Quantification of Cell Cycle Markers in Oral Precancer

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To

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To my parents and Lucie

Contents

List of Tables

Tables and figures are grouped at the end of each chapter in the sequence they are referred to in the text except for Chapter 3.

List of Figures

Abbreviations

AgNOR	silver stained nucleolar organiser region
bcl-2	tumour suppressor gene
Bcl-2	protein encoded for by the bcl-2 gene
BL	basement membrane length
BrdU	bromodeoxyuridine
CCND1	cyclin D1 gene
CDK	cyclin dependent kinase
CDKI	cyclin dependent kinase inhibitor
DAB	3,3'diaminobenzidine
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
EGF	epidermal growth factor
G_0	resting phase
G_1	presynthesis gap phase
G_2	post synthesis gap phase
GF	growth fraction
H&E	haematoxylin and eosin
HCl	hydrochloric acid
HPV	human papilloma virus
KS300	software used for quantification
Li%	labelling index per 100 total nucleated cells
LOH	loss of heterozygosity
MC	maturation compartment
MEM	minimal essential medium
MIB1	clone of Ki67 antibody
mRNA	messenger RNA
PAS	periodic acid schiff
PC	progenitor compartment
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
pRb	retinoblastoma protein
RNA	ribonucleic acid
S	DNA synthesis phase of the cell cycle
SB	suprabasal
SD	standard deviation
TBS	tris buffered saline
T_c	cell cycle duration

Glossary

atypia	term used to describe precancerous abnormalities of individual cells within an epithelium in this thesis often used synonymously with dysplasia
basal cells	those cells in an epithelium which are in contact with the basement membrane
bromodeoxyuridine	thymidine analogue which can be incorporated in the dna during synthesis
cyclin	cellular protein which forms complexes with cyclin dependent kinases to ensure correct passage through the cell cycle
dysplasia	the appearance of an epithelium which shows precancerous changes but no evidence of invasion
growth fraction	expression used to denote all of the cells within a population that are within the cell cycle
Ki67	cell cycle associated protein said to be expressed by all of those cells within the cell cycle

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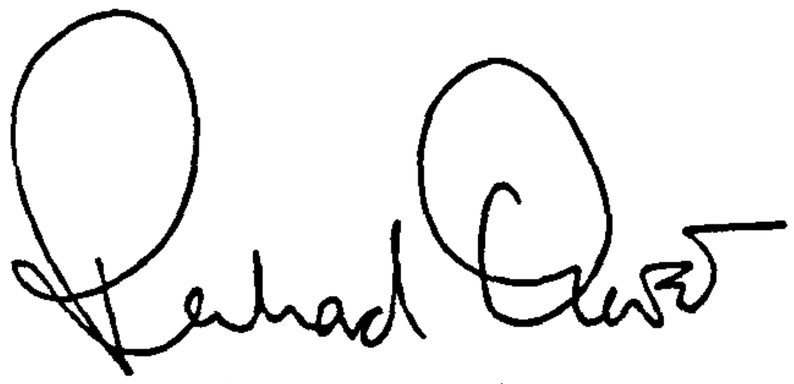
Declaration

This is the original work of the author



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Preface

The work of this thesis was undertaken while the author was working for Glasgow University as a Clinical Research Assistant. The research was undertaken in the Department of Oral Sciences at Glasgow Dental Hospital and School, Glasgow from October 1995 to April 1998.

The experimental work, with the exception of some of the histological staining, was carried out solely by the author. Some of the techniques are modifications of previously published work as indicated in the text. Some techniques and manipulations are the original ideas of the author.

Much of the work of this thesis has been presented by the author at the following scientific meetings. Part of the work from Chapter 2 was presented at the British Society for Dental Research annual conference in Bristol in April 1996. Further work from Chapter 2 was Presented at the annual meeting of the British Association of Head and Neck Oncologists in London in April 1996. The compartment analysis of Chapter 2 was presented at the annual meeting of the British Society of Oral Pathology in Manchester in September 1996. The work on post-mortem tongue mucosae was presented at the annual conference of the British Society for Dental Research in Brighton in April 1997. The work from Chapter 5 has been accepted for presentation, by the author, at the International Association of Dental Research to be held in Nice, France in June 1998 and the work of Chapter 6 has been accepted for presentation, by

Professor MacDonald, at the International Association Oral Pathology to be held in Cape Town, South Africa in August 1998.

Summary

The overall aims of the studies were to obtain objective measures of oral precancerous lesions based upon studies of the cell cycle and to investigate these parameters as possible prognostic indicators with regard to malignant transformation of these lesions. The majority of the precancerous lesions in the present study were dysplastic. These are the lesions which cause the greatest concern clinically with regard to malignant transformation.

The first part of the study investigated the S-phase, growth fraction and the relationship of these to each other and with the degree of dysplasia with the aim of achieving objective measurements of the dysplasia and prognostic information. This was achieved by the use of BrdU for labelling of cells in the S-phase and anti-Ki67 antibody as a marker of cells in the growth fraction. The BrdU labelling index was demonstrated to provide an objective assessment of the dysplastic lesions when compared to the semi-objective method of Smith and Pindborg (1969). The ratio of the S-phase to the growth fraction was higher in those lesions which progressed to malignancy and was cited as a possible prognostic indicator.

A number of methodological problems were identified from this first part of the study and these were investigated further by the development of techniques in Chapter 3. Firstly, a method was developed to enable the BrdU labelled tissues to be formalin fixed and then allow other cell

cycle associated markers to be studied on sequential sections of the same tissue block. Secondly, numerous antigen retrieval techniques were carried out in order to optimise the immunohistochemical staining of Ki67 and subsequently other antibodies utilised in later parts of the study.

Normal oral epithelium, derived post-mortem, was studied in Chapter 4 to investigate the apparent underestimation of proliferating cells identified by anti-Ki67 antibody in Chapter 2. It was apparent that, as in dysplastic epithelia, Ki67 significantly underestimated progenitor cells of the morphologically identified progenitor compartment. Ki67 did not identify all of those cells which would have been expected to be in the cell cycle as it was originally claimed to do. Also, this work demonstrated that post-mortem tissue was a useful source of control tissue in proliferation studies.

With the use of antibodies against the G_1 cyclins, cyclins D1, D3 and E, the studies of Chapter 5 investigated the possible lack of expression of Ki67 in the G_1 phase of the cell cycle. The results demonstrated that the expression of the D cyclins was high in oral epithelial dysplasia and was correlated with Ki67. The reasons for this were less clear. It may represent a true overexpression of the protein in these lesions or more likely a continued expression of the protein throughout other phases of the cell cycle in addition to G_1. The distribution of cyclin E was unexpected and largely appeared in cells that would be classified as belonging to the maturation compartment. There

was some association of the cyclin labelling indices to the degree of dysplasia but this was not as marked as the association of BrdU previously demonstrated.

Finally a further study of the relationship of the S-phase with the growth fraction was performed in Chapter 6. In addition to utilising the techniques developed in the methods chapter of formalin fixation of the BrdU labelled tissue, the S-phase associated cyclin A was investigated as a possible alternative to BrdU. A correlation of cyclin A with BrdU was demonstrated. However, the cyclin A labelling indices were much higher in some more severely dysplastic lesions suggesting this protein may be overexpressed as part of the carcinogenic process. The use of this marker for cells in the S-phase is probably only appropriate in normal or minimally dysplastic tissues.

Overall, although BrdU requires prospective collection of tissues, it consistently provided unequivocal positivity when detected immunohistochemically compared with some of the other antibodies studied. The relationship of the S-phase to the growth fraction appears to be a promising parameter to assess the prognosis of oral epithelial dysplasia. However, due to the often protracted natural history of such lesions, much longer term studies would be required to assess the validity of this. Post-mortem tissue appears useful as a source of truly normal epithelium. The G_1 cyclins have not contributed greatly to the present studies of oral epithelial dysplasia and until their precise

functions within the cell have been established their usefulness in proliferation studies of this kind is severely limited. Cyclin A, with its recognised limitations, appears to be a viable alternative to BrdU in the study of the S-phase.

Chapter 1

Introduction and Review of the Literature

1.1 Oral Cancer

1.1.1 Incidence and epidemiology of oral cancer

Oral cancer is estimated to be the sixth most prevalent cancer on a world-wide scale (Parkin *et al.*, 1988). There are, however, significant geographical and regional variations in incidence with oral cancer being prevalent in parts of Asia, but only accounting for about 2% of malignancies in Europe including the UK (Johnson, 1991). In the literature and in this thesis oral cancer is taken to refer to squamous cell carcinoma arising from the oral mucosa as this is by far the most common oral malignancy accounting for the majority of cases; carcinomas and sarcomas arising from salivary glands, bone and other soft tissues account for a relatively small number and have a different aetiology to oral squamous cell carcinoma.

In the UK, the majority of cases of oral cancer occur in older adults and more males than females are affected (Johnson, 1991). The incidence of oral cancer in England and Wales was decreasing in the first half of this century but there is evidence that the incidence is beginning to rise again (Hindle *et al.*, 1996; La Vecchia *et al.*, 1997; Macfarlane *et al.*, 1992). While there has been a decline in incidence and mortality in older age groups, there is an increasing incidence and mortality in younger males with evidence for clear birth cohort differences (particularly those born after about 1920) and implications for expected frequency in the future (Hindle *et al.*, 1996). This trend is also observed in Scotland

(Macfarlane *et al.*, 1992). The age-standardised rates for oral cancer showed the UK incidence in males to be 5.7 per 100 000 and 2.5 per 100 000 in females with mortality rates of 2.9 and 1.1 per 100 000 for males and females respectively in 1990 (Black *et al.*, 1997). A recent study based in the North-East of England suggested a possible link between oral cancer incidence and social deprivation (O'Hanlon *et al.*, 1997).

Intraoral cancer most commonly affects the lateral border of the tongue and the floor of the mouth with much lower incidences reported for the buccal mucosa, mandibular alveolus and soft-palate complex (Johnson, 1991). Cancer of the lip traditionally had a higher incidence in those manual workers who had worked for long periods outside but with fewer people employed in such tasks the incidence is declining.

1.1.2 Aetiology

There is overwhelming and consistent evidence linking tobacco to the aetiology of oral cancer. In developed countries tobacco is smoked predominantly in the form of cigarettes whilst in Asia (and, indeed, among Asian populations now residing in the West) a wide variety of methods are practised many involving combining tobacco with other substances which are often chewed or held for long periods in the mouth. An example of this is the betel quid or pan comprising tobacco mixed with areca nut and slaked lime and wrapped in betel leaf. This widespread practice accounts for the increased incidence and different sites involved (the buccal mucosa and retromolar areas) among these people (Johnson, 1991).

Alcohol is also strongly linked to the aetiology of oral cancer and appears to act synergistically with tobacco (Brugere *et al.*, 1986). Alcohol therefore probably contributes to many of the cases in the developed world where it is widely available.

The role of viruses in the aetiology of oral cancer is less clear. Despite being strongly implicated in some human carcinomas including the uterine cervix and nasopharynx there is little evidence for the role of viruses in oral carcinoma. Applications of recently developed molecular techniques such as *in situ* hybridisation and the polymerase chain reaction (PCR) have demonstrated the presence of human papilloma virus (HPV) DNA in oral cancer cells. This may be an incidental finding of a relatively ubiquitous virus or an effect rather than a cause as it is also found in a significant proportion of normal oral mucosae and in benign conditions (Miller and White, 1996).

The atrophic epithelium associated with nutritional deficiencies, particularly iron, has been shown to have an association with the development of carcinoma.

1.1.3 Treatment

The treatment of oral cancer is based on surgical resection and radiotherapy either alone or in combination for both the primary tumour and regional lymph node metastases (Brown and Langdon, 1995). Chemotherapy, although still practised by some, has not been proven as

effective as the other treatment modalities with no significant influence on survival (Stell and Rawson, 1990). The overall five year survival for oral cancer is in the region of 40% and despite advances in treatment this figure has not improved significantly over the last 20 years (Johnson, 1991). Failure of treatment often results in recurrence of the tumour at the primary site or development of metastases in the cervical lymph nodes, although, as treatment of the primary and regional sites improves there are increasing problems with distant metastases (Brown and Langdon, 1995). Similarly, the incidence of second primary tumours is increasing in the oral cavity, pharynx or larynx (Ogden, 1991). Such occurrences are often attributed to so called field cancerisation (Ogden, 1996; Slaughter *et al.*, 1953) or field change (Ogden, 1998) based on the theory that widespread exposure of the mucosa of the upper aero-digestive tract to carcinogenic substances may induce similar changes to cells in more than one site and, therefore, account for the occurrence of tumours at different sites.

1.1.4 Early oral cancer

The term "early oral cancer" has been applied to those lesions which are small and asymptomatic, located in the soft palate complex or floor of mouth and manifest most commonly as predominantly red lesions (Mashberg and Samit, 1995). These lesions are said to differ from more advanced cases in that they are not ulcerated or indurated and often resemble precancerous lesions (see below) although histological

examination reveals early invasion.

Early diagnosis of oral cancer should offer the best prognosis for the patient in terms of ease of removal with less ablative surgery, therefore less post-operative morbidity, and long-term survival. However, data to support this assumption are scarce and as Johnson *et al* (1995) point out, a small lesion may be such because it is slow growing with an inherently good prognosis. "Early" detection of a small lesion which is aggressive in terms of its histology and speed of growth may not confer any significant prognostic advantage.

Screening for oral cancer has received much attention recently but, as yet, there is no consensus of opinion to support such a measure in the UK despite the oral cavity being one of the most accessible sites to screen (Franceschi *et al.*, 1997; Speight *et al.*, 1992). There may be merit in some form of screening for those deemed to be in high risk groups on the basis of tobacco and alcohol habits but individuals of such groups are often those with low compliance with screening schemes (Jullien *et al.*, 1995).

1.2 Precancerous Lesions

1.2.1 Definitions

A precancerous (premalignant) lesion as defined recently by an international working group (Axéll *et al.*, 1996) is "a morphologically altered tissue in which cancer is more likely to occur than in its apparently

normal counterpart". This was updated from a previous definition from the same group (Kramer *et al.*, 1978) to attempt to overcome some of the problems identified with the previous definitions. Examples of such lesions are oral leukoplakia and erythroplakia. To clarify the terminology, a precancerous (premalignant) condition is a generalised state associated with a significantly increased risk of cancer, an example being oral submucous fibrosis (Axéll *et al.*, 1996). Morphological alterations referred to in this context are changes in clinical appearance and not histopathologically (MacDonald and Saka, 1991).

1.2.2 Leukoplakia

Oral leukoplakia is a predominantly white lesion of the oral mucosa that cannot be characterised as any other definable lesion (Axéll *et al.*, 1996). It is a purely clinical diagnosis and is based upon identifying and eliminating any possible aetiologic factors such as trauma or chemical agents (other than tobacco) and bears no relation to the histopathological features. Once the diagnosis of leukoplakia is established, histopathological examination is usually performed which serves two important purposes, namely to exclude other mucosal disease and determine the presence and degree of dysplasia (van der Waal *et al.*, 1997). A proportion of these lesions will transform into carcinoma. In the literature of the past there has often been erroneous use of leukoplakia in a histopathological sense which can lead to some confusion in interpreting results.

Three clinical variants of white and red lesions are proposed (Axéll *et al.*, 1996): homogenous leukoplakia which is a predominantly white lesion of uniform flat, thin appearance that may exhibit shallow cracks and has a smooth, wrinkled or corrugated surface with a consistent texture throughout. Non-homogeneous leukoplakia is a predominantly white or white and red lesion that may be irregularly flat, nodular or exophytic. A lesion showing white and red areas is referred to as erythroleukoplakia. Erythroplakia is analogous to leukoplakia in that it is a red patch that cannot be diagnosed as any other definable lesion.

1.2.3 Aetiology

As with oral cancer, tobacco and alcohol are strongly associated with leukoplakia. The relative importance of the two agents is difficult to assess since most patients with leukoplakia tend to indulge in both habits. If trauma or other agents are found to be responsible for oral white lesions (usually by exclusion) then the diagnosis of leukoplakia must not be applied. For example, frictional keratosis should be applied if friction is causative agent. Idiopathic leukoplakia has been proposed for those lesions which are not associated with tobacco use or other aetiology. However under the definition proposed these lesions should be included as leukoplakia along with the tobacco associated lesions.

1.2.4 Other white lesions

The term proliferative verrucous leukoplakia was coined by Hansen *et al* (1985) to denote a characteristic form of leukoplakia which is a

progressively expanding exophytic lesion which has a high risk of malignant transformation. The same group recently reported a follow-up study of 54 patients with this lesion (Silverman and Gorsky, 1997). They reported the lesion to be more common in women than men with no clear association to tobacco use and over 70% transformation to carcinoma over a period of nearly eight years.

Hairy leukoplakia is a bilateral white lesion usually found on the tongue, sometimes the buccal mucosa, in immunocompromised patients and is known to be caused by Epstein-Barr virus. It is not, however, a precancerous lesion despite the use of the term leukoplakia and the alternative term "Greenspan lesion" has been proposed (van der Waal, 1996).

Lichen planus and related lesions (lichenoid reactions, drug eruptions) often present as white patches, typically of the buccal mucosa and lateral borders of tongue. Controversy exists regarding the premalignant potential of these lesions which may in part be due to problems of interpreting the significance of dysplasia in some of these lesions. The term lichenoid dysplasia has been applied to those lesions exhibiting dysplasia which have some of the histological features of lichen planus (Eisenberg and Krutchkoff, 1992). Recently, Zhang *et al* (1997) using loss of heterozygosity (LOH) studies of chromosomes frequently involved in oral cancer, reported that LOH was not prevalent in lichen planus compared to epithelial dysplasia. They concluded that if malignant

change occurred in lichen planus it was due to different genetic changes than in dysplastic lesions.

1.2.5 Transformation to oral cancer

As stated in the definition of leukoplakia, a proportion of these lesions will transform into carcinoma. However the number of such lesions and in particular, which lesions will become malignant is the basis of much research and discussion and indeed one of the aims of this thesis. Also, opinions vary as to how many oral cancers are preceded by premalignant lesions, but it is thought that the majority of oral carcinomas arise from apparently clinically normal mucosa. Bouquot *et al* (1988) reported that only 36% of intraoral cancers were associated with leukoplakia while nearly twice as many lip cancers were. There is however, thought to be a marked geographic variation with the majority of lesions in the high risk counties of the Eastern subcontinent being preceded by precancerous lesions (MacDonald and Saka, 1991).

A proportion of the clinical lesions of leukoplakia demonstrate histopathological signs of epithelial dysplasia, however, the number of lesions doing so appears to be relatively few (Silverman *et al.*, 1984). The study of Silverman *et al* (1984) is, to date, one of the largest and most comprehensive studies of the behaviour of oral leukoplakia in developed counties and showed that only 22 out of 257 cases (8.5%) demonstrated epithelial dysplasia. Of this large group 17.5% developed carcinoma, but 36% of those exhibiting dysplasia transformed into squamous carcinoma.

Lummerman *et al* (1996) recently produced a retrospective study and found that 16%, 7 of 44 cases of epithelial dysplasia, developed invasive carcinoma and reviewing the literature of similar studies found an average transformation rate of 13.8%. This and other studies are summarised in Table 1.1. It should be noted that the aetiology, treatment and follow-up regimes for these lesions varied considerably and must influence the figures of such studies somewhat. The time scale over which this transformation occurs appears to vary considerably with periods of up to 20 years being recorded (Bánóczy, 1977). A further complication in interpreting these figures is introduced when consideration is given to the fact that the assessment of epithelial dysplasia is subjective, varies considerably between pathologists (Abbey *et al.*, 1995; Pindborg *et al.*, 1985) and is particularly difficult in atrophic epithelia (Pindborg *et al.*, 1985).

1.2.6 Treatment

Biopsy is often the first line of action in the management of leukoplakia. This should be regarded as mandatory for all lesions which clinically fit into the category of leukoplakia (i.e. cannot be categorised as any other definable lesion (Axéll *et al.*, 1996)). The histology report should always include an observation of the presence or absence of dysplasia and its severity (Axéll *et al.*, 1996). It is well known, however, that a biopsy may not be representative of the entire lesion and this raises the question of appropriate sampling of such lesions. Lummerman *et al* (1996)

observed skip areas where normal epithelium intervened between dysplastic epithelium and that a range of severity of dysplasia could be seen within various portions of the same specimen. Clinical experience and the knowledge of the natural history of such lesions indicates that lesions appearing as erythroplakia or erythroleukoplakia (speckled leukoplakia) have a higher incidence of dysplasia and malignant transformation (Speight and Morgan, 1993) and would, therefore, be the areas of choice for biopsy.

An old idea regaining popularity among some surgeons is the use of toluidine blue dye to aid in the selection of more dysplastic areas (Tradati *et al.*, 1997). A recent study concluded it was highly sensitive for detecting carcinomas but there was an alarming rate of false positives for detection of dysplasia (Martin *et al.*, 1998). This method may have some place in aiding the diagnosis in high-risk groups (Warnakulasuriya and Johnson, 1996) but it is not widely advocated for general use.

The definitive management of oral leukoplakia still has no standard protocol and variation has been demonstrated regarding the treatment of these lesions at different centres in the UK (Marley *et al.*, 1996). There is usually little doubt that those lesions showing more severe degrees of dysplasia histologically require active treatment. However, it is those lesions where dysplasia is minimal or absent where there are more varying practices.

Surgical excision or laser excision are the more popular modalities

of treatment and probably still the most widely used modalities (van der Waal *et al.*, 1997). A problem with the use of the latter is the inability to assess the margins histologically.

Vitamin A and its derivatives (retinoids) appear to have at least some protective effect regarding the development of cancer and are used in the treatment of premalignant lesions in their capacity as antioxidants (Kaugars *et al.*, 1996). McGregor *et al* (1997) have recently demonstrated loss of retinoic acid receptors in oral dysplastic lesions was accompanied by the development of an immortal phenotype associated with malignancy.

A relatively new approach to the treatment of leukoplakia is that of photodynamic therapy in which photosensitising drugs (porphyrin derivatives) are administered systemically and are then activated by the use of light and the presence of oxygen (Nauta *et al.*, 1996). There are as yet no long-term studies to prove the effectiveness of this treatment.

1.2.7 Histopathology

Histopathologically, epithelial dysplasia is characterised by the presence of features such as drop-shaped rete-ridges, disturbed epithelial stratification and changes in the size, shape and staining characteristics of the nuclei. There are at least 13 histological features described, a complete list of which can be found in Appendix 1. The above features relate just to the epithelium. Wright and Shear (1985) also included Russell bodies, eosinophils and inflammation to their assessments of

epithelial dysplasia; these being features of the reaction of the host to the abnormal epithelium. This aspect of dysplasia would seem to be important, particularly with regard to the prevention of invasion, but it appears to have received less attention in oral precancer than it has in oral cancer where it is one of the features assessed in the histological scoring system proposed by Anneroth *et al* (1987). Interestingly, the latest World Health Organisation publication on the histological typing of oral epithelial dysplasia includes prominent nucleoli as one of the features of dysplasia (Pindborg *et al.*, 1997). This is presumably as a reflection of the proliferative state of the tissue (see below).

The identification of the features of dysplasia is not difficult but the relative importance of the individual features and hence assessment of the severity of dysplasia is considerably more of a problem. Compared with the number of publications investigating other markers of potentially high risk lesions there are few investigating the histopathological features of oral epithelial dysplasia. This is surprising since this is the sole method employed by the majority of pathologists in routinely reporting these specimens. Kramer *et al* (1970) using a discriminant analysis investigated the histological features of oral leukoplakia that segregated those lesions that will undergo transformation to carcinomas. They identified nuclear hyperchromatism and loss of basal cell polarity as possible markers of such lesions.

Banoczy and Csiba (1976) found disturbed epithelial maturation in

75% of cases of epithelial dysplasia but this was not a predictor of malignant transformation. They found a lower incidence of basal cell hyperplasia, loss of basal cell polarity, loss of adherence and nuclear hyperchromatism in their series. The criteria for inclusion in their study, however, were cases which histologically showed as few as two features of dysplasia. Many pathologists would not classify such cases as dysplastic on this basis and this highlights the subjective nature of assessments.

Dysplasia is often found adjacent to established squamous cell carcinoma and on the assumption that dysplastic features in such a site may be better indicators of carcinoma development, Wright and Shear (1985) reported a high incidence of basal cell hyperplasia and disturbed epithelial maturation in these cases but a relatively low incidence of mitotic abnormalities.

An increased nuclear cytoplasmic ratio of basal and spinous cells is a histological feature of dysplastic epithelium but has been reported as being present in less than 50% of dysplasias adjacent to carcinomas (Wright and Shear, 1985). In recent studies the nuclear cytoplasmic ratios were less than those of non-dysplastic and benign lesions of buccal mucosa (White *et al.*, 1997) and palate (Jin *et al.*, 1995). The authors believed this was therefore of little predictive value for the assessment of potentially malignant lesions.

1.3 Cellular markers of transformation

While it is appreciated that those clinically precancerous lesions which show the histopathological features of epithelial dysplasia are of more concern and more likely than those not showing dysplasia to undergo transformation into carcinoma, it is still a minority of these lesions which do undergo this transformation (Lummerman *et al.*, 1996; Silverman *et al.*, 1984). Ideally there would be a "tumour marker" which would be present in those lesions which will become malignant but absent in those lesions which will not. Unfortunately matters are not that simple. Carcinogenesis is a multistage process requiring an accumulation of gene defects before a malignant clone is established with the ability to become autonomous with regard to its growth control, evade the host defence mechanisms and behave in a malignant fashion. The investigation of potential markers is based on the assumption that a key step in this process could be identified that will distinguish a lesion which will behave in a malignant fashion from one which will behave in a benign manner. By the multifactorial nature of the process it seems unlikely that such a perfect marker actually exists. To further compound studies of oral premalignant lesions, the sometimes protracted natural history of such lesions means meaningful data regarding malignant transformation would ideally require long-term follow up periods which are often neither practical or possible.

There now follows a review of the various parameters that have been investigated in relation to their differences in normal, dysplastic and malignant epithelia. It should be appreciated from the outset that there are various levels on which such an investigation can be performed. Until recent years many of the investigations involved either specialised histological techniques or immunohistochemistry which gave the investigator some indication of the expression of particular proteins. However, since it is defects at the level of the genes encoding such proteins which are important there followed the development of methods to enable this level of investigation. The latter methods necessitated the use of fresh tissue but developments have meant, that now, many molecular techniques can be applied to archival formalin-fixed, paraffin-embedded tissues. These developments may make it possible to study more lesions where the natural history is known and well documented.

Firstly there will be a brief overview of a vast literature concerning markers other than those directly associated with cell proliferation. This will be followed by a more detailed analysis of the investigations relating to the cell cycle which forms the basis of the work of this thesis.

1.3.1 Cell surface features

The loss of cell to cell and cell to tissue matrix adherence due to changes in the cell surface components is one characteristic of the malignant phenotype. The majority of work in this area has been achieved by the use of immunohistochemistry and has been comprehensively

reviewed with respect to oral precancer by Scully and Burkhardt (1993) and more generally with regard to human carcinomas by Dabelsteen (1996). A brief overview of the salient points now follows.

Cell surface carbohydrates have received much attention and it has been shown that the development of malignancy can be associated with the synthesis of new carbohydrates, the accumulation of precursors or the loss of complex carbohydrates from the cell surface (Hakomori, 1989). The ABH blood group antigens are found on oral mucosal cells as well as blood cells and are readily detected by the use of lectin histochemistry. Dabelstein *et al* (1983) have shown that in dysplastic oral epithelium there was an accumulation of the precursor, H-antigen, with the consequent loss of expression of the A and B antigens. This pattern of distribution was also seen in less aggressive squamous cell carcinomas (Dabelsteen *et al.*, 1983). Loss of the H-antigen has been reported in histologically benign epithelium which subsequently underwent malignant transformation but the predictive value in dysplastic lesions was not significant (Auclair, 1984). The Le^y antigen, a modified H-antigen, is present on all spinous cells in normal oral epithelium but is found only on the superficial cells in dysplastic lesions (Dabelsteen *et al.*, 1991). The expression of these antigens is, of course, related to the genetic background of the individual and varies between individuals of the same ABO blood group.

The Thomsen-Friedenreich antigen (T) is found in the sialosyl-T form on normal basal and parabasal oral epithelial cells and the

precursors Tn and sialosyl-Tn are seen in the cytoplasm of some spinous cells but not basal cells in normal oral epithelium (Mandel *et al.*, 1991). Sialosyl-T disappeared in basal cells in oral epithelial dysplasia while sialosyl-Tn expression appeared in the basal cells and these changes increased as the severity of dysplasia increased (Bryne *et al.*, 1991). These changes were not specific to precancerous lesions and, therefore, were of little value in predicting malignant transformation (Bryne *et al.*, 1991).

An epithelial-specific cell surface glycoprotein, MH99, has been reported to be expressed in dysplastic oral epithelium but not in normal or hyperkeratotic tissues and in relatively low amounts in squamous cell carcinoma and has been proposed as a potential marker of malignant transformation (High *et al.*, 1996).

Growth factors and receptors are known to be important in carcinomas. The most studied of these is epidermal growth factor receptor (EGFR) which is responsive to the polypeptide, epidermal growth factor (EGF) (Scully and Burkhardt, 1993). In dysplastic and malignant oral epithelia increased amounts of EGF were observed in the upper lamina propria in fibroblasts, the presumed source of EGF (Shirasuna *et al.*, 1991). The EGFR may be increased in oral epithelial dysplasia and carcinoma (Shirasuna *et al.*, 1991). The EGFR is related to the cellular oncogene c-erbB-2. Overexpression of c-erbB-2 is reported to be significantly correlated with poor survival in oral squamous cell carcinoma

(Xia *et al.*, 1997) as it has been for breast cancer (Ostrowski *et al.*, 1991).

Integrins are a family of adhesion molecules expressed by a variety of cells and are involved in cell to cell and cell to extracellular matrix interactions (Thomas *et al.*, 1997). In oral squamous cell carcinoma a considerable variation in integrin expression has been reported and no consistent relationship to tumour prognosis has been established (Thomas *et al.*, 1997). There have been no reported investigations of integrins in oral precancerous lesions to the author's knowledge.

1.3.2 Cytosolic features

The intermediate filaments of the cytoskeleton, the cytokeratins are found in epithelial cells. In a similar manner to cell surface antigen expression, there is a topographical distribution of many of the cytokeratins in normal oral epithelia. An altered distribution of cytokeratins was observed in oral epithelial dysplastic lesions (Heyden *et al.*, 1992; Morgan *et al.*, 1991). Morgan *et al* (1991) also reported a tendency for the expression of those keratins normally associated with simple epithelia particularly in lesions with histologically more severe dysplasia. These studies, however, lacked follow-up data and it was not possible to relate these observations to the subsequent behaviour of the lesions.

The proteins filaggrin and involucrin are normally found respectively in the keratinised layer and stratum spinosum of oral epithelia and can, therefore, be considered as markers of differentiation. However, their expression appears to be independent of the degree of dysplasia and

aggressiveness (Scully and Burkhardt, 1993).

Heat shock proteins (HSP) are ubiquitous, highly conserved proteins produced in cells under stress of many kinds, including tumour cells. HSP 70 expression in oral carcinoma was reported to be increased as it was in dysplastic lesions and benign oral mucosal lesions suggesting it is not a marker of malignant potential (Sugerman *et al.*, 1995). Kaur *et al* (1996) demonstrated the formation of a complex between HSP 70 and p53 in oral epithelial dysplasia and squamous cell carcinoma but not in normal oral mucosa and suggested this may be one mechanism of inactivation of p53 in the carcinogenic process.

1.3.3 Extracellular components

The basal lamina is composed primarily of laminin, type IV collagen and heparan sulphate. In carcinomas the basement membrane components may be absent but it has been suggested that laminin may promote invasion and metastasis through its role in adhesion to integrins on the surface of tumour cells (Pattaramalai and Skubitz, 1994; Scully and Burkhardt, 1993). Kumagai *et al* (1994) reported that carcinomas with a widespread loss of basement membrane components showed a high frequency of lymph node metastasis and suggested this may be due to the production of metalloproteinases, type IV collagenase in particular, by the tumour cells. In precancerous lesions, breaks in the continuity of the basement membrane were reported, assessed by immunohistochemical localisation of laminin and type IV collagen (Firth and Reade, 1996).

These breaks were more extensive in more severely dysplastic lesions but the study lacked follow-up data to support the notion that these findings may signify a progression to malignancy. Increased expression of laminin-5 mRNA was reported in oral epithelial dysplastic lesions and in the invasive front of carcinomas supporting a possible role in invasion (Kainulainen *et al.*, 1997).

Tenascin is an extracellular matrix protein involved in epithelial-mesenchymal interactions during embryogenesis and in adult tissues is expressed in inflammation, repair and tumourigenesis. Tenascin immunoreactivity was increased in the underlying stroma of oral epithelial dysplastic lesions particularly those with more severe dysplasia (Tiitta *et al.*, 1994). However, this was seen most prominently in those lesions containing Candida infection and hence associated with a prominent inflammatory reaction.

1.3.4 Nuclear features

The majority of nuclear parameters have an association with the cell cycle or cell proliferation and will therefore be discussed under those headings.

Genetic analyses of oral squamous cell carcinomas have revealed some consistent genetic alterations in these tumours. Loss of heterozygosity is suggestive of the existence of tumour suppressor genes and has been observed commonly in chromosomes 3p, 5q, 9p, 17p and 18q of oral carcinomas, some of which are also found in tumours with a

common aetiology to oral cancer such as the lung (Califano *et al.*, 1996; Papadimitrakopoulou *et al.*, 1998; Ransom *et al.*, 1996; Sakata, 1996; Wu *et al.*, 1994). A putative tumour suppressor gene, deleted in oral cancer-1 (doc-1), has been identified which altered the behaviour of malignant hamster keratinocytes reversing some of the malignant phenotypes (Todd *et al.*, 1995).

Sidransky's team in the USA have proposed a genetic progression model for head and neck cancer using PCR-based microsatellite analysis (Califano *et al.*, 1996). These workers have endorsed the field cancerisation theory, based on the finding that mucosal cells adjacent to tumours shared common genetic changes and that more histologically advanced areas showed additional changes (Califano *et al.*, 1996). These workers have proposed a further theory of a common clonal origin for the existence of multiple primary tumours. This is based upon the observation of identical genetic alterations in multiple tumours and it was suggested that this is due to the migration of a transformed clone through the mucosa to other sites (Bedi *et al.*, 1996). Clinically normal tissues some distance from the primary tumour site harboured identical genetic alterations and this was accounted for by so-called clonal expansion and migration of cells to these sites. This could account for the development of second primary tumours in these patients and could have far-reaching implications for the assessment of surgical clearance of tumours. Other workers have demonstrated differing genetic alterations in dysplastic

lesions compared with subsequent carcinomas at the same site (Partridge *et al.*, 1997)

With regard to oral epithelial dysplastic lesions, it has been suggested that loss of heterozygosity at chromosomes 3p and 9p is related to early carcinogenesis (Mao *et al.*, 1996). Certainly, chromosome 3p appears to be consistently affected in oral dysplastic lesions (Califano *et al.*, 1996; Mao *et al.*, 1996; Partridge *et al.*, 1997; Roz *et al.*, 1996).

1.3.5 Cancer-related antigens

No perfect "tumour marker" exists for oral cancer but a few workers have investigated this avenue. A monoclonal antibody, Ca 1, which recognises the malignancy associated Ca antigen was investigated in oral premalignant and malignant lesions (Masouredis *et al.*, 1987). The authors reported nearly three quarters of the squamous cell carcinoma cases were negative with this antibody as were all the dysplastic lesions. One benign keratotic lesion was positive indicating this antibody was unreliable.

Two monoclonal antibodies, 17.13 and 63.12, have been generated by immunising mice against human laryngeal and oral carcinoma cells respectively. In oral epithelial dysplasia a staining pattern resembling carcinoma was observed (Merrel *et al.*, 1997). However, associated inflammation also produced a similar altered reactivity in non-dysplastic lesions negating the prognostic use of these antibodies.

1.4 Cell proliferation

An increase in cell proliferation or a relative increase over cell loss has long been considered an important component in the pathogenesis of neoplasia. It must be noted that most of the methods currently available identify cells within a tissue at a "snap shot" in time and therefore give information on the proliferative "state" rather than the proliferation "rate", a term that is often referred to erroneously in the literature (Dover, 1992).

Traditionally, the numbers of mitoses per high power field have been assessed in conventional histological sections. This gives a parameter known as the mitotic count at a particular point in time but gives no indication as to the duration of mitosis, T_m, or of the cell cycle, T_c (Aherne *et al.*, 1977). Furthermore, this is a non-standardised measurement, it makes no account of cell size or that the area of a high power field varies between microscopes and it is open to interobserver variation (Quinn and Wright, 1992).

Many workers have focused their attention on markers of cell proliferation which identify cells that are within the cell cycle either at a specific phase (or phases) or throughout all phases of the cell cycle. The majority of these provide a proliferation state rather than proliferation rate. Central to the understanding and rationale of the use of these agents is the cell cycle, a review of this follows.

1.4.1 The cell cycle

The cell cycle is traditionally described as comprising of four

phases as follows. The presynthesis gap phase (G_1) is the interval which follows mitosis and precedes the DNA synthesis phase (S) which typically lasts 6 to 16 hours (Dover, 1992). During this phase the DNA is replicated before the cell enters a second gap phase (G_2) lasting between 4 and 8 hours (Dover, 1992). Mitosis (M) follows to yield two daughter cells which then enter the G_1 phase. In the G_1 phase of the cell cycle the cell will either continue to cycle through the phases already described or will leave the cell cycle in to a resting phase (G_0). G_1 is the most variable phase in terms of length of time spent and could be very long in the case of stem cells.

From G_0 the cell can either re-enter the cell cycle into G_1 phase or become terminally differentiated. The G_0 phase was initially suggested by Lajtha (1963) to describe stable liver cells which only proliferate when stimulated to do so (a conditionally renewing or stable tissue). The G_0 phase may be a phase of every cell cycle or may be a completely separate state but in those cells with a long G_1 phase it is not possible to distinguish between the two (Dover, 1992). Indeed, some authors doubt the existence of G_0 at all (Cooper, 1991). The growth fraction (GF) is a parameter used to describe the proportion of cells actually in the cell cycle at a particular point in time.

Heteroprotein dimers comprising a protein kinase (the cyclin-dependent kinases or CDK) and a cyclin act as regulatory proteins for progression through certain check points in the cell cycle and ensuring the

correct chronological order of events. The levels of CDKs remain relatively constant throughout the cell cycle and are only activated by binding of the cyclins, hence the cyclins have relatively short half lives and are therefore potential markers of cells at various stages of the cell cycle. The cell cycle and its regulatory proteins is demonstrated in Figure 1.1.

1.4.2 Restriction point

The first control point of the cell lies in the G_1 phase and the continued passage through this phase is influenced by growth factors, for example PDGF, EGF, IL3, and adequate nutrients. There is a point in the G_1 phase referred to as the restriction point, or START, after which the cell is committed to enter the S phase and DNA replication. Transition through this point requires the formation of a complex between the D cyclins and CDK4 or CDK6 with the transcription factor E2F and pRb (the retinoblastoma gene product and equivalent to p105) (Goodger *et al.*, 1997). The cyclin D-CDK complex phosphorylates pRb leading to the dissociation of E2F allowing transcription of genes for further cycle progression (Ewen *et al.*, 1993). Once the cells have passed this point they become refractory to extrinsic stimuli and further cell cycle events are governed by intrinsic factors.

1.4.3 S-phase control

A complex formed between cyclin E and CDK2 controls the transition from G_1 into S-phase (Koff *et al.*, 1991). This complex may also phosphorylate pRb releasing E2F as well as being involved with DNA

replication (Goodger *et al.*, 1997). Cyclin A is produced at the beginning of S-phase and complexes with CDK2 appearing to control DNA replication (Goodger *et al.*, 1997).

1.4.4 Mitosis

The CDK $p34^{cdc2}$ appears central to the control of G_2/M transition (mitosis) and is, in fact, a component of the so-called maturation promoting factor (MPF) as is histone H1 kinase (Brooks, 1992). $p34^{cdc2}$ has a close association with the B cyclins. Phosphorylation and dephosphorylation of MPF components controls this transition (Brugere *et al.*, 1986). The complex moves from the cytosol to the nucleus to trigger chromosome condensation, nuclear lamina breakdown and the formation of mitotic spindles at the onset of the prophase of mitosis.

Following metaphase, cyclins A and then B are broken down via a ubiquitin-dependent pathway (Goodger *et al.*, 1997). Dividing cells then continue through anaphase and telophase before the two daughter cells exit from mitosis into G_1.

1.4.5 Inhibition of cyclin/CDK complexes

The CDKs can be activated by cyclin binding and phosphorylation (Morgan, 1995). Phosphorylation can also inhibit the CDK-cyclin complexes as can another group of proteins, the CDK inhibitors (CDKI) (Morgan, 1995). Two families of inhibitory proteins, the ink4 proteins and the cip/kip group of proteins exert negative effects upon the cyclin/CDK complexes (Goodger *et al.*, 1997).

The ink4 family of proteins, which include p15 and p16, has a negative control on CDK4 and CDK6 therefore preventing them from binding cyclin D which inhibits the phosphorylation of pRb and progression through the restriction point (Hall *et al.*, 1995). The loss of p16 function is implicated in the process by which neoplastic cells develop an immortal phenotype, along with mutation or deletion of p53 (Loughran *et al.*, 1994).

$p21^{waf1/cip1}$ is a member of the cip/kip group and acts upon the cyclin component of the complexes (cyclins A, B, D and E), a broader range than the ink4 group (Hall *et al.*, 1995). Overexpression of this protein is associated with arrest of cells in G_1 (Harper *et al.*, 1993). The tumour suppressor gene, p53, causes G_1 arrest in cells with DNA damage by up-regulation of $p21^{waf1/cip1}$ (Jacks and Weinberg, 1996). p53 will be discussed further in relation to apoptosis.

1.4.6 Apoptosis

Apoptosis is programmed death of individual cells in a tissue and is now known to be important in both physiological and pathological situations. Pathologically, most apoptosis occurs in non-proliferating tissues but, particularly in regard to neoplasia, it has a strong association with cell proliferation and is a target for therapy. Apoptosis is the mechanism by which most cytotoxic drugs exert their effect (Evan *et al.*, 1995). Paradoxically it seems, some of the inducers of cell proliferation such as the adenovirus early gene, E1A, and the oncogene c-myc, can trigger apoptosis in certain situations (Evan *et al.*, 1995). Indeed, some of

the CDK-cyclin complexes described above, including cyclin A-CDK2, are also activated in apoptosis (Evan *et al.*, 1995).

A number of tumour suppressor genes have been shown to be important in apoptosis. pRb suppresses apoptosis by an, as yet, unknown mechanism but possibly by preventing transition through the restriction point into S-phase (Evan *et al.*, 1995). Bcl-2, the protein encoded for by the tumour suppressor gene bcl-2, directly suppresses apoptosis. The mechanism by which Bcl-2 inhibits apoptosis is unclear. Alteration of mitochondrial function has been suggested (Hockenbery *et al.*, 1990) as has regulation of intracellular calcium ions (Baffy *et al.*, 1993). Localisation of Bcl-2 to the nuclear membrane and mitotic nuclei has lead to the suggestion that it may protect DNA against nucleases (Lu *et al.*, 1994). Conversely, from the same family as Bcl-2, the oncogene *bax*, promotes apoptosis.

Perhaps the most widely studied gene in relation to apoptosis is p53. This tumour suppressor gene is a nuclear phosphoprotein which functions as a transcription factor. Other functions are now being attributed to it (Hall *et al.*, 1996). The normal form (or wild type) is thought to be responsible for the arrest of the cell in G_1 of the cell cycle when DNA damage is detected and can act to induce apoptosis or encourage DNA repair (Lane, 1992). In so doing, p53 prevents the replication of cells with damaged DNA which could be oncogenic and for this role it has been given the title of "guardian of the genome" (Lane, 1992). Mutation or loss

of p53 is considered to be one of the key stages in the malignant transformation of cells (Lane, 1993) and p53 has been shown to be the most commonly mutated gene, inactivated in almost half of all human tumours (Greenblatt *et al.*, 1994).

More recently, Ambrosini *et al* (1997) have described a gene expressed in foetal tissues and a variety of human cancers but not normal adult cells which inhibits apoptosis which they have designated *survivin*.

As noted above in regard to interpretations of proliferation markers, caution is required in the interpretation of data on apoptosis, in particular, the widely quoted apoptotic index (Potten, 1996). The duration of apoptosis is not known precisely, but is thought to be short. Many apoptotic fragments are small and on histological examination may be missed. A single cell undergoing apoptosis characteristically forms many membrane-bound fragments, the number of these observed histologically does not necessarily equate to the number of cells that have undergone apoptosis (Potten, 1996).

In oral epithelial dysplasia and squamous cell carcinoma, apoptosis was reported by Birchall *et al* (1996) to remain at a static level in relation to mitosis as the severity of dysplasia increased and in carcinomas. However, the same authors previously reported an increase in the apoptotic index to mitotic index ratio as the severity of dysplasia increased up to carcinoma in situ and then a reduction in carcinoma (Birchall *et al.*, 1995). More recently the same group have curiously reported a highly

significant correlation between apoptotic and mitotic indices (Birchall *et al.*, 1997). From the limited number of studies available it is felt that the role of apoptosis in oral epithelial dysplasia is neither consistent nor as significant as that of proliferation.

1.4.7 The cell cycle and neoplasia

When all the above factors are taken into account a possible genetic pathway leading to increased cell proliferation and also cancer development can be envisaged as proposed by Sladek (1997) and summarised in Figure 1.2. Clearly alterations to any of the factors discussed above could potentially lead to an increase of cell proliferation or a suppression of apoptosis. Accumulation of genetic damage and replication of cells with these defects could lead to the formation of carcinoma. The cell cycle, therefore, has an important and central role in the development of carcinoma.

In addition to these events, the acquisition of an immortal phenotype is thought to be the action of telomerase (Ershler and Longo, 1997). The reactivation of this enzyme prevents the shortening of the telomeres that normally occurs at every cell division which eventually leads to cellular senescence and growth arrest.

1.5 Cell cycle associated markers in oral precancer

1.5.1 Nucleolar organisation regions

Nucleolar organiser regions (NORs) are loops of ribosomal DNA

(rDNA) found in the nucleolus that transcribe to rRNA and thus ribosomes and later to protein (Crocker, 1990). The relationship of NORs to cell activity has prompted investigations into the size and number of NORs as possible indicators of cell proliferation and transformation. Demonstrated by a colloidal silver staining technique that binds to NOR-associated proteins, they are often referred to as AgNORs. A small number of investigations have looked at AgNORs in oral epithelial dysplasia. A correlation between the DNA index (assessed by flow cytometry) and the mean number of AgNORs has been reported (Kahn *et al.*, 1993) and a significant difference between the mean AgNORs in oral epithelial dysplasia and oral squamous cell carcinoma has been demonstrated (Kobayashi *et al.*, 1995).

Migaldi *et al* (1998) reported significant differences in AgNOR values between oral epithelial dysplastic lesions and benign epithelial lesions and squamous cell carcinoma. These authors also described the same findings of another nucleolar protein, p120, which can be identified immunohistochemically.

MacDonald and Milne (1997) reported a correlation between intranucleolar AgNOR counts and atypia scores in oral epithelial dysplastic lesions. The overall AgNOR counts however did not show any significant association with the degree of dysplasia.

1.5.2 Flow cytometry

Flow cytometry is a rapid and objective method of analysing large

numbers of cells or their constituents. For example, nuclear DNA content, S-phase fraction and detection of DNA aneuploid cell populations are most commonly assessed with regard to malignancy (Kahn *et al.*, 1992; Kahn *et al.*, 1993). The S-phase fraction is simply the percentage of cells in the S-phase. The DNA index is a ratio of the peaks, obtained from the flow cytometeric analysis, of a DNA aneuploid G_0/G_1 population to normal G_0/G_1 cells. The flow cytometer, with the aid of monoclonal antibodies, can be used to assess the presence of specific proteins or molecules.

A correlation between DNA index and AgNOR staining was reported by Khan *et al* (1993) in oral epithelial dysplasia but surprisingly, no correlation existed between AgNOR counts and S-phase fraction. More recently Saito *et al* (1995) reported a higher incidence of aneuploidy in severely dysplastic leukoplakia than in mildly dysplastic lesions. On the basis that those lesions showing a higher degree of dysplasia are more likely to undergo malignant transformation, these authors suggested that these findings may be of predictive value, but as yet, they lack any follow-up data to support this (Saito *et al.*, 1995).

1.5.3 Proliferating cell nuclear antigen

Proliferating cell nuclear antigen (PCNA) is a 36kD nuclear protein, an auxiliary protein of DNA polymerase-δ which is essential for DNA synthesis (Bravo *et al.*, 1987). Originally it was designated as PCNA/cyclin. Fortunately, the latter suffix has been dropped by most users, but can lead to some confusion with the cyclins when used

incorrectly. PCNA is synthesised in late G_1 and S-phase of the cell cycle and is detectable by immunohistochemical methods. However, although it is related to the cell cycle it is not specific to a particular aspect of the cell cycle. It has a relatively long half-life of approximately 20 hours. Immunoreactivity appears to be dependent upon fixation and it has been detected in cells that have left the cell cycle (Yu *et al.*, 1992). In oral premalignant lesions, PCNA staining was reported to be significantly higher than in non-dysplastic mucosa (Kobayashi *et al.*, 1995; Tsuji *et al.*, 1992). Yama *et al* (1996) reported no correlation between PCNA expression and the severity of dysplasia in lesions of the buccal mucosa and gingiva but an apparent confinement of staining to the basal third of the epithelium in severely dysplastic lesions. A higher PCNA labelling index was found in hyperplastic oral lesions compared with dysplastic lesions and squamous cell carcinoma by Birchall *et al* (1997).

1.5.4 p53

The p53 literature in oral lesions is vast and was comprehensively reviewed in recent publications (Raybaud-Diogène *et al.*, 1996; Slootweg, 1997). Only the main points will be discussed below. Mutations of p53 have been reported to be present in most oral squamous cell carcinomas (Sakai and Tsuchida, 1992) and to be greater in the normal oral mucosa of smokers than non-smokers suggesting that p53 mutations may be an early event in the pathogenesis of oral cancer in these patients (Colucci *et al.*, 1997). Overexpression of the p53 gene has been reported in oral

squamous cell carcinoma (Nylander *et al.*, 1995; Ogden *et al.*, 1992; Sakai and Tsuchida, 1992; Warnakulasuriya and Johnson, 1994; Warnakulasuriya and Johnson, 1992) and it correlated with heavy smoking (Field *et al.*, 1991).

In oral epithelial dysplasia, p53 overexpression has been demonstrated immunohistochemically (Coltrera *et al.*, 1992; Girod *et al.*, 1993; Kerdpon *et al.*, 1997; Nishioka *et al.*, 1993; Regezi *et al.*, 1995; Shin *et al.*, 1994; Warnakulasuriya and Johnson, 1994; Warnakulasuriya and Johnson, 1992). The degree of p53 expression has been reported as being greater in those lesions showing moderate or severe dysplasia compared to mild dysplasia (Kerdpon *et al.*, 1997; Sauter *et al.*, 1994; Shin *et al.*, 1994). Regezi *et al* (1995) reported that half of the cases exhibiting p53 expression in oral epithelial dysplasia progressed to p53 positive carcinomas although one p53 negative dysplasia progressed to carcinoma. Ogden *et al* (1992) failed to demonstrate p53 expression in formalin fixed specimens of oral epithelial dysplasia but did not use any form of antigen retrieval, a factor the same author has subsequently demonstrated significantly increases the detection of the protein immunohistochemically (Dowell and Ogden, 1996).

Wild-type p53 protein has a short half life (Reihsaus *et al* 1990) and therefore cannot be detected immunohistochemically. Conversely, the mutated form is a more stable protein and is demonstrable by immunohistochemical techniques. It has been suggested recently that p53

positive tissues demonstrated immunohistochemically do not necessarily contain p53 mutations but a stabilised form of the wild-type p53 protein (Battifora, 1994; Wynford-Thomas, 1992). The wild-type p53 may be stabilised by complexing with the MDM2 protein and therefore is demonstrable by immunohistochemistry (Oliner *et al.*, 1992). MDM2 is the product of an oncogene which inactivates p53-mediated transcription as part of an autoregulatory loop (Levine, 1997). This action, however, still removes p53 from its role outlined in Section 1.4.6 in a similar manner to the E6 protein of the HPV. p53 expression has also been reported in reactive lesions with no propensity for malignant change (Kerdpon *et al.*, 1997). The immunohistochemical results must therefore be interpreted with some caution. Likewise, a loss of expression of the p53 gene is possible in carcinogenesis and this would result in its failure to be demonstrated immunohistochemically, a point not addressed in many studies.

Cruz *et al* (1998) recently showed that in both dysplastic and non-dysplastic oral precancerous lesions suprabasal p53 expression was a positive predictor of malignant transformation compared to those oral mucosae where there was only basal or no p53 immunoreactivity.

1.5.5 bcl-2 and bax

Although originally studied extensively in haematopoietic cell lines there is evidence that bcl-2 has a role in regulating cell death in epithelium.

The bcl-2 gene is expressed in most developing tissues at specific stages of differentiation. High levels of Bcl-2 expression in human embryonic skin have been reported in the basal cells committed to hair follicle development; whilst other skin areas express very low or undetectable levels of the protein (Lu *et al.*, 1993).

bcl-2 appears to be associated with proliferative compartments but not directly associated with proliferation. Lu *et al* (1996; 1993) postulate, on the basis of their observations of bcl-2 expression in foetal tissues, that bcl-2 expression is associated with the transition from undifferentiated stem cell to committed precursor and that bcl-2 is down regulated once cells are terminally differentiated.

bcl-2 expression in epithelial malignancy appears to be variable; high levels of expression being reported in nasopharyngeal, breast and prostate carcinomas but lower levels of expression in lung, bladder and gastric carcinomas (Lu *et al.*, 1996). The topographical distribution of Bcl-2 expression in well differentiated tumours, including oral carcinomas, closely resembled that in the corresponding normal epithelium (Jordan *et al.*, 1996). Harada *et al* (1998) recently reported that oral squamous cell carcinoma cell lines transfected with bcl-2 DNA failed to show morphological signs of differentiation in culture and did not express the cytokeratins usually observed in differentiated epithelial cells. The authors postulated that bcl-2 is possibly more important in preventing differentiation than preventing apoptosis.

Jordan *et al* (1996) reported increased immunohistochemical staining of Bcl-2 in oral epithelial dysplasia adjacent to tumours, often throughout the full thickness of the epithelium compared to non dysplastic epithelium where staining was restricted to basal cells. This combined with decreased expression of the apoptosis permitting protein, bax, in dysplastic lesions lead the authors to believe that cells may acquire selective growth advantage (Jordan *et al.*, 1996). Birchall *et al* (1997) found reduced bcl-2 expression in squamous cell carcinoma and adjacent normal epithelium and suggested that this may represent a constitutive suppression in the patients, possibly accounting for multiple tumours in patients with oral cancer.

1.5.6 Tritiated thymidine

Thymidine is the nucleoside of the pyrimidine base thymine which has the advantage over the other bases in that it is only found in DNA and not in RNA. Synthetically produced tritiated thymidine (^{3}H-thymidine) can be incorporated into DNA during the S-phase and the emission of β-particles can detected by means of an autoradiographic film placed over a treated tissue section. Quantification of the ^{3}H-thymidine labelling index gives an estimation of the number of cells in the S-phase at the time of labelling and thus an indication of the proliferative state of the tissue (Dover, 1992).

Regarding oral mucosa, Warnakulasuriya and MacDonald (1993) have demonstrated a diurnal variation in the ^{3}H-thymidine labelling index

(LI) in normal buccal mucosa. Also in buccal mucosa, an increase in the ^{3}H-thymidine LI in biopsies of oral leukoplakia has been demonstrated (Alvares *et al.*, 1972; Warnakulasuriya and MacDonald, 1995a). These investigations will be discussed further in Chapter 2.

The rate of entry of cells into the S-phase can be measured by a double-labelling method which can be achieved either by labelling the same tissue with two different isotopes or two differing concentrations of the same isotope. Alternatively, two separate pieces of tissue can be labelled at differing times (Dover, 1992). In oral leukoplakia the length of time spent in the S-phase was not significantly different to that of normal oral mucosa (Warnakulasuriya and MacDonald, 1995a). However, in the tongue the length of the S-phase was reported as being shorter in iron deficiency (Rennie and MacDonald, 1984).

1.5.7 Bromodeoxyuridine

Bromodeoxyuridine (BrdU) is a pyrimidine analogue that can be incorporated into the DNA of replicating cells in a similar manner to ^{3}H-thymidine and therefore can be used as a marker of those cells in the S-phase of the cell cycle. This process has been facilitated by the development of a monoclonal antibody to BrdU and negates the need for autoradiography which can be technically demanding. BrdU also has the potential, albeit limited, to be used *in vivo* as well as *in vitro*. Indeed, it is used therapeutically in certain patients to enhance the sensitivity of tumours to radiotherapy (Barnes and Gillett, 1995). ^{3}H-thymidine has been

used *in vivo* but essentially only in experimental animal models.

In common with ^{3}H-thymidine, the BrdU labelling method requires fresh tissue and cannot be used on archival material unlike some of the other proliferation markers discussed below. These potentially time consuming methods have not been routinely adopted in diagnostic histopathology as has been the case with some other markers of cell proliferation.

In squamous cell carcinoma of the oral cavity, BrdU labelling has been reported to be greater in T3 tumours than T1 or T2 tumours and higher also in those tumours with cervical lymph node metastases (Hemmer, 1990).

1.5.8 Ki67

Much attention has been paid to this nuclear protein since Gerdes *et al* (1983) described the production of a monoclonal antibody that recognised an antigen in cycling cells but not those in G_0. It potentially gives a marker of cells within the growth fraction in a tissue. However, its precise function within the cell cycle is still poorly understood (Ross and Hall, 1995). Ki67 now appears to be two nuclear proteins with molecular weights of approximately 345 and 395 kDa that have no detectable precursor (Heidebrecht *et al.*, 1996). An association of the protein with the nucleolus and chromosomes during mitosis has suggested a structural role (Ross and Hall, 1995). Recently, Gerdes' group (Gerlach *et al.*, 1998) have demonstrated that Ki67 protein is a target for autoantibodies in

systemic lupus erythematosis.

The gene for Ki67, which is located on chromosome 10q25 (Fonatsch *et al.*, 1991), was cloned in 1993 (Schlüter *et al.*, 1993). The gene is highly conserved throughout mammals (Falini *et al.*, 1989). This has enabled detailed study of the protein. Ki67 arises *de novo* in cycling cells and has a short half-life in the region of 60 to 90 minutes (Bruno and Darzynkiewicz, 1992; Heidebrecht *et al.*, 1996). Synthesis of Ki67 appears to begin at the start of the S-phase rising to a peak during mitosis and expression decreases during G_1 which may correspond to degradation (Lopez *et al.*, 1991) or decreased synthesis (Bruno and Darzynkiewicz, 1992). These results support the nucleolar and mitotic associations of the protein. Furthermore, cells with a long G_1 phase may not express detectable levels of Ki67 which may lead to an underestimation of cells within the growth fraction (Lopez *et al.*, 1991).

The original Ki67 antibody described by Gerdes *et al* (1983) was limited by nature of the fact that it could only be used on fresh frozen tissue. Subsequently new antibodies (MIB1 and MIB3) were developed that could be used on formalin-fixed, paraffin embedded tissue following antigen retrieval methods (Cattoretti *et al.*, 1992; Key *et al.*, 1993). It has been claimed that the original Ki67 antibody is equally effective in formalin-fixed tissue following antigen retrieval (Cuevas *et al.*, 1993). The development of an antibody effective in formalin fixed, paraffin embedded tissues has resulted in a multitude of reports utilising Ki67, notably MIB1,

in proliferation studies particularly in relation to neoplasia.

In oesophageal squamous cell carcinoma, the number of MIB1 positive cells correlated with the differentiation as well as with prognosis in stage III tumours (Lam *et al.*, 1996).

In laryngeal carcinoma MIB1 counts were higher in more advanced stage tumours and correlated with prognosis as did PCNA and tumour front grading (Welkoborsky *et al.*, 1995).

Premalignant lesions of the uterine cervix are graded as cervical intraepithelial neoplasia (CIN) and graded 1-3 which roughly equates to mild dysplasia through to carcinoma in situ according to what proportion of the epithelium is occupied by atypical cells and this relates to some extent to the clinical outcome of these lesions (Anderson *et al.*, 1991). Bulten *et al* (1996) reported that MIB1 labelling indices correlated closely to CIN grade and suggested it was a useful adjunct to the traditional subjective assessment. However, Payne *et al* (1996) observed a similar trend but noted that the MIB1 labelling did not correlate closely enough with the CIN grade to be of use in the assignment of a grade.

There are relatively few studies of Ki67 in oral mucosa, particularly oral epithelial dysplasia and normal mucosa. Kannan *et al* (1996) reported similar Ki67 labelling indices in dysplastic and non-dysplastic lesions of leukoplakia, the sites of the lesions were not specified. In epithelial dysplasia from the floor of the mouth, Kushner *et al* (1997) reported significantly higher Ki67 labelling than in non-dysplastic lesions.

1.5.9 D Cyclins

In normal cells the D cyclins are involved with cell progression through G_1 and committing the cell to DNA replication (Hunter and Pines, 1994). They associate with CDK4 and CDK6 to phosphorylate the pRb, inactivating it. This allows the dissociation of the transcription factor E2F from this complex and hence transition of the cell through the restriction point (START) and into the S-phase (Weinberg, 1995). There are three subtypes identified in the cyclin D group designated cyclins D1, D2 and D3.

The D cyclins are reported as amplified and overexpressed in a variety of tumours including head and neck cancers (Bartkova *et al.*, 1995; Callender *et al.*, 1994; Davidson *et al.*, 1996; Gimenez-Conti *et al.*, 1996; Masuda *et al.*, 1996; Mineta *et al.*, 1997; Worsley *et al.*, 1996; Yatabe *et al.*, 1996) where cyclin D is functionally inactivating the inhibitory effect of pRb. Juan *et al* (1996) suggest that there is an unscheduled expression of the D cyclins (D1 and D3) rather than overexpression in tumour cells. In human tumour cell lines these workers demonstrated expression of cyclins D1 and D3 in the S, G_2, and M phases as well as the G_1 phase of the cell cycle. It was suggested that the content of the D cyclins may even be lower than in normal cells (Juan *et al.*, 1996).

It has been suggested that the three D cyclins are cell-type specific; cyclin D1 predominating in fibroblasts, while being negative in lymphocytic cells in which cyclins D2 and D3 are expressed (Ando *et al.*, 1993; Tam *et*

al., 1994). Skin keratinocytes express cyclin D1 in G_1 (Javier *et al.*, 1997). Thus, some of the D cyclins may be redundant in some cell types or may have separate but overlapping functions (Lahti *et al.*, 1997).

The gene for cyclin D1 (CCND1) is located at 11q13 and was initially described as the oncogene Bcl-1, as it was found at the translocation of B-cell lymphomas, and PRAD 1, an oncogene amplified in parathyroid adenomas (Rosenberg *et al.*, 1991). Later it was shown that both these genes were in fact CCND1.

In an immunohistochemical study of squamous cell carcinoma of the hypopharynx, Masuda *et al* (1996) reported a correlation between cyclin D1 immunoreactivivty (interpreted as CCND1 gene overexpression) and regional lymph node metastasis while those with negative expression had a better prognosis. In oral verrucous carcinoma the positive immunohistochemical expression of cyclin D1 has been shown to be present in moderately invasive lesions but not in more aggressive cases or in normal oral mucosa which demonstrated negative staining (Gimenez-Conti *et al.*, 1996). Interestingly, in none of these cases was there any loss of expression of pRb. The interpretation of this was that the gene was functioning normally and it could be postulated that in these cases overexpression of CCND1 gene was overriding the tumour suppressor effects of pRb.

Southern blotting was used to investigate the amplification of CCND1 in frozen specimens of head and neck squamous cell carcinoma,

including oral tumours (Mineta *et al.*, 1997). Amplification of CCND1 was seen in 16% (6 of 39) of the tumours, all of these having p53 mutations. The tumours were described as being clinically advanced, perhaps indicating that CCND1 abnormalities are a late event in tumour progression as a further 12% of the cases had p53 mutations but no CCND1 amplification (Mineta *et al.*, 1997). In a series of cultured head and neck tumour cell lines, CCND1 gene amplification was shown in some cell lines but most of these had normal p53 genes while most of those with p53 mutations had a normal CCND1 gene copy number leading to the thought that either p53 mutation or CCND1 amplification may be sufficient to overcome the cell cycle control (Xu *et al.*, 1994). Using Southern blotting, again on frozen head and neck squamous cell carcinoma samples (over half from the oral cavity), CCND1 amplification was identified in 23% (13 of 57) of cases. This was up to five-fold in two cases which also showed a significantly higher level of mRNA (Davidson *et al.*, 1996). In these cases, normal mucosa adjacent to tumours showed a similar CCND1 expression to those tumours without CCND1 amplification. Tobacco exposure was also significantly higher in those cases in which there was CCND1 amplification (Davidson *et al.*, 1996). The cyclin D1 gene was found to be amplified in over a third of laryngeal carcinomas where it correlated with advanced local invasion and lymph node metastasis as well as with mRNA overexpression (Jares *et al.*, 1994). Again, in a series of largely laryngeal carcinomas, CCND1 amplification

was identified in a third of cases (including one base of tongue tumour) and this was more noted in high grade aneuploid tumours (Callender *et al.*, 1994).

Fewer investigations have been reported of cyclin D3. The precise mode of action of cyclin D3 has not been fully elucidated but it has been shown to have a greater affinity for pRb in vitro (Ewen *et al.*, 1993). Usuda *et al* (1996) reported cyclin D3 positivity in 71% of lung carcinomas by immunohistochemistry but this did not relate to any pathological variables and the proportion of cyclin D3 positive nuclei was small in many cases. The authors also reported cyclin D3 positive cells in normal tissues including reactive pneumocytes, transitional epithelium, squamous mucosa from the oesophagus and endothelium.

1.5.10 Other cell cycle associated proteins

The retinoblastoma gene product was infrequently observed in oral carcinomas using immunohistochemistry and western blotting indicating a normal cell cycle-dependent function. However, those tumours not expressing the protein were associated with a poorer survival rate (Pavelic *et al.*, 1996). Yoo *et al* (1994) reported that despite a frequent loss of heterozygosity in the chromosomal region of the Rb locus, there was normal pRb suggesting the presence of another tumour suppressor gene in this region.

$p16^{INK4a}$, a member of the INK4 family of CDK inhibitors, blocks the binding of CDK4/6 to cyclin D1 causing G_1 arrest. It therefore acts as a

tumour suppressor gene (Goodger *et al.*, 1997). Loss of heterozygosity at the gene locus or loss of expression immunohistochemically of $p16^{INK4a}$ has been reported in nearly half of the cases of oral precancerous lesions (Papadimitrakopoulou *et al.*, 1997). However, Matsuda *et al* (1996) did not find any $p16^{INK4a}$ abnormalities in oral precancerous lesions and only 10% of advanced oral carcinomas showed deletions or mutations of the gene. These results are consistent with those of González *et al* (1997) who reported $p16^{INK4a}$ deletions in 20% of head and neck carcinomas. LOH of the locus at 9p21 where the CDKI, p16, is mapped to has been shown to be associated with an immortal phenotype in head and neck cancer cells in culture (Loughran *et al.*, 1994).

Another CDK inhibitor, $p21^{WAF1/CIP1}$, which is the downstream moderator of p53 has not yet been studied in oral epithelia. In the normal squamous epithelia of the tonsil and cervix positive immunoreactivity was observed mainly in the suprabasal cells with accompanying p53 positivity but no Ki67 reactivity (Mateo *et al.*, 1997). This suggests $p21^{WAF1/CIP1}$ may be a marker for those cells in G_0. In cells with mutant p53, $p21^{WAF1/CIP1}$ expression was not seen in carcinoma of the skin (Inohara *et al.*, 1996) or the oesophagus (Ohashi *et al.*, 1997). In the latter case, the distribution of $p21^{WAF1/CIP1}$ positive cells was almost the same as that of apoptotic cells (Ohashi *et al.*, 1997). However, $p21^{WAF1/CIP1}$ expression has been reported in carcinomas of the larynx independent of p53 suggesting an alternative trigger for the expression of the gene (Cox, 1997).

Jordan *et al* (1998) recently reported reduced levels of $p27^{Kip1/Cip1}$ determined immunohistochemically in oral epithelial dysplastic lesions and squamous cell carcinoma compared with normal epithelium from the floor of the mouth. These authors reported a significant decrease in $p27^{Kip1/Cip1}$ expression between low and high grade dysplasias with a concurrent increase in Ki67 and cyclin A expression and suggested that the loss of expression of $p27^{Kip1/Cip1}$ may be an early event in carcinogenesis of these lesions.

The CDK, $p34^{cdc2}$, which forms part of the MPF is observed throughout the cell cycle of dividing cells but shows low levels in non-dividing cells. In normal oral epithelia it was observed in the progenitor compartment and into the prickle cell layers and a similar pattern of immunoreactivity was seen in well differentiated carcinomas (Goodger *et al.*, 1996). In this study (Goodger *et al.*, 1996), a higher intensity of staining and a higher proportion of positive cells were observed in poorly differentiated carcinomas. However, there seems to be little value of this parameter in regard to assessing premalignant lesions.

A relatively new proliferation marker, HsMCM2, a member of the family of minichromosome maintenance proteins thought to be involved with DNA replication, has recently been studied in normal and neoplastic tissues (Todorov *et al.*, 1998). These authors, interestingly, reported a labelling index for antibodies against this antigen between those of Ki67 and PCNA which sounds promising and may prove to truly represent

cycling cells, something which Ki67 and PCNA appear not to do. Furthermore, antibodies raised against the protein did not require antigen retrieval. However, until commercial antibodies become available and more comparative studies have been carried out its usefulness in proliferation studies will be limited.

1.6 Conclusions

From the data presented in the literature review it can be seen that there are many limitations in the previous work in the field of oral precancer. There has been a lack of standardisation by many workers in the presentation of their results making comparisons between centres difficult.

There are clearly problems associated with the assessment of dysplasia. Although Smith and Pindborg (1969) attempted to address this, the technique is laborious and, judging from the few studies that have used the method, unlikely to be used widely. Some aspects of the research in the present thesis will attempt to address this.

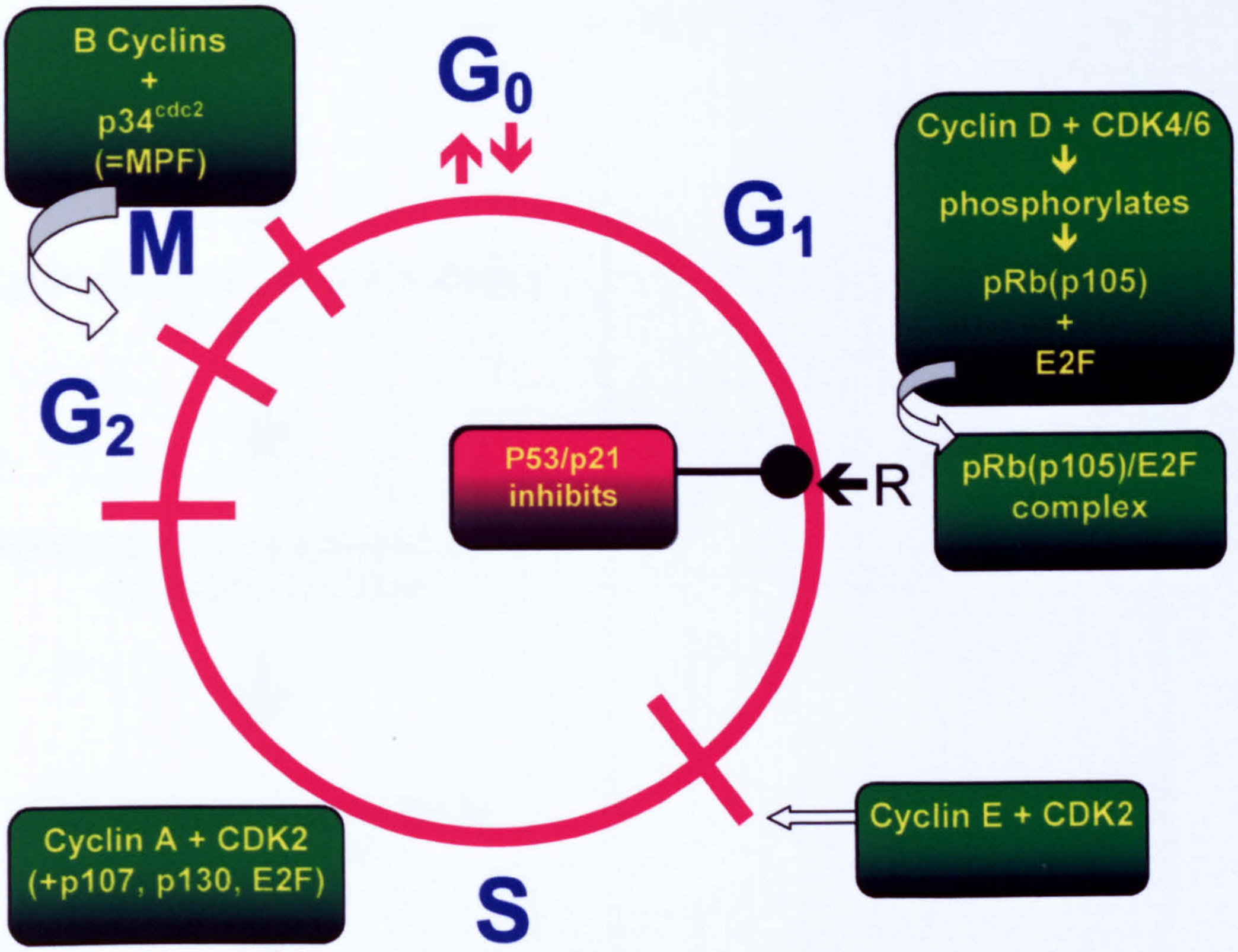
One approach has been to be use immunohistochemistry the interpretation of which is inherently subjective; this is often compounded by workers expressing their results based on a subjective assessment alone. In the studies presented in this thesis the author will attempt to make these results more objective.

Many of the oncogenes involved in the carcinogenic process are related to the cell cycle or intimately involved in the cell cycle. With division of the cell necessary in the development of malignancy it would appear appropriate to study the cell cycle in more detail. This motivated the cell cycle work in this thesis.

Table 1.1 Transformation of oral epithelial dysplasia to squamous cell carcinoma

Author	Country	Number of cases	Number of carcinomas (%)	Transformation time
Mincer *et al* 1972	USA	45	5 (11.1)	up to 8 years
Banoczy and Csisba 1976	Hungary	68	9 (13.2)	1-20 years
Pindborg *et al* 1977	India	61	4 (6.6)	upto 7 years
Silverman *et al* 1984	USA	22	8 (36)	mean 8.1 years
Lumerman *et al* 1995	USA	44	7 (16)	upto 6.5 years
Cowan *et al* 1996	N. Ireland, UK	164	29 (17.6)	data not available

Figure 1.1 The eukaryotic cell cycle



G_1	pre-synthesis gap phase
S	DNA synthesis phase
G_2	post-synthesis gap phase
M	mitosis
G_0	resting phase or terminal differentiation
R	restriction point

Gene and pathway	Effect of component on cell proliferation
p53	-
↓	
Cyclin dependent kinase inhibitors e.g. p16, p21	-
↓	
Cyclins and cyclin-dependent kinases e.g. cyclin D1/CDK4	+
↓	
Retinoblastoma gene family e.g. pRb, p107	-
↓	
Transcription factors e.g. E2F	+
↓	
Cell proliferation ?carcinoma	

Figure 1.2 A possible genetic pathway to carcinoma (Sladek, 1997)
The effects in the right-hand column occur in normal cells to control cell proliferation. In the carcinogenic process, those factors with a plus sign effectively increase their stimulatory effect on the cell cycle, therefore acting as oncogenes. Those factors associated with a negative sign normally exert a negative effect on the progression of the cell cycle, if these effects are lost through deletion, mutation or loss of expression, this inhibitory effect is lost. These therefore act as tumour suppressor genes. In the schema of events above it can be seen that alterations to these factors may lead to an increase in cell proliferation and eventually carcinoma

Chapter 2

Growth fraction and S-phase analysis in oral epithelial dysplasia

2.1 Introduction

2.1.1 Objective assessment of oral epithelial dysplasia

In the practice of histopathology there is clearly a requirement for more objective methods of assessment of oral epithelial dysplasia. It has been shown that it is difficult for pathologists to reach a consensus and even the same pathologist viewing the same section at different times can assess dysplasia differently (Abbey *et al.*, 1995; Abbey *et al.*, 1998; Pindborg *et al.*, 1985). Abbey *et al* (1998) recently showed that consensus was not improved by the inclusion of clinical information and in their study may have even decreased the accuracy attained by a series of examiners.

A semi-objective method of assessing oral epithelial dysplasia is available (Smith and Pindborg, 1969) which has been shown to offer more standardisation in assessing such lesions (Katz *et al.*, 1985). However, it is rather time consuming and has not been adopted by the majority of histopathologists, or indeed researchers, as a means of comparison for parameters being investigated with the histological severity of dysplasia.

Furthermore, there is no histological or other reliable marker of those lesions that will progress to frank malignancy although, in the recognised precancerous lesions of leukoplakia and erythroplakia, there appears to be a higher incidence of malignant transformation in those lesions which show dysplasia histologically (Lummerman *et al.*, 1996).

2.1.2 Prediction of malignant change and cell proliferation

An increased rate of cell proliferation is associated with an

increased risk of malignant transformation and potentially more aggressive behaviour (Iversen, 1993). An increased or uncontrolled cycling of cells allows replication of cells which may harbour genetic mutations or aberrations; one of the necessary steps in the process of carcinogenesis. To bring this into context Farber (1995) doubted the concept of an increase in cell proliferation as a risk in cancer development. The author pointed out that many genotoxic agents are actually inhibitors of proliferation and observed that cell proliferation is increased in many organs and diseases not considered to be precancerous notably the lactating breast, the endometrium and the skin in psoriasis.

^{3}H thymidine was one of the earliest markers to be used in the investigation of the cell cycle. It labels cells in the S-phase of the cell cycle being incorporated into newly formed DNA. As well as giving information on the cell proliferation state it can also give information on the rate of entry into S-phase and mitosis by the use of double labelling techniques (Dover, 1992). However, the technique requires the process of autoradiography for the demonstration of ^{3}H thymidine labelled nuclei which can be both time consuming and a technique sensitive procedure. In lesions of leukoplakia from the buccal mucosa, the ^{3}H thymidine labelling index (LI) has been reported as being increased (Alvares *et al.*, 1972a; Warnakulasuriya and MacDonald, 1995a).

Bromodeoxyuridine (BrdU) is another thymidine analogue which can be incorporated into the replicating DNA, labelling cells in the S-

phase. Using a monoclonal antibody against BrdU an estimate of the cells in the cells in the S-phase can be achieved and give another parameter of the proliferative state of the tissue (Gratzner, 1982). The relative ease of use of immunohistochemistry in the detection of incorporated BrdU over autoradiography makes this method a potentially more attractive and simpler option for the study of the S-phase. The studies of oral epithelial dysplasia to date have been based upon *in vivo* administration of BrdU (Kotelnikov *et al.*, 1996). There are no reported studies using the *in vitro* method described below.

The cell cycle associated and endogenously produced antigen Ki67 can be detected immunohistochemically. Ki67 is reported to be expressed by all cells within the cell cycle (the growth fraction) but not those cells resting or which have left the cell cycle in the G_0 phase (Gerdes *et al.*, 1983). The advent of the production of an antibody against Ki67 which can be detected in formalin-fixed, paraffin-embedded tissue (Cattoretti *et al.*, 1992; Key *et al.*, 1993) has made this parameter very popular among investigators for the study of cell proliferation. However, the studies of oral epithelia are limited in both quantity and quality.

2.1.3 Aims

The aims of the studies described in this chapter were to investigate the S-phase fraction and growth fraction in a series of oral epithelial dysplastic lesions using BrdU and Ki67 and to attempt to correlate these parameters with the semi-objective dysplasia scoring

system of Smith and Pindborg as a way of developing a more objective assessment. By attempting to correlate these indices with clinical outcome, their use as prognostic indicators of malignant transformation was investigated.

2.2 Materials and Methods

2.2.1 Collection of samples

Incisional biopsies of 20 cases were taken from 19 patients with lesions of the tongue or floor of mouth who presented at the Oral Medicine Department at the Glasgow Dental Hospital. The lesions were clinically diagnosed as leukoplakia, erythroplakia or smokers keratosis. The tissue obtained was divided into two halves; the first half was routinely formalin fixed and paraffin-embedded for the diagnostic histopathology service and used subsequently for Ki67 investigation. The other half was transported in the minimal essential medium (MEM) with glutamax (Gibco BRL, 41090-028) for the BrdU labelling which was started promptly within 20 minutes. Sections were examined for the presence of fungal mycelia using diastase PAS.

2.2.2 Bromodeoxyuridine incubation

The method for incubation of the tissue with BrdU was adapted from that previously described by Thornton *et al* (1988). Each individual block of tissue was incubated in 15ml of MEM containing 4×10^{-4} M BrdU (5'-bromo-2-deoxyuridine, Sigma B5002) at 37°C for 15 minutes and 95%

oxygen/ 5% carbon dioxide was bubbled through the solution. The tissue was then fixed in Carnoy's solution (Appendix 2) for one hour and processed through graded alcohols to xylene and embedded in paraffin wax.

Four micrometre sections were cut from the BrdU and routinely processed blocks, mounted on silane-coated slides (Polysine BDH, 406/0178/00) and dried overnight in a 60°C oven.

2.2.3 Preliminary studies

Prior to progressing, the optimal concentrations and incubation times of the anti-BrdU and anti-Ki67 antibodies and antigen retrieval conditions for Ki67 detection were established as follows.

Monoclonal mouse anti-bromodeoxyuridine antibody (M0744, DAKO clone Bu20a (Magaud *et al.*, 1989)) was used. To determine the optimum antibody concentration and incubation time a series of experiments was undertaken with the following variables:

Primary antibody concentration	Time and temperature of incubation
1:20 1:50 1:100 1:150	1 or 2 hours at room temperature or overnight (approx. 16 hours) at 4°C

A qualitative assessment of the staining appearances with these primary antibody dilutions and incubation times was performed to determine the optimal conditions for the tissue studied.

Similarly for Ki67 immunohistochemistry a range of times of antigen

retrieval of between 5 and 50 minutes in five minute intervals was undertaken. Antibody concentrations and incubation times were as for anti-BrdU above.

2.2.4 Bromodeoxyuridine immunohistochemistry

For BrdU immunohistochemistry, slides were dewaxed in xylene and then rehydrated through graded alcohols to water. Endogenous peroxidase was blocked by incubating the sections in 3% hydrogen peroxide in methanol for 20 minutes. The sections were washed in water for 10 minutes which was followed by a 2 x 5 minute wash in Tris-buffered saline (TBS, pH 7.6, Appendix 2). DNA denaturation was performed by immersing the slides in 1M hydrochloric acid at 60°C for 8 minutes and this was followed by two 5 minute washes in TBS. Non-specific antibody binding was blocked by incubation of the sections with normal horse serum for 20 minutes at room temperature in a humidified chamber.

The primary antibody was applied to the BrdU processed sections at the optimal antibody concentration (1:50) for the optimal incubation time (overnight at 4°C) as assessed above.

Following primary antibody incubation, the sections were rinsed in TBS for 10 minutes and incubated in biotinylated anti-mouse IgG antibody (Vectastain ABC elite, Vector PK6102) for 30 minutes at room temperature. Following washing in TBS they were incubated in avidin-biotinylated horseradish peroxidase complex for 30 minutes at room temperature.

Sections were then developed in 0.1% 3,3'diaminobenzidine (D5905, Sigma) in TBS containing 0.1% hydrogen peroxide for 5 minutes.

The sections were counterstained in Mayers haematoxylin (Appendix 3) for 2 minutes and dehydrated through graded alcohols, cleared in xylene and mounted .

2.2.5 Antigen retrieval

For Ki67 immunohistochemistry, the sections were dewaxed and rehydrated through graded alcohols to distilled water. Microwave antigen retrieval was performed by placing the sections in a plastic slide rack in a plastic beaker containing 0.01M sodium citrate buffer (pH 6.0). Sections were heated in a microwave oven (Tricity MT930, 600W) for the optimal time (15 minutes) as described above in periods of 5 minutes. After each 5 minute period the level of the buffer was restored to a predetermined level with distilled water. After heating, the container was removed from the oven and allowed to cool at room temperature for 30 minutes.

2.2.6 Ki67 immunohistochemistry

Sections were washed in TBS buffer for 5 minutes before endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide in methanol for 20 minutes. Sections were washed in distilled water for 2 x 5 minutes followed by TBS for 2 x 5 minutes.

Non-specific antibody binding was blocked by incubation of the sections with normal horse serum for 20 minutes at room temperature in a humidified chamber.

The Ki67 antibody (NCL-Ki67-MM1, Novocastra, clone MM1) was applied to the microwave treated sections at the optimal antibody concentration (1:100) and incubation time (2 hours).

Following primary antibody incubation, the sections were rinsed in TBS for 10 minutes and incubated in biotinylated anti-mouse IgG antibody (Vectastain ABC elite) for 30 minutes at room temperature. Following washing in TBS they were incubated in avidin-biotinylated horseradish peroxidase complex for 30 minutes at room temperature.

Sections were then developed in 3,3'diaminobenzidine and counterstained, dehydrated cleared and mounted as described above.

2.2.7 Controls

Positive controls were sections of tissue previously incubated with BrdU in this laboratory and sections of human tonsil were used for Ki67 staining. Negative controls were prepared by way of omitting the primary antibody from the sections.

2.2.8 Quantification

The quantitative assessment of the labelled nuclei was performed using a Leitz Ortholux microscope and a locally developed computerised planimetry system (Image Analysis System version 4.01, ©Video Vector Dynamics Ltd 1989). An eye-piece graticule was used to select a column of the epithelium at right angles to the epithelial surface, the width of one graticule in from the left hand side of the section, at an objective magnification of x16. Three adjacent, non-overlapping columns were

counted in three levels of the tissue in each case according to a method previously reported (Al-Damouk and MacDonald, 1987). The same selection procedure was adopted for the subsequent studies in the thesis. The immunohistochemically labelled cells, total number of viable keratinocyte nuclei and the number of labelled suprabasal cells (i.e. those not in contact with the basement membrane) were counted. The length of the basement membrane (BL) in the field was measured by means of a light spot projected into the microscope field from a mouse on a digitiser pad. The labelling index (LI) for BrdU was expressed in terms of the total nucleated cells (LI%) and per millimetre (mm) basement membrane length (LI/BL). The percentage of suprabasal labelled cells was calculated (%SB). Likewise, the growth fraction (GF) for Ki67 was expressed in the same units. In order to ensure consistency of counting the first five cases were re-quantified at the end of the initial quantification.

A compartment analysis was performed on haematoxylin and eosin (H&E) stained (Appendix 3) sections of the same blocks, preceding those used for the growth fraction analysis. Using the computerised planimetry system at an objective magnification of x25, again in defined fields and close to those used for the GF analysis, the cells of the progenitor (PC) and maturation (MC) compartments were identified on morphological grounds as previously described (Eveson and MacDonald, 1978) and then counted. A distinction was made in the progenitor compartment between cells which were basal and those which were suprabasal and the length of

the basement membrane was measured. The areas of the viable cell compartments were measured. The area values were divided by the width of the counting graticule to provide values for mean epithelial thickness as well as for the individual compartments. The keratinised layer was not quantified (when present) as it was frequently observed to be partially absent or fragmented.

The degree of dysplasia was assessed using the semi-objective atypia scoring technique of Smith and Pindborg (1969). Sections stained with H&E from both the BrdU processed and routinely processed tissue blocks were scored. The sections were examined by the author and another examiner. For each case, the mean score for each of the individual features from each observer was used to reach the final atypia score. The details of the relative weightings applied to each of the 13 features of dysplasia assessed are listed in Appendix 4. A subjective report was provided by the routine diagnostic service assessing the degree of dysplasia as mild, moderate, severe or carcinoma in-situ. Sections were examined for the presence of fungal mycelia using diastase PAS (Appendix 3).

Correlations between the LI, GF, compartment sizes and atypia scores were investigated statistically using the "Spearman-Rank" correlation test. The values of significance were all taken from the monograph by Siegel and Castellan (1988).

2.2.9 Shrinkage of Carnoy's fixed tissue

Whilst quantification was being performed an appreciable shrinkage of the Carnoy's fixed tissue was observed compared to the formalin fixed tissue. To quantify this process the areas of 20 lymphocyte nuclei were measured in both the Carnoy's fixed and formalin fixed tissues using the interactive mode of the Kontron KS300 image analysis software (described in more detail in Chapter 4). Lymphocytes were chosen as it was assumed these would be the most consistent cells in terms of size. The means of these measurements were calculated for each tissue.

2.2.10 Clinical parameters

The following clinical details of the patients were obtained at the time of biopsy: smoking and alcohol habits, medical history and drug history. Additionally, haematologic and biochemical profiles for each patient were available. The patients records were consulted at regular intervals throughout the study period to determine the progression or regression of the lesions.

2.3 Results

2.3.1 Preliminary studies

For BrdU immunohistochemistry, an antibody concentration of 1:50 was deemed the minimum dilution to give optimal and consistent staining results and this was best after an overnight incubation at 4°C. Immunohistochemical staining of the sections with BrdU gave little

discernible background staining, generalised crisp nuclear staining was observed as demonstrated in Figure 2.1.

The Ki67 staining was deemed optimal with an antibody concentration of 1 in 100 incubated at room temperature for 2 hours but was more variable than BrdU staining with a number of differing patterns being observed; a nucleoplasmic staining, strong nucleolar staining and finely speckled cytoplasmic staining (Figure 2.2). In several cases, a clear lack of Ki67 positivity was evident in the basal layers of the epithelium (Figure 2.3) and occasionally mitoses were conspicuously lacking in positivity (Figure 2.4) . Positive Ki67 cells were counted if they exhibited the staining patterns of strong nucleolar and nucleoplasmic staining but not when they showed only weak cytoplasmic staining. Occasional positively stained lymphocytes were observed in the epithelium and these were excluded from the counts.

2.3.2 Patient details

The patient clinical details, subjective grading of dysplasia, atypia scores and epithelial thickness are presented in Table 2.1. The mean age of the patients was 59.6 years (range 31 to 84 years), 12 of whom were female (mean age 56.5) and 7 male (mean age 65.6). All the patients except case 12 admitted to being smokers or previous smokers consuming at least five cigartettes per day or half an ounce of tobacco per day. Alcohol consumption was admitted by half of the patient group. Mild iron deficiency was found in Case 2, all of the other cases had

haematologic and biochemical values within normal ranges. Only two cases showed the presence of fungal mycelia (Cases 11 and 15) which were from the same patient taken four months apart.

2.3.3 Quantitative results

An example of how the results were obtained is shown in Table 2.2. The BrdU labelling indices, growth fraction and compartment analysis are shown in Table 2.3. These results were obtained by adding the measurements obtained for each of the three fields at each level and taking an average for the three levels.

The mean BrdU LI% was 10.80% (SD 3.23) and the mean LI/BL was 46.58/mm (SD 20.75) and an average of 40.51% (SD 14.30) labelled cells were suprabasal.

The mean GF%, as identified by Ki67, was 27.82% (SD 16.31) and the GF/BL was 141.31/mm (SD 116.58) with a mean of 71.49% (SD 6.05) Ki67 stained cells being suprabasal.

The mean measurements and indices were similar for each of the three levels, it was therefore deemed unnecessary in further studies to quantify at three levels.

From the compartment analysis it was possible to calculate a mean epithelial thickness as described above which gave an average value of 229.09µm (SD 104.73). The mean PC% was 71.49 (SD 6.05) and the mean PC thickness was 127.53µm (SD 69.85) while the mean MC% was 28.51 (SD 6.05) and mean MC thickness was 101.56µm (SD 48.72). The

mean ratio of the PC:MC was 2.72 (SD 1.08). The morphological PC was clearly greater than the GF identified by Ki67 and is illustrated in Figures 2.5 and 2.6.

Using the Spearman rank correlation test, significant correlations were demonstrated between the BrdU labelling index per mm basement membrane length (LI/BL) and the atypia score (r_s=0.6836, $p<0.01$). The GF/BL correlated with the LI/BL (r_s=0.7292, $p<0.001$) but the growth fraction did not correlate with the atypia scores. By contrast, the PC% correlated with the atypia score ($p<0.02$).

The mean ratio of labelling index to growth fraction using the data LI% and GF% was 0.46 within a wide range of 0.11 to 0.94. This index did not correlate with the atypia scores. Comparisons of the indices expressed in relation to the basement membrane length were not felt to be valid because of the varied shrinkage due to the two fixatives.

2.3.4 Shrinkage of Carnoy's fixed tissue

The mean areas of the lymphocytes in the Carnoy's fixed tissues and the formalin-fixed tissues were 18.54 μm^2 and 23.23 μm^2 respectively equating to mean diameters of 4.85 µm and 5.43 µm. The mean ratio of the Carnoy's fixed tissues to the formalin fixed tissues was 0.89 indicating an average linear shrinkage of 11% of the Carnoy's fixed tissues more than the formalin fixed tissues.

2.3.5 Clinical follow-up

The 20 patients of this series were followed up for at least 12

months, the first eight patients for a period in excess of 40 months. Cases 4, 7, 12, 14 and 15 underwent surgical excision of their lesions. Case 6 whose lesion showed severe dysplasia refused further treatment and has been lost to follow-up.

Of the patient series, one lesion (Case 14) has undergone clinically evident malignant transformation at the same site as the biopsy over a period of 6 months (Figures 2.7a and 2.7b). This case had a LI:GF of 0.6 which was one of the higher of the series despite having low atypia score, labelling index and growth fraction in comparison to the rest of the series.

One case (Case 12) had early invasive squamous cell carcinoma evident on surgical excision of the lesion. The LI:GF of this case was 0.49, again above average. Interestingly, this was the patient who did not report to any previous or current smoking habits.

Two of the lesions studied were taken from the same patient (Cases 11 and 15). Over the four month period between the two biopsies, the lesion progressed from homogenous leukoplakia to erythroleukoplakia. The histology of the two biopsies was similar but the LI:GF reduced by half during this period from 0.40 to 0.20.

2.4 Discussion

2.4.1 S-phase analysis

The pyrymidine analogue, BrdU was used to label cells undergoing DNA synthesis to enable an estimate of those cycling cells which were in

the S-phase.

Previous proliferation studies in oral leukoplakia of the buccal mucosa using tritiated thymidine have reported a higher LI% compared to clinically normal epithelium (Alvares *et al.*, 1972a; Warnakulasuriya and MacDonald, 1995a). In addition Warnakulasuriya and MacDonald (1995a) reported a significant correlation between a raised LI and the severity of dysplasia which is supported by the results of the present study where it has also been demonstrated that the LI/BL was correlated with the atypia score. The LI% in the present study was higher than that reported for tritiated thymidine labelling, but this could reflect differing cell turnover rates of buccal mucosa and tongue/ floor of mouth.

A diurnal variation in the labelling index in human buccal epithelium was demonstrated using tritiated thymidine (Warnakulasuriya and MacDonald, 1993). This has been shown to relate to a variable rate of transition of cells from G_1 to S-phase (Warnakulasuriya and MacDonald, 1995b). Since all of the biopsies in the present series were taken during a small window of time (approximately 10am to 12 midday) it is unlikely this phenomenon would have influenced the labelling indices.

A recent study investigated the LI% of non-involved oral mucosa adjacent to tumours using *in vivo* administration of BrdU or iododeoxyuridine (IrdU) (Kotelnikov *et al.*, 1996). These workers reported a low LI% (1.6%) for the basal cell layers but much higher LI% for the suprabasal layers (31.6%) in cytologically normal epithelia in 14 untreated

patients. Seven cases of dysplastic epithelium showed a mean LI% of 1.4% and of 36.6% for the basal and suprabasal layers respectively. These figures were significantly different from those calculated for the present study of 6.26% and 4.54% for the two respective layers and probably reflects the different mode of administration of halogenated pyrimidines to the samples. *In vitro* labelling may underestimate that of *in vivo* labelling (Chavaudra *et al.*, 1979) but the duration of *in vivo* administration is longer than the short pulse labelling technique *in vitro*. Also, there is a delay in harvesting of the tissues following administration of BrdU in most *in vivo* studies and it could be expected that the basal cells would have moved into suprabasal positions by this time to account for this. There may also possibly be differences in sampling for quantification. The study of Kotelnikov *et al* (1996) used both enzymatic digestion and 4M HCl for the denaturation of the DNA compared to the present study using 1M HCl only. The use of the former has been shown to produce more intense staining than 1M HCl in other studies (Frank *et al.*, 1995).

Using homogenised samples of oral squamous cell carcinoma, one study found a median BrdU LI% of 2.6% (Hemmer, 1990), much less than the present study (9.89%). Kotelnikov *et al* (1995) using *in vivo* BrdU administration reported a mean LI% almost double that of previous investigations they reviewed using similar techniques, with a BrdU LI% of over 50% for one poorly differentiated carcinoma. The authors did not

comment upon the clinical behaviour of such lesions

2.4.2 Growth fraction analysis

The present study utilised an antibody against the proliferation antigen Ki67, which is said to be expressed only in cells within the cell cycle, to give an estimate of the growth fraction of the lesions. The vast majority of studies involving Ki67 to date have utilised the MIB1 antibody. The present study used a relatively new Ki67 antibody said by the manufacturers to be equivalent to MIB3 as characterised by Key *et al* (1993).

The distribution of Ki67 during the cell cycle has been described (van Dierendonck *et al.*, 1989) and shows a very distinct localisation pattern. During interphase there is prominent nucleolar staining with diffuse nucleoplasmic staining. As the chromosomes condense, staining becomes nucleoplasmic and concentrated around the chromosomes. In metaphase, there is staining of a reticular network of fibrils around the chromosomes which has a more granular appearance. The density of Ki67 staining is greatest during anaphase, still granular around the chromosomal network. As the daughter nuclei form and the chromosomes condense in telophase, the staining becomes speckled throughout the nucleoplasm and returns to become nucleolar as the cell enters the interphase. These observations suggest an association of Ki67 with the RNA of the nucleolus and a structural role in nucleoli and during mitosis in association with the chromosomes (Ross and Hall, 1995).

The use of Ki67 monoclonal antibodies has been made possible on formalin fixed, paraffin embedded material by the use of antigen retrieval either by microwave, autoclave or pressure cooker (Shi *et al.*, 1991). A degree of caution should be exercised when interpreting results following antigen retrieval as varying staining patterns have been reported (Dowell and Ogden, 1996; Gee *et al.*, 1995; Mighell *et al.*, 1995). Indeed, in the present study, Ki67 demonstrated variable staining patterns as described above. The subject of antigen retrieval for immunohistochemistry is discussed further in Chapter 3.

Ki67 is reported to be expressed in all phases of the cell cycle except G_0 and early G_1 (Gerdes *et al.*, 1983). Hence, it can provide an estimate of the growth fraction defined as those cells proliferating or growing in a population. Clearly in this series of cases the GF identified by Ki67 was less than that identified morphologically by means of the compartment analysis. As will be discussed in more detail in Chapter 4, it is becoming evident that Ki67 has a shorter half-life than originally believed and is actively produced from the beginning of the S-phase reaching a peak around the G_2/M transition; thereafter its production being below immunohistochemically detectable levels (Bruno and Darzynkiewicz, 1992; Lopez *et al.*, 1991). Therefore cells in the G_1 phase may not be detected by Ki67 antibodies and this may account for the discrepancy noted in the present study. Cells in the G_0 phase and early post-mitotic maturing cells are not part of the growth fraction but are

indistinguishable from cycling cells on morphological grounds (Hume, 1981). Furthermore, stem cells are slowly cycling cells with a long G_1 phase (Lajtha, 1983) found in the basal epithelial layers and therefore on the basis of the information presented above may not express detectable Ki67.

A high proportion (68%) of the cycling cells were demonstrated to be suprabasal using the Ki67 antigen in the present study. Also, it was observed that several cases demonstrated areas lacking Ki67 positivity in the basal layers (Figure 2.3). This has been reported previously in dysplastic oral epithelium (Bryne *et al.*, 1995; Girod *et al.*, 1993; Kannan *et al.*, 1996) and in both normal and dysplastic cervical epithelia (Steinbeck, 1997) but the significance of this is not known. It is also not known whether this phenomenon occurs in normal oral epithelium although it was frequently observed in the epithelium of the tonsils in the positive controls. The studies in Chapter 4 will attempt to address this.

Kushner *et al* (1997) recently studied Ki67 labelling indices in oral epithelial dysplasia of the floor of the mouth. A mean GF/BL of 85.06 was reported by the authors which is considerably less than the present study. However, this may be accounted for by the differing sampling methods. Kushner *et al* (1997) measured the basement membrane length and performed cell counts of the entire length of the epithelium. The biopsy specimens would have been different sizes and measuring the epithelium in its entirety may have included areas of normal mucosa, a phenomenon

observed in the present study as well as by others (Lummerman *et al.*, 1996). Additionally, measuring and expressing such indices per millimetre basement membrane length takes no account of the epithelial thickness. Indeed, in the present study a wide range of epithelial thickness was observed from 59.51 to 437.57 µm.

The progenitor compartments were large in the present study, being as much as six times as large as the maturation compartments, and correlated with the atypia scores. Histologically, this would be reflected in the observation of basal cell hyperplasia. A high incidence of basal cell hyperplasia was reported by Wright and Shear (1985) in their series of cases of oral epithelial dysplasia adjacent to squamous cell carcinomas. This epithelial feature has a relatively low weighting in the atypia scoring system of Smith and Pindborg (1969) used in the present study (Appendix 4).

2.4.3 Relation of the S-phase to growth fraction

Using the labelling index as an estimate of cells in the S-phase it was possible to estimate the proportion of cycling cells that were in the S-phase. The cases that have progressed to clinically overt malignancy (Cases 12 and 14) had a higher than average proportion of cycling cells in the S-phase despite case 14 having a labelling index, growth fraction and atypia score in the middle of the range. The LI:GF may therefore be of prognostic significance. There are certain shortcomings to this assumption; firstly the two parameters were measured on different blocks

of tissue, albeit from the same biopsy, and shrinkage was observed in the Carnoy's fixed tissue. Secondly, the variation in antigen detection using microwave antigen retrieval has already been mentioned above. The follow-up period of the cases in the present study was relatively short, it is well known that the natural history of leukoplakia is very variable and often protracted with some lesions undergoing malignant transformation over time periods in excess of 20 years (Lummerman *et al.*, 1996). A further consideration is that of sampling of lesions for the biopsy as it was possible that a carcinoma was already present in the lesion shown in Figure 2.7a at a different site to that of the biopsy. In lesions of this appearance and size there maybe an indication for more than one biopsy from different parts of the lesion. Interestingly, from another study performed in Glasgow which included some of the same cases as the present study, cells from the original biopsy of Case 14 proved to have an immortal phenotype when grown in culture (F.McGregor, personal communication).

Warnakulasuriya and Johnson (1993) on studies of murine ventral tongue epithelium using *in vitro* BrdU and PCNA (estimating the growth fraction) reported a LI:GF of 0.25, approximately half of that obtained in the present study. However, PCNA has the opposite drawback to Ki67 in overestimating cycling cells and may account for this discrepancy.

In the present study, case 12 was found to have early invasive carcinoma on excision of the entire lesion. This is a case which clinically

would have been labelled as idiopathic leukoplakia by virtue of the fact that there was no association of the lesion with tobacco or any other agent(van der Waal *et al.*, 1997). The most recent WHO classification of leukoplakia (Axéll *et al.*, 1996) has dismissed this term for reasons of clarity and consistency. However, as this case demonstrates, the aetiology of such lesions clearly differs from the so-called tobacco-associated lesions and the natural history differs, with the possibility that such lesions have a greater tendency towards malignant transformation. There appears to be a justification for retaining the use of this term. With this being only one case out of a series of 20, it also demonstrates that such idiopathic leukoplakias are much fewer in number making larger scale studies potentially more difficult. It certainly warrants further study. Koch and McQuone (1997) recently reviewed and discussed the subject of oral squamous cell carcinoma development in the non-smoker and non-drinker. These authors quoted studies where development of carcinoma was significantly greater in non-smoking females and it was suggested this may be due to chronic anaemia associated with menstruation. Also, there may be inherited defects in enzymes required for metabolising carcinogens and DNA repair as well as a family history of cancer but the association is not as strong as in other sites such as the colon and breast (Koch and McQuone, 1997).

Fungal mycelia were demonstrated in two lesions (11 and 15) and as may be expected the proliferative indices of these cases were above

the mean for the group as a whole. The number of cases with demonstrable fungal infection was comparable with the studies of Barrett *et al* (1998) who recently found 12.3% of dysplastic lesions showed fungal infection in a series of nearly 600 cases. These authors also noted that in subsequent biopsies of such lesions the degree of dysplasia worsened in severity in more instances compared to cases in which fungi were never demonstrated.

Cruchley *et al* (1998) investigated cell proliferation in oral hairy leukoplakia using the same markers as the present study. These workers reported mean LI/BL and GF/BL values substantially lower than the present series and found no difference between lesional and normal mucosae but observed a reduction of basal BrdU positive cells in the presence of EBV. By contrast, cytomegalovirus (CMV) has been shown to increase immunohistochemically detectable Ki67 (Mate *et al.*, 1998a).

In primary squamous cell carcinoma of the head and neck, an *in vivo* BrdU LI accounted for up to half the growth fraction as assessed by Ki67 in one study (Wilson *et al.*, 1996). These workers also noted a wide intra- and inter-patient variation in the BrdU LI particularly. This, again, raises the problem in studies of this kind of adequate and representative sampling of lesions.

The GF/BL correlated with the LI/BL but these indices did not correlate with each other when expressed as a percentage of the total cell numbers. This may be due to the fact that the length of the basement

membrane does not take into account the wide variation in epithelial thickness which was observed in these cases.

Recent investigations using Ki67 and BrdU in prostate carcinoma demonstrated a correlation between LI and GF (Limas and Frizelle, 1994) and a high GF measured by Ki67 was predictive for clinical progression (Bubendorf *et al.*, 1996). The prostate is a stable tissue in terms of cell proliferation whereas the oral epithelium is labile and perhaps these results are not surprising. In a separate publication Limas *et al* (1993) inferred that the variable staining intensity of Ki67 positive nuclei may have introduced inconsistencies and subjectivity even when performed in fresh frozen tissue.

Langford *et al* (1996) reported a correlation between GF and LI assessed by Ki67 and BrdU in meningiomas. However this study was primarily concerned with investigating the use of Ki67 as an alternative marker of proliferation to BrdU rather than for interpreting the cell cycle in detail.

2.5 Summary and conclusions

In the present chapter, the BrdU labelling index has provided a further objective parameter in the assessment of oral epithelial dysplasia. The use of this in a routine laboratory may be limited due to the lengthy processing time, however, formalin fixation of the tissue would reduce this somewhat and will be investigated in Chapters 3 and 6.

The LI:GF ratio may prove useful as a prognostic indicator with regard to malignant transformation in the lesions of leukoplakia. Due to the nature of such lesions, longer-term follow-up is required to support this further. Also, these deductions were made upon tissue sections some distance from each other with an appreciable tissue shrinkage occurring in the Carnoy's fixed tissue. Again, formalin fixation of the BrdU processed tissue would overcome this shortfall.

Lesions of leukoplakia that are not associated with tobacco usage may behave in a more aggressive manner undergoing malignant transformation relatively early in their natural history. A larger study of a cohort of such lesions would be useful.

Finally, the immunohistochemical staining obtained following antigen retrieval for Ki67 was not ideal. This may be overcome by further experimentation with this technique including the use of alternative retrieval buffers and methods of heating. This will be addressed in Chapter 3.

Table 2.1. Patient clinical details, subjective grading, epithelial thickness and atypia scores

	Sex	Age	Clinical Lesion	Site of biopsy	Histology	Epithelial thickness (μm)[1]	Atypia score
1	F	52	speckled leukoplakia	fom[2]	mild dysplasia/ lichenoid	220.93	19
2	F	31	leukoplakia/ erythroplakia	fom	mild dysplasia/ lichenoid	119.74	12
3	M	61	leukoplakia/ erythroplakia	fom	severe dysplasia	166.33	55
4	F	63	smokers keratosis	fom	mild-moderate dysplasia	173.00	19
5	F	74	leukoplakia	fom	mild dysplasia	120.91	10
6	F	65	leukoplakia	fom	moderate-severe dysplasia	221.63	41
7	F	32	erythroplakia	fom	moderate dysplasia	242.17	24
8	F	69	leukoplakia/ erythroplakia	fom	mild-moderate dysplasia	161.31	19
9	M	47	speckled leukoplakia	fom	severe dysplasia/ carcinoma in situ	360.24	57
10	F	54	smokers keratosis	fom	mild to moderate dysplasia	179.31	37
11	M	77	leukoplakia[3]	ton	moderate dysplasia	314.78	35
12	F	62	leukoplakia	ton	severe dysplasia	425.56	47
13	F	62	leukoplakia	ton	mild dysplasia	184.78	26
14	F	84	leukoplakia	ton	mild dysplasia	437.57	22
15	M	77	erythroplakia[3]	ton	moderate dysplasia	325.81	34
16	M	70	smokers keratosis	fom	mild to moderate dysplasia	180.89	18
17	M	83	leukoplakia	ton	mild to moderate dysplasia	59.51	25
18	F	55	leukoplakia	fom	minimal dysplasia	165.41	14
19	F	31	leukoplakia	fom	mild to moderate dysplasia	173.73	17
20	M	44	keratosis	ton	minimal dysplasia	348.17	19

Notes:

1. epithelial thickness refers to the viable cell layers only (progenitor and maturation compartments)
2. fom= floor of mouth, ton= tongue
3. biopsies 11 and 15 were from adjacent areas of the tongue in the same patient obtained at different time

Table 2.2 Derivation of labelling indices

Case	Level One Summary			
	Total BL	Total LC	Total LC SB	Total Cells
1	3150.71	158	55	1162
2	1968.55	67	25	613
3	2321.68	229	77	928

Case	Level Two Summary			
	Total BL	Total LC	Total LC SB	Total Cells
1	3133.82	108	48	1420
2	2508.16	133	59	1200
3	2896.09	138	66	958

Case	Level Three Summary			
	Total BL	Total LC	Total LC SB	Total Cells
1	4256.70	149	43	1385
2	2220.08	71	14	859
3	2957.58	239	115	1034

Case	BrdU Summary: Average of 3 Levels			
	BL	LC	LC SB	TNC
1	3513.74	138.33	48.67	1322.33
2	2232.26	90.33	32.67	890.67
3	2725.12	202.00	86.00	973.33

BrdU Summary

Case	LI%	LI/BL	%SB
1	10.46	39.37	35.18
2	10.14	40.47	36.16
3	20.75	74.13	42.57

BL: basement membrane length (μm)
LC: BrdU labelled cells
SB: suprabasal
TNC: total viable nucleated cells
LI%: BrdU labelling index expressed per 100 viable nucleated cells
LI/BL: BrdU labelling index expressed per millimetre basement membrane length

Table 2.3 Estimated labelling indices, growth fraction and compartment analysis.

Case	BrdU Summary			Ki67 Summary			Compartment analysis				LI:GF	PC:MC
	LI%	LI/BL	%SB	GF%	GF/BL	%SB	PC%	MC%	PC (µm)	MC (µm)		
1	10.46	39.37	35.18	18.38	79.62	51.67	65.03	34.97	129.52	91.41	0.57	1.86
2	10.14	40.47	36.16	20.89	122.71	82.56	62.55	37.45	56.41	63.33	0.49	1.67
3	20.75	74.13	42.57	21.97	111.21	89.22	76.98	23.02	112.19	54.15	0.94	3.34
4	9.00	39.53	27.80	21.64	99.75	55.23	68.74	31.26	58.24	114.76	0.42	2.20
5	7.04	32.44	40.13	28.44	158.30	69.98	70.53	29.47	45.56	75.35	0.25	2.39
6	8.23	59.03	58.74	73.12	553.35	92.76	69.77	30.23	126.07	95.56	0.11	2.31
7	7.85	39.46	23.02	25.14	90.11	58.23	67.11	32.89	114.17	128.00	0.31	2.04
8	10.36	57.65	37.74	23.92	132.01	58.22	66.94	33.06	86.85	74.46	0.43	2.02
9	15.48	114.28	87.63	56.15	283.43	86.56	80.88	19.12	229.00	131.24	0.28	4.23
10	9.20	44.46	45.38	23.40	130.86	76.49	71.93	28.07	94.06	85.26	0.39	2.56
11	14.51	52.85	40.85	36.73	153.07	78.80	76.47	23.53	187.04	127.74	0.40	3.25
12	9.10	42.14	28.15	18.70	89.53	80.33	86.16	13.84	167.04	258.52	0.49	6.22
13	11.14	43.66	32.97	19.11	90.06	78.99	67.50	32.50	88.13	96.65	0.58	2.08
14	7.57	34.36	34.20	12.63	65.75	70.65	72.17	27.83	267.93	169.65	0.60	2.59
15	12.21	59.46	47.57	60.05	294.99	65.53	73.92	26.08	205.61	120.20	0.20	2.83
16	8.98	29.92	48.24	14.21	69.74	46.20	65.09	34.91	97.25	83.63	0.63	1.86
17	9.42	17.02	20.21	21.74	58.68	68.18	67.35	32.65	28.89	30.62	0.43	2.06
18	12.66	38.04	43.90	21.99	84.19	53.47	67.89	32.11	87.02	78.39	0.58	2.11
19	12.24	37.60	39.93	22.99	97.90	50.26	73.04	26.96	109.62	64.11	0.53	2.71
20	9.64	35.82	39.869	15.17	60.85	51.08	79.81	20.19	260.02	88.14	0.64	3.95
Mean	**10.80**	**46.58**	**40.51**	**27.82**	**141.31**	**68.22**	**71.49**	**28.51**	**127.53**	**101.56**	**0.46**	**2.72**
SD	3.23	20.75	14.30	16.31	116.58	14.52	6.05	6.05	69.85	48.72	0.19	1.08
Min	7.04	17.02	20.21	12.63	58.68	46.20	62.55	13.84	28.89	30.62	0.11	1.67
Max	20.75	114.28	87.63	73.12	553.35	92.76	86.16	37.45	267.93	258.52	0.94	6.22

Indices expressed as labelled cells per 100 total nucleated cells (LI% and GF%), basement membrane length (LI/BL and GF/BL), the proportion of suprabasal labelled cells (%SB) and the progenitor (PC) and maturation (MC) compartments expressed as percentage nucleated cells and as thickness for each compartment

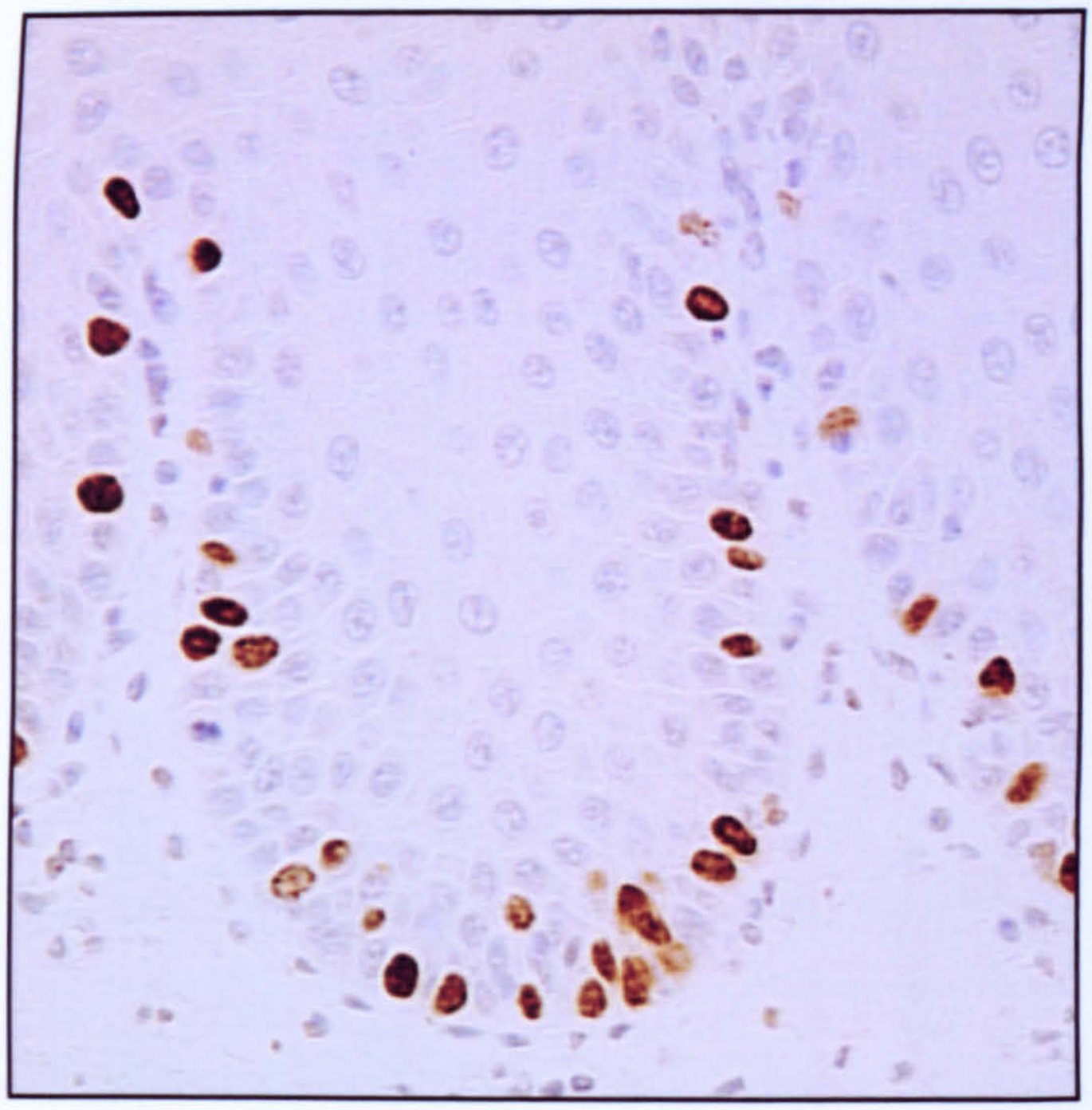


Figure 2.1 Demonstration of BrdU immunohistochemistry in Case 7 (magnification x478)

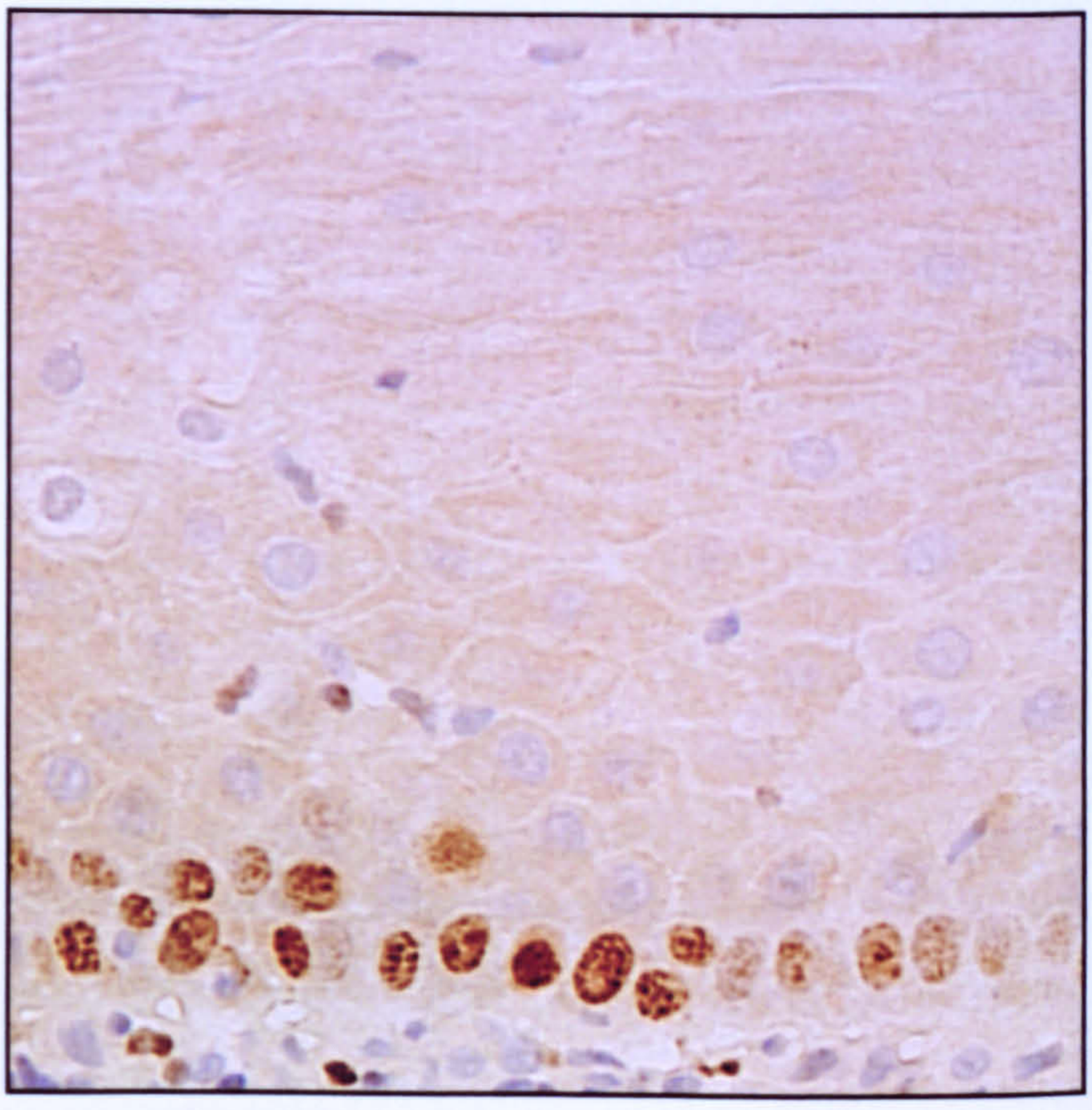


Figure 2.2 Demonstration of Ki67 immunohistochemistry in Case 4 (magnification x478)

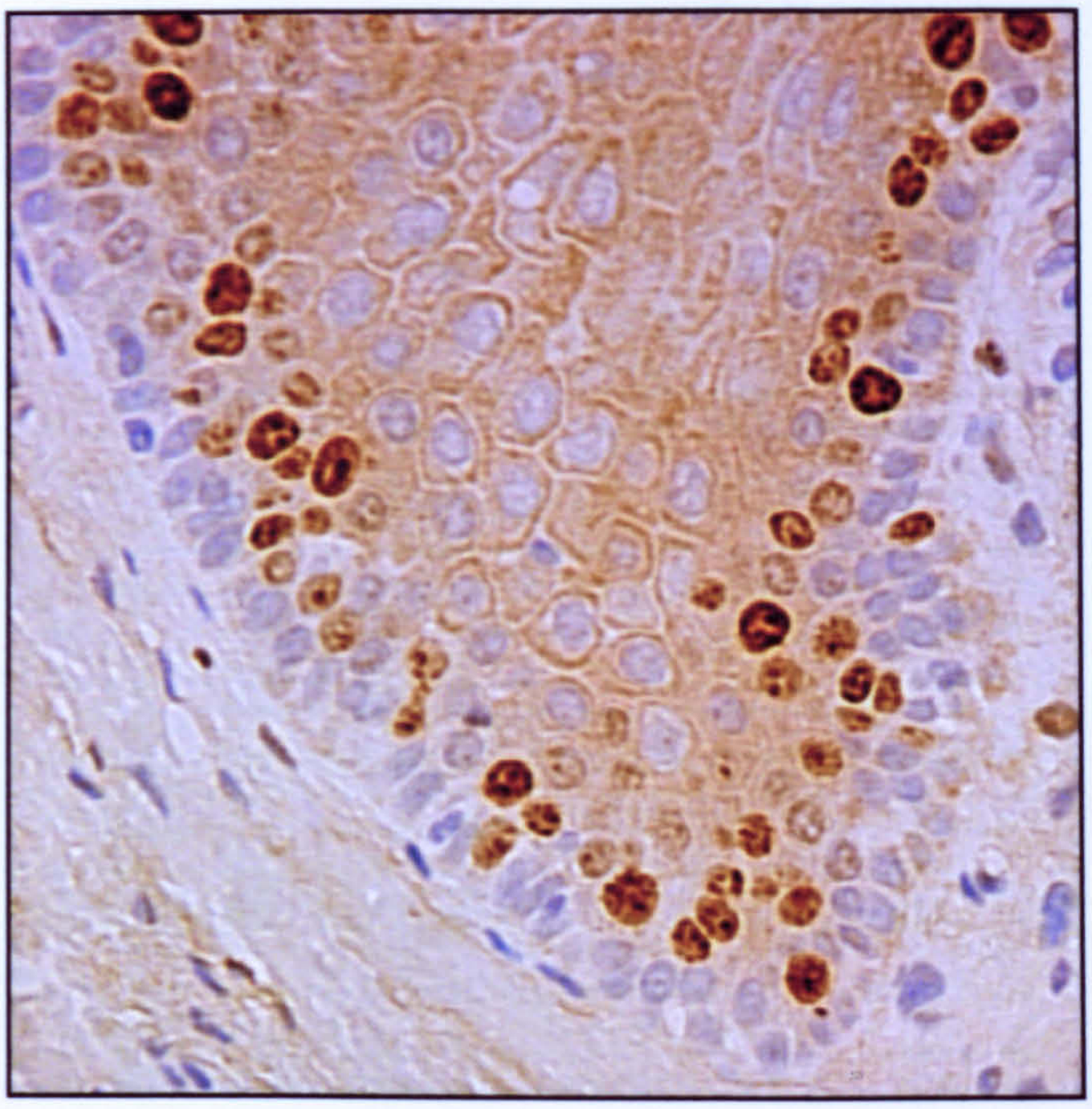


Figure 2.3 Lack of basal staining with Ki67 antibody in case 12 (magnification x478)

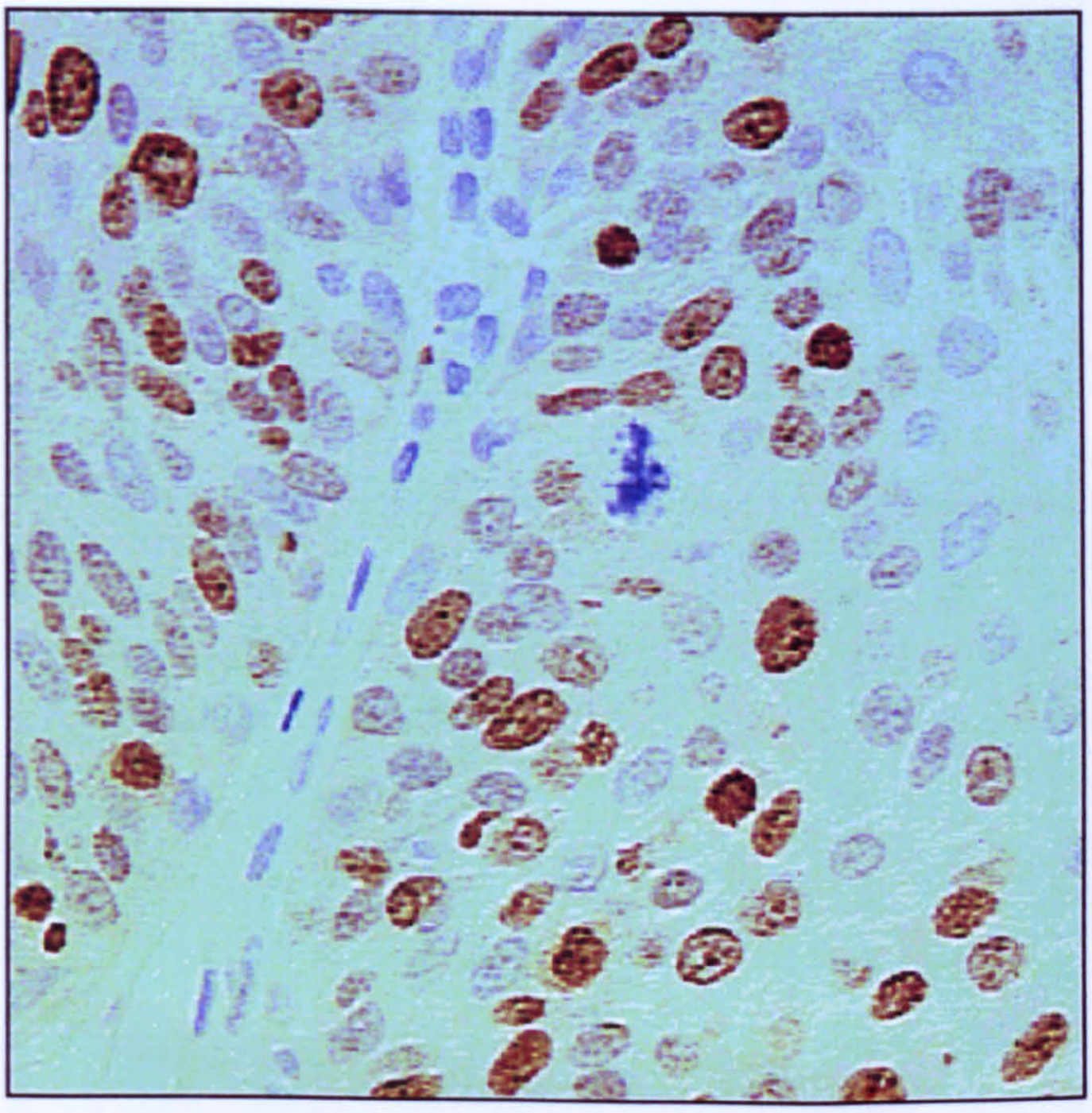


Figure 2.4 Lack of Ki67 positivity of a mitosis in Case 9 (magnification x478)

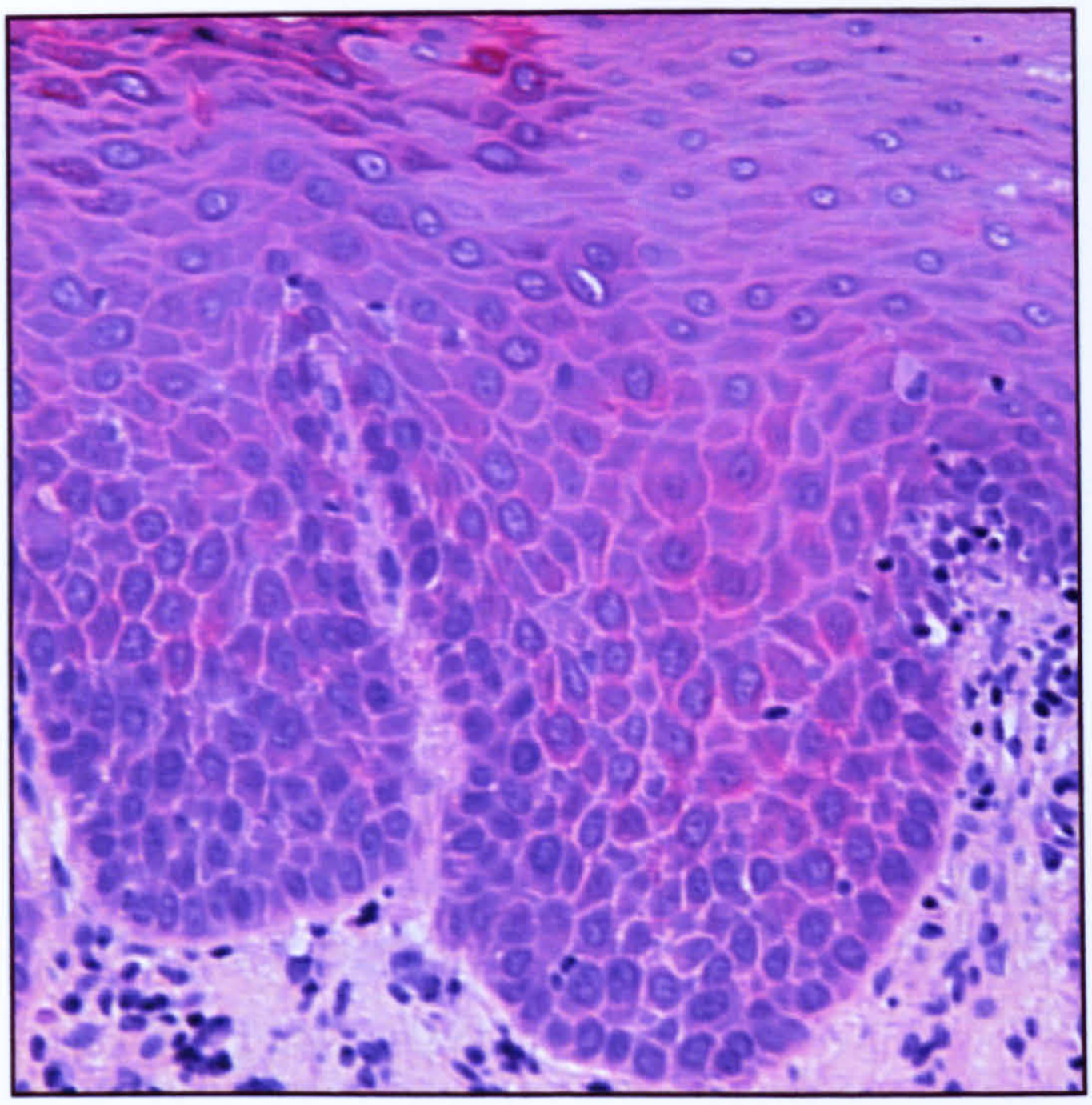


Figure 2.5 Haematoxylin and eosin stained section of Case12 (magnification x236)

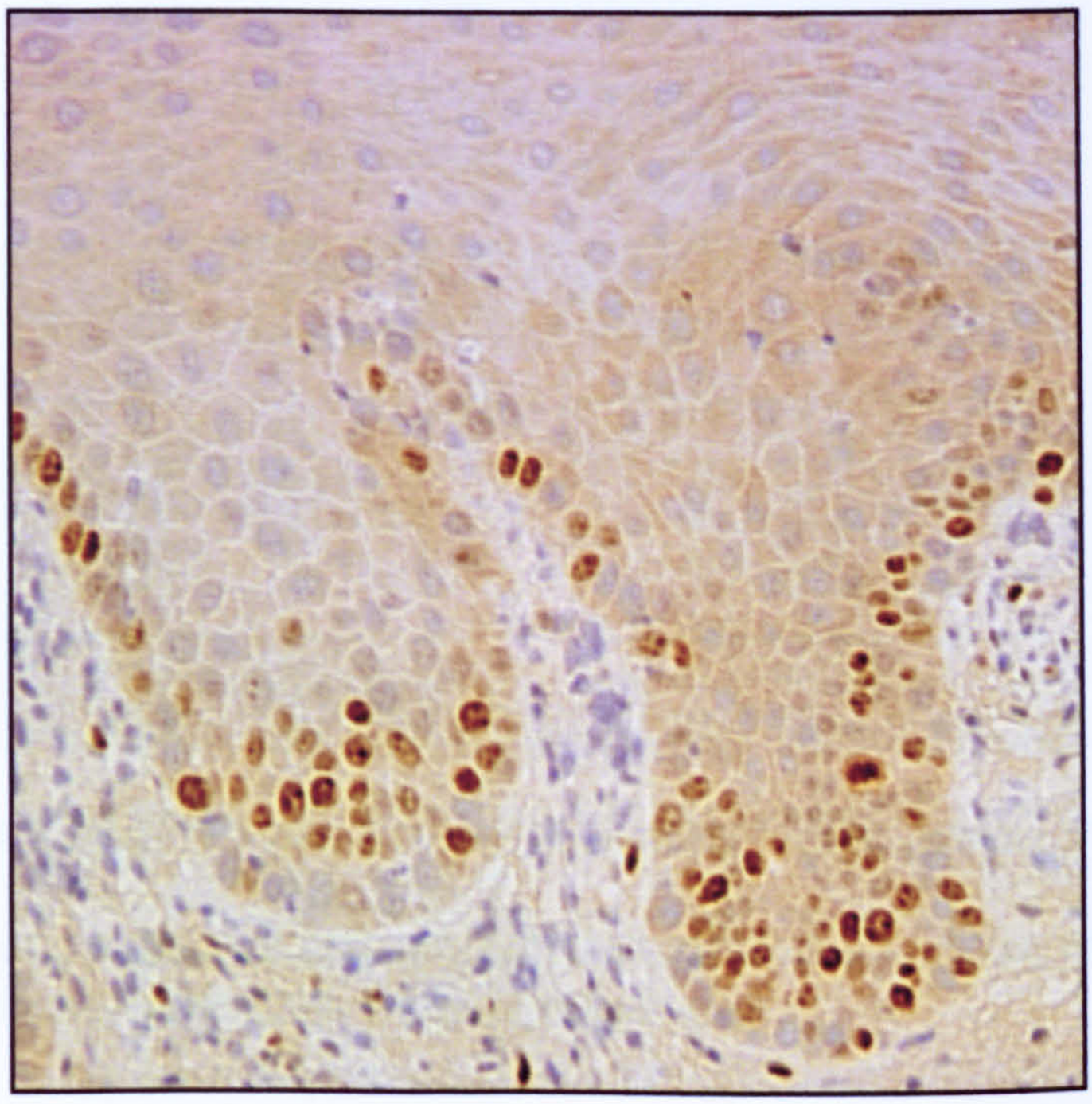
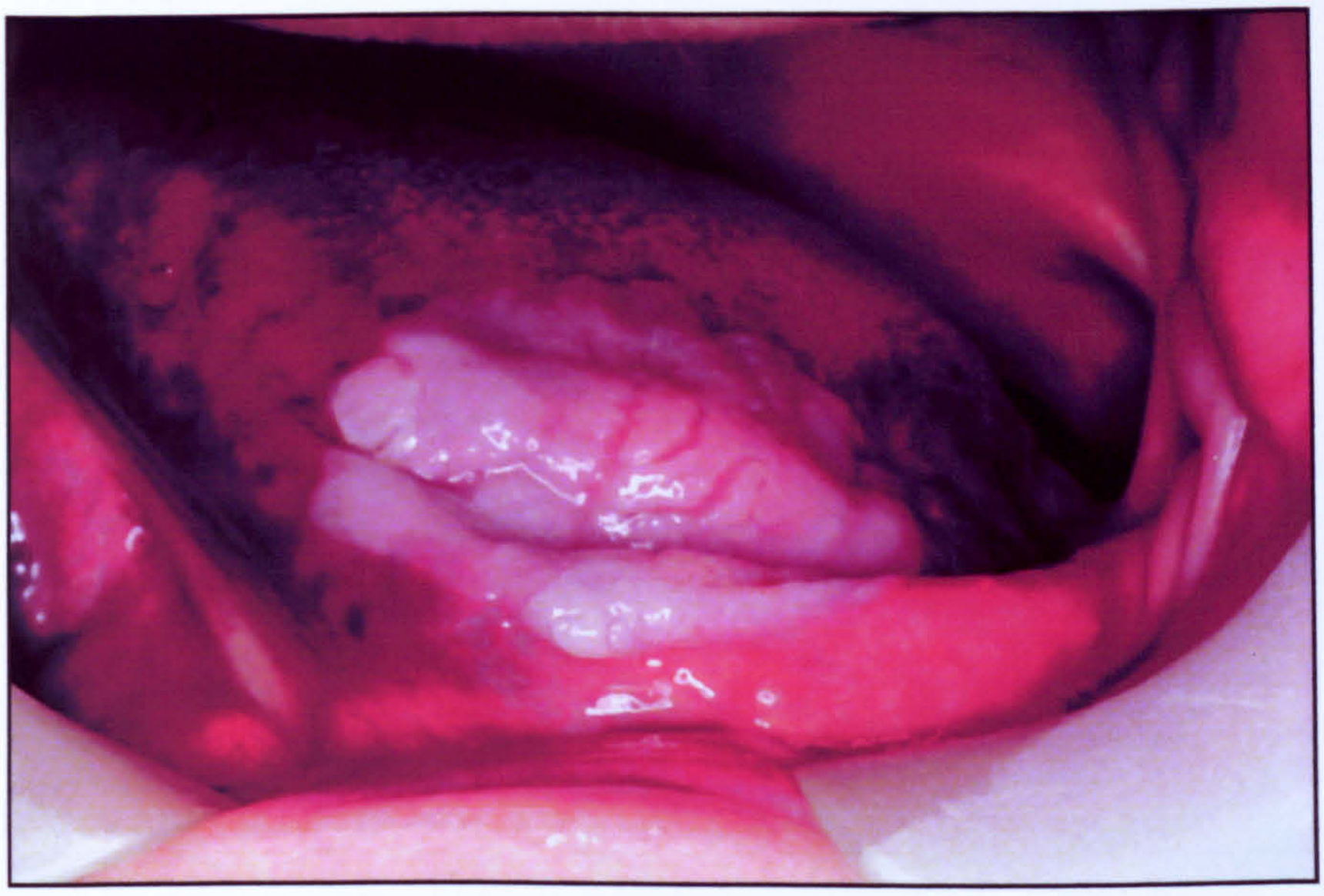
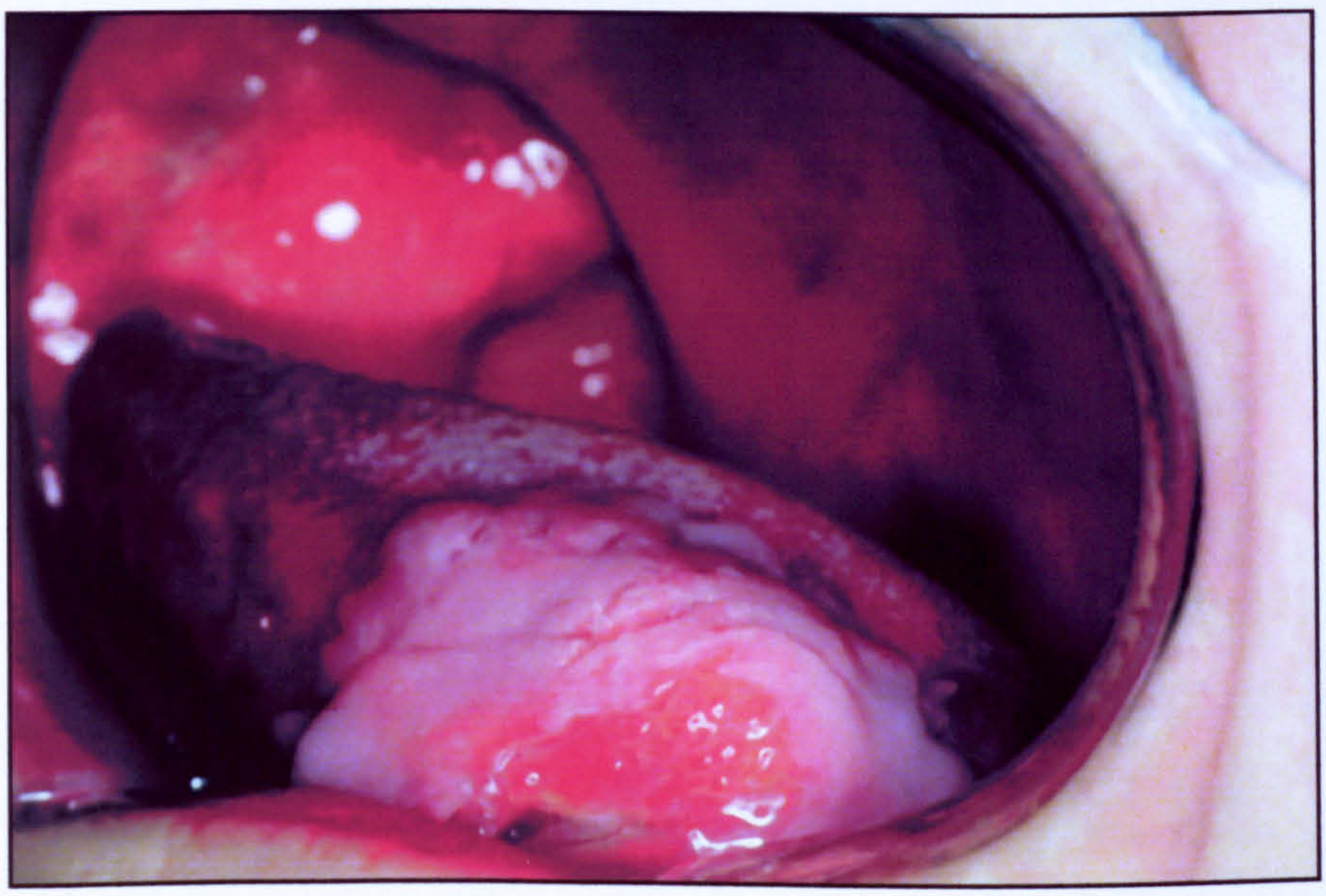


Figure 2.6 Corresponding field of Case 12 demonstrating the growth fraction identified by Ki67 ((magnification x236)



A. At initial presentation



B. Clinical appearance six months after initial presentation

Figure 2.7 Leukoplakia of left lateral border of the tongue of case 14 which underwent malignant transformation

Chapter 3

Investigation of methods

3.1 Introduction

3.1.1 Fixation

In Chapter 2, the comparisons of the BrdU LI and the GF identified by Ki67 were performed on different tissue blocks and hence some distance apart and clearly on different cell populations despite coming from the same biopsy specimen. Carnoy's solution was initially chosen as it is said to give clearer nuclear detail and is recommended for the study of nucleic acids (Pearse, 1980), a factor important when assessing labelled nuclei. However, such fixation does not allow the rather rigorous procedure of antigen retrieval required for Ki67 immunohistochemistry and results in loss of the tissue section from the slide. For convenience of processing formalin would be a desirable alternative but it is known to mask antigens in tissues, a potential problem for immunohistochemistry.

3.1.2 Antigen retrieval

Shi *et al* in 1991 first reported a new method of unmasking antigens in formalin-fixed, paraffin-embedded tissues and allowing their demonstration by immunohistochemistry following microwave oven heating of tissue sections. Antigen retrieval has recently been extensively reviewed by the original author (Shi *et al.*, 1997).

The subject of antigen retrieval has received much attention recently in the literature with many varied methods being proposed. It is evident that conditions must be optimised by laboratories for each different tissue and antibody used rather than employing the same method

for every study (Shi *et al.*, 1997).

3.1.3 Aims

The first aim of the studies in the present chapter was to attempt to develop a method whereby formalin fixation could be used in the preparation of BrdU labelled tissues.

Secondly, a qualitative study was undertaken to determine the optimum heating conditions and solutions for antigen retrieval in the tissues used in the studies reported in this thesis.

3.2 Materials and Methods

3.2.1 Formalin fixation of BrdU processed tissue

Incisional or punch biopsies were obtained from 4 patients (2 male, 2 female) presenting with the clinical lesions of leukoplakia of the buccal mucosa or tongue, the details are presented in Table 3.1.

Table 3.1 Patient details

Case	Age	Sex	Site of biopsy	Histology
1	80	M	tongue	frictional keratosis
2	45	M	tongue	keratosis
3	74	F	buccal	keratosis
4	46	F	tongue	frictional keratosis

The tissue was divided and one half pulse labelled with BrdU as described in Section 2.2.2, the other half being processed for the routine diagnostic service. The BrdU incubated tissue was then further divided; half was fixed in Carnoy's solution and processed as described previously (Section 2.2.2) and the other half fixed in 10% buffered formalin and routinely

processed through graded alcohols to chloroform in an automated Vacuum Infiltration Processor (VIP, Bayer Diagnostics). The tissue was embedded in paraffin wax and 4µm sections were cut and mounted on silane coated slides.

3.2.2 Immunohistochemistry

To enable the incorporated BrdU to be identified a number of procedures were investigated. Firstly, incubation in 2M HCl at 60°C was performed as previously on both the Carnoy's fixed and formalin fixed tissue sections. Microwave antigen retrieval was performed on further tissue sections, fixed in each fixative. The buffers used were 0.01M sodium citrate buffer and 1mM ethylenediaminetetra-acetic acid (EDTA, pH 8.0) for periods of 3, 5, 7, 10, 15 and 20 minutes followed by cooling at room temperature for 30 minutes. Also, a combination of microwave antigen retrieval and hydrochloric acid denaturation was attempted.

The remainder of the immunohistochemistry procedure has been described previously in Chapter 2.

Once optimum conditions for antigen retrieval in the formalin fixed BrdU processed tissue were established the process was repeated on further tissue sections with Ki67 immunohistochemistry being performed on adjacent sections as previously described to ensure there was no contraindication to the use of this antibody on these tissues.

3.2.3 Quantification

For each case the stained sections were quantified as previously

but using the interactive mode of the KS300 image analysis software (Version 2.0, Kontron, Germany) on a standard IBM-compatible computer coupled to an Olympus BX50 microscope via a JVC KYF30E colour video camera. The BrdU labelled nuclei were counted in three defined fields at an objective magnification of x20 and expressed as cells per 100 total nucleated cells (LI%) and per millimetre basement membrane length (LI/BL).

3.2.4 Comparison of antigen retrieval techniques

The following tissues were selected for the analysis, normal human tonsil, oral squamous cell carcinoma and oral epithelial dysplastic lesions from the routinely processed blocks used in Chapter 2.

The buffers used for antigen retrieval were as follows, 0.01M sodium citrate (pH 6.0), 1mM EDTA (pH 8.0) and acetic acid (pH 2.0) as previously described in the literature (Shi *et al.*, 1996a). The detailed constituents of these buffer are listed in Appendix 2.

Four micrometre sections of each of the above tissues were cut and mounted on silane coated slides. Following dewaxing in xylene and rehydrating through graded alcohols, slides were placed in each of the three buffers and heated in either a microwave oven for three periods of 5 minutes or in a domestic pressure cooker (Model number 6182, Prestige Ltd, UK). The procedure for the latter was as follows; 600 ml of buffer was brought to the boil on a gas ring, the sections, held in a metal staining rack, were then placed in the boiling buffer and the lid placed on to seal

the container. Once both of the pressure valves had risen to indicate the vessel had reached maximum pressure, it was held at pressure, in separate experiments, for periods of 1, 2 and 5 minutes. The vessel was then placed under cold running water until the pressure valves fell, the lid removed and the vessel filled with cold water. The slide rack was removed and placed in TBS for 5 minutes. The manufacturer's operating instructions quote an operating pressure of 103 kPa for this device although the temperature achieved is not known.

Immunohistochemistry was then performed on the sections using a Ki67 antibody (NCL-Ki67-MM1) and the procedure as described in Section 2.2.6.

The stained sections were evaluated subjectively and qualitatively for intensity of staining, crispness of nuclear detail, background staining and other artefacts.

3.3 Results

3.3.1 Formalin fixation of BrdU processed tissue

Very weak BrdU staining was observed in the formalin fixed tissue when pre-treated with hydrochloric acid. The staining was considered inadequate for quantification. Microwave treatment of the Carnoy's fixed tissue caused severe tissue destruction and loss of tissue from the slide. Weak BrdU reactivity was observed after 3 minutes of microwave antigen retrieval and became optimal after 7 minutes remaining at a similar

intensity without any increase in background staining up to a period of 20 minutes. The immunohistochemical staining intensity and crispness of the formalin fixed material following antigen retrieval were similar the Carnoy's fixed material (Figure 3.1). Indeed, in some cases the intensity of the staining following antigen retrieval was superior to that of the Carnoy's fixed material. Regarding the formalin fixed material and antigen retrieval, the results were similar for both buffers studied, although there was a marginal increase in intensity using EDTA. Figure 3.1 also demonstrates the shrinkage of the Carnoy's fixed material in comparison to the formalin fixed tissue.

Table 3.2 BrdU labelling indices

Case	Carnoy's fixed HCl denaturation		Formalin fixed/ antigen retrieval Citrate		EDTA	
	LI/TNC	LI/BL	LI/TNC	LI/BL	LI/TNC	LI/BL
1	7.45	35.27	8.65	57.75	9.16	59.38
2	8.02	23.14	8.74	37.70	10.18	39.86
3	11.19	48.87	5.62	20.49	6.76	23.68
4	12.43	50.95	8.07	31.37	14.59	36.32
Mean	**9.77**	**39.56**	**7.77**	**36.83**	**10.17**	**39.81**
SD	2.42	12.97	1.46	15.65	3.28	14.78

The quantitative results for the four cases are summarised in Table 3.2. The mean labelling indices are in a close range in each of the groups. The Carnoy's fixed tissue had labelling indices and standard deviation closest to the formalin-fixed tissue that underwent microwave antigen retrieval using EDTA. Using the Mann-Whitney U test, no pairs of these sets reached significance ($p>0.443$) indicating they are from similar

populations. Meaningful statistics on these results are probably not possible, but, using the Kendall rank correlation test a tendency towards correlation was observed between the Carnoy's-fixed group and the formalin-fixed group using EDTA as an antigen retrieval buffer (p=0.375).

Immunohistochemistry using Ki67 on BrdU incubated formalin-fixed sections was positive and qualitatively similar to previously observed cases.

3.3.2 Antigen retrieval

Immunohistochemistry with Ki67 following microwave antigen retrieval using the three buffers can be seen in Figure 3.2. EDTA buffer resulted in the strongest staining at the expense of some increase in background staining. Acetate buffer gave inferior antigen retrieval to the two other buffers.

Citrate buffer combined with pressure cooker heat treatment gave the next best antigen retrieval, slightly superior to the same buffer used with the microwave, at the expense of some increase in background staining (Figure 3.3). EDTA using the pressure cooker tended to produce tissue destruction and loss of the tissue from the microscope slide and acetate buffer gave inferior results to the microwave treatment.

Heating in the pressure cooker for periods of 1 and 2 minutes gave equivocal staining results. Heating for longer periods produced tissue destruction and loss of tissue from the slide.

3.4 Discussion

3.4.1 Formalin fixation of BrdU processed tissue

In the present chapter a method was developed by which formalin fixation of BrdU processed tissue was seen to give qualitatively equivalent staining to that of Carnoy's fixed tissue when microwave antigen retrieval was applied. The protocol upon which this study was initially based reported that Carnoy's fixative was superior to formalin with regard to identification of immunohistochemical demonstration of BrdU labelled cells (Thornton *et al.*, 1988); this was based upon using hydrochloric acid as the DNA denaturation agent without any other form of antigen retrieval. Magaud *et al* (1989) who developed the monoclonal anti-BrdU antibody (Bu20a) used in the present study also reported a lack of staining of formalin fixed tissue sections using hydrochloric acid alone. Other workers have successfully utilised hydrochloric acid to denature DNA in formalin fixed tissues but in these cases the BrdU was administered *in vivo* (Frank *et al.*, 1995; Kotelnikov *et al.*, 1996; Langford *et al.*, 1996; Wilson *et al.*, 1996). Tischler (1995) reported the use of microwave antigen retrieval in citrate buffer for the demonstration of BrdU in formalin fixed tissue, but again this was in an *in vivo* model.

Other studies fixed BrdU processed tissue in ethanol prior to embedding in paraffin wax (Browman *et al.*, 1991; Limas *et al.*, 1993; Limas and Frizelle, 1994). While this negates the use of antigen retrieval its potential use in a diagnostic laboratory would be limited because of the

need for lengthy manual processing of the tissue rather than automated as formalin fixation would allow.

Although not attempted in this study, enzymatic antigen recovery is possible in formalin fixed tissue but has been reported to decrease the specificity of some anti-BrdU antibodies (Bak and Panos, 1997). Interestingly, Bak and Panos (1997) reported more specificity using the Bu20a clone (as used in the present study) compared to other antibodies when protease digestion was used.

3.4.2 Antigen retrieval

Formalin (formaldehyde) is probably the most popular fixative in use today and continues to be used for the fixation of specimens for histological examination as most of the criteria for diagnosis have been established by the examination of sections fixed this way (Shi *et al.*, 1997). Formalin appears to act by forming intermolecular bridges between proteins and formalin as well as cross-links between protein end-groups (Pearse, 1980). Consequently, there is a loss of antigen expression for many forms of immunohistochemistry when tissues are fixed in formalin.

Williams *et al* (1997) conducted an elaborate series of experiments to investigate variable factors in the whole course of tissue processing on the immunostaining of a panel of antibodies. These authors reported various traditional preparations of formalin to be superior to some preparations recommended for immunohistochemistry such as B5 fixative and Bouin's fixative. Optimal immunoreactivity was obtained after 12

hours of formalin fixation and some antibodies were affected by the pH of the fixative, particularly those of high pH values. Interestingly, the temperature and duration of tissue section drying was found to affect the immunoreactivity of some antibodies, a factor that is not normally considered important. Many variables in the processing procedure had no effect including, delay prior to fixation, use of vacuum, xylene contamination of formalin and prolonged storage of cut sections.

Since the original report by Shi *et al* (1991), a vast range of publications has appeared featuring microwave antigen retrieval; it has been particularly instrumental in allowing proliferation studies using Ki67 notably using the MIB1 antibody (Key *et al.*, 1993).

The precise mechanism by which the epitopes are unmasked by this method is unclear, although the heat element is believed to be the most critical factor (Shi *et al.*, 1996b). Heat may provide the energy to free protein bound calcium from the complexes formed during formalin fixation (Morgan *et al.*, 1994). The degree of antigen retrieval appears to be a function of temperature (T) and time (t) in the relationship "T x t" (Shi *et al.*, 1996a).

3.4.3 The pH and buffer constituents in antigen retrieval

More recently the pH of the antigen retrieval solution has been investigated and appears to be important for certain antigens (Evers and Uylings, 1994; Shi *et al.*, 1996). Low pH solutions tend to increase background staining when used for antigen retrieval of some antigens and

a solution of a higher pH is recommended (Shi *et al.*, 1997). Initially and probably the most widely used buffer for antigen retrieval was sodium citrate buffer at pH 6.0 (Shi *et al.*, 1991), however, subsequent reports suggest chelating agents such as EDTA, pH 8.0, may be more effective as antigen retrieval solutions (Morgan *et al.*, 1994). Other buffers including distilled water, Tris buffered saline and even simple commercially available surfactants have all been used in certain situations with success (Shi *et al.*, 1997). The molarity of such buffers is thought not to be critical (Shi *et al.*, 1997). Norton *et al* (1994) reported fluctuation of pH from 6.0 to 9.1 when using citrate buffer in an aluminium pressure cooker, thought to be due to the liberation of aluminium ions from the vessel, but this appeared not to affect the results suggesting pH control was not critical.

In the present study, antigen retrieval of Ki67 in oral epithelium appeared to be optimal with the use of EDTA at pH 8.0. Morgan *et al* (1994) also reported EDTA to be more effective than citrate for the retrieval of Ki67 in formalin fixed tissue sections of human tonsil and suggested chelation or precipitation of calcium ions was the critical step in the antigen retrieval process. These workers used an autoclave for administration of heat. EDTA has also been reported to be superior to citrate when using a panel of antibodies, including Ki67, and pressure cooker (Pileri *et al.*, 1997).

3.4.4 Methods of heat application

Antigen retrieval has also been achieved using other forms of heat

treatment including the autoclave (Bánkfalvi *et al.*, 1994; Hunt *et al.*, 1996; Mighell *et al.*, 1995; Piffkó *et al.*, 1995; Shi *et al.*, 1996) and a domestic pressure cooker (Kushner *et al.*, 1997; Norton *et al.*, 1994). These methods are claimed to have an advantage over the microwave method in that they preserve the nuclear morphology (Hunt *et al.*, 1996) which may be advantageous when quantifying proliferation by the use of nuclear antigens such as BrdU, Ki67 and the cyclins.

Bussolati *et al* (1997) recently reported restoration of the reactivity of endogenous biotin following antigen retrieval, particularly with the pressure cooker, resulting in an intense and finely granular staining of the cytoplasm when using the streptavidin-biotin peroxidase method. This effect was most pronounced in liver, kidney, adrenal cortex, adipose tissue and in tissues or tumours rich in mitochondria but was not observed in normal skin or skin tumours (Bussolati *et al.*, 1997) and therefore would probably not be a significant problem in oral mucosa.

For convenience and practicality, the use of the microwave or pressure cooker is recommended for most research and diagnostic applications (Shi *et al.*, 1997). Indeed, the pressure cooker method has a number of advantages over the microwave; larger batches of slides are capable of being processed simultaneously, the equipment is cheap and easy to use, and it avoids the occurrence of hot and cold spots that are found during microwave heating (Pileri *et al.*, 1997). It was noted in the present studies that over a period of time the rubber seals in the pressure

cooker appear to become less effective. Therefore, these probably require regular replacement to ensure adequate pressure is maintained within the vessel, something that does not appear to have been addressed in the literature. Autoclave antigen retrieval was attempted in this study but resulted in tissue destruction, was more difficult to control and was considered inappropriate for use (data not shown).

3.4.5 Standardisation of antigen retrieval

When using antigen retrieval it is sometimes observed that a range of staining intensities is produced which makes the interpretation of positively stained cells more difficult (Dowell and Ogden, 1996; Gee *et al.*, 1995) and makes a subjective elimination of the weakly staining cells necessary (Gee *et al.*, 1995). Indeed, for each antigen and tissue studied, the optimum antigen retrieval conditions both in terms of time of heating and the buffer used as well as on occasion enzymatic pre-treatment should be thoroughly investigated to minimise these difficulties in interpretation and introduce a degree of standardisation between investigators (Cattoretti, 1994; Mighell *et al.*, 1995). One technique is to introduce a "test battery" approach for each new antibody studied as recently described (Shi *et al.*, 1996a) in which three levels of heating times and three pH values of buffer are used to determine the optimal antigen retrieval for each antibody used. Ideally, additional studies comparing the intensity of staining following antigen retrieval with that of frozen specimens of the same tissue should be performed for each antibody (Shi

et al., 1997). Gee *et al* (1995) performed such a study using Ki67 on breast carcinoma tissue and suggested a cut-off point of staining intensity be applied. This would, however, further increase the degree of subjectivity of assessment of immunohistochemically stained sections.

3.4.6 Signal amplification in immunohistochemistry

The avidin-biotin complex (ABC) method (Hsu *et al.*, 1981) utilised in the present studies is perhaps the most widely used technique for amplification of antibody labelled cells in tissue sections. Other methods are available and have recently been reviewed by McNicol and Richmond (1998). A reversed ABC technique has been developed in which there is an excess of biotinylated peroxidase rather than avidin which has been shown to be superior to the original technique (Grumbach and Veh, 1995). Additionally, heavy metals such as nickel can be used to enhance DAB staining (McNicol and Richmond, 1998).

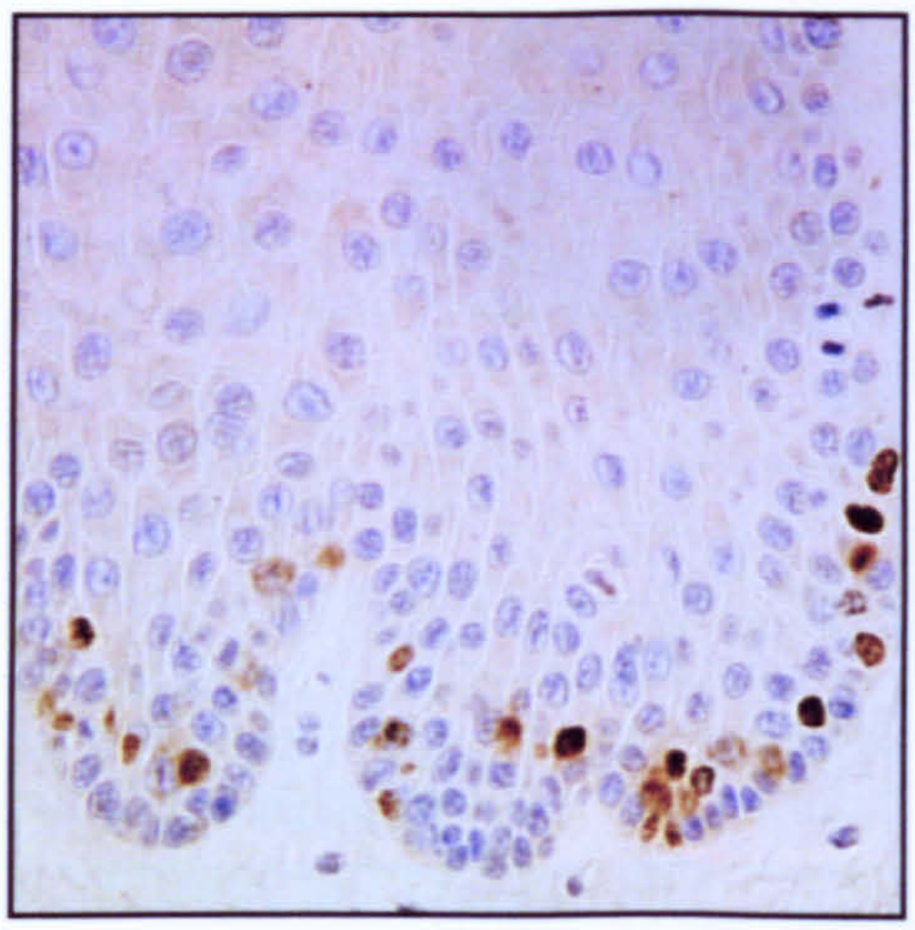
A newer approach to amplification of signals is that of catalysed reporter deposition (CARD) whereby horseradish peroxidase catalyses the deposition of biotinylated tyramine at and around antibody binding sites. This can then be detected using already established enzyme methods (von Wasielewski *et al.*, 1997). A number of studies have demonstrated the increased sensitivity and reproducibility of this method which has even enabled antigens to be visualised in formalin fixed tissues that had previously been apparently unreactive (King *et al.*, 1997; Van heusden *et al.*, 1997; von Wasielewski *et al.*, 1997). Additionally, with the emergence

of commercially available kit forms of this method (Merz *et al.*, 1995) this is likely to become increasingly utilised in immunohistochemistry.

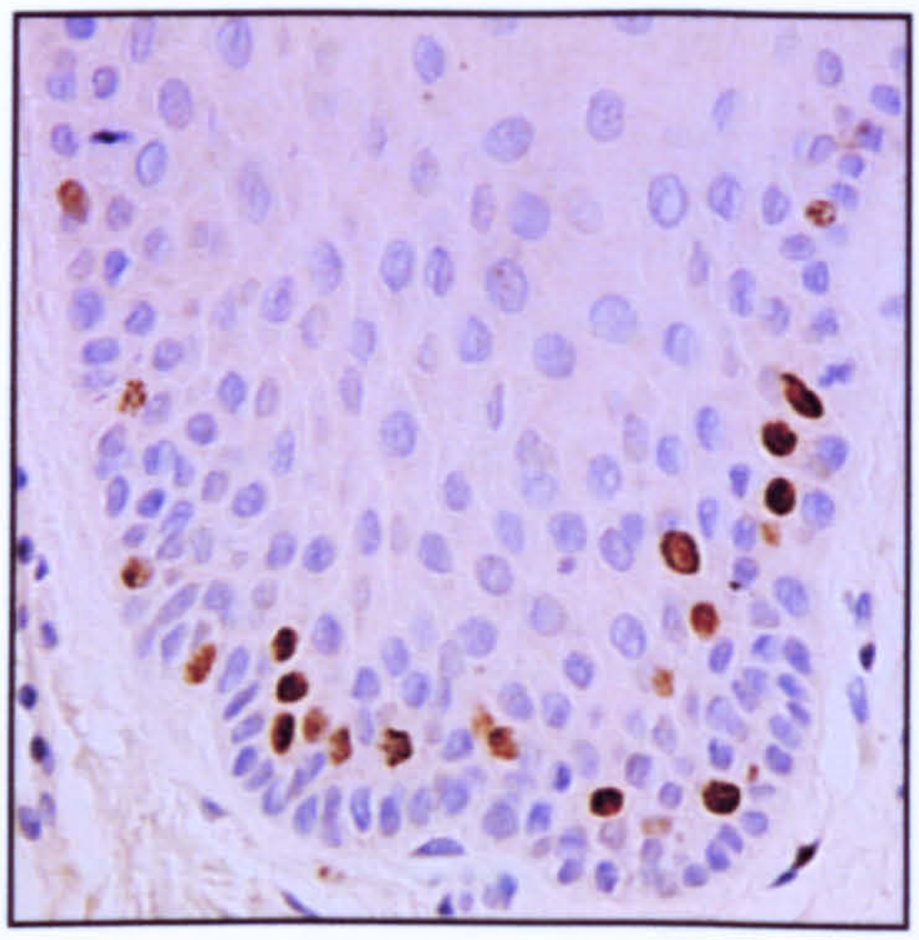
3.5 Summary and conclusions

BrdU immunohistochemistry was demonstrated in formalin fixed oral epithelium following antigen retrieval. To the author's knowledge this has not been performed before using *in-vitro* administration of BrdU to biopsy specimens. This allows the use of automated tissue processing, therefore, reducing manual laboratory time. The use of formalin fixation will permit other antibodies to be used on sequential sections of the same tissue block, perhaps enabling more meaningful comparisons of the parameters being measured. This aspect will be further investigated in Chapter 6.

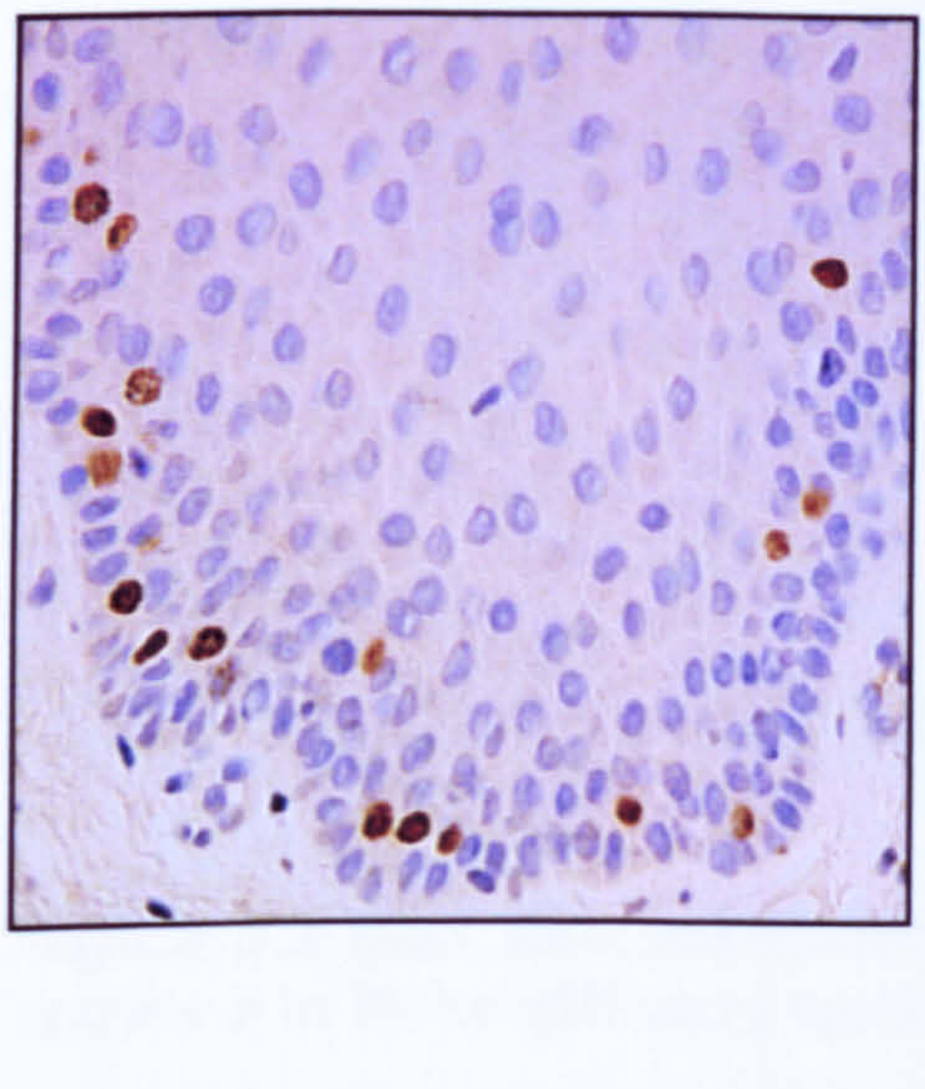
Antigen retrieval in oral epithelia was investigated by a variety of methods to enable optimal results for the Ki67 antibody which has been widely reported as being problematic. The underlying methods employed for this investigation will be of value in optimising antigen retrieval involving other antibodies in this thesis.



A. Carnoy's fixed tissue. DNA denaturation with HCl



B. Formalin fixed tissue. Antigen retrieval in citrate buffer (pH 6.0)

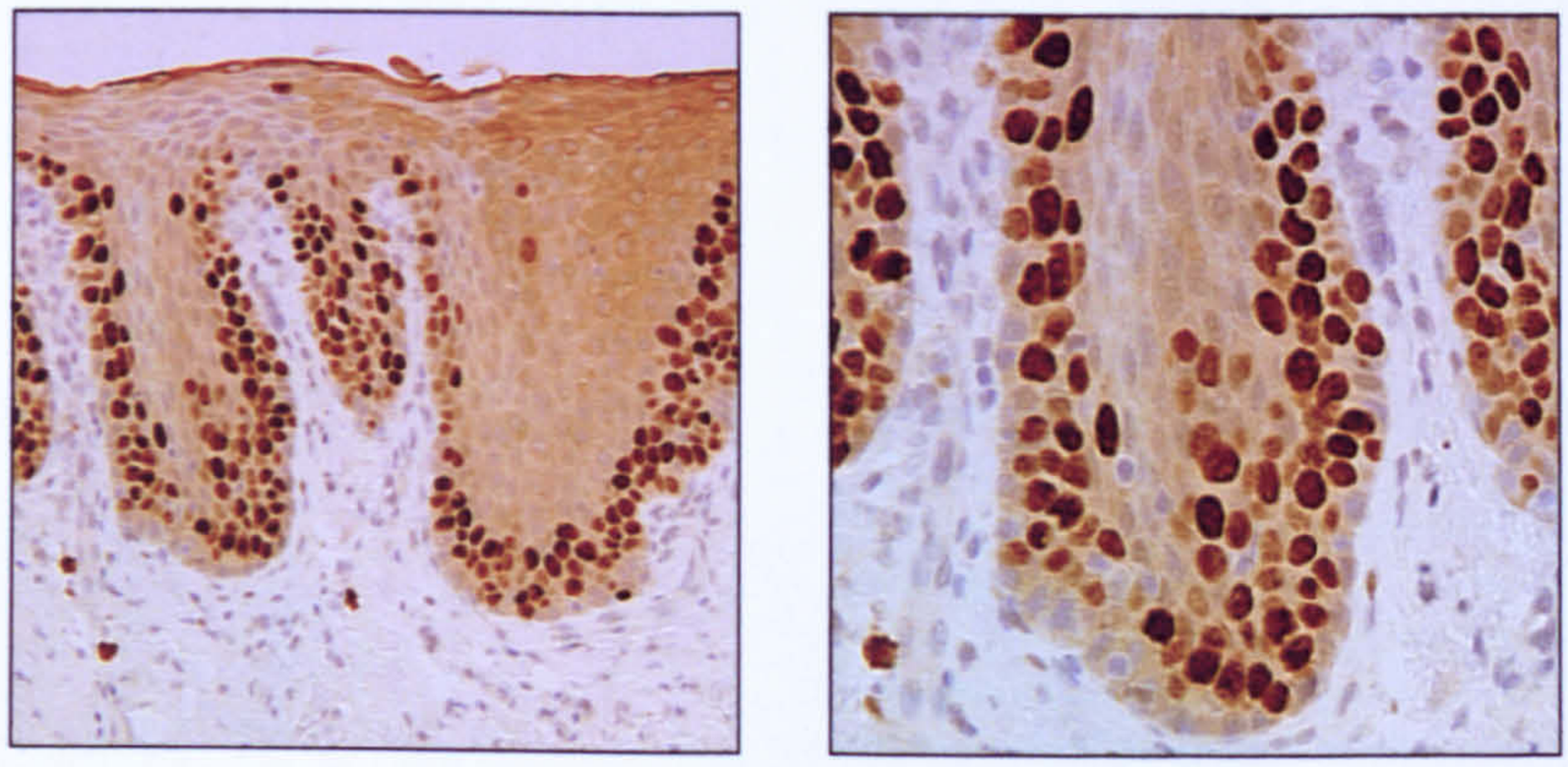


C. Formalin fixed tissue. Antigen retrieval in EDTA buffer (pH 8.0)

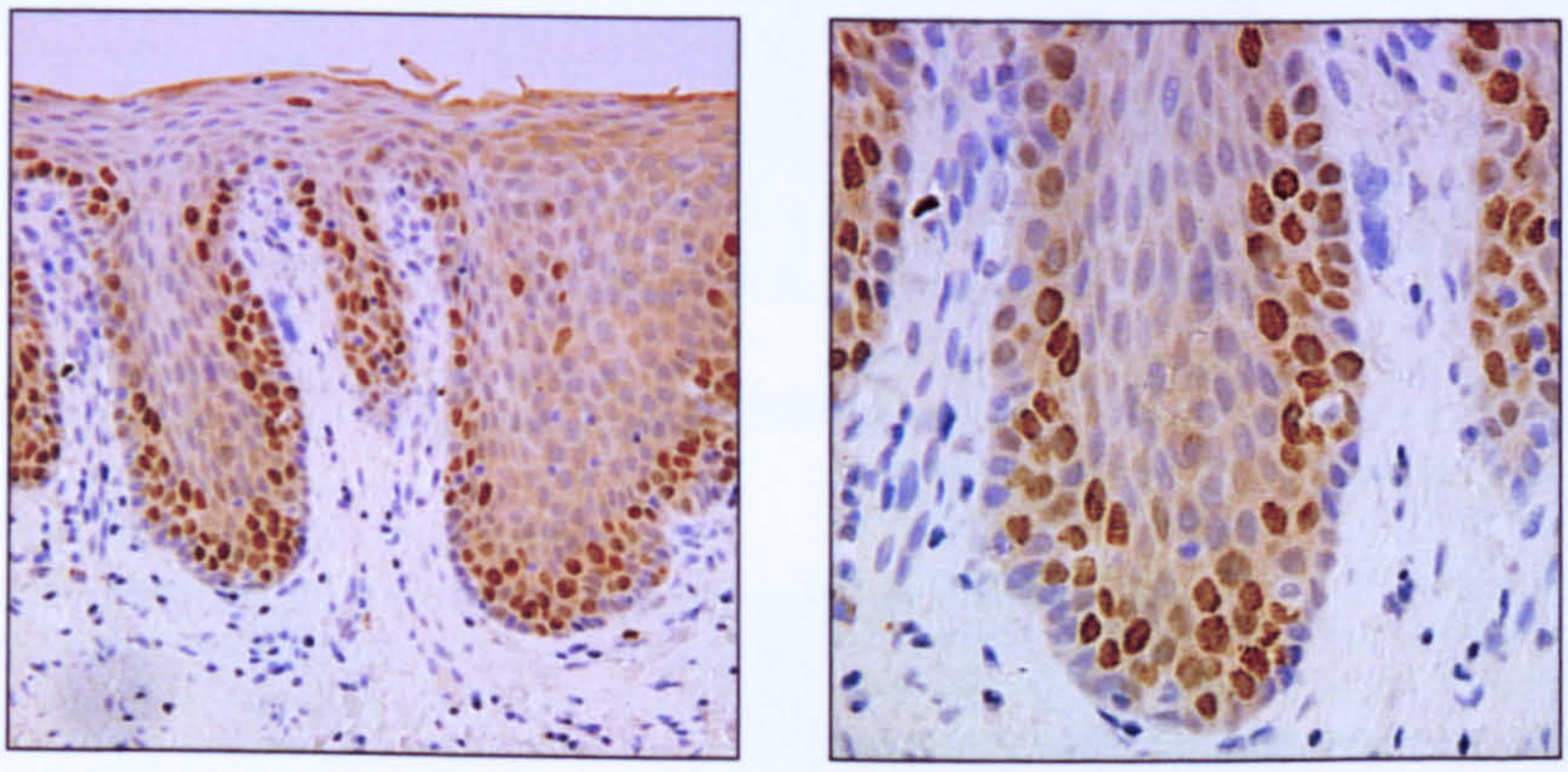
Figure 3.1 BrdU immunohistochemistry in Carnoy's fixed tissues and formalin fixed tissues (magnification x319)

x131 x265

A. EDTA



B. citrate



C. acetate

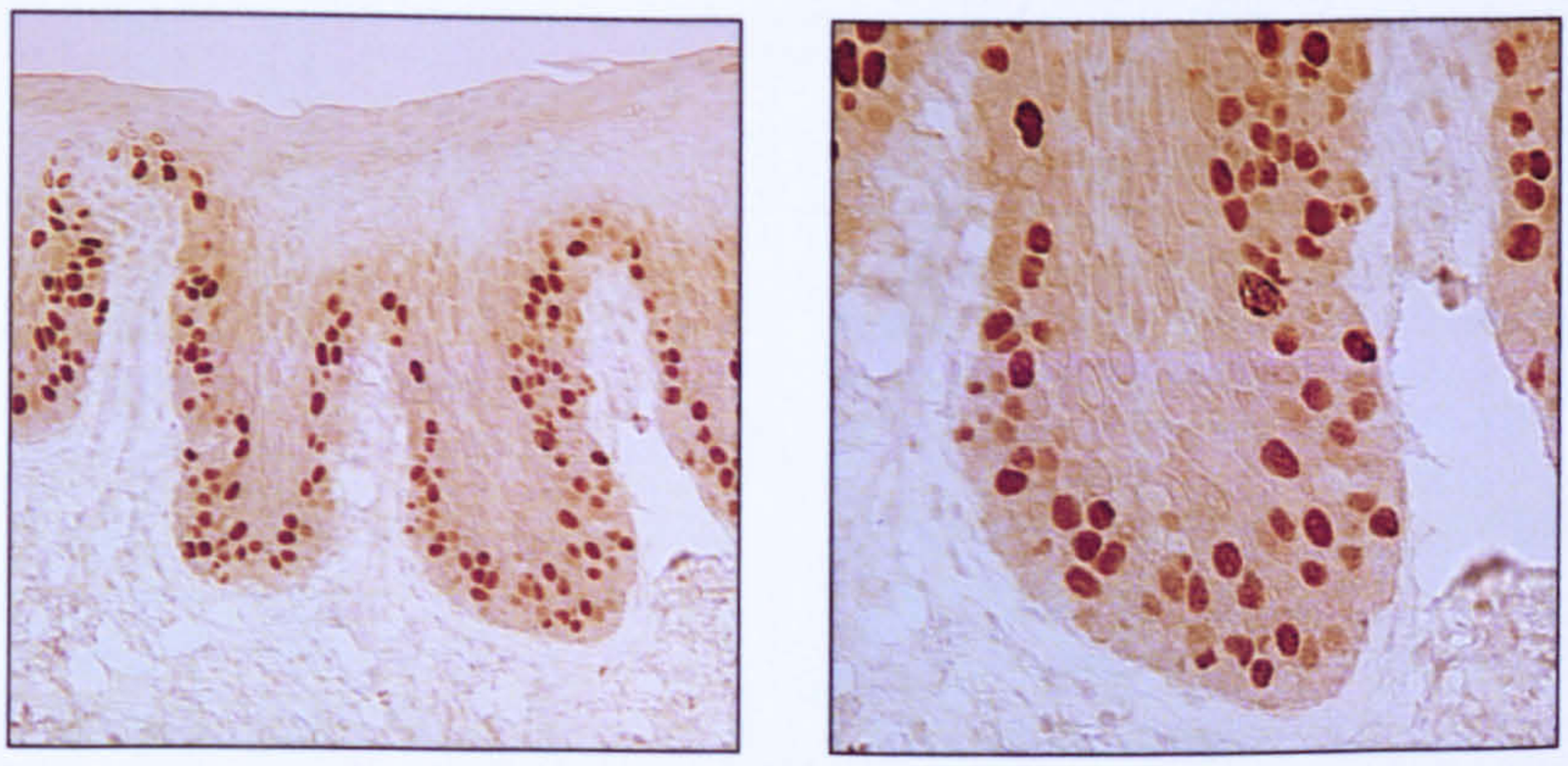
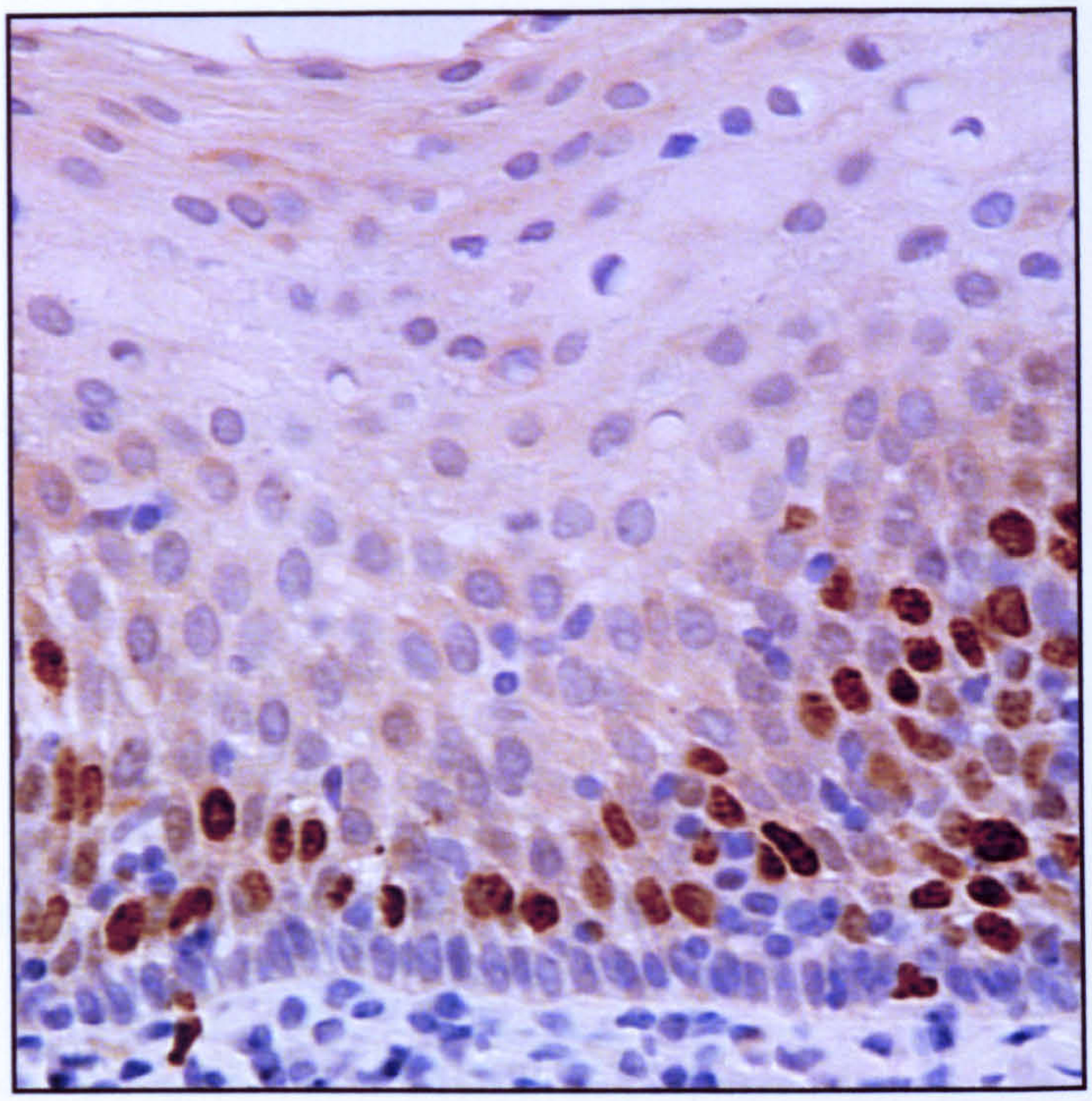
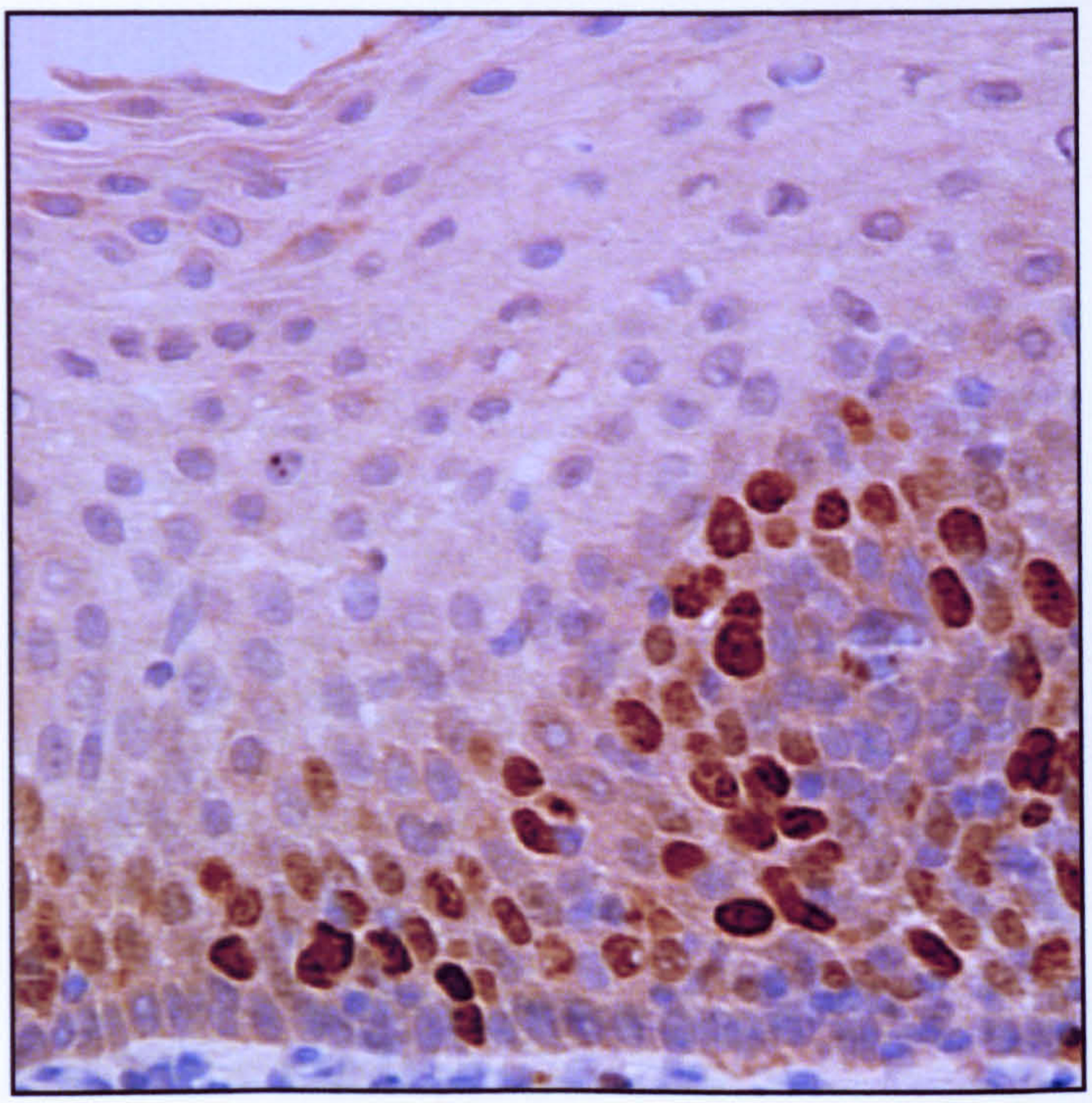


Figure 3.2 Ki67 immunohistochemistry following microwave antigen retrieval in three different buffers



A. in the microwave



B. in the pressure cooker

Figure 3.3 Ki67 immunohistochemistry following antigen retrieval in citrate buffer

Chapter 4

Growth fraction and compartment analysis in post-mortem oral mucosa

4.1 Introduction

4.1.1 Ki67

In the study of dysplastic oral epithelium in Chapter 2 it was observed that Ki67 antibody did not identify all the cells which would be categorised morphologically as progenitor compartment cells and therefore be expected to be in the cell cycle. Ki67 antibody is claimed to identify the growth fraction (GF) by reacting with cells at all stages within the cell cycle (Gerdes *et al.*, 1983). However, from the studies of dysplastic lesions in Chapter 2 this clearly was not the case and there may be a significant proportion of cells, probably those in the G_1 phase of the cell cycle that are not identified by Ki67 (Bruno and Darzynkiewicz, 1992; Tsurusawa and Fujimoto, 1995).

4.1.2 Post-mortem tissues

In order to investigate the apparent discrepancy of Ki67 reactivity, a study of normal oral epithelium was an option. Many workers utilise tissue from benign, often hyperplastic, lesions of the oral cavity as surrogates for normal tissue. However, such tissues by the nature of their aetiology would be expected to have higher proliferation rates compared to truly normal oral mucosa. Obtaining biopsies of normal tissue is difficult and would ideally require subjects without any oral mucosal pathology. Post-mortem tissue is an alternative which is relatively readily available. However, to date there are no reports of studies using Ki67 on post-mortem tissues. It is known that Ki67 has a relatively short half-life in

normal cells and might be broken down before harvesting of tissue post-mortem. Similarly, cells undergo autolysis post-mortem which could have deleterious effects both on the Ki67 protein and the overall tissue architecture.

4.1.3 Aims

The aims of the present study were to investigate the GF identified by Ki67 and perform a compartment analysis in normal oral mucosa to see if, as in dysplastic oral epithelium, Ki67 significantly underestimates the morphological progenitor compartment. Secondly the use of post-mortem tissue as a possible source of material for the study of Ki67 and as future normal control epithelium was investigated.

4.2 Material and methods

4.2.1 Collection of material

Samples of lining mucosa were obtained from the lateral border of the tongue from 20 cadavers at post-mortem and routinely formalin fixed and processed. Five micrometre sections were cut and stained with haematoxylin and eosin (H&E). These were examined to exclude the possibility of any mucosal pathology. Consecutive sections were cut and mounted on silane-coated slides (BDH) for Ki67 immunohistochemistry.

4.2.2 Preliminary studies

As described in Chapter 3, prior to progressing with the main study, a modified test battery approach was applied to determine the optimal

conditions for antigen retrieval. Sections were subjected to each of the following four combinations prior to immunohistochemistry.

Buffer	Method of heat application
0.01M sodium citrate or 1mM EDTA	microwave oven for multiples of 5 minutes or domestic pressure cooker for 1,2 or 5 minutes

The sections were assessed qualitatively following Ki67 immunohistochemistry for clarity and intensity of staining and the presence and degree of artefacts. Once the optimal conditions had been achieved the procedure was continued as described below.

4.2.3 Immunohistochemistry

Following antigen retrieval the Ki67 antibody (NCL-Ki67-MM1, Novocastra, clone MM1) was applied to the sections at a dilution of 1 in 100 for 2 hours at room temperature. The remainder of the procedure was as previously described in Section 2.2.6.

Positive controls were sections of human tonsil and negative controls were the test tissue omitting the primary antibody from the incubation sequence.

4.2.4 Quantification

Quantification was undertaken using the interactive mode of the Kontron KS300 image analysis system. Keratinocyte compartments were identified morphologically in the H&E stained sections on the basis of

nuclear-cytoplasm ratio and orientation of nuclei as previously described (Eveson and MacDonald, 1978). Nuclear counts were undertaken in the progenitor compartment (PC) and maturation compartment (MC) and the basement membrane length was measured. The corresponding fields were identified in the immunohistochemically stained sections for the GF analysis. The labelled nuclei and the total number of viable keratinocyte nuclei were counted and the length of the basement membrane measured as previously. Distinction was made between those cells which were basal, i.e. in contact with the basement membrane, and those which were suprabasal. Three non-overlapping fields were examined at two levels from one block in each case to provide adequate sampling.

4.3 Results

4.3.1 Sample material

Histological examination of the sections showed two cases had keratinised epithelium and features of mild dysplasia. These were therefore excluded. Of the remaining 18 cases, tissues were obtained between 3 and 108 hours (mean 38.36 hours) post-mortem from subjects with an average age of 73.28 years. Eleven of the subjects were female and seven were male. The details are listed in Table 4.1.

4.3.2 Preliminary studies

The optimal antigen retrieval conditions were deemed to be heating the sections in 1mM EDTA buffer for three periods of 5 minutes in a

microwave oven. Use of the pressure cooker consistently caused loss of tissue sections from the slide either in part or completely. Generally it was observed that there was an increase in background in the necropsy tissue staining compared with biopsy material. H&E stained sections and the immunohistochemical stained sections of the corresponding fields are demonstrated in Figures 4.1 to 4.4.

4.3.3 Quantitative results

The results are summarised in the Table 4.1. The mean epithelial thickness was 176.81 μm. The morphologic PC comprised 68.17% of the total nucleated cells in the epithelium compared with the GF 26.23% identified by Ki67. The mean ratio of GF:PC, therefore, being 0.40. Expressed per millimetre basement membrane length these figures were 330.05/mm and 123.93/mm respectively. Of the total basal cell population, Ki67 identified just 26.70% of cells to be in the GF. No correlation was demonstrated between the GF and the number of hours post-mortem.

Figures 4.1 and 4.3 shows the location of Ki67 positivity in the progenitor compartment and Figure 4.3 demonstrates the lack of staining of many cells in the basal layer.

4.4 Discussion

4.4.1 Ki67

The monoclonal antibody Ki67 reacts with a nuclear antigen said to be expressed in cycling cells, but not those in the resting state (G_0)

(Gerdes *et al.*, 1983). Despite widespread use for a number of years the molecular characterisation of the antigen recognised by Ki67 has only recently been identified (Duchrow *et al.*, 1995) and its function is still not completely understood. Ki67 appears to be an absolute requirement for cell proliferation (Schlüter *et al.*, 1993). It has even been suggested that it is a potential oncogene or involved in apoptosis (Duchrow *et al.*, 1995) and because of the location of the gene at 10q25-ter (Fonatsch *et al.*, 1991) close to the end of the chromosome, translocations or rearrangements are quite likely. Another proposed function is a role in rRNA metabolism since Ki67 is located predominantly in the nucleolus (Bruno and Darzynkiewicz, 1992)

If Ki67 is expressed in all cycling cells it would be expected it to be present in all the cells in a progenitor compartment of a tissue and hence the growth fraction. In the present study, the mean ratio of the GF:PC was 0.40, so clearly in normal oral mucosal epithelium Ki67 antibody is identifying significantly less cells than in the progenitor compartment morphologically. A similar proportion (GF:PC 0.39) was also found in the studies in dysplastic oral epithelium of Chapter 2.

More detailed analysis of the expression of Ki67 at various stages in the cell cycle has revealed that the half life of the antigen is about one hour and the level of the protein synthesis increases during the S-phase reaching a peak during the G_2/M transition (Bruno and Darzynkiewicz, 1992; Lopez *et al.*, 1991). In cells with a long G_1 phase, when production

of the Ki67 antigen is minimal, the antigen may be below immunohistochemical detection levels (Tsurusawa and Fujimoto, 1995). Therefore, cells not demonstrating Ki67 immunoreactivity may still be cycling, but erroneously identified as in the resting state (Bruno and Darzynkiewicz, 1992; van Dierendonck *et al.*, 1989). Oral epithelial cells probably are in this category with a long G_1 phase.

4.4.2 Post-mortem tissues

To the authors knowledge, there are no previous reports of the use of Ki67 in post-mortem tissues. Its reactivity and staining properties appear to be similar to those in biopsies of oral mucosa. It is not known how long individual cells of different tissues continue to cycle after death of the patient. The continued presence of immunoreactivity in the cases in this series would suggest either that cells continue to cycle for some considerable time or alternatively that the Ki67 antigen avoids the normal breakdown mechanisms in the cell post-mortem.

Post-mortems tend to be performed largely on older age groups as is evidenced in Table 4.1. This may influence results slightly with regard to proliferation studies.

4.5 Summary and conclusions

As in dysplastic mucosa, Ki67 immunostaining appears to significantly underestimate the progenitor cells of the morphologically identified progenitor compartment. It remains possible to detect the Ki67

antigen in post-mortem oral mucosa. The latter point may prove useful for providing normal tissue for comparison with dysplastic oral epithelium in future studies.

Table 4.1 Case details, epithelial thickness, compartment and growth fraction analysis

Case No.	Hours post-mortem	Age	Thickness (µm)	PC%	GF%	MC%	PC/BL	GF/BL	MC/BL
1	72	73	237.94	69.10	44.69	32.26	273.69	175.94	130.86
2	22	61	185.56	78.01	42.27	21.74	283.24	186.71	81.16
3	19	68	196.78	74.76	33.21	25.43	265.79	108.57	89.69
4	13	72	167.67	66.12	17.64	33.90	316.08	74.92	161.65
5	34	80	191.49	65.14	20.88	34.87	354.43	101.34	199.26
6	3	72	269.28	72.14	27.12	27.82	377.89	153.51	150.82
7	34	66	180.65	68.16	19.29	31.66	359.18	99.28	167.31
8	8	64	197.93	70.47	26.21	29.58	395.31	152.30	164.81
9	28	80	146.04	73.10	19.58	27.04	268.32	71.87	98.87
10	108	75	106.57	71.64	22.92	28.13	358.28	113.62	139.85
11	72	72	150.45	77.34	23.96	22.70	354.63	107.13	104.19
12	31	85	166.66	66.63	22.55	33.35	438.93	145.75	219.74
13	40	75	199.02	60.54	13.96	39.67	351.10	79.79	234.91
14	64	90	114.04	65.01	18.56	35.00	287.89	75.96	155.00
15	67	59	133.35	63.65	32.54	36.57	336.78	168.77	194.56
16	32	66	175.21	58.73	22.67	41.77	279.64	111.73	195.90
17	6	77	215.70	67.10	33.21	32.99	322.97	157.73	158.62
18	37	84	148.23	59.49	30.83	40.65	316.79	145.85	216.75
Mean	**38.36**	**73.28**	**176.81**	**68.17**	**26.23**	**31.95**	**330.05**	**123.93**	**159.11**
SD	28.05	8.48	41.20	5.70	8.42	5.80	48.38	37.33	45.93
Min	3	59	106.57	58.73	13.96	21.74	265.79	71.87	81.16
Max	108	90	269.28	78.01	44.69	41.77	438.93	186.71	234.91

PC: progenitor compartment; **GF**: growth fraction; **MC**: maturation compartment; **BL**: basement membrane length in mm

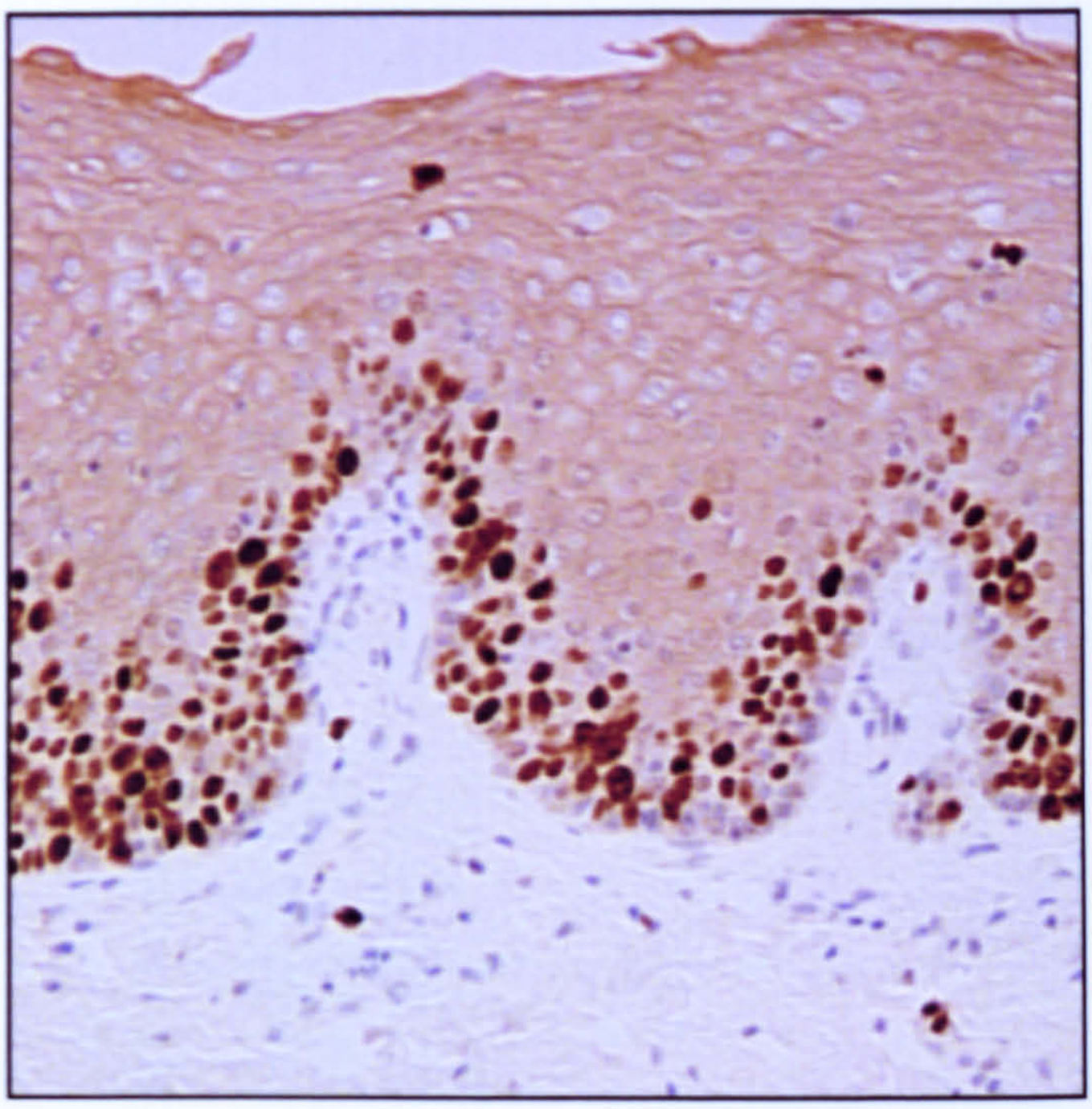


Figure 4.1 Case 1 demonstrating the growth fraction identified by Ki67 (magnification x236)

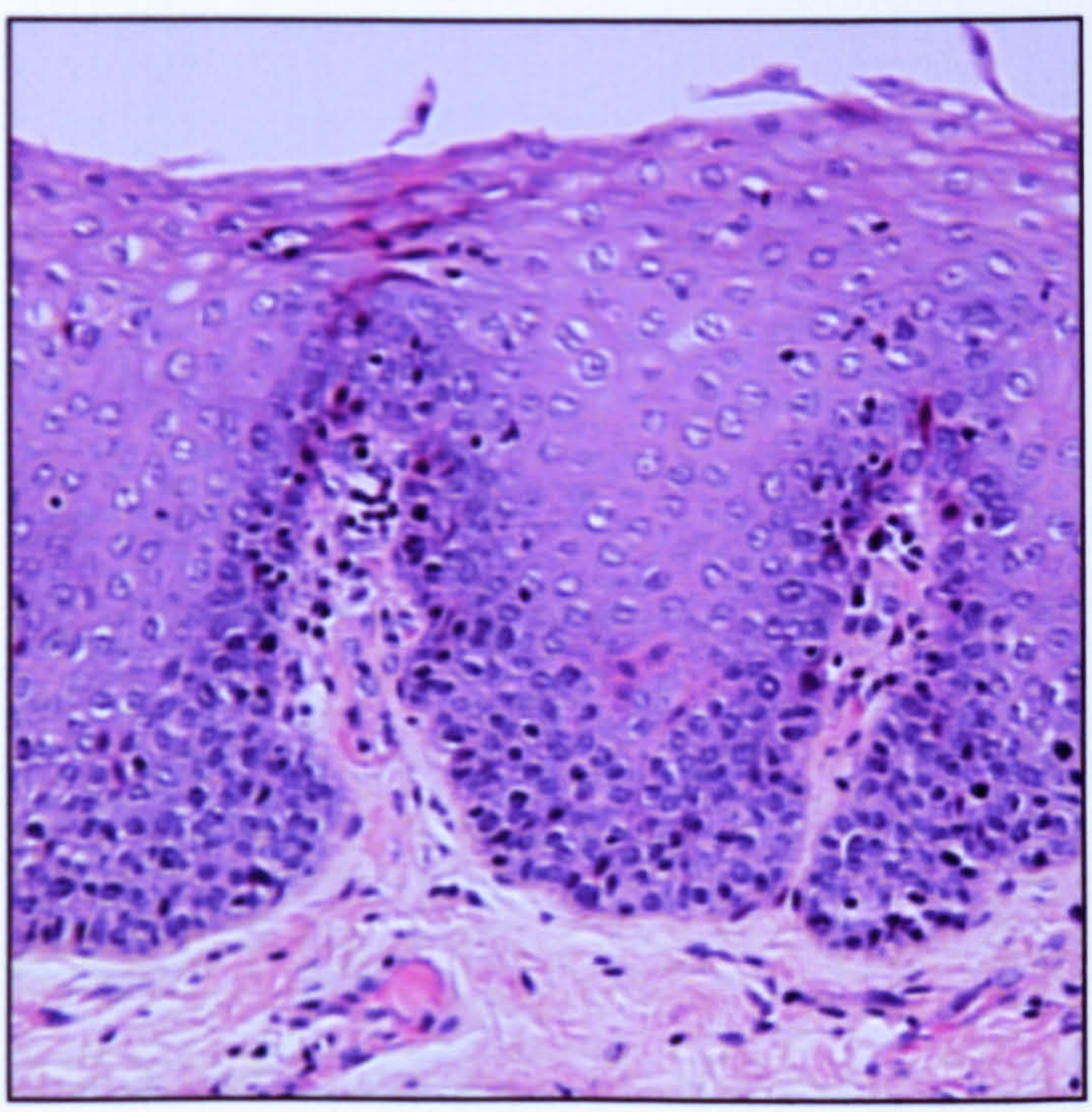


Figure 4.2 Corresponding field of Case1 stained with haematoxylin and eosin (magnification x236)

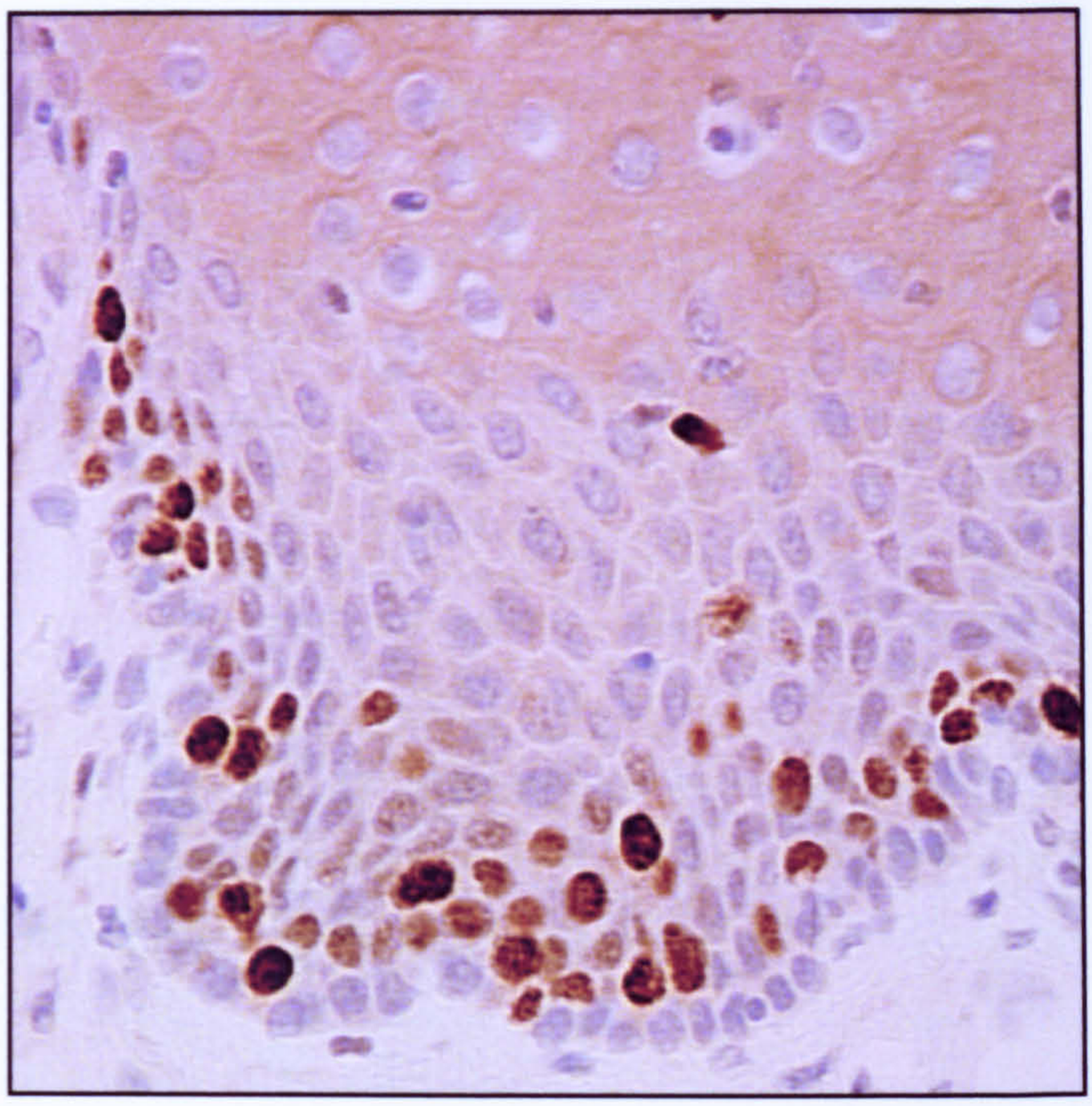


Figure 4.3 Case 6 demonstrating the growth fraction identified by Ki67 (magnification x478)

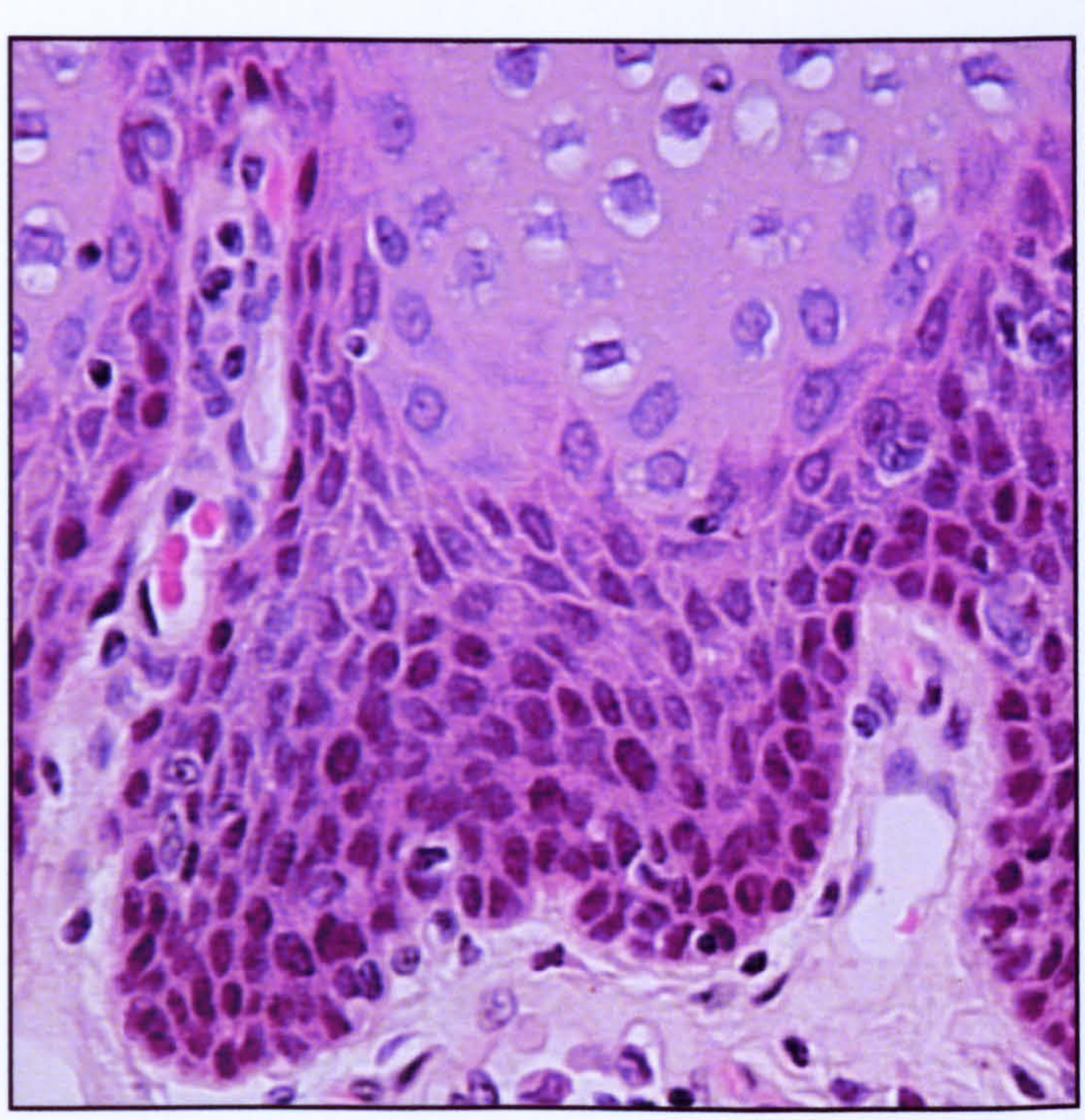


Figure 4.4 Corresponding field of Case 6 stained with haematoxylin and eosin demonstrating the morphological progenitor compartment (magnification x478)

Chapter 5

The G_1 cyclins in oral epithelial dysplasia

5.1 Introduction

The cyclins are part of an elaborate and highly conserved group of proteins concerned with the progression of the cell through the cell cycle. In association with CDKs they control the passage of the cell through the four active phases of the cell cycle and maintain the correct sequence of events. A further family of proteins, the CDK inhibitors (CDKI), exert a negative control upon the CDK-cyclin complexes. Unlike the CDKs, the cyclins are only transcribed and expressed at specific points within the cell cycle.

At least two of the cyclins have been shown to be important in the G_1 phase of the cell cycle, cyclins D and E. These two cyclins are concerned with overcoming the inhibitory effect of pRb and allowing progression through the restriction point and entry into S-phase.

5.1.1 The D cyclins

The family of D cyclins are, perhaps, the most studied of the cyclins. They specifically associate with CDK4 or CDK6 and phosphorylate pRb thereby releasing the transcription factor E2F during the mid to late G_1 phase (Sherr, 1994). The CDK-cyclin activation by phosphorylation is catalysed by an upstream enzyme, CDK-activating kinase (CAK) comprising CDK7 and cyclin H (Sherr, 1994). CDKIs can also bind to the CDK-cyclin complex to inhibit the phosphorylation of pRb and prevent transition through the restriction point and hence the progression into S-phase.

Three D cyclins have been described, cyclins D1, D2 and D3, the expression of which may be cell type specific (Ando *et al.*, 1993; Tam *et al.*, 1994). To date the majority of investigators have studied cyclin D1 which was initially discovered as a putative oncogene in parathyroid adenomas and located at a translocation break point in B-cell lymphomas hence the earlier names of PRAD1 and Bcl-1 (Rosenberg *et al.*, 1991). Cyclin D1 is also reported as being overexpressed or amplified in head and neck squamous cell carcinomas in a growing number of papers, as cited in Section 1.5.9, supporting its role as an oncogene. The role of cyclin D1 in human neoplasia has recently been reviewed by Donellan and Chetty (1998). Additionally, it has been suggested that cyclin D1 may have a role in apoptosis (Kotelnikov *et al.*, 1997).

5.1.2 Cyclin E

Cyclin E appears later in the G_1 phase of the cell cycle than the D cyclins and associates with CDK2 which then phosphorylates pRb enabling the release of E2F (Sherr, 1993). Maximal levels of cyclin E-CDK2 activity are observed at the G_1-S-phase transition and this appears to act as the rate limiting step for this progression; overexpression of cyclin E shortens the duration of the G_1 phase but not the overall cell-cycle time (Sherr, 1993). It is not yet known if cyclin E acts upstream of the D cyclins or in parallel with them. Indeed, Gong *et al* (1995b) suggested the D cyclins may have additional roles in the cell and showed either an invariable expression of D cyclins throughout the cell cycle or some D-

cyclin negative cells but a consistent expression of cyclin E confined to the G_1-S-phase transition.

It has been suggested that the three CDK-cyclin complexes that interact with pRb, namely CDK4-cyclin D1, CDK2-cyclin E and CDK2-cyclin A, all have a role in the differential phosphorylation of pRb which is necessary for the transition through the restriction point (Zarkowska and Mittnacht, 1997).

5.1.3 Aims

The studies in the present chapter investigated the expression of the G_1 cyclins, D1, D3 and E, in oral epithelial dysplastic lesions. The aim of this was to see if these were truly representative of the G_1 phase and in this capacity if they were useful as markers of this phase where Ki67 expression may be lacking as found in Chapters 2 and 4. The results were related to Ki67 expression. In an attempt to further the objective assessment of such lesions the correlations with the atypia scores were also investigated.

5.2 Materials and Methods

5.2.1 Case details

From the diagnostic histopathology files of Glasgow Dental Hospital, 18 cases of dysplastic lesions from the floor of mouth or tongue were selected. Because the use of the antibodies did not require prospective collection of tissues archival specimens were used in the first

instance. In order to investigate the findings further in another group of lesions, the 20 cases used in Chapter 2 were included and compared for part of this study.

5.2.2 Immunohistochemistry

Preliminary studies were performed to optimise the antigen retrieval conditions using a modified test battery approach as described in Chapter 3. This involved the use of either microwave or pressure cooker heating and either citrate or EDTA as the buffers

The primary antibodies applied are listed in Table 5.1, along with the clone and recommended working dilution. All the antibodies used were supplied by Novocastra (Newcastle, UK). The sections were incubated for periods of 1,2 or 4 hours at room temperature or overnight at 4°C in a humidified chamber at varying concentrations to optimise the antibody concentrations and incubation times.

Sections of human tonsil, lymph node, lymphoma and oral squamous cell carcinoma with overlying normal epithelium were used as positive controls and omission of the primary antibody was a negative control.

5.2.3 Quantification

Once the optimal antigen retrieval and staining was achieved, the sections of oral epithelial dysplasia were immunohistochemically stained. Sequential sections from each tissue block were used for the 18 archival cases, the antibodies being applied in the following order: cyclin D1, cyclin

D3, Ki67, cyclin E. Quantification was performed using the KS300 image analysis software and an objective magnification of x20. The contrast of the digitally acquired images required enhancement in order to confidently quantify the cyclin antibody immunohistochemically stained cells in the majority of cases as demonstrated in Figure 5.1.

Three corresponding fields were selected for each case and the antibody labelled cells counted and expressed per total nucleated cell population (LI%) and per millimetre basement membrane length (LI/BL). This was facilitated by the virtue of the software used having an image gallery in which the previous images could be viewed in order to accurately locate corresponding fields as demonstrated in Figure 5.2. To aid the quantification of the large number of sections in the study part of the procedure was automated by the use of a macro custom written by the author. Essentially this is a short program written to run under the KS300 software to consistently reproduce the software functions (Appendix 6).

The degree of dysplasia for each case was assessed on haematoxylin and eosin stained sections both subjectively and by the method of Smith and Pindborg (1969). The first third of the cases were quantified by another observer in order to ensure consistency.

The Spearman-Rank correlation test was used to assess the existence of correlation between the labelling indices and atypia scores.

5.3 Results

5.3.1 Case details

The clinical details of the series of 18 cases studied are shown in Table 5.2. The mean age of the patients was 57.56 (range 31 to 85). There were eight females (mean age 57.5) and 10 males (mean age 57.6). Ten of the lesions were from the lateral border of the tongue and eight were from the floor of the mouth. Since these lesions were archival and obtained from a number of different centres in the West of Scotland, data were not readily available regarding the clinical follow-up and subsequent behaviour.

5.3.2 Immunohistochemistry

Antigen retrieval for the D cyclin antibodies was deemed optimal following heating in the pressure cooker with citrate buffer for two minutes under pressure. Using microwave antigen retrieval, periods in excess of 35 minutes were required for any detectable immunoreactivity of the D cyclins. This also resulted in some tissue destruction particularly of the underlying connective tissue. The conditions deemed optimal for cyclin E were heating in EDTA buffer for 20 minutes (4 x 5 minutes) with the microwave as the heat source. The intensity of the D cyclins and cyclin E staining overall was low relative to Ki67 (Figures 5.3 to 5.6). The staining intensity was not noticeably improved by increasing the primary antibody concentration or length of incubation times. Also, in some cases the

staining intensity varied between batches of slides which were stained at the same time and even between different regions of the same slide. Whether this is a function of the antigen retrieval or a true variation in protein expression is not known. Quantification was only possible following the enhancement of contrast which improved the image beyond what even the naked eye could detect prior to this operation (Figure 5.1).

An interesting observation was made with regard to cyclin D3 immunoreactivity in that positivity was seen in the smooth muscle layer around blood vessels, interpreted as arteries (Figure 5.7). This was thought to be cross-reactivity of the antibody with this tissue. Interestingly, the nuclei of the endothelial cells were also positive for cyclin D3 in some cases.

The distribution of cyclin E was unexpected. In the majority of cases reactivity was confined to cells which would be classified as belonging to the maturation compartment (Figure 5.8). In some of the more dysplastic lesions the intensity of the cyclin E staining was particularly marked (Figure 5.9).

Ki67 immunoreactivity was consistent with previously observed cases following antigen retrieval for 1 minute in EDTA using the pressure cooker (Figure 5.5).

5.3.3 Quantitative results: LI%

The results for the 18 archival cases are summarised in Table 5.3. The mean LI% for cyclin D1 was 32.05% (SD 11.72), for cyclin D3 34.36%

(SD 15.93), for cyclin E was 24.06% (SD 12.31) and 31.57% (SD 9.22) for Ki67. The D cyclin labelling indices were highly significantly correlated with each other ($p<0.005$) while cyclin D1 correlated with Ki67 ($p<0.01$) and cyclin D3 correlated with Ki67 ($p<0.05$). The cyclin E indices did not correlate significantly with any of the other indices but there was a tendency for a correlation with the atypia scores ($r_s=0.3333$, $p<0.1$). These correlations are detailed in Table 5.4a.

The labelling indices of the cases from the series studied in Chapter 2 are summarised in Table 5.5. The mean LI% indices were not appreciably different than those for the 18 case group.

When the results of the two groups were combined to form a larger group (38 cases), the mean labelling indices were as follows. The mean LI% for cyclin D1 was 31.28% (SD 12.34), for cyclin D3 was 31.32% (SD 14.74), for cyclin E was 26.39% (SD 12.11) and 29.60% (SD 13.39) for Ki67. In this larger group the D cyclin indices per total nucleated cells no longer correlated with Ki67 or with the atypia scores. Again in this larger group the cyclin E LI% did not correlate with any of the other indices. These correlations are summarised in Table 5.6a.

5.3.4 Quantitative results: LI/BL

For the archival group of 18 cases the results are summarised in Table 5.3. The mean LI/BL for cyclin D1 was 139.61/mm (SD 62.75), for cyclin D3 was 153.03/mm (SD 96.86), 90.30/mm (SD 51.63) for cyclin E and 143.03/mm (SD 53.80) for Ki67. These labelling indices all correlated

with each other significantly and with the atypia scores ($p<0.05$). These correlations are summarised in Table 5.4b.

The LI/BL indices for the series of cases from Chapter 2 are summarised in Table 5.5. The LI/BL for the D cyclins were noticeably different in this group compared to those of the group of 18 cases (Table 5.3). This could be accounted for at least in part by the fact that Case 6 of this series was consistently negative when stained with D cyclin antibodies. There may also be a contribution to this difference due to differing basement membrane lengths.

When the two groups were combined to give a larger group of 38 cases the mean labelling indices were as follows. The mean LI/BL for cyclin D1 was 127.19/mm (SD 62.48), for cyclin D3 was 129.34/mm (SD 80.17), for cyclin E was 99.29/mm (SD 55.04) and for Ki67 was 142.12/mm (SD 91.16). The Ki67 LI/BL correlated significantly with all of the cyclin LI/BL indices and the D cyclin indices correlated with each other. Only the cyclin E LI/BL correlated with the atypia scores in this group. The details of these correlations are shown in Table 5.6b.

5.3.5 Quantitative results: ratios and distributions

The percentages of the total basal cell population labelled with cyclin D1 and D3 were 29.14% (SD 17.87) and 33.25% (SD 14.36) respectively compared with 40.26% (SD 14.19) of the basal cell population identified with Ki67. When the larger group was considered, these figures did not alter significantly. These results are summarised in

Table 5.7. Reflecting the staining pattern of cyclin E, there were relatively few of the basal cells (8.24%) identified by this antibody compared to the other antibodies. It was not possible to make the same comparison with the earlier group as the Ki67 labelling indices were counted differently, viz., the total number of basal cells was not assessed as a single parameter.

To further investigate the relationship between the D cyclins and Ki67, the ratios of the Ki67 and cyclin labelling indices were examined and are shown in Table 5.8. These ratios were close to unity for the labelling indices expressed as a percentage. However, when the labelling indices per mm basement membrane length were expressed as a ratio the values were higher. These findings are not readily explainable.

When the results of the group of 38 cases was examined by the two sites of origin of the biopsies the mean labelling indices are shown in Table 5.9. Using the Mann-Whitney U test, the only significant differences between the two groups were for the cyclin E LI/BL.

5.4 Discussion

5.4.1 The D cyclins

Although designated as G_1 cyclins, there is evidence that the expression of the D cyclins continues throughout much of the cell cycle (Gong *et al.*, 1995a). However, some workers have demonstrated a clear association of cyclin D1 with the G_1 phase (Baldin *et al.*, 1993; Bartkova *et*

al., 1994). Unlike cyclin E, there appears to be no threshold level of expression required for entry into S-phase (Gong *et al.*, 1995b). Certainly, in the present study the level of D cyclin expression was demonstrated to be roughly equivalent to that of Ki67.

The degree of expression of the D cyclins in the present study could be interpreted a number of ways. Firstly, it could represent a true overexpression of the protein. This could be determined by assessing the mRNA content of the cells either by *in situ* hybridisation or reverse transcriptase PCR. Indeed, the latter approach was attempted (data not shown) but it was not possible to successfully extract RNA from the paraffin-embedded tissues. Secondly, it could represent a continued production of the protein through other phases of the cell cycle in addition to the G_1 phase. Peters (1994) suggested that there was a redistribution of the protein through the S-phase rather than an apparent enhanced activity in the G_1 phase. A further assumption could be that the protein half life is increased. At the gene level this could be due to an up-regulation of the gene expression or the expression of a modified gene product due to point mutation, chromosomal rearrangement or DNA amplification with the D cyclins, therefore, acting as an oncogene (Bates and Peters, 1995). In the present study, in the majority of cases, the intensity of immunohistochemical staining was not strong when compared to other antigens studied and therefore was not thought likely to be due to overexpression. It could be speculated that this represented a continuing

expression of the protein beyond the G_1 phase of the cell cycle rather than a true overexpression.

A recent paper by Mate *et al* (1998b) demonstrated similar staining intensities, using the same cyclin D1 antibody as the present study, when investigating psoriasis in the skin but failed to detect the protein in condyloma acuminatum and normal human foreskin epithelium.

Rey *et al* (1998) recently reported a strong association between cyclin D1 expression and that of $p21^{WAF1/Cip1}$, a CDKI, in breast carcinoma. These authors found this to be independent of p53 expression and suggested a possible role for the CDKI in the modulation of cyclin D1.

The role of cyclin D1 within cells is slowly becoming clearer and appears to be more than just responsible for the G_1/S-phase transition. In a series of elaborate experiments involving spliced forms of cyclin D1 protein Sawa *et al* (Sawa *et al.*, 1998) recently reported some significant findings. These workers found some forms of the cyclin D1 protein caused suppression of cell growth in culture by preventing entry into the S-phase as evidenced by the cells lack of BrdU uptake and Ki67 expression. The mechanism for this appeared to be related to up-regulation of pRb, p53 or $p21^{WAF1}$ depending on the conditions. These genes are intimately involved with apoptosis supporting a role for cyclin D1 in this process as suggested by Kotelnikov *et al* (Kotelnikov *et al.*, 1997). Sawa *et al* (Sawa *et al.*, 1998) also cite evidence that cyclin D1 is involved in senescence.

It is not yet clear what the functions of the three D cyclins are within

cells. There appears to be some cell type specificity of cyclins D1 and D2. Cyclin D1 has been confirmed to be present in oral keratinocytes. Cyclin D3 has been demonstrated in all cell lines studied so far (Bates and Peters, 1995).

Already cited above, cyclin D1 is overexpressed or amplified in oral carcinoma where it is often associated with advanced clinical stage. At other sites overexpression of cyclin D1 has been associated with a poor prognosis. For example, in bladder carcinoma it correlated with early recurrence (Shin *et al.*, 1997), in pancreatic carcinoma it was associated with reduced survival periods (Gansauge *et al.*, 1997) and in oesophageal squamous cell carcinoma cyclin D1 overexpression correlated with reduced survival, particularly in patients with simultaneous pRb expression (Ishikawa *et al.*, 1998). In hormone-dependent breast carcinoma, however, cyclin D1 overexpression appears to correlate with increased survival and patients who responded well to anti-oestrogen therapy (Barnes, 1997). Indeed, the anti-oestrogen drugs, such as tamoxifen, appear to exert their action via oestrogen receptors on the cyclin D1/CDK/pRb complex thereby preventing the phosphorylation of pRb and transition into the S-phase (Barnes, 1997).

Nichols *et al* (1996) used *in situ* hybridisation to demonstrate cyclin D1 mRNA overexpression in cervical malignant and premalignant lesions in the absence of immunohistochemically detectable protein. This approach would be an option for studying the apparent overexpression of

the protein in the present study, although, the results of *in situ* hybridisation are difficult to interpret and difficult to quantify compared to immunohistochemistry.

Less is known regarding cyclin D3 although Bartkova *et al* (1996) suggest from their studies that overexpression of cyclin D3 is less frequent than cyclin D1. These authors, who developed the antibody against cyclin D3 used in the present study, showed a clear overexpression of the protein in ductal and lobular breast carcinomas. In squamous cell carcinomas of the head and neck, however, only weak or moderately intense immunoreactivity was demonstrated (Bartkova *et al.*, 1996) which equates to the findings in the present study.

The gene encoding cyclin D3 is located at chromosome 6p21 (Hall and Peters, 1996). However, as yet there are no known alterations or translocations mapped to this site in human cancers.

Like cyclin D1 the levels of cyclin D3 protein throughout the cell cycle are not phase specific and it may be present at all stages of the cell cycle suggesting an additional role other than in the G_1 phase transition into S-phase (Gong *et al.*, 1995a). However, these studies used immunocytochemical and immunoblotting methods rather than immunohistochemistry for detection of the proteins. Kang *et al* (1997) suggested cyclin D3 may have a role in meiosis in male germ cells.

The observation of cyclin D3 immunoreactivity in the smooth muscle of blood vessels appears to be a novel finding. It was interpreted

as cross-reactivity of the antibody with these tissues. The significance of cell nuclei staining positive with cyclin D3 is less clear.

5.4.2 Cyclin E

Unlike the D cyclins, there is a clear increase in cyclin E expression towards the late G_1 phase before entry into S-phase and a discontinuous expression of this cyclin through the rest of the cell cycle (Gong *et al.*, 1995b)

Mate *et al* (1998b) studied cyclin E in their series of skin lesions. These workers demonstrated cyclin E in the suprabasal layers but not the basal layers in condyloma acuminatum and psoriasis. They failed to demonstrate cyclin E immunoreactivity in normal human foreskin. The pattern and intensity of immunoreactivity pictured in their paper were very similar to that seen in the present study. The significance of cyclin E immunoreactivity predominantly in cells that are in the maturation compartment is not known. Indeed, this appears contrary to their supposed function.

Patel *et al* (1997) reported overexpression of cyclin E in head and neck squamous cell carcinoma cell lines with additional overexpression of its related CDK, CDK2. These authors also demonstrated increased levels of CDK4 and CDK6 as well as cyclin A but surprisingly, contrary to many other reports, failed to find any abnormalities of cyclin D1.

In breast tissue, cyclin E immunoreactivity was lacking in normal and benign tissues but was significantly correlated with poor differentiation

in carcinomas (Scott and Walker, 1997). Cyclin E immunoreactivity correlated with the degree of dysplasia in colorectal adenomas and was significantly greater in adenocarcinomas (Yasui *et al.*, 1996).

5.4.3 Correlations with Ki67

In the present study, the D cyclin and Ki67 labelling indices correlated with each other. The indices were also quantitatively similar; this could be interpreted as a continued expression of the D cyclins through the cell cycle as the D cyclin positive cells were almost exclusively observed in the progenitor compartment. Or, it could be that the D cyclins were identifying a population of cells in the G_1 phase that were not identified by Ki67 in addition to a population of cells also identified by Ki67. In support of the former theory is the finding that the D cyclin and Ki67 labelling indices correlated with each other and the fact that fewer basal cells were identified by the D cyclin antibodies than with Ki67. This could be investigated by the use of a double immunohistochemical staining applying the two antibodies sequentially. As cyclin D1 is commonly overexpressed in malignancy, this could be interpreted as an overexpression of the gene. However, this seems unlikely here.

Kotelnikov *et al* (1997) failed to demonstrate a correlation between cyclin D1 and the S-phase fraction, as assessed by *in vivo* administration of IrdU, but demonstrated apoptosis, identified by *in situ* end labelling was significantly more prevalent in cyclin D1 positive carcinomas of the head and neck. These authors suggested this finding might indicate a role for

cyclin D1 in apoptosis. In normal oral mucosa from the uvula the authors noted that cyclin D1 staining was predominantly suprabasal and surprisingly labelling indices were higher in these mucosae compared with non-involved mucosa from the carcinoma patients.

Using the same antibodies as the present study in non-small cell carcinoma of the lung, Mate *et al* (1996) demonstrated a correlation between cyclin D1 and Ki67 labelling indices but there was no association of cyclin D1 with cell cycle phase as assessed by flow cytometry.

5.5 Summary and conclusions

The expression of the D cyclins in oral epithelial dysplasia was high and correlated with that of Ki67 but this was weaker and not correlated in the larger group. This may be due to a continued expression of the D cyclins through phases of the cell cycle in addition to G_1. It appears unlikely that the D cyclins are identifying those cells in G_1 that were not identified by Ki67 described previously for two reasons. Firstly, the ratios of the labelling indices for the D cyclins and Ki67 were close to unity suggesting they are identifying similar groups of cells. Secondly, in support of this theory, the D cyclin antibodies were identifying less cells in the basal layer than Ki67.

Cyclin D3 appears to cross-react with the smooth muscle of blood vessels, a previously unreported phenomenon.

The distribution of cyclin E immunoreactivity was unexpected, being chiefly in cells of the maturation compartment. Such cells are normally

considered to have left the cell cycle and be terminally differentiated. This suggests that there might be an additional role for this cyclin in addition to G_1/S-phase transition.

There was some association of the cyclin labelling indices with the degree of atypia. However, this was not as strong as BrdU in Chapter 2 and with the inferior staining intensities using these antibodies, was not considered a useful adjunct in the objective assessment of oral epithelial dysplasia.

Table 5.1 Primary antibodies

Antibody	Catalogue Number	Clone	Working dilution	Optimal antigen retrieval
Cyclin D1	NCL-CYCLIN D1-GM	P2D11F11	1:50	p.c./citrate/ 2 minutes
Cyclin D3	NCL-CYCLIN D3	DCS-22	1:40	p.c./citrate/ 2 minutes
Cyclin E	NCL-CYCLIN E	13A3	1:40	m.w./EDTA/ 20 minutes
Ki67	NCL-Ki67	MM1	1:100	p.c./EDTA/ 1 minute

p.c. pressure cooker
m.w. microwave oven

Table 5.2 Patient clinical details, subjective grading and atypia scores

Case No.	Sex	Age	Clinical lesion	Site	Histology	Atypia scores
1	M	80	speckled leukoplakia	ton	severe dysplasia	29
2	M	85	erythroplakia	ton	severe dysplasia	52
3	M	65	erythroleukoplakia	ton	carcinoma in situ	31
4	M	42	speckled leukoplakia	ton	mild dysplasia	9
5	F	31	smokers melanosis	ton	mild dysplasia	8
6	M	46	leukoplakia	fom	moderate dysplasia	21
7	F	65	leukoplakia	fom	moderate dysplasia	15
8	M	49	leukoplakia	ton	moderate dysplasia	15
9	M	54	erythroplakia	ton	severe dysplasia	47
10	F	53	erythroleukoplakia	fom	moderate dysplasia	12
11	M	40	leukoplakia	fom	mild dysplasia	9
12	F	51	leukoplakia	fom	moderate dysplasia	14
13	F	68	erythroleukoplakia	ton	moderate dysplasia	16
14	F	76	leukoplakia	ton	moderate dysplasia	16
15	M	63	leukoplakia	fom	moderate dysplasia	13
16	F	62	leukoplakia	ton	mild dysplasia	9
17	M	52	leukoplakia	fom	mild dysplasia	7
18	F	54	leukoplakia	fom	mild dysplasia	18

fom= floor of mouth, ton= tongue

Table 5.3 Summary of results for 18 archival cases

	LI%				LI/BL			
Case	D1	D3	E	Ki67	D1	D3	E	Ki67
1	38.97	43.52	48.58	32.95	191.80	220.59	180.15	146.74
2	54.96	41.77	17.05	52.28	312.09	187.83	93.21	208.61
3	59.41	53.96	47.97	50.32	182.33	212.10	127.26	166.62
4	32.63	33.33	22.01	22.54	123.77	122.20	68.49	96.32
5	49.09	27.42	13.78	39.58	212.85	120.26	51.93	151.80
6	34.63	23.40	23.93	35.14	124.31	77.98	75.56	109.54
7	27.63	27.11	43.32	21.97	127.71	129.95	201.96	136.07
8	20.26	13.68	10.98	18.15	66.36	41.04	31.07	63.12
9	30.21	27.24	17.24	40.79	187.89	159.35	72.94	292.12
10	31.17	76.24	16.16	28.30	182.51	449.01	77.44	162.24
11	23.63	33.05	13.56	26.18	110.30	134.34	50.26	124.86
12	18.93	22.72	39.43	30.24	88.02	98.14	193.77	181.25
13	24.06	37.16	19.02	30.22	102.85	155.08	66.51	160.26
14	27.12	20.39	20.90	24.27	78.28	54.06	60.64	75.37
15	27.96	49.30	23.82	26.63	113.13	230.28	90.50	134.64
16	20.55	22.58	26.11	29.81	80.09	80.01	78.72	101.99
17	23.40	14.99	10.85	26.90	75.91	48.61	36.52	91.32
18	32.35	50.52	18.36	32.08	152.78	233.68	68.53	171.75
Mean	**32.05**	**34.36**	**24.06**	**31.57**	**139.61**	**153.03**	**90.30**	**143.03**
SD	11.72	15.93	12.31	9.22	62.75	96.86	51.63	53.80
Min	18.93	13.68	10.85	18.15	66.36	41.04	31.07	63.12
Max	59.41	76.24	48.58	52.28	312.09	449.01	201.96	292.12

Table 5.4 Values of the Spearman-Rank correlation coefficient for the 18 archival cases

a. percentage labelling indices

LI%	Cyclin D1	Cyclin D3	Cyclin E	Ki67
Cyclin D3	0.6987¶			
Cyclin E	0.2002	0.3117		
Ki67	0.6130§	0.4665*	0.1620	
Atypia	0.4252*	0.3653	0.3333	0.5521*

b. labelling indices per mm basement membrane length

LI/BL	Cyclin D1	Cyclin D3	Cyclin E	Ki67
Cyclin D3	0.6873¶			
Cyclin E	0.4262*	0.4912‡		
Ki67	0.6894§	0.6883§	0.5160‡	
Atypia	0.4572*	0.4314*	0.4180*	0.5449¶

¶ $p<0.005$
§ $p<0.01$
‡ $p<0.025$
* $p<0.05$

Table 5.5 Summary of results for 20 cases from Chapter 2

	LI%				LI/BL			
Case	D1	D3	E	Ki67	D1	D3	E	Ki67
1	29.18	38.06	28.57	18.38	125.75	152.42	94.41	79.62
2	29.71	39.37	24.19	20.89	127.51	188.02	78.60	122.71
3	42.84	29.06	37.42	21.97	158.08	114.53	109.83	111.21
4	36.72	29.49	26.67	21.64	167.41	127.03	109.26	99.75
5	37.76	24.28	39.41	28.44	178.10	105.84	199.13	158.30
6	0.00	0.00	30.08	73.12	0.00	0.00	143.99	553.35
7	50.16	47.01	5.24	25.14	112.99	108.91	12.63	90.11
8	53.50	53.53	18.92	23.92	241.32	235.67	79.40	132.01
9	17.54	31.34	36.38	56.15	58.28	103.33	193.03	283.43
10	45.23	22.06	40.50	23.40	238.82	96.14	193.69	130.86
11	45.84	47.56	48.02	36.73	136.46	158.13	169.10	153.07
12	20.31	24.65	32.39	18.70	70.87	95.23	119.32	89.53
13	28.46	13.09	5.19	19.11	123.48	51.68	19.29	90.06
14	25.66	7.62	33.00	12.63	61.42	20.63	92.60	65.75
15	26.64	27.86	34.31	60.05	85.50	87.31	87.82	294.99
16	27.11	23.41	33.12	14.21	79.34	74.11	86.90	69.74
17	23.58	15.55	22.08	21.74	77.90	47.92	46.40	58.68
18	31.42	27.47	22.81	21.99	139.49	105.41	82.74	84.19
19	28.31	37.08	41.69	22.99	99.54	165.75	190.57	97.90
20	11.76	33.44	9.96	15.17	37.99	122.29	38.67	60.85
Mean	**30.59**	**28.60**	**28.50**	**27.82**	**116.01**	**108.02**	**107.37**	**141.31**
SD	13.13	13.38	11.85	16.31	61.65	55.79	58.04	116.58
Min	0.00	0.00	5.19	12.63	0.00	0.00	12.63	58.68
Max	53.50	53.53	48.02	73.12	241.32	235.67	199.13	553.35

Table 5.6 Values of the Spearman-Rank correlation coefficient for the combined group of 38 cases

a. percentage labelling indices

LI%	Cyclin D1	Cyclin D3	Cyclin E	Ki67
Cyclin D3	0.5434¶			
Cyclin E	0.0942	0.0453		
Ki67	0.2049	0.2328	0.1406	
Atypia	0.1285	0.0073	0.2996*	0.1873

b. labelling indices per mm basement membrane length

LI/BL	Cyclin D1	Cyclin D3	Cyclin E	Ki67
Cyclin D3	0.6793¶			
Cyclin E	0.2140	0.1905		
Ki67	0.4023§	0.4272¶	0.4289¶	
Atypia	0.0236	-0.0782	0.3122*	0.2057

¶ $p<0.005$
§ $p<0.01$
* $p<0.05$

Table 5.7 Percentages of basal cells identified by the antibodies

	18 cases	38 cases
cyclin D1	29.14% (5.24-60.29)	31.80% (5.24-90.29)
cyclin D3	33.25% (9.66-69.03)	32.17% (9.66-69.03)
cyclin E	8.24% (1.57-17.42)	9.83% (1.57-17.42)
Ki67	40.26 (18.59-78.82)	N/A

N/A not available

Table 5.8 Ratios of Ki67 to cyclin labelled cell cells (38 cases)

	LI%	LI/BL
Ki67:cyclin D1	0.97	1.15
Ki67:cyclin D3	1.00	1.21
Ki67:cyclin E	1.43	1.82
cyclin D1:cyclin D3	1.15	1.17

Table 5.9 Mean labelling indices by site

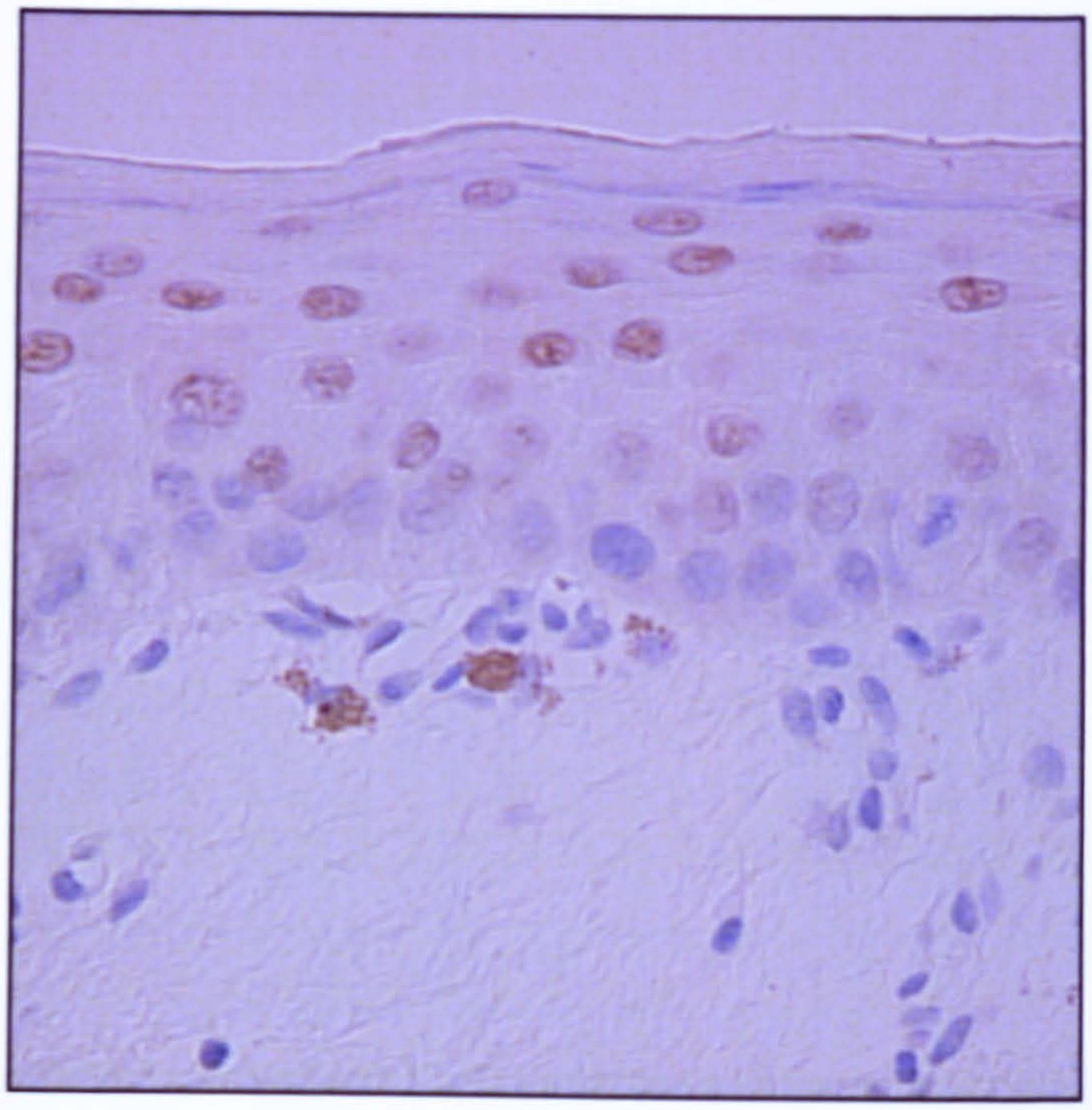
A. Tongue (17 cases)

	LI%				LI/BL			
	D1	D3	E	Ki67	D1	D3	E*	Ki67
Mean	**30.91**	**33.31**	**27.35**	**28.56**	**128.63**	**141.87**	**112.80**	**148.80**
SD	11.74	16.02	11.03	13.14	56.26	92.21	59.60	103.87
Min	0.00	0.00	5.24	14.21	0.00	0.00	12.63	69.74
Max	53.50	76.24	43.32	73.12	241.32	449.01	201.96	553.35

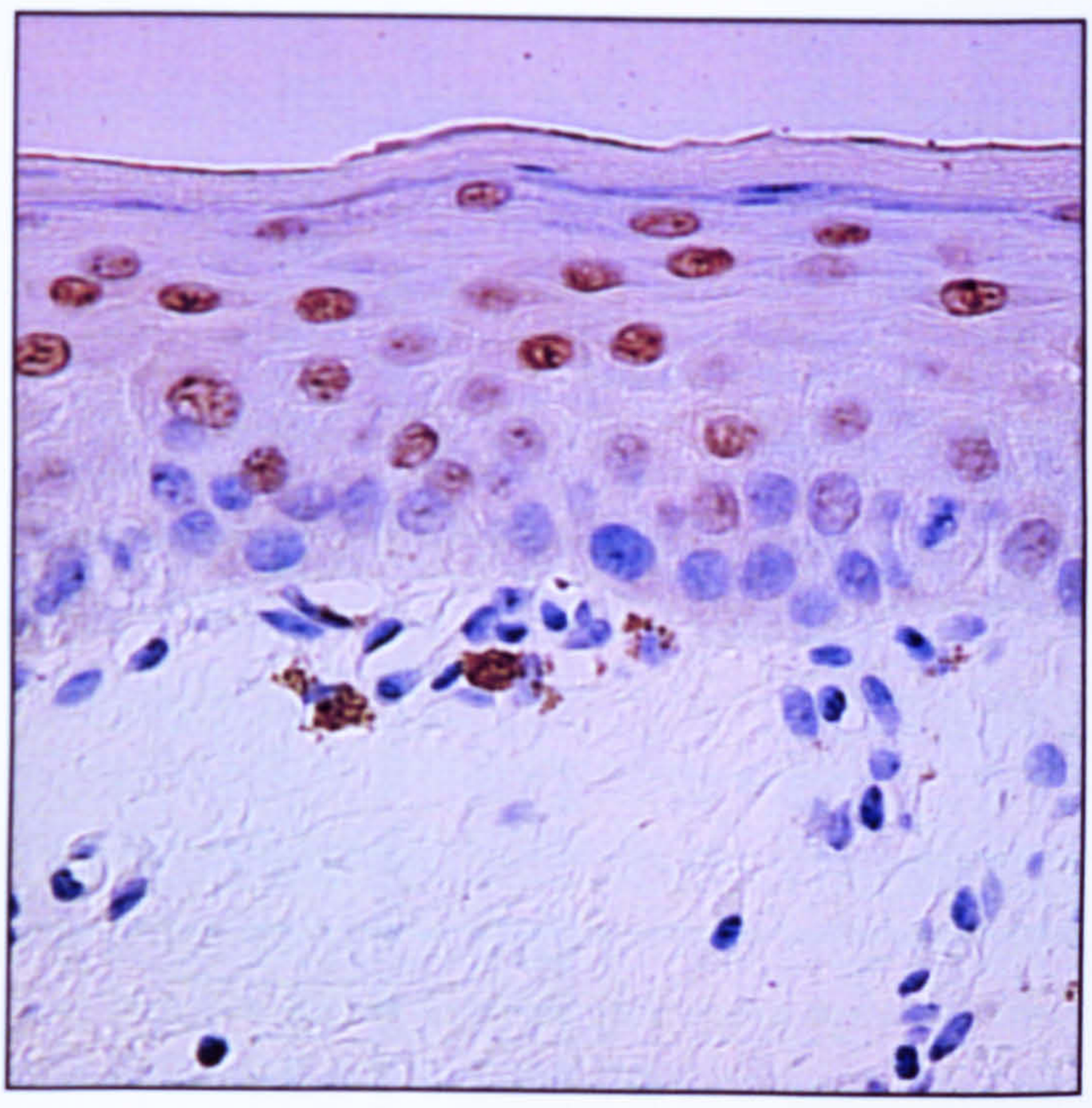
B. Floor of mouth (21 cases)

	LI%				LI/BL			
	D1	D3	E	Ki67	D1	D3	E*	Ki67
Mean	**31.74**	**28.87**	**25.21**	**30.88**	**125.41**	**113.86**	**82.60**	**133.87**
SD	13.39	13.02	13.59	13.98	71.15	61.44	45.05	74.82
Min	11.76	7.62	5.19	12.63	37.99	20.63	19.29	58.68
Max	59.41	53.96	48.58	60.05	312.09	220.59	180.15	294.99

*significantly different groups (p=0.0401)



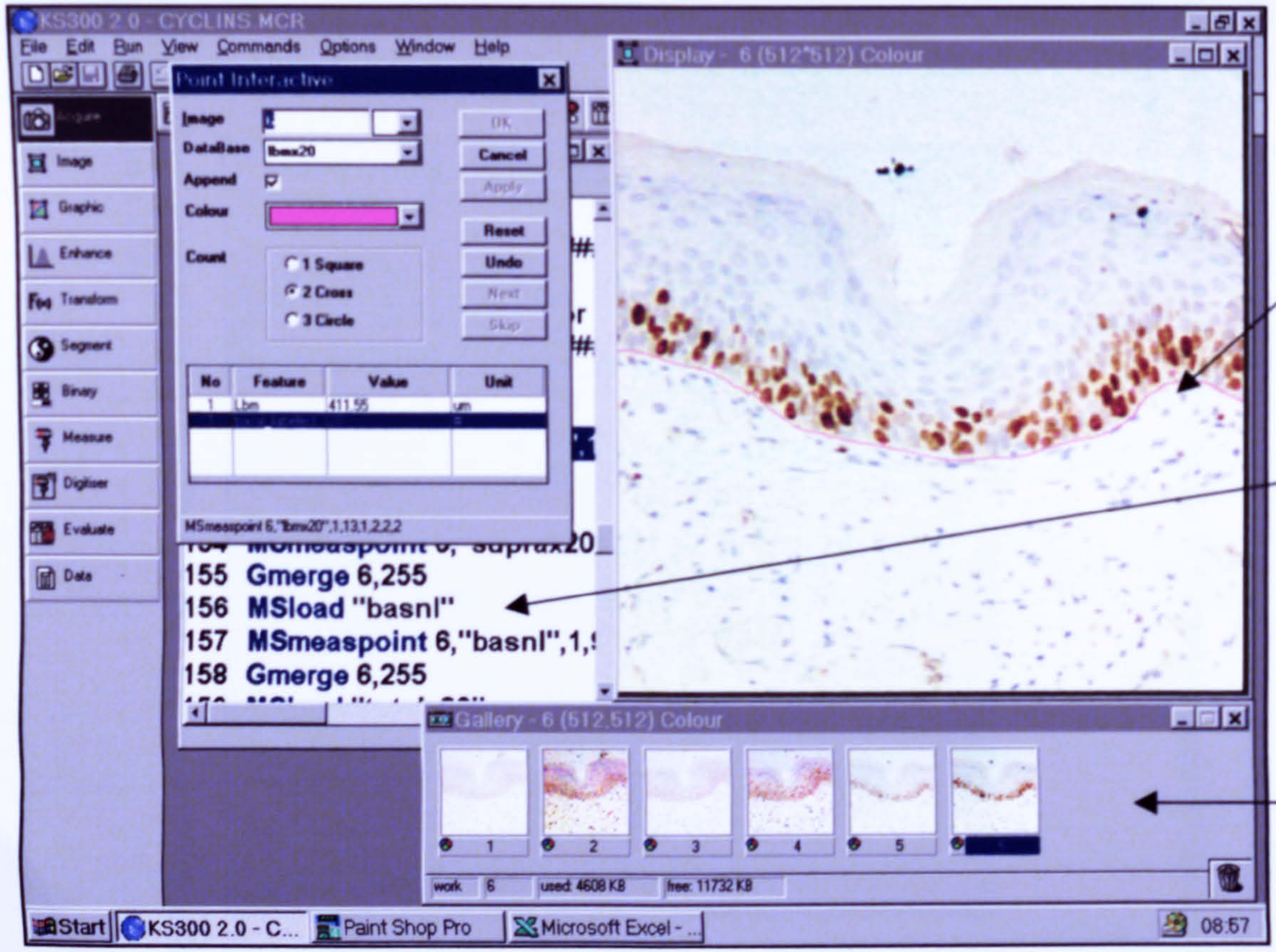
A. Initial captured image.



B. Image following enhancement of contrast.

Figure 5.1 Enhancement of digital image by the Kontron KS300 image analysis software. (Anti-cyclin E, magnification x478)

Figure 5.2 Kontron KS300 image analysis software



Main display image used for quantification. Here measurement of the basement membrane length and counting of basal cells identified by Ki67 was being performed

The customised macro program runs in the background

Image gallery to aid in the location of corresponding fields. For each of the preceding cases the images were captured and the contrast enhanced

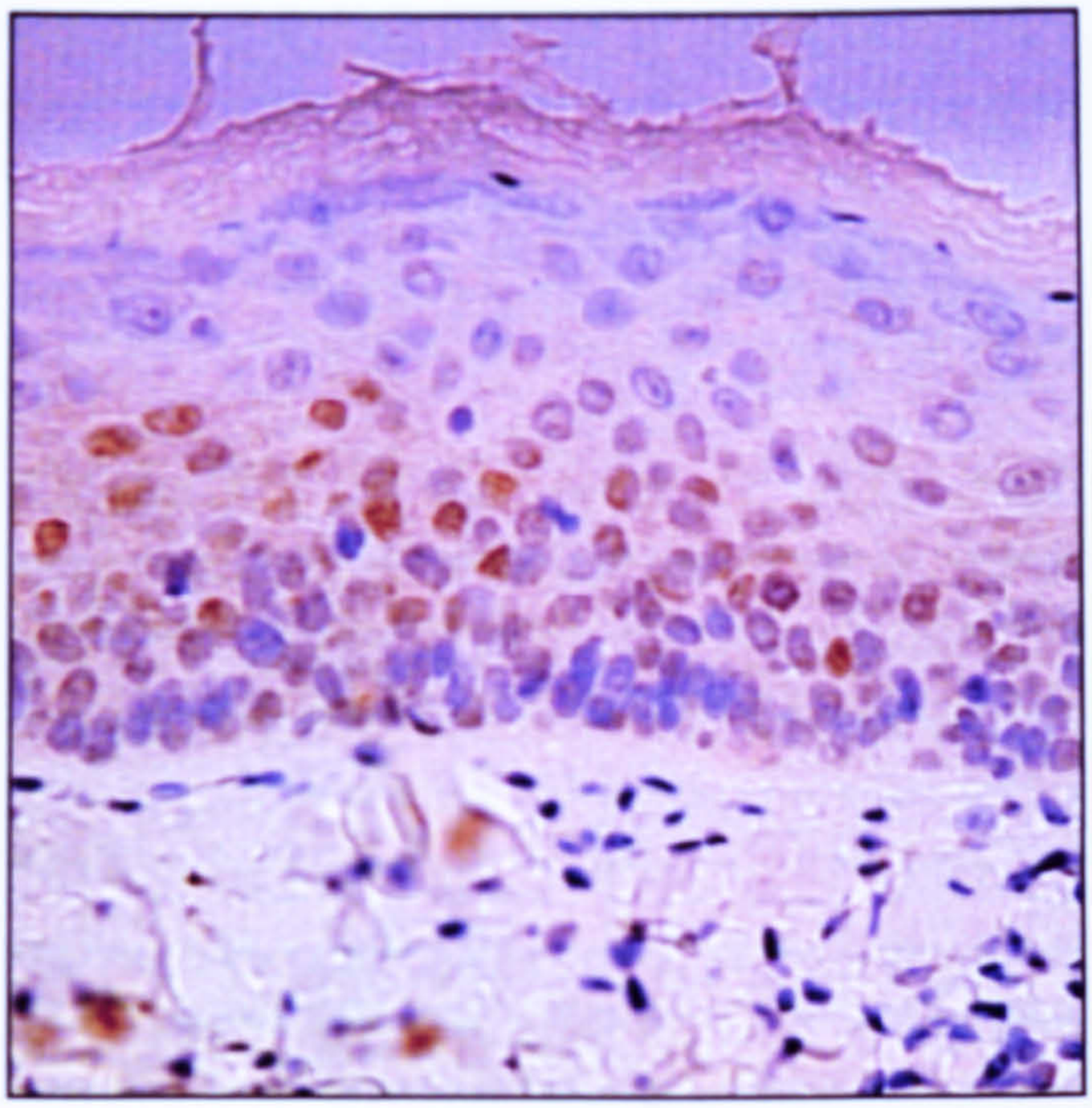


Figure 5.3 Demonstration of cyclin D1 immunoreactivity in Case 8 (magnification x478)

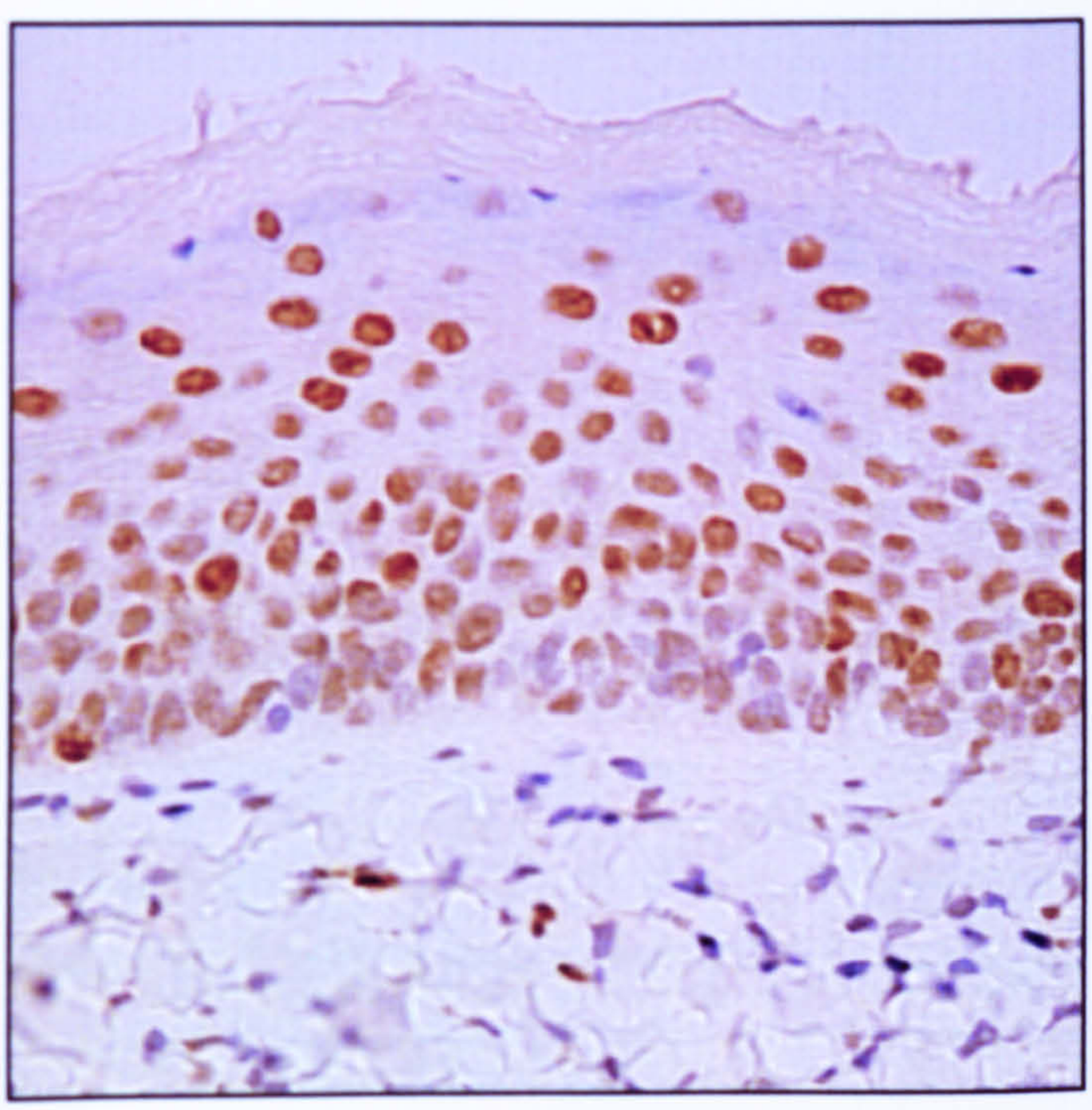


Figure 5.4 Demonstration of cyclin D3 immunoreactivity in Case 8 (magnification x478)

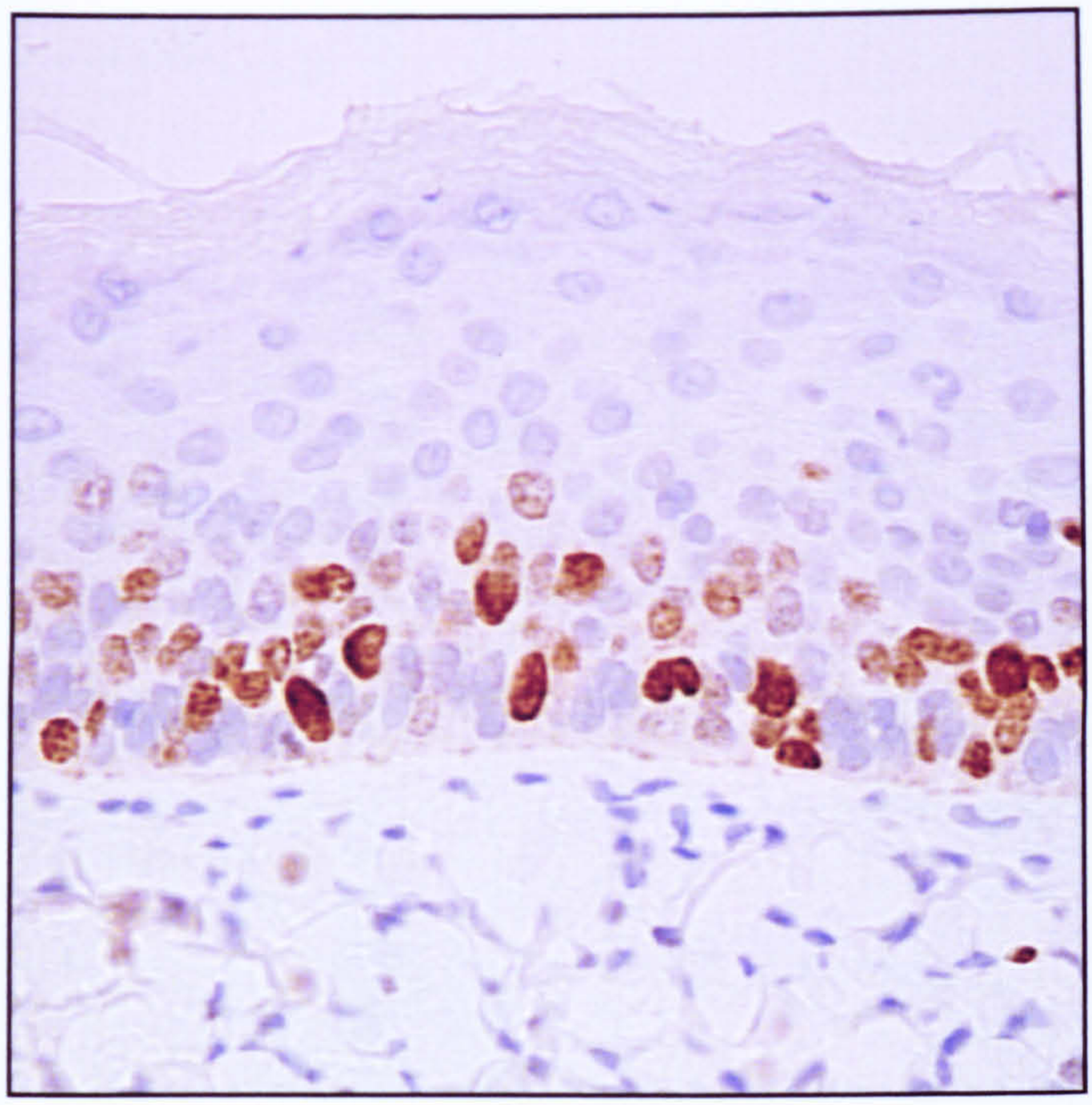


Figure 5.5 Demonstration of Ki67 immunoreactivity in Case 8 (magnification x478)

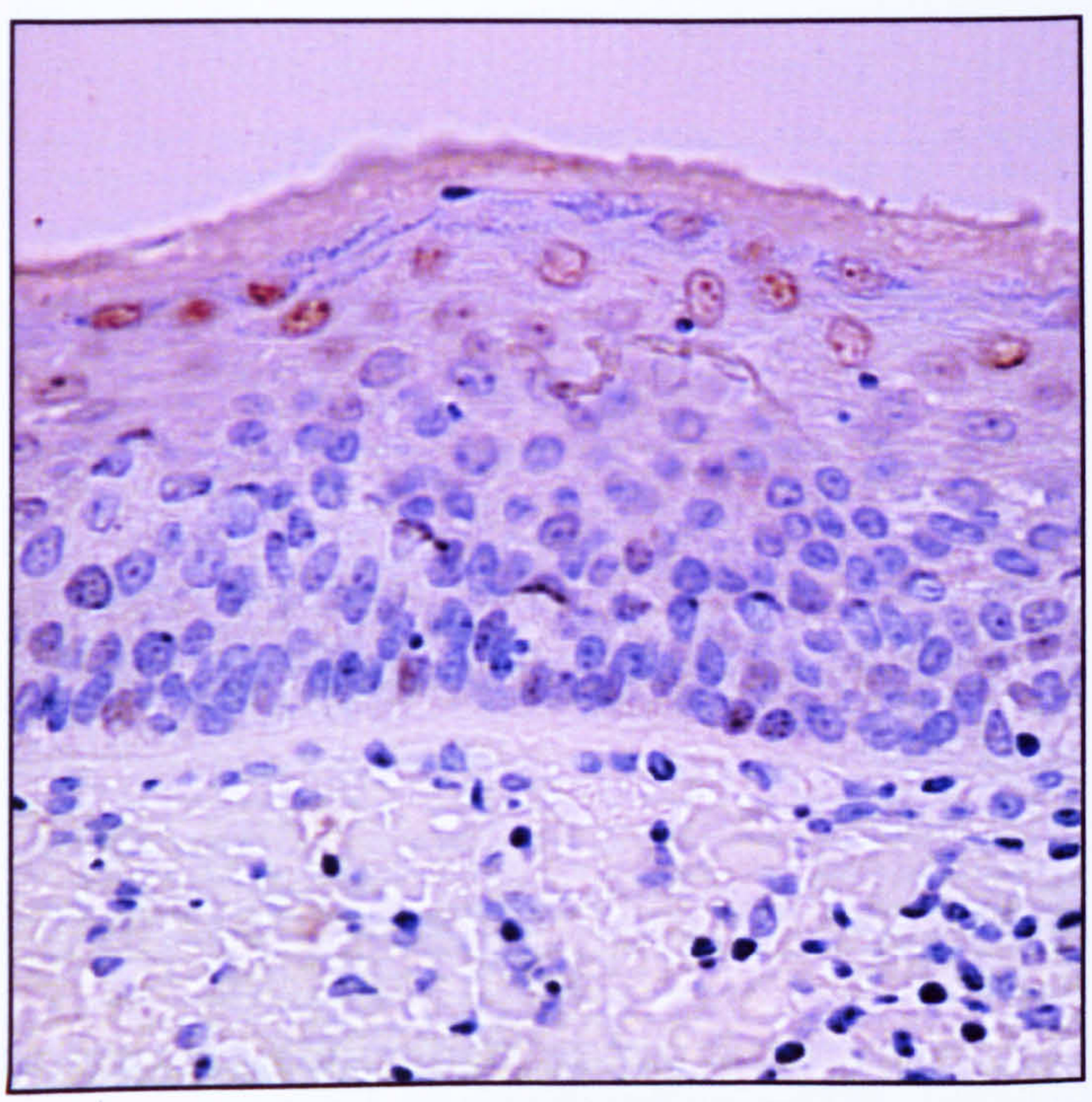
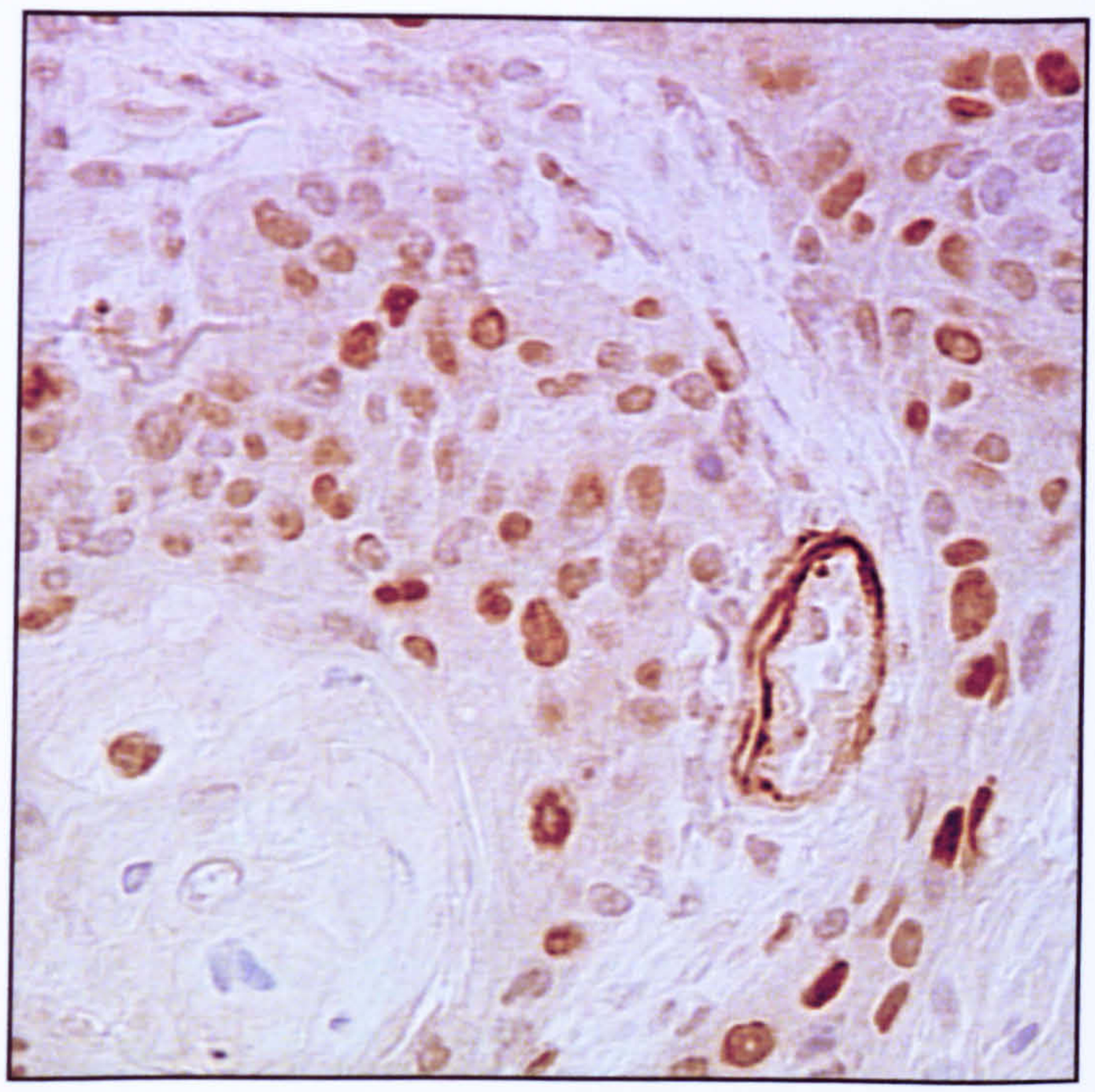


Figure 5.6 Demonstration of cyclin E immunoreactivity in Case 8 (magnification x478)

A. In the connective tissue of a dysplastic lesion (magnification x236)



B. A blood vessel within squamous cell carcinoma of a positive control (magnification x478)

Figure 5.7 Cyclin D3 cross-reactivity in the smooth muscle layer of blood vessels

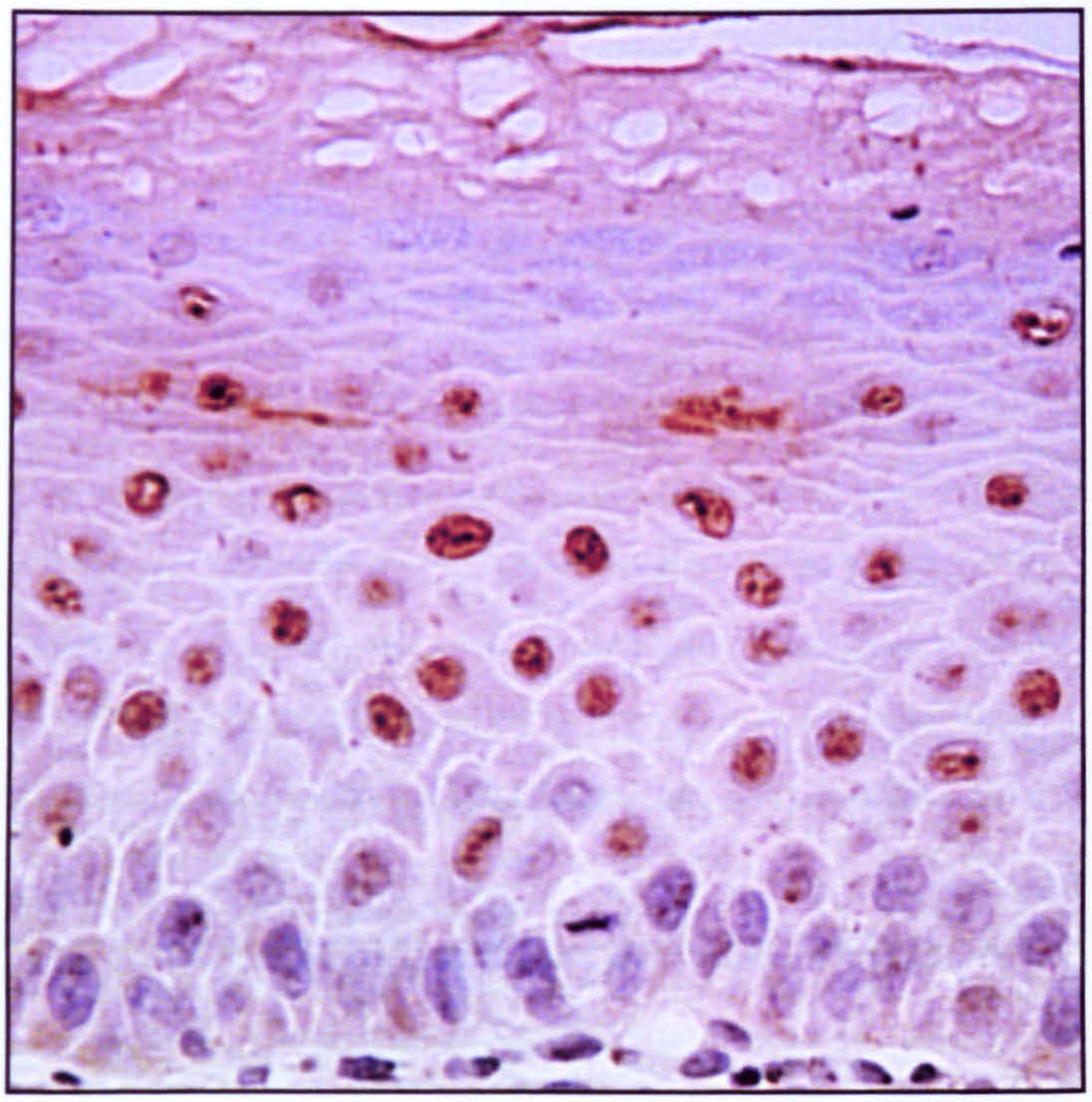


Figure 5.8 Demonstration of cyclin E immunoreactivity in the maturation compartment of the epithelium of Case 7 (magnification x478)

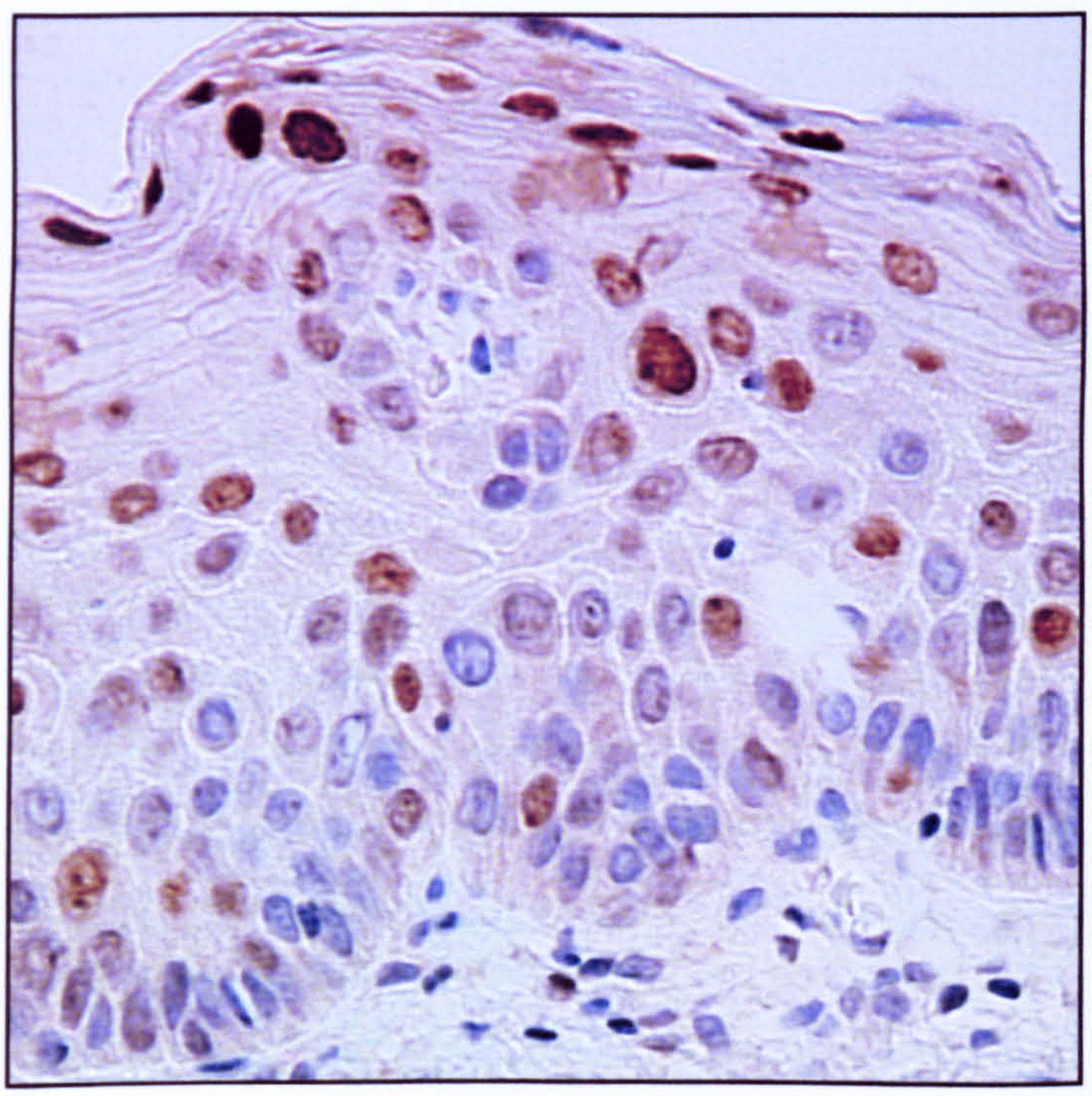


Figure 5.9 Increased cyclin E immunoreactivity in Case 3 (magnification x478)

Chapter 6

Further investigation of the S-phase and growth fraction

6.1 Introduction

6.1.1 Cyclin A

One of the S-phase cyclins, cyclin A, is concerned with the transition of the cell through the S-phase, into G_2 and into mitosis. Cyclin A, like cyclin E, complexes with CDK2 but unlike the other complexes appears to bind directly to the transcription factor E2F (Pines, 1995). Cyclin A also binds to $p34^{cdc2}$ involved in the transition from G_2 to M-phase (Pagano *et al.*, 1992; Roy *et al.*, 1991). It has been suggested that cyclin A transcription is stimulated by a signal from surface adhesion molecules late in the G_1/early S-phase (Pines, 1995). The levels of cyclin A increase from the beginning of the S-phase reaching a peak in G_2 (Gong *et al.*, 1995a) and it is then broken down by the ubiquitin-proteasome pathway just prior to metaphase (Spataro *et al.*, 1998). At this stage cyclin B1 becomes associated with $p34^{cdc2}$ and the cell continues through mitosis. Compared to the other cyclins there are only a limited number of publications on cyclin A, particularly in regard to the histopathology of neoplasms.

6.1.2 Cyclin A and the S-phase

Cyclin A production begins early in the S-phase and has been shown to correlate strongly with DNA synthesis as assessed by BrdU incorporation (Juan *et al.*, 1997). Overexpression of cyclin A has been shown to advance cells into the S-phase, shortening the cell cycle time (Rosenberg *et al.*, 1995)

The specificity of cyclin A to the S-phase makes it a possible alternative to BrdU labelling.

6.1.3 Aims

The aims of the studies in this chapter are two-fold; firstly a comparison of BrdU and cyclin A, as another potential marker of cells in the S-phase with the possibility of utilising this marker in place of BrdU with the advantage of being able to use it on archival tissues. At the same time a further study upon the relationship between the S-phase and the growth fraction was carried out to continue the work of Chapter 2. Following the development of the methods in Chapter 3, these cases were all fixed in formalin subsequent to BrdU incubation. This, therefore, allowed sequential sections to be studied with other proliferation markers.

6.2 Materials and Methods

6.2.1 Case selection

A series of 15 biopsies of oral leukoplakia or erythroplakia from the floor of the mouth or tongue were collected prospectively from patients attending the Oral Medicine Department at Glasgow Dental Hospital. The tissue was divided at the time of biopsy as described in Chapter 2. The fresh tissue was transported to the laboratory in MEM and BrdU incubation commenced within 20 minutes. In order to investigate the findings of these studies further, the series of patients from Chapter 2 were also included in parts of the study.

6.2.2 BrdU

The BrdU administration was performed as previously described in Section 2.2.2 but the tissues were fixed in 10% buffered formalin. Sequential sections were cut from the paraffin embedded tissues and mounted on silane coated slides. Immunohistochemistry was performed following optimisation of antigen retrieval as described in Section 3.2.3.

6.2.3 Cyclin A immunohistochemistry

Sequential sections were cut from BrdU processed blocks and preliminary studies were performed to optimise antigen retrieval, antibody concentration and incubation times as previously described. Immunohistochemistry was then performed as described in Chapter 2. The primary antibody used in this instance was a monoclonal mouse anti-cyclin A antibody (NCL-CYCLIN A, clone 6E6, Novocastra, Newcastle, UK).

The section in between the BrdU and cyclin A was stained with Ki67 as described previously. Additionally, to the two series of cases described above, the series of cases described in Chapter 5 was available for the evaluation of cyclin A and Ki67 but not the BrdU.

6.2.4 Quantification

The procedure described in Chapter 5 was adopted for the quantification of the immunohistochemically stained sections. The sections were quantified in the following order: BrdU, Ki67, cyclin A. For each case three corresponding fields were identified and measured at an objective

magnification of x20. The quantification of labelled cells was expressed as a percentage of the total nucleated cell population (LI%) and per mm basement membrane length (LI/BL). For ease of use, the term labelling index was used to express the quantified cyclin A cells, although this is not strictly a labelling index as in the case of BrdU labelled cells. As previously, the labelling indices for Ki67 were expressed as the growth fraction (GF). An H&E section from each case was graded according to the method of Smith and Pindborg (1969)

Statistical analysis was performed on the data using the Spearman rank correlation coefficient, the Wilcoxon matched-pairs signed-ranks test and the Mann-Whitney U test as appropriate.

6.3 Results

6.3.1 Case details

The clinical details of the 15 new patients studied are shown in Table 6.1. The mean age was 51.9 (range 31 to 78 years). Eight of the patients were male (mean age 54.0) and seven of the patients were female (mean age 49.4). The histology of four of the lesions (cases 3, 7, 10 and 15) showed no or minimal dysplasia when graded subjectively. These were still included in the study. Three of the biopsies of this series (cases 6, 10 and 12) were from the same patients as those studied in Chapter 2. These will be highlighted in the results below.

6.3.2 Immunohistochemistry

BrdU immunohistochemistry was optimal following antigen retrieval in EDTA for 15 minutes using microwave heating as was that for Ki67.

Cyclin A required 15 minutes of microwave antigen retrieval in EDTA and an antibody concentration of 1 in 100 at room temperature for 1 hour for optimal staining results. The intensity was equivalent to that of BrdU immunostaining (Figure 6.1). The staining pattern was notable for this antibody; there was strong nuclear staining but also a fine granular staining throughout the cytoplasm of most positive cells (Figure 6.2). When cells were apparently suprabasal, often an extension of their cytoplasm to the basement membrane was observed. Figure 6.3 shows an apparent over expression of cyclin A.

6.3.3 Quantitative results: LI%

The results for the two series of cases are summarised in Tables 6.2 and 6.3. Considering first the group of 15 new cases, the mean LI% for the BrdU labelled cells was 11.24% (SD 2.83) and for cyclin A 12.76% (SD 3.88). These indices correlated with each other significantly (r_s=0.5268, z=1.9711, p=0.0244). The GF% for Ki67 of this series was 29.25% (SD 11.88). There was no significant correlation between the GF% and BrdU LI% (r_s=0.1571, z=0.5880, p=0.2810) nor between GF% and cyclin A LI% (r_s=0.3911, z=1.4633, p=0.0721). This indicates that a variable fraction of the GF are in the S-phase. As in the series described in Chapter 2, the BrdU and Ki67 indices did not correlate (p=0.2810). The atypia scores of this group ranged from three to 22. Of these indices, only

the GF% correlated with the atypia scores significantly (r_s=0.7802, z=2.9193, p=0.0018).

The mean LI% for BrdU of the original series of 20 cases was 10.80% as determined in Chapter 2. The mean LI% for cyclin A of this series was 19.84% (SD 9.96). The quantification here, it should be noted, was performed on different tissue blocks, the former Carnoy's fixed and the latter formalin fixed. These indices did not correlate with each other significantly (r_s=0.3165, z=1.3798, p=0.0853) nor with the atypia scores (p<0.1). These results show a quantitatively similar mean BrdU LI% for both of the series of cases but the mean LI% of cyclin A labelled cells was larger in the original BrdU series from Chapter 2 and this difference was significant (z=2.0203, p=0.0217). The relationship of the labelling indices to each other and to the atypia scores is illustrated in Figure 6.4.

The results from the series of 18 cases studied in Chapter 5 are shown in Table 6.4. The mean Ki67 LI% as determined in Chapter 5 was 31.57% compared to the cyclin A LI% determined in this study of 16.50% (SD 6.29). These indices correlated with each other significantly (r_s=0.5418, z=2.2339, p=0.0132) and the cyclin A LI% correlated with the atypia scores (r_s=0.4613, z=1.9020, p=0.0287). The mean LI% for cyclin A in this series was in between the two other series described above.

6.3.4 Quantitative results: LI/BL

The mean LI/BL for the series of 15 cases was 40.93/mm (SD 11.88) for BrdU and 47.59/mm (SD 18.28) for cyclin A. These indices were

highly significantly correlated (r_s=0.6911, z=2.5858, p=0.0049) but did not correlate with the atypia scores. The mean GF/BL for this group was 110.72/mm (SD 49.30) and this was significantly correlated with the atypia scores (r_s=0.9648, z=3.6101, p<0.0001).

From the results in Chapter 2, of the initial series of 20 cases the mean LI/BL for BrdU was 46.58/mm (SD 20.28) which correlated with the atypia scores. The mean LI/BL for cyclin A in this series was 67.98/mm (SD 34.21). These indices were correlated with each other significantly (r_s=0.6992, z=3.0480, p=0.0012). The mean GF/BL for this group was 141.31/mm and this correlated with BrdU LI/BL as determined in Chapter 2. The GF/BL also correlated with the cyclin A LI/BL (r_s=0.8662, z=3.7755, p<0.00011).

As with the LI%, the mean BrdU LI/BL were quantitatively similar but the mean cyclin A LI/BL was higher in the first series of 20 cases. This difference did not reach statistical significance (z=1.3131, p=0.0951)

The series of 18 cases studied in Chapter 5 gave a mean GF/BL of 67.16. The mean cyclin A LI/BL for this group was 143.03 (SD 53.80) which correlated with the GF/BL highly significantly (r_s=0.8215, z=3.3870, p=0.00005) and with the atypia scores (r_s=0.5232, z=2.1573, p=0.0158).

6.3.5 Ratios and distributions

With regard to the new series of 15 cases studied, of the total basal cell population, an average of 19.00% (SD 8.70) of the cells were identified by BrdU compared with an average of 21.40% (SD 9.99) of the

cells identified by cyclin A. From a different angle, a mean 39.29% (SD 18.15) of all of the BrdU labelled cells were basal and a mean 38.42% of the cyclin A labelled cells were basal. Ki67 identified an average of 39.64% (SD 26.70%) of the basal cell population equating to 30.17% (SD 16.01) of the total Ki67 labelled population.

The mean ratios of cyclin A to BrdU positive cells were 1.14 (0.63 to 1.86) and 1.16 (0.64 to 2.09) for the percentage and per mm indices respectively indicating that cyclin A was identifying just over 15% more cells than BrdU. This compares with the original series where the mean ratios were 1.86 (1.13 to 3.71) for the percentage indices and 1.47 (0.93 to 2.88) for the per mm indices.

The S-phase to growth fraction ratios are listed in Tables 6.2 and 6.3. If the S-phase represented by BrdU is S_{BrdU} and that represented by cyclin A is $S_{cyclinA}$ and the growth fraction identified by Ki67 is GF the means are as follows. The mean S_{BrdU}:GF was 0.43 (0.22 to 0.62) and the mean $S_{cyclinA}$:GF was 0.48 (0.22 to 0.82) based on the labelling index percentages. These equated to a mean S_{BrdU}:GF of 0.41 (0.21 to 0.65) and a mean $S_{cyclinA}$:GF of 0.47 (0.24 to 0.84) based upon the labelling indices per mm, the latter being valid here as the measurements were performed on sequential sections and identical areas were measured. The mean S_{BrdU}:GF, for the percentage labelling indices, of the original series of 20 cases was 0.46 (0.11 to 0.94) as determined in Chapter 2. The mean $S_{cyclinA}$:GF was 0.75 (0.42 to 1.12) reflecting the higher cyclin A LI% of this

group.

6.3.6 Quantitative results by site

By combining the results of the two series of cases, the mean labelling indices for the two sites, tongue and floor of mouth, are listed in Table 6.5, the sites of origin of the first group of cases can be found in Table 2.1. There was no significant difference between the two sites for the percentage indices assessed by the Mann-Whitney U test. There was, however, a statistically significant difference between the two sites with regard to their labelling indices per mm basement membrane length for all three of the markers; BrdU ($p=0.0375$), Ki67 ($p=0.0170$) and cyclin A ($p=0.0322$). The mean epithelial thickness of the two groups were 217.91μm (SD 128.07) for the tongue and 164.39μm (SD 64.10) for the floor of the mouth but these were not significantly different due to the large range of thickness at the two sites. When the basement membrane lengths measured in each of the quantified fields for each of the three markers (nine fields) for each of the cases was considered the following was found. The mean basement membrane length (3 fields) for those cases from the tongue was 2260.01μm (SD 675.37) and for the floor of mouth was 1855.81μm (SD 585.45). These were significantly different ($p=0.0239$) and account for the different labelling indices for the two sites when expressed per mm basement membrane length.

6.4 Discussion

6.4.1 Cyclin A

There are conflicting reports regarding the cellular localisation of the cyclin A protein. Pines and Hunter (1991) reported cyclin A as being a predominantly nuclear protein but indicated it was also found dispersed throughout the cell following prophase. In the present study, cyclin A immunoreactivity was commonly observed in the cytoplasm, but rarely in the absence of strong nuclear staining. However, in the present study, the immunohistochemical detection of the protein was in cells presumed to be pre-mitotic and would appear to contradict the above report. Carbonaro-Hall *et al* (1993) reported in immunocytochemical studies that cyclin A was present in the cytoplasm but not the nucleus of late G_1 cells and both the cytoplasm and nucleus of S-phase cells. Cytoplasmic staining with cyclin A antibodies in pre-mitotic cells has been reported in tonsillar epithelium (Bodey *et al.*, 1994).

Jordan *et al* (1998) reported a mean LI% for cyclin A of 20.5% and a mean Ki67 GF% of 30.2% in a series of 36 oral epithelial dysplastic lesions, these figures being within the range of values derived from the present study. However, these authors reported no significant difference between the dysplastic group and the normal epithelium except their severely dysplastic group. The group of cases studied by these authors included more cases with severe epithelial dysplasia than the present study.

The mean cyclin A labelling indices were higher in the original series of 20 cases from Chapter 2 than they were for the series collected for the present study. This probably reflects the generally lower severity of dysplasia (atypia scores from 3 to 22) of the smaller series compared with the original series from Chapter 2 (atypia scores 10 to 57). Additionally, the gene for cyclin A as with other cyclins could be amplified, mutated or the protein overexpressed, therefore acting as an oncogene, as part of the carcinogenic process.

Certainly, apparent overexpression of the protein detected immunohistochemically was evident in some cases in this study, particularly cases from the original series which were severely dysplastic (Figures 6.3 and 6.4). Degradation of cyclin A is also required for exit from mitosis (Roy *et al.*, 1991) and it has proved difficult experimentally to produce constitutive cyclin A overexpression as it may not be compatible with cell viability (Rosenberg *et al.*, 1995). However, Chao *et al* (1998) recently reported overexpression of cyclin A protein in hepatocellular carcinoma, some of which had amplified DNA or increased mRNA, and this was correlated with tumour recurrence and decreased overall survival.

Cyclin A for use as an investigative tool for cell kinetics may, therefore, be of value only in normal tissues because of the possibility of it being overexpressed in precancerous or cancerous lesions.

The curious observation of immunohistochemically detectable cyclin A protein in the cytoplasm often extending to the basement

membrane in cells that were apparently suprabasal raises an important issue (Figure 6.2). The histological section is only a two dimensional representation of a three dimensional structure and although cells are apparently suprabasal, above or below the plane of section these cells could be in contact with the basement membrane. The thin cytoplasmic processes observed with cyclin A not normally being discernible in histological sections.

6.4.2 Cyclin A and the S-phase

Juan *et al* (1997) described a series of experiments investigating the relationship between BrdU uptake and cyclin A expression assessed immunocytochemically and by flow cytometry in MOLT-4 human leukaemic cells. These authors found a significant correlation between BrdU labelled cells and cyclin A labelled cells. Moreover, cyclin A identified cells of the early S-phase that were not yet labelled by BrdU. However, cyclin A expression continues through G_2 and the protein is degraded at the prometaphase and theoretically would overestimate the number of cells in the S-phase. Assuming all the cells that enter the S-phase actually exit into G_2 it nevertheless provides a useful marker of cells in this stage of the cell cycle. A potential method for increasing the specificity of cyclin A to the S-phase would be to double-stain sections for both cyclin A and cyclin B1. The latter is said to be produced at the G_2/M phase of the cell cycle (Pines and Hunter, 1991) and would indicate those cells which have entered mitosis.

Cyclin A has been shown to bind to both CDK2 and $p34^{cdc2}$ and therefore to be important at two points in the cell cycle namely the S-phase and the G_2/M phase (Pagano *et al.*, 1992). It could therefore be expected that cyclin A labelling indices are greater, as found in the present study, than those for BrdU which by its nature can only be expressed in the S-phase.

However, the study of BrdU presented here did not take into account the possibility of endogenous thymidine synthesis which could potentially reduce the number of BrdU labelled cells. The protocol upon which this was based (Thornton *et al.*, 1988) used fluordeoxyuridine to block such de novo synthesis. However, these workers labelled their endometrial specimens for periods of one to three hours, much longer periods than in the present study; in such situations de novo thymidine synthesis may be more significant.

The oncogene B-*myb* appears to be closely related to the S-phase and may have a role in the downstream activation of transcription factors (Saville and Watson, 1998). It is activated by cyclin A and appears essential for S-phase progression. This may be a further avenue of research with regard to the S-phase.

6.4.3 S-phase and growth fraction

Jordan *et al* (1998) reported a significant correlation between cyclin A and Ki67 percentage labelling indices in normal and dysplastic oral epithelium. The LI% for BrdU did not correlate with the GF% for Ki67 in

the 20 cases of Chapter 2 nor did the LI% for cyclin A determined in the present study. This probably reflects the variable proportion of the growth fraction in the S-phase as evidenced by the S-phase:growth fraction (Tables 6.2 and 6.3).

The variations of the labelling indices at the two different sites when expressed with regard to the basement membrane length raised an important issue with regard to quantification. The morphology of the epithelium differs from the floor or mouth and tongue. The latter has more pronounced rete ridges and therefore normally has a longer basement membrane length than that of the floor of the mouth. Similarly this is often observed in dysplastic oral epithelia. When the indices based upon the total cell population were considered, the indices for both sites were quantitatively similar. This indicates the need for total nuclear counts in addition to basement membrane measurement. The latter is often performed in addition to labelled nuclear counts to provide a labelling index as it is relatively quick to perform. This can be flawed as it does not take into account the thickness of the epithelium which can be shown to be variable as shown in the present study. Total nuclear counts can take considerably longer to achieve, but give more valid indices for comparison of epithelia of differing thickness and basement membrane configuration.

6.5 Summary and conclusions

The studies presented in this chapter have demonstrated a correlation between cyclin A and BrdU labelling indices. The former, as was to be expected, were higher than the BrdU labelling indices. There was also the possibility that the cyclin A protein was being overexpressed or abnormal, especially in more severely dysplastic lesions. This may limit the application of this technique for studies of the kinetics of tissues but in normal or non dysplastic tissues it would appear that cyclin A is a viable alternative to BrdU in the study of the S-phase.

Expanding the work of Chapter 2, it was demonstrated that BrdU could be used successfully with formalin fixation and antigen retrieval and its use could be combined with other antibodies that recognise cell cycle-associated proteins such as Ki67. From the very limited follow-up times of the new series of BrdU incubated cases it is not yet possible to apply the data regarding S:GF which appeared promising in Chapter 2 as a possible marker of prognosis. The alternative use of cyclin A and Ki67 was not investigated on archival material where long term clinical data would be available but this is a possible avenue of further study.

Table 6.1 Patient clinical details and subjective histological grading

Case	Sex	Age	Clinical lesion	Site of biopsy	Histology
1	f	67	leukoplakia	fom*	mild dysplasia
2	m	50	erythroplakia	fom	mild/mod dysplasia
3	f	39	leukoplakia	ton	hyperplasia
4	m	31	leukoplakia	fom	mild dysplasia
5	m	50	leukoplakia	ton	moderate dysplasia
6[a]	f	34	leukoplakia	fom	moderate dysplasia
7	m	58	leukoplakia	fom	keratosis
8	f	47	leukoplakia	ton	severe dysplasia
9	m	63	leukoplakia	ton	mild dysplasia
10[b]	m	78	leukoplakia	ton	keratosis
11	m	52	leukoplakia	fom	mild dysplasia
12[c]	f	31	leukoplakia	fom	mild/mod dysplasia
13	f	66	leukoplakia	ton	mild/mod dysplasia
14	f	62	leukoplakia	fom	mild dysplasia
15	m	50	leukoplakia	ton	keratosis

*fom= floor of mouth, ton= tongue

[a]biopsy from same patient as case 7 in original series (Chapter2)

[b]biopsy from same patient as cases 11 and 15 of the original series

[c]biopsy from same patient as case 19 of the original series

Table 6.2 Summary of results for series of 15 cases

Case	Epithelial thickness (μm)[§]	Atypia	BrdU		Ki67		Cyclin A		S_{BrdU}:GF		$S_{cyclin\ A}$:GF	
			LI%	LI/BL	GF%	GF/BL	LI%	LI/BL	LI%	LI/BL	LI%	LI/BL
1	114.62	4	11.39	40.69	18.27	77.78	7.17	26.03	0.62	0.52	0.39	0.33
2	192.88	17	12.39	53.46	35.75	158.58	14.51	61.13	0.35	0.34	0.41	0.39
3	164.51	6	14.09	32.41	34.61	85.91	14.90	34.35	0.41	0.38	0.43	0.40
4	153.07	22	11.18	57.96	26.44	149.84	15.28	82.26	0.42	0.39	0.58	0.55
5	62.61	17	12.67	27.62	26.78	60.05	9.38	23.46	0.47	0.46	0.35	0.39
6[a]	149.11	14	13.08	44.10	60.09	214.00	14.19	52.95	0.22	0.21	0.24	0.25
7	79.07	5	8.56	46.93	37.29	176.50	8.30	42.35	0.23	0.27	0.22	0.24
8	150.15	21	17.34	51.64	28.93	103.92	20.96	75.04	0.60	0.50	0.72	0.72
9	100.32	17	9.98	43.84	38.82	137.63	12.20	48.59	0.26	0.32	0.31	0.35
10[b]	170.87	3	6.21	21.89	11.17	35.28	8.12	23.84	0.56	0.62	0.73	0.68
11	81.11	22	9.46	29.16	32.80	114.07	17.55	61.02	0.29	0.26	0.54	0.53
12[c]	89.87	13	14.31	62.27	23.39	96.32	15.21	60.51	0.61	0.65	0.65	0.63
13	124.24	20	8.42	31.34	14.25	54.51	11.64	45.64	0.59	0.57	0.82	0.84
14	106.80	17	10.41	35.29	21.36	78.33	9.23	29.12	0.49	0.45	0.43	0.37
15	181.89	20	9.12	35.39	28.76	118.10	12.78	44.97	0.32	0.30	0.44	0.38
Mean	**128.07**		**11.24**	**40.93**	**29.25**	**110.72**	**12.76**	**47.42**	**0.43**	**0.41**	**0.48**	**0.47**
SD	41.04		2.83	11.88	11.88	49.30	3.88	18.28	0.15	0.14	0.18	0.18
Min	62.61	3	6.21	21.89	11.17	35.28	7.17	23.46	0.22	0.21	0.22	0.24
Max	192.88	22	17.34	62.27	60.09	214.00	20.96	82.26	0.62	0.65	0.82	0.84

[§]epithelial thickness refers to the viable cell layers only (progenitor and maturation compartments)
[a]biopsy from same patient as case 7 in original series (Chapter2)
[b]biopsy from same patient as cases 11 and 15 of the original series
[c]biopsy from same patient as case 19 of the original series

Table 6.3 Summary of results for series of 20 cases from Chapter 2

Case	Epithelial thickness (μm)[§]	Atypia	BrdU		Ki67		Cyclin A		S_{BrdU}:GF	$S_{cyclinA}$:GF
			LI%	LI/BL	GF%	GF/BL	LI%	LI/BL		
1	220.93	19	10.46	39.37	18.38	79.62	15.13	46.52	0.57	0.82
2	119.74	12	10.14	40.47	20.89	122.71	16.14	47.77	0.49	0.77
3	166.33	55	20.75	74.13	21.97	111.21	24.18	90.40	0.94	1.10
4	173.00	19	9.00	39.53	21.64	99.75	18.72	75.38	0.42	0.87
5	120.91	10	7.04	32.44	28.44	158.30	20.18	93.28	0.25	0.71
6	221.63	41	8.23	59.03	73.12	553.35	30.53	139.37	0.11	0.42
7*	242.17	24	7.85	39.46	25.14	90.11	14.58	39.14	0.31	0.58
8	161.31	19	10.36	57.65	23.92	132.01	16.37	69.73	0.43	0.68
9	360.24	57	15.48	114.28	56.15	283.43	48.56	154.03	0.28	0.86
10	179.31	37	9.20	44.46	23.40	130.86	17.44	70.00	0.39	0.75
11*	314.78	35	14.51	52.85	36.73	153.07	30.14	70.35	0.40	0.82
12	425.56	47	9.10	42.14	18.70	89.53	11.73	47.37	0.49	0.63
13	184.78	26	11.14	43.66	19.11	90.06	15.91	51.19	0.58	0.83
14	437.57	22	7.57	34.36	12.63	65.75	14.17	32.06	0.60	1.12
15*	325.81	34	12.21	59.46	60.05	294.99	38.93	106.64	0.20	0.65
16	180.89	18	8.98	29.92	14.21	69.74	11.35	34.35	0.63	0.80
17	59.51	25	9.42	17.02	21.74	58.68	10.67	28.78	0.43	0.49
18	165.41	14	12.66	38.04	21.99	84.19	15.29	63.16	0.58	0.70
19*	173.73	17	12.24	37.60	22.99	97.90	14.02	51.78	0.53	0.61
20	348.17	19	9.64	35.82	15.17	60.85	12.72	48.39	0.64	0.84
Mean	**229.09**		**10.80**	**46.58**	**27.82**	**141.31**	**19.84**	**67.98**	**0.46**	**0.75**
SD	104.73		3.23	20.28	16.31	116.58	9.96	34.21	0.19	0.17
Min	59.51	10	7.04	17.02	12.63	58.68	10.67	28.78	0.11	0.42
Max	437.57	57	20.75	114.28	73.12	553.35	48.56	154.03	0.94	1.12

[§]epithelial thickness refers to the viable cell layers only (progenitor and maturation compartments)
*see Table 6.2

Table 6.4 Summary of results for series of 18 cases from Chapter 5

Case	Ki67		Cyclin A	
	LI%	LI/BL	LI%	LI/BL
1	32.95	146.74	17.74	74.91
2	52.28	208.61	26.98	102.12
3	50.32	166.62	31.23	99.84
4	22.54	96.32	7.17	22.09
5	39.58	151.80	16.47	60.19
6	35.14	109.54	14.92	46.79
7	21.97	136.07	16.88	73.89
8	18.15	63.12	11.71	35.41
9	40.79	292.12	13.85	94.31
10	28.30	162.24	13.19	74.28
11	26.18	124.86	18.84	74.79
12	30.24	181.25	22.01	106.89
13	30.22	160.26	24.87	112.73
14	24.27	75.37	10.66	28.94
15	26.63	134.64	13.92	61.31
16	29.81	101.99	10.50	33.41
17	26.90	91.32	11.14	34.80
18	32.08	171.75	14.85	72.12
Mean	**31.57**	**143.03**	**16.50**	**67.16**
SD	9.22	53.80	6.29	28.80
Min	18.15	63.12	7.17	22.09
Max	52.28	292.12	31.23	112.73

Table 6.5 Mean labelling indices by site

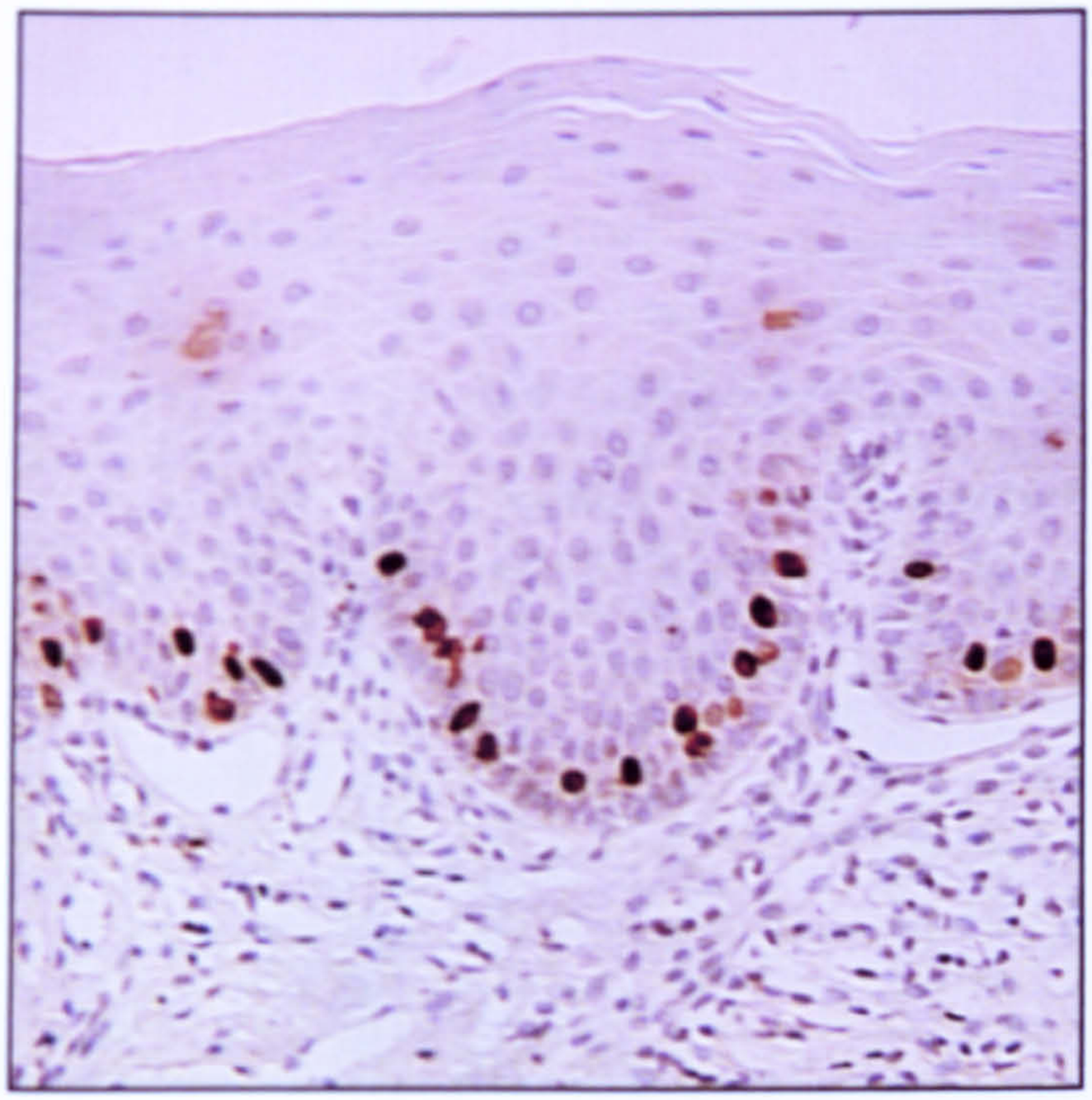
A. Tongue (14 cases)

	Epithelial thickness	BrdU		Ki67		Cyclin A	
		LI%	LI/BL	LI%	LI/BL	LI%	LI/BL
Mean	**217.91**	**10.82**	**37.82***	**26.25**	**100.59***	**16.02**	**48.62***
SD	128.07	3.03	11.96	13.31	65.20	8.60	22.68
Min	59.51	6.21	17.02	11.17	35.28	8.12	23.46
Max	437.57	17.34	59.46	60.05	294.99	38.93	106.64

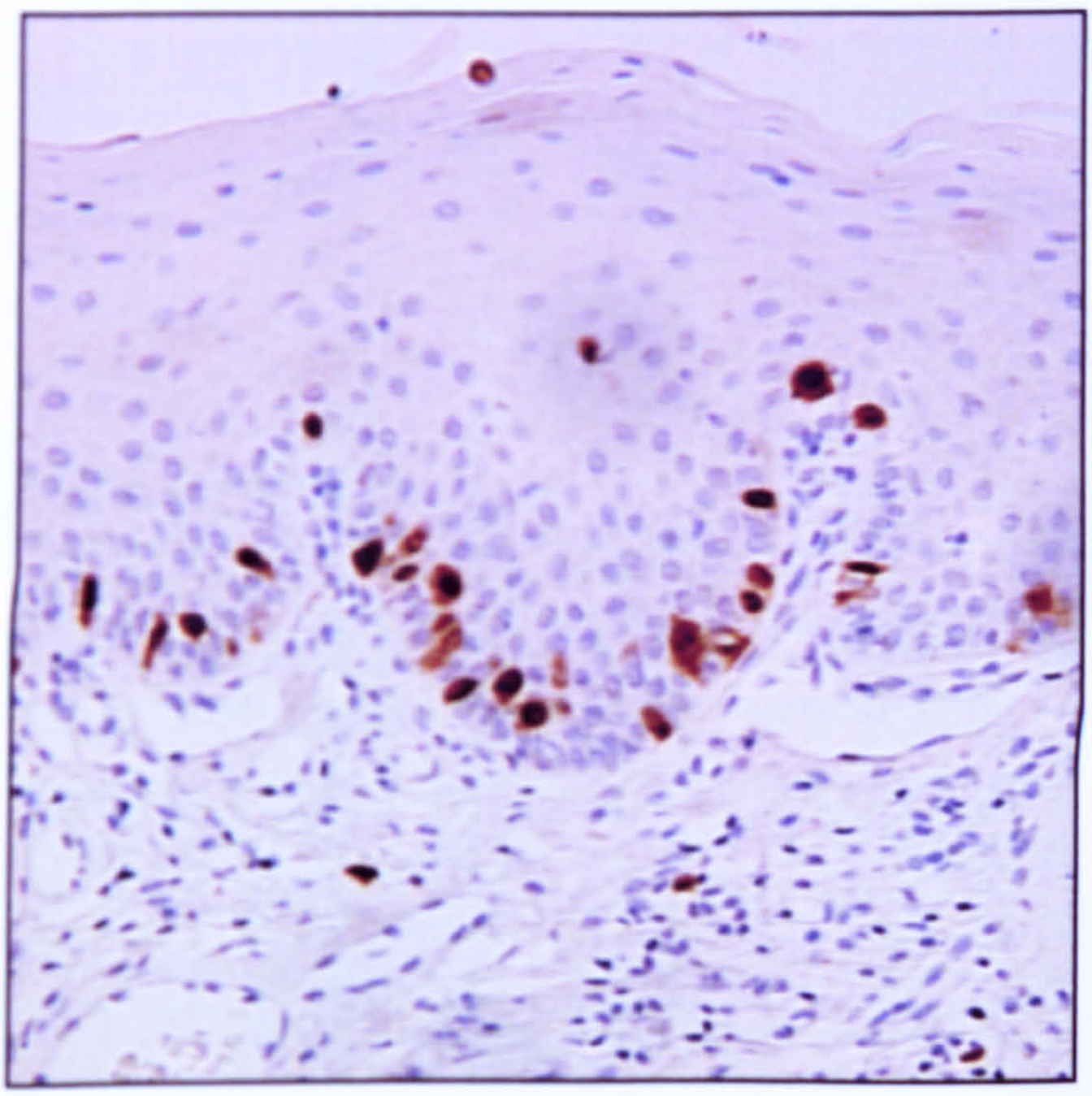
B. Floor of mouth (21 cases)

	Epithelial thickness	BrdU		Ki67		Cyclin A	
		LI%	LI/BL	LI%	LI/BL	LI%	LI/BL
Mean	**164.39**	**11.10**	**48.39***	**29.89**	**146.60***	**17.33**	**66.20***
SD	64.10	3.09	19.07	15.22	106.59	8.80	32.61
Min	79.07	7.04	29.16	14.21	69.74	7.17	26.03
Max	360.24	20.75	114.28	73.12	553.35	48.56	154.03

*significantly different groups ($p<0.05$)



A. BrdU



B. cyclin A

Figure 6.1 Comparison of anti-BrdU and anti-cyclin A antibody (magnification x236)

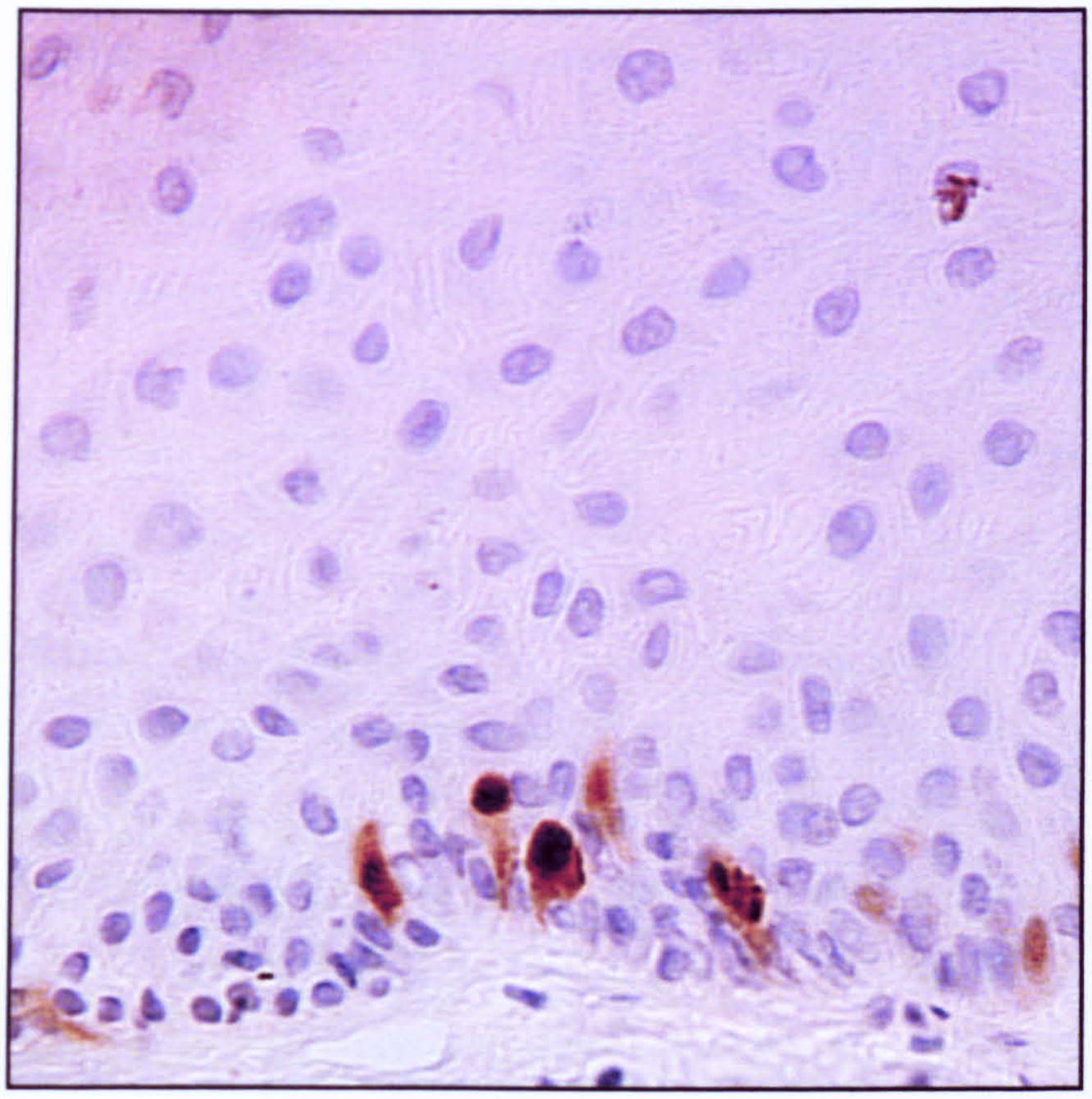


Figure 6.2 Illustrates the characteristic cytoplasmic staining obtained with anti cyclin A antibody (magnification x478)

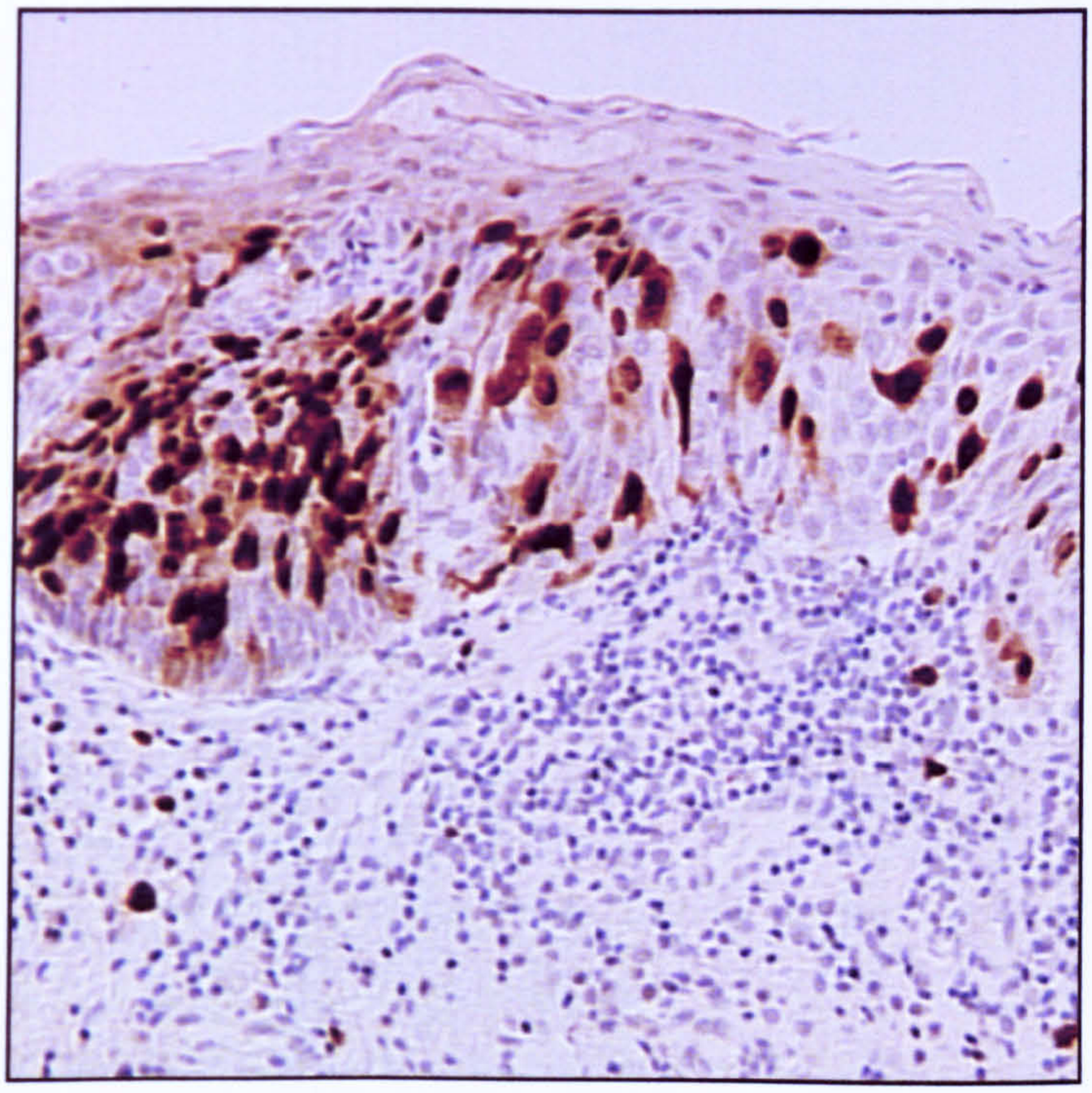
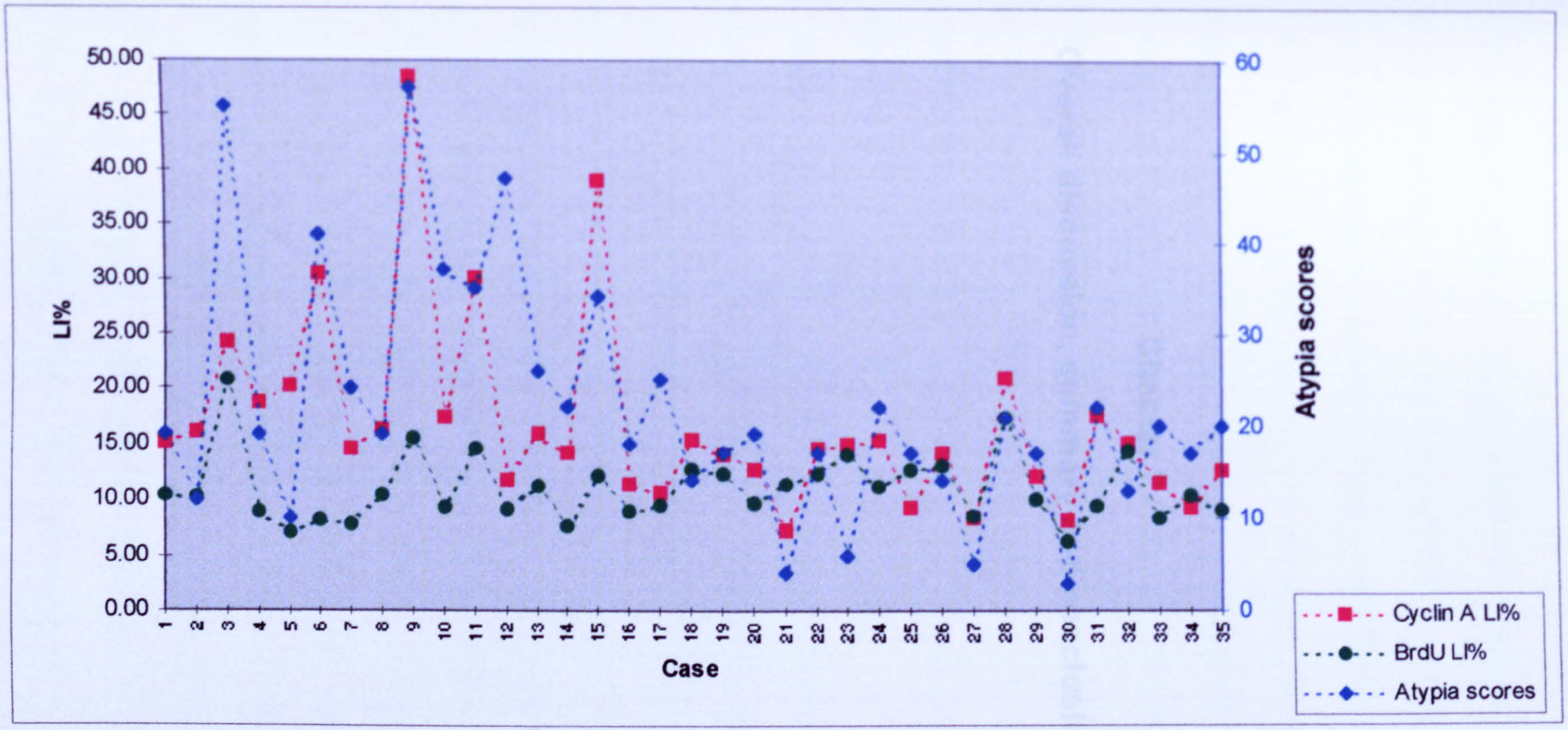


Figure 6.3 Illustrating case number 3 from the original BrdU series of Chapter 2 with a high cyclin A labelling index and lack of basal staining (magnification x236)

Figure 6.4 S-phase labelling indices and atypia scores



The BrdU and cyclin A labelling indices are expressed per 100 total viable nucleated cells for the series of cases from Chapter 2 (1 to 20) and from Chapter 6 (21 to 35).

Chapter 7

Overall discussion, summary and conclusions

7.1 Introduction

The work presented in the preceding five chapters has centred around the cell cycle in oral precancerous lesions. The initial aims of the work were to investigate more objective assessments of dysplastic lesions in oral epithelium with a possible view to their use as prognostic indicators. During the course of the thesis the emphasis has changed a little with the kinetic events of the cell cycle being investigated but still where possible being related to the dysplasia.

7.2 Materials and methods

7.2.1 Material

Three series of cases of dysplasia were studied during the course of the thesis. Two of these were samples collected prospectively because fresh tissue was required for incubation with BrdU. The third series was obtained from archival tissues in an attempt to study cell cycle associated markers without the need to collect fresh tissue. One aim of this was to negate the requirement for prospective collection of samples. Despite oral cancer being prevalent in the West of Scotland, the prevalence of precancerous lesions is surprisingly low. The archival series of cases used in Chapters 5 and 6 represented nearly all of the dysplastic lesions of the tongue and floor of mouth for the year of 1996 with the exception, of course, of those lesions for which biopsies were collected for the BrdU study. These were carefully selected, however, to exclude those lesions

that had associated pathology most commonly lichenoid reactions and fungal infection. As discussed previously, the relative importance of dysplasia in lichenoid lesions and lichen planus is the subject of much debate in the literature and warrants a study of this size in its own right. Similarly, the number of cases collected during the course of the study was relatively few. However, with the potentially vast archives of tissue for which clinical follow-up data would be available the development of methods applicable to this form of material would be desirable when the prognostic implications of such investigations are of prime importance.

A further potential source of material for the study of oral epithelial dysplasia would be that related to existing carcinomas, an approach adopted by Wright and Shear (1985) but not generally adopted by the majority of researchers. Indeed, for the present studies this was investigated. However, on observing the sections from the files it was felt that in many of the specimens the epithelium was poorly orientated and often sectioned obliquely which would not be ideal for the kind of studies performed here.

The study of normal human oral mucosa is a potential problem as by nature of a pathology department biopsies are taken due to a pathological lesion being present. Studies that do use "normal" tissue often utilise the epithelium overlying such lesions as reactive fibrous lesions, mucoceles or amalgam tattoos. By the nature of some of these lesions the epithelium could be expected to have an increased rate of

proliferation and would not be the ideal control for studies of this kind. Also, the tobacco and alcohol consumption of patients from whom such tissues are obtained are never quoted; it is now widely accepted that such habits affect the whole oral mucosa and could reasonably affect the epithelial cell proliferation in apparently clinically normal mucosa. The ideal normal tissue would be obtained from healthy non-smoking volunteers. For an invasive procedure such as a biopsy, volunteers might be difficult to obtain. As demonstrated in the study of Chapter 4, post-mortem tissue is an attractive option. Admittedly, in this study the tobacco habits were *unknown* but could have been obtained. However, this was not relevant to the aim of this study which was to *investigate* the apparent anomaly of Ki67 immunoreactivity observed in dysplastic lesions in histologically normal epithelium. Investigating the use of post-mortem material for cell proliferation studies does not appear to have been performed previously. This potentially fruitful source of material may be somewhat limited by the declining number of post-mortem examinations being performed in the UK over recent years (Start *et al.*, 1993) and the fact that most post-mortems are performed on individuals of an older age group.

7.2.2 Methods

One of the basic methods used extensively in this thesis, immunohistochemistry, is one that is well established and widely utilised in studies of oral epithelial dysplasia. It has the advantage over the more

recently developed molecular techniques of allowing the visualisation of the proteins *in situ* and their relationship to the adjacent cells and tissues.

The field of immunohistochemistry has been revolutionised by the development of antigen retrieval (Shi *et al.*, 1991) which has enabled many antigens to be visualised in fixed and processed tissue which had previously not been possible. However, numerous workers have rightly highlighted some of the potential problems with this procedure. Most notably is the variation of staining intensities achieved using different methods of antigen retrieval with different antibodies and tissues as was observed and demonstrated in the present studies. An attempt to solve this problem has been the use of a so called test battery to be performed with each new antibody or tissue. One drawback of antigen retrieval experienced in the present study was the occasional loss of tissue from the slides. When this did occur it was consistently with the same cases and may reflect some factor related to the processing of the tissue. Related to this is the common observation of increased artefacts following antigen retrieval.

Another method developed during the course of this thesis is the use of computer assisted technology for the digital capture, manipulation and quantification of images. As demonstrated in Chapter 5, this enabled the enhancement of images to render them quantifiable where this would have previously not been possible using a conventional microscope alone. Although the software potentially allows automated counting of cells the

subjective nature of sometimes interpreting positively antibody labelled cells made this approach undesirable for the present study. Also, the time taken to set the parameters for this kind of automation is similar to the time taken to measure and count the images manually. The most time consuming aspect of this procedure was actually locating the fields and capturing the images.

7.3 Discussion of the results

The studies presented in this thesis have generated large amounts of numerical data despite the relatively small numbers of cases. A potential problem is the interpretation of this data with respect to the cell kinetics of the tissues. Indeed, the kinetic aspects of the data have been cautiously interpreted, the data have largely been used as a comparison with other cell cycle associated markers, the atypia scores and have been related where possible to the clinical outcome.

There is no easily adopted approach for the objective assessment of oral epithelial dysplastic lesions. Although a number of the markers studied in this thesis have shown associations with the atypia scores their routine use in assessments seems unlikely. The semi-objective scoring technique of Smith and Pindborg (Smith and Pindborg, 1969) used throughout this thesis has not been widely adopted by the majority of researchers. From the author's experience of the method this is not surprising since it is a very time consuming procedure if performed

correctly. However, this remains the only reliable method to introduce a degree of objectivity into such studies.

The results from Chapter 2 demonstrated that BrdU labelling indices provided a possible objective assessment of oral epithelial dysplasia. Although the finding that a high S-phase to growth fraction ratio was in the first series of cases associated with malignant transformation of the lesions this was interpreted cautiously. A further longer term follow-up of these patients is required and due to the often prolonged natural history of such lesions this would ideally be for as long as possible. Indeed, the aspect of follow-up of these patients has highlighted some problems with the current system employed in the Glasgow Dental Hospital. It will be interesting to follow up the additional series of patients from whom biopsies were collected in Chapter 6 and to see if cyclin A really has a role as an alternative to BrdU labelling in assessment of the S-phase. The development of formalin fixation for the BrdU processed tissue enabled sequential sections to be used with other proliferation markers and should have provided more accurate comparisons than the previous studies in Chapter 2.

From a practical point of view with regard to quantification, BrdU was the least subjective of all the markers studied, although cyclin A immunohistochemical staining was equivalent in most cases, generally producing definite nuclear staining despite cytoplasmic staining being common. Once antigen retrieval was optimised the staining characteristics

with Ki67 antibody were generally improved. The staining with the other cyclin antibodies was generally less clear-cut and required image enhancement to enable quantification which despite this was sometimes open to subjective interpretation of positive cells.

From the studies of Chapters 2 and 4, it was evident that Ki67 was not identifying all those cells which would have been expected to be in the growth fraction and hence in the active phases of the cell cycle as it was claimed to do (Gerdes *et al.*, 1983). The generally high expression of the G_1 cyclins and distribution of the D cyclins similar to that of Ki67 meant these were probably not identifying those cells of the G_1 phase which were not being identified by Ki67. Indeed, the studies of the D cyclins and cyclin E probably created more questions than answers in the present study. Until the precise role of these proteins is established in the cell they probably are of little value in the study of cell proliferation.

The cross-reactivity of the anti-cyclin D3 antibody with the smooth muscle of the blood vessels was unexpected and to the author's knowledge has not been reported previously.

7.4 Conclusions

Throughout this thesis numerical data has been obtained from immunohistochemically stained tissue sections. The author appreciates the limitations of making judgements based on this due to the inherently subjective nature of the interpretation of positivity. The figures obtained are, at best, only an estimation and in reality reflect a range of values. It is

also appreciated that there are many potential sources of error that could have been introduced at each stage. Firstly there is the matter of sampling of the lesions which may not be truly representative of the lesion as a whole. Secondly within the biopsy a limited number of fields were examined sometimes only in one tissue section which again may not be truly representative of the lesion as a whole.

From the studies reported here it was concluded that BrdU remains the "gold standard" for the investigation of the S-phase in precancerous and cancerous lesions, although cyclin A is promising for the study of the S-phase in minimally dysplastic and normal tissues. BrdU studies provided some objectivity with regard to assessment of the degree of dysplasia within the precancerous lesions studied but this will probably not be of great value in diagnostic practice.

An ideal study would have compared the results obtained in Chapter 2 with normal mucosa but the problems of obtaining such tissues has been discussed in Section 7.2.1.

Once antigen retrieval conditions were optimised, Ki67 was a useful adjunct in the study of cell proliferation states in oral precancerous lesions despite its now known drawback of not identifying all of the cycling cells. Although an attractive idea, the ratio of the S-phase to the growth fraction requires more study before its usefulness as a prognostic indicator can be proven.

The G_1 cyclins, D1, D3 and E, did not contribute at this stage

specifically to the study of oral epithelial dysplasia, but have contributed to the growing body of data on cyclins and various disease states. The specificity of the antibodies used in this study requires further investigation.

7.5 Suggestions for future research

From the preceding chapters and the review of the literature it is clear that long term follow up of oral precancerous lesions will be essential to determine the possible prognostic value of the parameters studied in this thesis. One possible approach to this is to study groups of archival cases where the outcome of the lesions is known. Retrospective studies often have the disadvantage of incomplete data recording and properly constructed prospective studies are much more likely to be helpful.

If sufficient lesions of idiopathic leukoplakia could be identified, studies of these lesions either with cell cycle-associated markers or other markers are advocated to investigate their differing pathogenesis and apparently greater potential for malignant transformation.

In the field of cell cycle research, a number of areas highlighted in this thesis require further study. First there is the expression of the Ki67 protein and its precise function within cells. Once this has been established it may be of more value particularly in cell kinetic studies. Further studies of the HsMCM/BM28 antigen (Todorov *et al.*, 1998) as discussed in Section 1.5.10 are advocated to see if this truly represents all

cycling cells unlike Ki67.

With regard to the cyclins, the expression of the D cyclins in oral epithelial dysplastic lesions could be studied by other techniques. In the first instance, looking at the D cyclin mRNA levels within the cells would give a good initial indication whether or not the protein was being overexpressed. Reverse transcriptase PCR or in situ hybridisation would be appropriate techniques for this kind of study.

Other antibodies have become available for studying proteins related to the cyclins, such as the CDKs or the CDKIs. Investigation of these may enhance the understanding of their related cyclins.

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Appendices

Appendix 1. Features of dysplasia

1. drop-shaped rete ridges
2. irregular epithelial stratification
3. keratinisation below the keratinised layer and individual cell keratinisation
4. basal cell hyperplasia
5. loss of intercellular adherence
6. loss of polarity of the basal cell layer
7. hyperchromatic nuclei
8. increased nuclear:cytoplasmic ratio
9. anisonucleosis and anisocytosis
10. pleomorphic nuclei and cells
11. increased numbers of mitoses
12. mitoses present above the basal layers
13. bizarre or abnormal mitoses

The above features are those cited by Smith and Pindborg (1969) forming the basis for their scoring system. The current WHO guidelines include the above features and add the presence of prominent nucleoli (Pindborg *et al.*, 1997).

Appendix 2. Working solutions

Carnoy's fixative (Pearse, 1980)

Ethyl alcohol	60 ml
Chloroform	30 ml
Glacial acetic acid	10 ml

10% buffered formalin

Made from neutral buffered formaldehyde concentrate (Genta Medical, York, UK) diluted 1 in 5 with distilled water. Constituents when reconstituted (pH 7.0):

formaldehyde solution	10% v/v
sodium phoshphate	0.65% w/v
sodium acid phosphate	0.35% w/v

Bromodeoxyuridine (M_w 307.1)

- used in a solution of 4×10^{-4} M (=0.0004M)
- 15ml of solution used per incubation
- measure out 1.84mg BrdU into glass universal containers, store at -*20°C*
- add 15ml minimum essen*tial medium (MEM)* to container prior to use

TBS (Tris-buffered Saline)

Comprises 0.05M Tris/HCl
0.15M NaCl
at pH 7.6

	1l	5l	10l
Trizma HCl (Sigma)	6.06g	30.3g	60.6g
Trizma Base (Sigma)	1.39g	6.95g	13.9g
NaCl	8.76g	43.8g	87.6g

Antigen retrieval buffers

Citrate buffer pH 6.0

- Dissolve 2.1gm citric acid monohydrate in 1l of water.
- Adjust pH with sodium hydroxide to pH 6.0

1mM EDTA pH 8.0

- Mw EDTA disodium salt 372.24
- to make 5l , dissolve 1.96g in 5l dH_2O
- adjust to pH 8.0 with sodium hydroxide

0.01M acetate pH 2.0

- 1M sodium acetate solution prepared by dissolving 82.04 g anhydrous (136.09g crystals) in distilled water and making up to 1l.
- to make pH 2 solution, 100 ml of sodium acetate and 105 ml of 1M HCl and make up to 500 ml with distilled water

Appendix 3. Histological methods (Bancroft and Stevens, 1990)

Haematoxylin and Eosin

Mayers haematoxylin solution

haematoxylin	2.5g
water	2500ml
potassium alum	125g
sodium iodate	0.5g
citric acid	2.5g
chloral hydrate	125g

1% acid alcohol solution

hydrochloric acid	25ml
alcohol	2475ml

Scott's tap water substitute

magnesium sulphate	50g
sodium bicarbonate	8.75g
water	2500ml
thymol	1 crystal

1% eosin

calcium chloride	12.5g
water	2500ml
eosin	25g

Method

1. dewax sections in xylene and rehydrate through graded alcohols to water
2. stain nuclei with haematoxylin for 10 minutes
3. rinse in water
4. differentiate in 1% acid alcohol for 5 sec
5. rinse in water
6. blue in Scott's tap water substitute
7. wash in water
8. counterstain in eosin for 10 seconds
9. dehydrate, clear and mount

Periodic acid Schiff (PAS)

1. sections to water
2. oxidise in 1% periodic acid for 10 minutes
3. wash well with water
4. wash in water
5. immerse in Schiff reagent (Sigma) for 30 minutes
6. wash well with water
7. counterstain with haematoxylin
8. wash with water
9. differentiate with 1% acid alcohol
10. wash with water
11. blue in Scotts tap water solution
12. rinse in water
13. dehydrate, clear and mount

Appendix 4 Smith and Pindborg atypia scoring technique

Feature	Objective magnification	Score
1. "drop-shaped" rete ridges	x10	0/2/4
2. irregular epithelial stratification	x10	0/2/5
3. keratinisation below the keratinised layer	x10	0/1/3
4. basal cell hyperplasia	x10	0/1/4
5. loss of intercellular adherence	x10	0/1/5
6. loss of polarity	x10	0/2/6
7. hyperchromatic nuclei	x10	0/2/5
8. increased nuclear-cytoplasmic ratio	x40	0/2/6
9. anisocytosis and anisonucleosis	x40	0/2/6
10. pleomorphic cells and nuclei	x40	0/2/6
11. mitotic activity	x40	0/1/5
12. level of mitotic activity	x40	0/3/10
13. bizarre mitoses	x40	0/6/10
14. hyperplasia/ atrophy		
15. surface characteristics		
Total score		

For each of the individual features sections are examined, compared with photographic standards and assigned a grade; 0 if the feature is not present, the middle score if the feature is slight and the highest score if the feature is marked. The sum of the individual scores produces the atypia score. Features 14 and 15 are not given numerical scores.

Appendix 5. Reagents

Reagent	Full Name	Company	Code
Anti-BrdU	Monoclonal mouse Anti-bromodeoxyuridine clone Bu20a	Dako	M0744
Anti-Cyclin A	Monoclonal mouse anti-cyclin A antibody	Novocastra	NCL-CYCLIN A
Anti-Cyclin D1	Monoclonal mouse anti-cyclin D1 antibody	Novocastra	NCL-CYCLIN D1-GM
Anti-Cyclin D3	Monoclonal mouse anti-cyclin D3 antibody	Novocastra	NCL-CYCLIN D3
Anti-Cyclin E	Monoclonal mouse anti-cyclin E antibody	Novocastra	NCL-CYCLIN E
Anti-Ki67	Monoclonal mouse anti-human Ki67 antigen clone Ki67	Dako	M0722
Anti-NCL Ki67 MM1	Mouse monoclonal antibody NCL-Ki67-MM1 clone MM1	Novacastra	NCL-Ki67-MM1
BrdU	5'-bromo-2-deoxyuridine	Sigma	B5002
Citric Acid	Citric Acid monohydrate	BDH	
DAB	3,3'diaminobenzidine tetrahydrcholoride	Sigma	D5905
DAB Substrate Kit	DAB Substrate Kit for peroxidase	Vector	SK4100
EDTA	Ethylenediaminine tetraacetic acid disodium salt	BDH	44388-2G
HCl	1M HCl CONVOL Concentrate	BDH	18036/5D
Hydrogen Peroxide		BDH	10128N
MEM	Minimum essential medium (Eagles) with glutamax-1 with Earles salts	GIBCO	41090-028
NaCl	Sodium Chloride	BDH	30123-50
NAP-5-Columns	NAP-5-column Sephadex G25	Pharmacia	17-0853-01
Polysine	Silane coated slides	BDH	406/0178/00
SG Substrate Kit	SG Substrate Kit for peroxidase	Vector	SK4700
Superfrost Plus	Microscope slides	BDH	406/0179/00
Tizma HCl	Tris[hydroxymethyl] amino-methane hydrochloride	Sigma	T3253
Trizma base	Tris[hydroxymethyl] amino methane	Sigma	T1503
Trypsin		Dako	S2012
Vectastain Elite ABC Kit	Mouse IgG	Vector	PK6102
VIP Substrate Kit	VIP Substrate Kit for peroxidase	Vector	SK4600

Appendix 6. KS300 macro for the quantification of cyclins (Chapter 5)

```
1. ##################################
2. ##Cyclin Study###################
3. ##Oral Epithelial dysplasia#####
4. ##################################
5. ##Richard Oliver################
6. ##Glasgow Dental Hospital#######
7. ##February 1997#################
8. ##################################
9. # start
10.tvframe 6,28,512,512
11.################################
12.##First-delete all images in gallery
13.imgdelete "*"
14.Gclear 0
15.################################
16.##Open display windows
17.################################
18.showwindow "Display",1
19.showwindow "Messages",1
20.showwindow "Gallery",1
21.################################
22.##Capture image
23.case = "96_"
24.tvlive
25.! tvwbalance 1
26.tvinput 1
27.imgdisplay 1
28.################################
29.path = "c:/ks300/conf/images/cyclinD1"
30.imgsetpath path
31.read case,"Enter case number"
32.write
33.write
34.write
35.write " Case number:", case
36.write " Cyclin D1"
37.imgsave 1,case+".bmp"
38.################################
39.##enhance contrast
40.################################
41.normalize 1,2,9
42.##Now measurements for cyclinD1
43.################################
44.MSload "x20"
45.MSload "basalx20"
46.MSmeaspoint 2,"lbmx20",1,13,1,2,2,2
47.Gmerge 2,255
48.MSload "s_basal"
49.MSmeaspoint 2,"suprax20",1,10,1,2,2,2
50.Gmerge 2,255
51.MSload "basnl"
52.MSmeaspoint 2,"basnl",1,12,1,2,2,1
53.Gmerge 2,255
54.MSload "totalx20"
55.MSmeaspoint 2,"total",1,1,1,2,2,1
56.Gclear 0
57.################################
58.write " Case number:", case
59.write " Cyclin D3"
60.################################
61.tvlive
```

```
62.! tvwbalance 3
63.tvinput 3
64.imgdisplay 3
65.#####################################
66.path = "c:/ks300/conf/images/cyclinD3"
67.imgsetpath path
68.imgsave 3,case+".bmp"
69.#####################################
70.normalize 3,4,4
71.#####################################
72.##Now measurements for cyclinD3
73.#####################################
74.MSload "x20"
75.MSload "basalx20"
76.MSmeaspoint 4,"lbmx20",1,13,1,2,2,2
77.Gmerge 4,255
78.MSload "s_basal"
79.MSmeaspoint 4,"suprax20",1,14,1,2,2,2
80.Gmerge 4,255
81.MSload "basnl"
82.MSmeaspoint 4,"basnl",1,9,1,2,2,1
83.Gmerge 4,255
84.MSload "totalx20"
85.MSmeaspoint 4,"total",1,1,1,2,2,1
86.#####################################
87.Gclear 0
88.#####################################
89.tvlive
90.! tvwbalance 5
91.tvinput 5
92.imgdisplay 5
93.#####################################
94.path = "c:/ks300/conf/images/Ki67"
95.imgsetpath path
96.imgsave 5,case+".bmp"
97.#####################################
98.write " Case number:", case
99.write " Ki67"
100.####################################
101.normalize 5,6,9
102.##Now measurements for Ki67
103.####################################
104.MSload "x20"
105.MSload "basalx20"
106.MSmeaspoint 6,"lbmx20",1,13,1,2,2,2
107.Gmerge 6,255
108.MSload "s_basal"
109.MSmeaspoint 6,"suprax20",1,14,1,2,2,2
110.Gmerge 6,255
111.MSload "basnl"
112.MSmeaspoint 6,"basnl",1,9,1,2,2,1
113.Gmerge 6,255
114.MSload "totalx20"
115.MSmeaspoint 6,"total",1,1,1,2,2,1
116.####################################
117.Gclear 0
118.####################################
119.write " Cyclin E"
120.####################################
121.tvlive
122.! tvwbalance 7
123.tvinput 7
124.imgdisplay 7
```

```
125.#####################################
126.path = "c:/ks300/conf/images/cyclinE"
127.imgsetpath path
128.imgsave 7,case+".bmp"
129.#####################################
130.#####################################
131.normalize 7,8,9
132.##Now measurements for cyclinE
133.#####################################
134.MSload "x20"
135.MSload "basalx20"
136.MSmeaspoint 8,"lbmx20",1,13,1,2,2,2
137.Gmerge 8,255
138.MSload "s_basal"
139.MSmeaspoint 8,"suprax20",1,14,1,2,2,2
140.Gmerge 8,255
141.MSload "basnl"
142.MSmeaspoint 8,"basnl",1,9,1,2,2,1
143.Gmerge 8,255
144.MSload "totalx20"
145.MSmeaspoint 8,"total",1,1,1,2,2,1
146.#####################################
147.Gclear 0
148.##close down windows
149.#####################################
150.showwindow "Display",0
151.showwindow "Gallery",0
152.showwindow "Messages",0
153.#####################################
154.stop
```