment-free period [17, 18].

MATERIALS AND METHODS

The project was approved by the Animal Experimentation Ethics Committee of the University of Melbourne. One-hundred and eight outbred male Sprague–Dawley rats, aged approximately 45 days at the start of the experiment, were used in the present study. Carcinogenesis was induced by the thrice weekly application of 4NQO (Sigma Chemical Company, St Louis, Missouri, U.S.A.) at a concentration of 0.5% m/v in propylene glycol (PG) (Ajax Chemicals, Sydney, Australia) following the method of Wallenius and Lekholm [13]. While the animals were lightly anaesthetised by inhalation of halothane vapour (Fluothane, ICI Australia Operations Pty. Ltd., Melbourne, Australia) in air, the carcinogen was applied to the palatal mucosa. Groups of six animals in the 4NQO and PG control groups were painted thrice weekly for six time periods (1, 2, 4, 8, 12 and 16 weeks) and there were 36 unpainted controls (UP). The rats were maintained under routine conditions in the Dental Alumni Research Centre (School of Dental Science, University of Melbourne). They were weighed fortnightly and macroscopic observations and photographs were made at monthly intervals. Twenty-four weeks after the final application all experimental and control animals were killed by an overdose of halothane followed by cervical dislocation.

The palatal mucosa bound by the molar teeth was removed

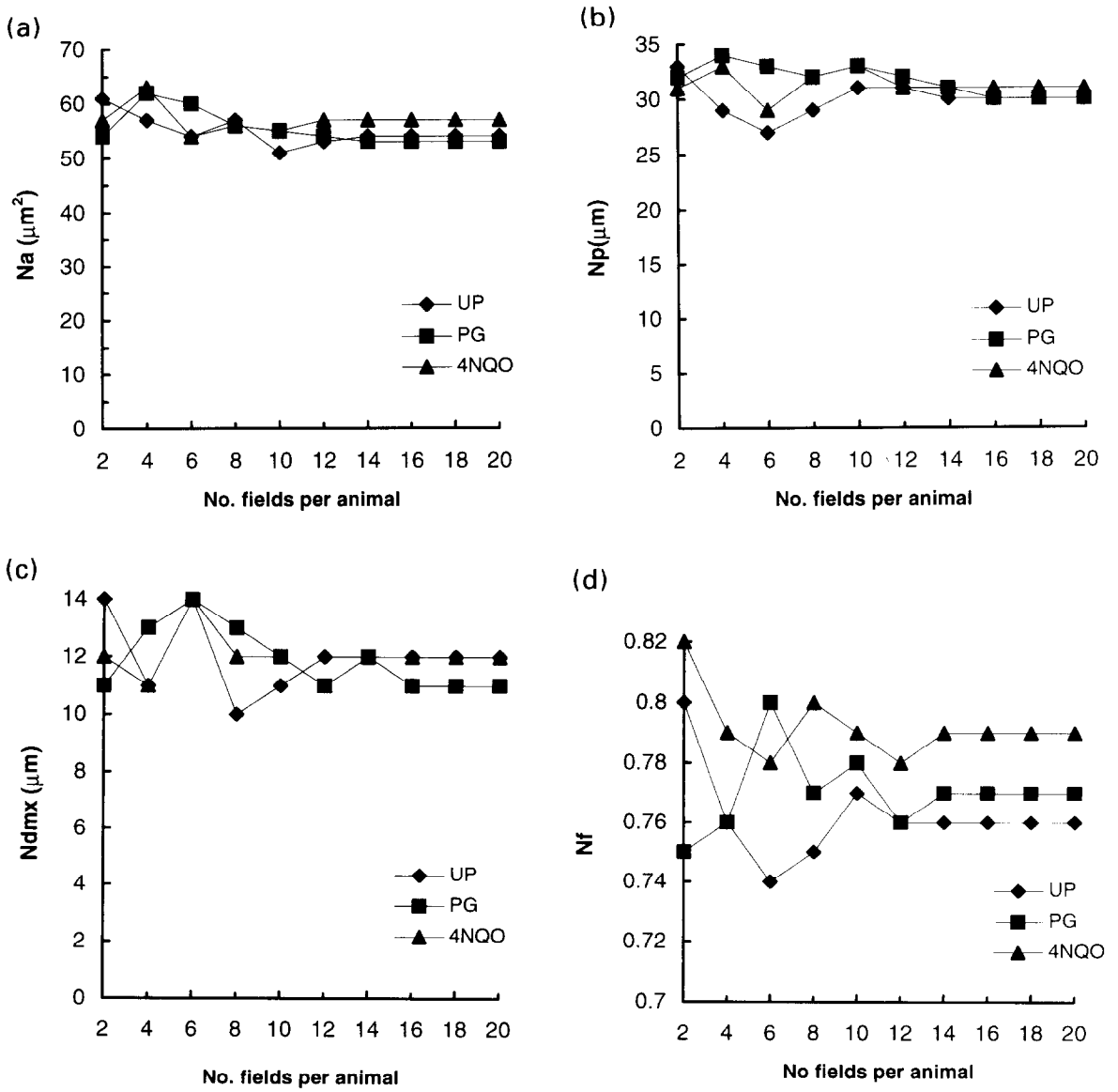

Fig. 1. Accumulative mean plots from pilot calibration study.

Table 1. Histological assessment of dysplasia

Group		No dysplasia	Mild dysplasia	Moderate dysplasia	Severe dysplasia	SCC
1-week	UP	6/6				
	PG	6/6				
	4NQO	6/6				
2-week	UP	6/6				
	PG	6/6				
	4NQO	6/6				
4-week	UP	6/6				
	PG	6/6				
	4NQO		4/6	2/6		
8-week	UP	6/6				
	PG	6/6				
	4NQO			3/6	3/6	
12-week	UP	6/6				
	PG	6/6				
	4NQO					6/6
16-week	UP	6/6				
	PG	6/6				
	4NQO					6/6

UP, unpainted control group; PG, propylene glycol-treated control group; 4NQO, carcinogen-treated group.

surgically, the tissue was placed into Bouin's fixative and, following fixation, the tissue was trisected antero-posteriorly at right angles to the epithelial surface and processed routinely. Sections were cut with a rotary microtome set at 5 μm. Every tenth section was stained with Ehrlich's haematoxylin and eosin to provide three representative sections per animal.

An assessment of the degree of epithelial dysplasia was made using a modification of the criteria of Smith and Pindborg [7] as previously described [16].

A Zeiss MOP 30 Image Analysing System (Carl Zeiss Inc., West Germany) with an Olympus BH microscope (Olympus Optical Co. Ltd., Tokyo, Japan) and a drawing tube (Leitz Wetzler, Germany) was used for all measurements. The basal cell nuclei were outlined with a light projected on to the microscopic field from the light emitting diode in the cursor. The cursor was linked to the digitising tablet and microcomputer of the image analysis system. All sections were examined by one author without prior knowledge of the experimental group from which they came. Features measured were Na, Np, Ndmx and Nf. The form factor was a ratio between the area and the perimeter of the measured object, calculated directly by the image analysis system using the formula: form $= 4\pi a/p^2$ (a = area of nuclear profile, p = perimeter of nuclear profile). With this system the form factor of a perfect circle was one; deviations from the circular profile resulted in a lower number, with very irregular structures having a form factor of close to zero [19]. The purpose of the present study was not to obtain absolute measurements, but to have relative values for comparisons between the groups, and for this reason no correction factors were applied.

Beginning at the anterior aspect of the palate, at the base of the first ruga, a field in every inter-rugal region delineated by an eyepiece grid was assessed until the soft palate was reached; this provided five inter-rugal fields for examination. Eight basal cells at the centre of each field were measured, giving 40 measurements per section and 120 measurements per animal. A 40 × planapo objective (Carl Zeiss Inc., West Germany) was used for these observations. Accumulative means tests from a pilot calibration study using slides from the 2-week UP, PG and 4NQO groups showed that this number of measurements provided stable mean values (Fig. 1). If necessary, for the 12- and 16-week carcinogen-painted groups in which carcinomas had occurred, measurements were made adjacent to areas of invasion.

The Minitab programme (Minitab Inc., Pennsylvania, U.S.A.) was used for statistical analysis. The Kruskal–Wallis (KW) test was used for comparisons of multiple groups of data. If a significant difference was found with this test, pairwise comparisons were undertaken with the Mann–Whitney (MW) test [20, 21]. A value of $P<0.05$ was taken to indicate statistical significance.

RESULTS

Rat weights at the commencement of the experiment ranged from 187 g to 230 g, with a mean weight of 204 g. The mean weight of the 16-week UP group at the time the rats were killed was 445 g, standard deviation (S.D.) 40 g, range 381–494 g. The mean weight of the 16-week PG group was 454 g, S.D. 40 g, range 398–493 g and that of the 4NQO group was 425 g, S.D. 57 g, range 361–502 g. There was no significant weight difference between the three groups at this time (KW: $H=1.1$, $P=0.59$) and no significant differences were found in the groups of rats involved in the experiment for shorter periods.

The results of the assessment of dysplasia are presented in Table 1. In brief, the palatal epithelium of all UP and PG-treated control animals and those painted with 4NQO for 1 and 2 weeks respectively and killed after a further 24 weeks showed no dysplasia. Rats painted with 4NQO for 4 weeks showed mild to moderate dysplasia and those painted for 8 weeks

Table 2. Mean measurements of basal cell nuclear profiles

	UP	PG	4NQO
1-*week*			
Area (μm^2)	$50.4^{b-e} \pm 1.2$*,†	$49.2^{b-e} \pm 1.7$	$51.2^{b-e} \pm 1.4$
Perim. (μm)	28.6 ± 1.1	$29.1^{d} \pm 0.7$	$29.9^{b,c} \pm 1.0$
Max diam. (μm)	11.4 ± 0.6	11.4 ± 0.5	$11.5^{d} \pm 0.7$
Form factor	$0.76^{b-e} \pm 0.01$	$0.76^{b,c,e} \pm 0.01$	$0.78^{b-e} \pm 0.02$
2-*week*			
Area (μm^2)	$51.2^{g-i} \pm 1.6$	$50.5^{g-i} \pm 1.5$	$53.3^{f-i} \pm 1.4$
Perim. (μm)	28.6 ± 1.6	$28.4^{h,i} \pm 0.9$	$28.9^{f-h} \pm 1.0$
Max diam. (μm)	11.6 ± 0.5	11.7 ± 0.4	$11.7^{g} \pm 0.4$
Form factor	$0.78^{f} \pm 0.01$	$0.76^{f,g} \pm 0.02$	$0.78^{f-i} \pm 0.01$
4-*week*			
Area (μm^2)	$52.4^{j-l} \pm 1.2$	$51.6^{j-l} \pm 1.5$	$57.1^{a,j,k} \pm 1.9$
Perim. (μm)	29.7 ± 0.9	29.5 ± 0.8	$32.1^{a,j-l} \pm 1.6$
Max diam. (μm)	11.8 ± 0.4	11.7 ± 0.5	12.0 ± 0.6
Form factor	$0.80^{j} \pm 0.01$	0.79 ± 0.01	$0.80^{j-l} \pm 0.01$
8-*week*			
Area (μm^2)	$57.8^{m} \pm 1.4$	58.6 ± 1.0	$62.3^{a,m,n} \pm 2.2$
Perim. (μm)	29.7 ± 1.1	$28.8^{m,n} \pm 0.7$	$33.0^{a,m,n} \pm 1.2$
Max diam. (μm)	11.8 ± 0.3	11.9 ± 0.5	$12.6^{a,m} \pm 0.4$
Form factor	0.78 ± 0.01	$0.79^{m,n} \pm 0.01$	$0.75^{a} \pm 0.02$
12-*week*			
Area (μm^2)	58.8 ± 1.1	58.4 ± 1.2	59.5 ± 1.6
Perim. (μm)	29.8 ± 0.7	30.1 ± 0.8	30.3 ± 1.0
Max diam. (μm)	11.8 ± 0.4	12.0 ± 0.6	12.1 ± 0.4
Form factor	0.79 ± 0.01	0.78 ± 0.02	$0.74^{a} \pm 0.02$
16-*week*			
Area (μm^2)	58.8 ± 1.2	58.3 ± 1.6	59.3 ± 1.9
Perim. (μm)	29.7 ± 0.9	30.2 ± 0.8	30.2 ± 1.2
Max diam. (μm)	11.8 ± 0.4	12.0 ± 0.8	12.1 ± 0.5
Form factor	0.79 ± 0.01	0.78 ± 0.02	$0.75^{a} \pm 0.02$

*Measurement $\pm$ S.D.

†$P < 0.05$ in all cases for: [a]4NQO versus UP; [b]1 week versus 4 weeks; [c]1 week versus 8 weeks; [d]1 week versus 12 weeks; [e]1 week versus 16 weeks; [f]2 weeks versus 4 weeks; [g]2 weeks versus 8 weeks; [h]2 weeks versus 12 weeks; [i]2 weeks versus 16 weeks; [j]4 weeks versus 8 weeks; [k]4 weeks versus 12 weeks; [l]4 weeks versus 16 weeks; [m]8 weeks versus 12 weeks; [n]8 weeks versus 16 weeks.

moderate to severe dysplasia when analysed 24 weeks after the final carcinogen application. All animals treated with 4NQO for 12 weeks or longer developed well differentiated, keratinising SCC of the palatal mucosa.

The results of the measurements of basal cell nuclear features are shown in Table 2. No significant difference between the control groups and carcinogen painted groups was found in any of the nuclear parameters of rats involved in the experiment for 1 week then killed 24 weeks later. In the animals killed 24 weeks after 2 weeks of 4NQO application, the only significant difference was in the Na, with the Na of the 4NQO group being significantly larger than the PG control group, but not the UP control group. The Na and the Np of the rats painted with carcinogen for 4 weeks and killed 24 weeks later (when the epithelium showed mild to moderate dysplasia) were significantly greater than those of both control groups. No differences in Ndmx or Nf were found. At 8 weeks, Na, Np and Ndmx were significantly greater in the 4NQO group than in the control groups. The Nf of the 4NQO group was significantly less regular than that of both control groups (MW: UP versus 4NQO, $W = 52.5$, $P = 0.03$; PG versus 4NQO, $W = 55.5$, $P = 0.01$). Carcinomas had developed in all rats killed 24 weeks after being painted with carcinogen for 12 weeks and 16 weeks. There was no significant difference between the nuclear features of the epithelium adjacent to the carcinomas in the 12-week and 16-week groups. There was no significant difference in the Na (KW: $H = 3.6$, $P = 0.17$), Np (KW: $H = 1.1$, $P = 0.58$) or Ndmx (KW: $H = 1.4$, $P = 0.49$) in the animals painted with 4NQO for 12 weeks and then killed 24 weeks later (by which time carcinomas had developed) by comparison with the control groups. The Nf of the 4NQO group was significantly less regular than that of both control groups (MW: UP versus 4NQO, $W = 55.0$, $P = 0.01$; PG versus 4NQO, $W = 54.0$, $P = 0.02$). Similar results were found in the 16-week group.

There was a significant difference in Na of the UP control group in relation to time (KW: $H = 23.3$, $P = 0.0$), with an increase in Na with increasing time (Table 2, Fig. 2a). The Na of the PG groups and 4NQO groups also increased with time until 8 weeks. There was a significant decrease in Na in the rats killed 24 weeks after the application of 4NQO for 15 weeks, by comparison with 8 weeks of painting (Table 2, Fig. 2a).

No variation with time was found in the Np of the UP group (KW: $H = 6.7$, $P = 0.15$), (Table 2, Fig. 2b). There was no

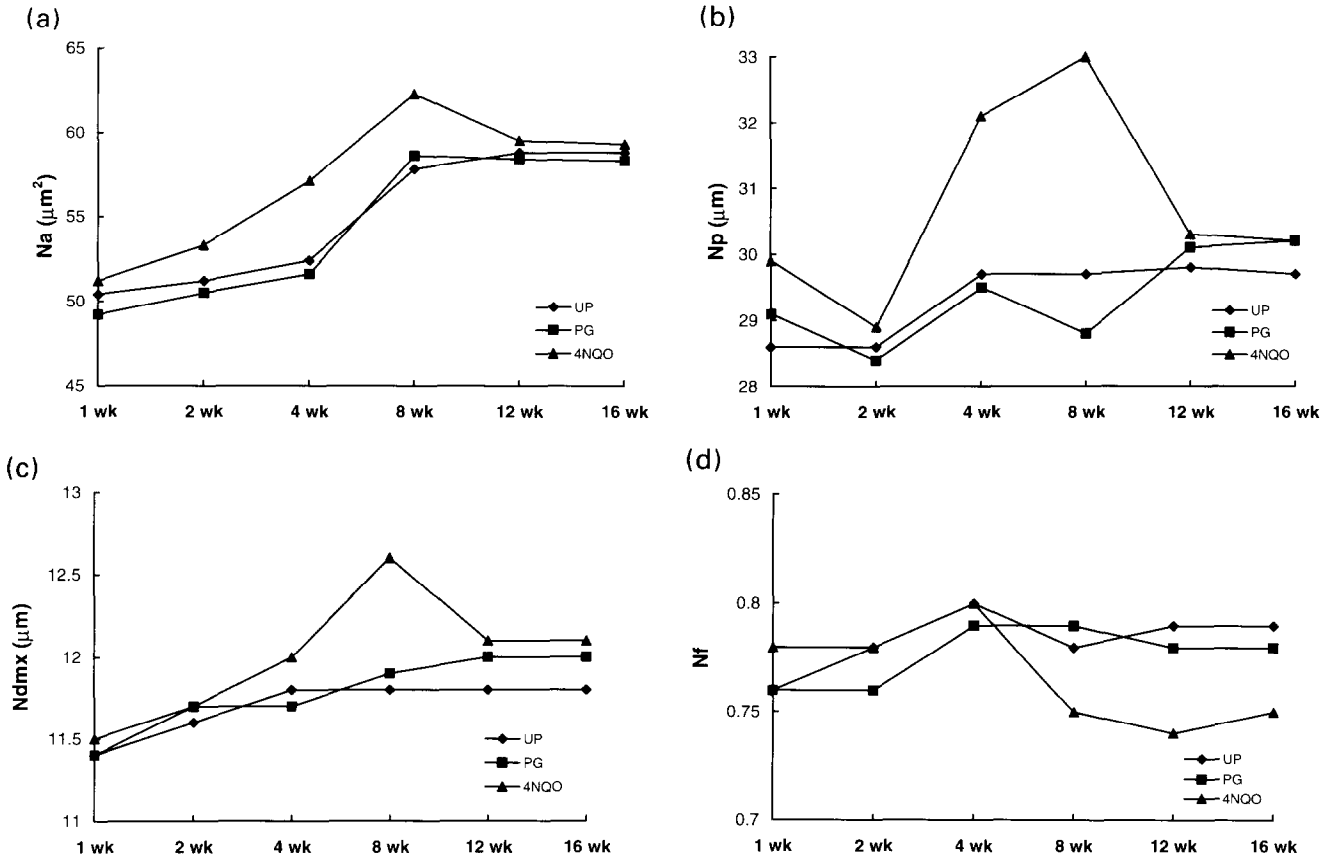

Fig. 2. Nuclear morphometry with time.

consistent pattern to the variations detected in the PG groups, but the Np of the 12-week PG group was significantly greater than the 1-week group. The Np of the 4NQO group increased to 8 weeks, then decreased.

Nuclear profile diameter did not alter with time in either the UP or PG control group. In the 4NQO groups there were significant differences, with an increase in Ndmx to 8 weeks, then a decrease at 12 weeks and 16 weeks (Table 2, Fig. 2c).

There was a significant variation in Nf with time in the UP group; the Nf was greater, i.e. the nucleus more closely resembled a sphere, in the rats killed 24 weeks after being involved in the experiment for 12 weeks and 16 weeks compared to those involved for 1 week (MW: UP, 1 week versus 12 weeks and 1 week versus 16 weeks, $W=23.0$, $P=0.01$) (Table 2, Fig. 2d). In the 4NQO treated rats there was a significant increase in nuclear roundness in the 4-week group by comparison with the 1- and 2-week groups, but the Nf of the 8-, 12- and 16-week groups was significantly reduced by comparison with the 1- and 2-week groups (Table 2, Fig. 2d).

DISCUSSION

Histometric techniques have been shown to produce accurate, reproducible results [22, 23]. The main source of error is usually in the inability of the operator to delineate the object to be measured; this is best overcome by appropriate magnification [22].

Nuclear features of cells in the basal compartment, but not the maturation compartment, were assessed. The basal cell population of the oral mucosa is heterogeneous and includes stem cells and amplifying or proliferating cells [24]. Stem cells are slowly cycling cells that divide to produce daughter stem cells as well as cells committed to differentiate. It is likely that stem cells are the target for agents that cause alterations in epithelial cell differentiation and changes in these cells may alter future cell behaviour, including the development of neoplasia [25]. As carcinogenesis progresses, basal cell nuclei are likely to be the first to show morphological alterations. For these reasons, only basal cell nuclei were measured in the present study.

There have been a number of studies assessing cell/nuclear size in human oral mucosal precancer and cancer [8, 9, 26, 27]. These studies have used tissue from patients of varying age, from various oral mucosal sites and other factors that may affect nuclear size, such as tobacco smoking, iron, folate and vitamin B_{12} status, have not always been taken into account [28–32].

Animal studies have the advantage that conditions can be carefully controlled. Using a model of experimental oral mucosal carcinogenesis it has been shown previously that results pertaining to the nuclear size of basal cells were variable and could not always be attributed to the change from normal to neoplastic disease [33]. The use of animal models of this sort has disadvantages as pointed out by Johnson [34], in that some of the changes detected may not be specific for carcinogenesis. The use of animals with relatively few applications of 4NQO and with the tissue harvested after a 24-week treatment-free observation period was intended to address this difficulty. Any non-specific changes related to the irritant effect of the

solution which might have affected cell size and profile would have resolved in this treatment-free period [17, 18]. In the present study, there was an indication that basal cell nuclear area became larger, by comparison with control nuclei, as the epithelium became dysplastic, then as the carcinoma developed the nuclear area decreased, as has been previously reported in colon pathology [6]. It should be noted, however, that the unpainted control animals showed significant variation in nuclear area in relation to length of time in the experiment, thus the results of studies that have not used age-matched controls should be interpreted with care. The results relating to Np and, to a lesser extent Ndmx, mirrored those of Na and did not provide useful, independent means of distinguishing between the groups.

A significant variation in nuclear profile has been found between oral mucosal lesions that eventually underwent malignant transformation by comparison with lesions that did not transform [8, 9], but the factors mentioned above relating to site, tobacco smoking and nutritional status were not controlled. The nuclei of floor of mouth SCC cells were significantly less round than normal cells, but the Nf could not be used to distinguish patients with or without cervical metastases [35]. Bryne *et al.* [36] found that semi-quantitative grading of nuclear pleomorphism from 76 cases of buccal mucosal SCC correlated with prognosis. An association between the nuclear profile of tumour cells and prognosis has been established for prostatic carcinoma [10, 11] and for colorectal carcinoma [12]. The results in relation to breast cancer have been variable [37, 38].

In the present study, the basal cell nuclei of rats with moderate or severe dysplasia (8-week group) and those with carcinoma (12- and 16-week groups) were more irregular in form by comparison with controls. Thus, in this animal model, nuclear profile appeared to vary between lesions which were clearly dysplastic or cancerous and those that were not, but these changes were not apparent in the early phases of carcinogenesis. These findings are of interest in relation to molecular aspects of carcinogenesis. Adhesion receptors such as integrins have been shown to be involved in determining cell profile; integrin density has been found to be altered in transformed cells [39, 40]. Alteration in cell profile reflected alteration to the cytoskeleton; this, in turn, might affect gene expression [41]. Boyd *et al.* [42] suggested that variation in the profile of tumour cells might be a reflection of "structural alterations in the nuclear architecture and skeleton (nuclear matrix)". The nuclear matrix is a nuclear component that is thought to regulate nuclear morphology and play a role in DNA organisation and repair [43]. Non-tumorigenic cell lines have been established which, after hybridisation with a tumour cell line, either retained or lost the ability to suppress the tumorigenic phenotype [42]. They found that the clone that lost the ability to suppress the tumorigenic phenotype had nuclei with a greater variation from roundness than those that retained their suppressor activity. Thus, alteration in nuclear profile appeared to be related to a fundamental nuclear alteration, i.e. loss of tumour suppressor gene function, in that experimental system.

The long-recognised variation in the nuclear profile of cancer cells was confirmed in the rat palatal model of oral mucosal carcinogenesis used in the present study. The diagnosis of human oral cancer is relatively straightforward and assessment of the nuclear profile is not likely to be of any practical value. It would be of considerable interest, however, to extend this work to determine whether there is a correlation between the nuclear profile and the prognosis of human oral mucosal SCC and whether suppressor gene activity can be related to nuclear profile.

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