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c-*myc* Oncoprotein Expression in Oral Precancerous and Early Cancerous Lesions

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Squamous cell carcinoma of the oral cavity is often preceded by clinically evident precancerous lesions. These lesions exhibit progressive cytomorphologic changes connoting cellular transformation to malignant neoplasia. Molecular events underlie the microscopically identifiable cytological changes that herald transformation. Various proto-oncogenes are activated, overexpressed or mutated in the process of transformation. This investigation was designed to determine whether the nuclear binding c-*myc* oncoprotein, an activator of cell division, parallels or precedes cytomorphological changes at various histologically defined stages in oral precancerous lesions. Employing immunohistological methods, it was determined that c-*myc* nuclear labelling paralleled the progressive histological changes among various stages of transformation. It was also noted that some, yet not all, instances of benign keratoses which lacked any cytologic evidence of atypia harboured c-*myc* oncoprotein positive nuclei. It is concluded that c-*myc* expression is correlated with progressive cell transformation in oral cancerous lesions.

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INTRODUCTION

SQUAMOUS CELL carcinoma of the oral mucosa usually arises from clinically characteristic precancerous lesions including leukoplakia, erythroplakia, or combined leukoerythroplakia [1, 2]. Microscopic changes that indicate malignant transformation are well defined. Precancerous lesions exhibit progressively more advanced cytological atypia as they temporally progress from benign keratosis, to dysplasia, to carcinoma *in situ* ultimately eventuating in invasive carcinoma [3–6]. During the evolution of these clinically recognisable lesions and in conjunction with the appearance of cytomorphological indices of malignant transformation, molecular events are ensuing which account for activation of the cell cycle. The biochemical perturbations that favour uncontrolled proliferation, often with a loss of cellular differentiation, involve a wide array of genes and gene products, many of which represent protooncogenes/oncoproteins, growth factor receptors/ligands, and internal signalling pathway proteins and kinases [7, 8].

It is well established that most solid tumours are not characterised by the activation of a single oncogene or growth factor; rather, combinations of proteins that affect growth and differentiation are perturbed [see review by Field, 9]. The perturbations that attend malignant transformation include oncogene amplification, mutations, rearrangements, gene product upregulation and suppressor gene defects. One such gene that has been associated with neoplasia is the *myc* family of oncogenes [7–9]. Specifically, c-*myc*, a nuclear binding dimeric leucine zipper protein with putative cell cycle regulatory activity, has been shown to be amplified and/or overexpressed in various solid tumours [10–16]. In carcinomas of the head and neck, genital tract, colon and breast, c-*myc* oncoprotein overexpression has been correlated with a poor prognosis [17–21]. c-*myc* Transcripts and oncoprotein have been shown in many studies to be elevated in head and neck squamous carcinomas and gene amplification has been reported by some, yet not all investigators who have assayed this phenomenon [22–29]. It has been concluded by some, that since elevated c-*myc* oncoprotein is found in more anaplastic lesions or lesions associated with a poor prognosis, this oncoprotein exerts an influence late in the course of malignancy [9, 21]. In this immunohistochemical study we show that in humans, expression of c-*myc* oncoprotein is an early event, occurring at the inception of oral carcinogenesis.

MATERIALS AND METHODS

From the archives of the UCLA Oral Pathology Service, neutral formalin fixed, paraffin embedded specimens representing five categories of premalignant lesions were retrieved. The diagnostic designations included (1) benign hyperorthokeratosis (HOK), (2) benign hyperparakeratosis (HPK), (3) dysplasia (DYSP), (4) carcinoma *in situ* (CIS), and (5) invasive carcinoma (CA). The histopathological criteria for each of the diagnostic categories as employed by other investigators [1, 4, 6] is as follows: benign hyperkeratosis—increased thickness of the cornified layer, either orthokeratin or parakeratin, with no evidence of cytological atypia; dysplasia—cellular atypia including hyperchromatism, pleomorphism, increased and bizarre mitotic figures and tear drop rete ridges confined to the basilar and lower spinous strata (included in this group were cases originally diagnosed as mild to moderate dysplasia); carcinoma *in situ*—cellular atypia involving most to all strata of the epithelium (included in this group were cases originally diagnosed as severe dysplasia to carcinoma—*in situ*); invasive carcinoma—identification of invasive cords and islands of epithelial cells in the submucosa with evidence of origin from the surface and showing cytologically atypical cells (Fig. 1). Normal oral mucosal specimens

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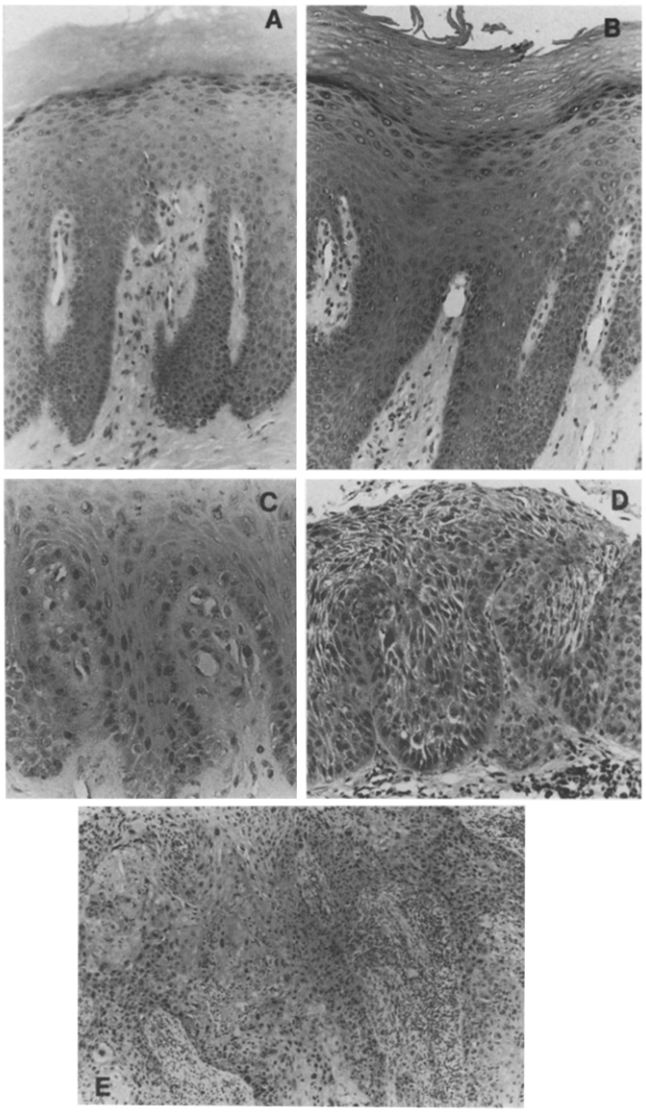

Fig. 1. Histopathological features of the five groups. (a) Hyperorthokeratosis (100×). (b) Hyperparakeratosis (100×). (c) Mild dysplasia (200×). (d) Severe dysplasia (200×). (e) Invasive squamous cell carcinoma (100×). Haematoxylin/eosin.

were selected from orthokeratinised and parakeratinised locations to serve as controls. Ten tissue blocks obtained from different subjects from each diagnostic and control category were used. Three 6 μm sections from each tissue block were adhered to polylysine coated glass slides; one section was routinely stained with haematoxylin and eosin and the remaining slides were utilised for immunohistochemistry [ ].

The sections were dewaxed, and hydrated in Tris–HCl pH 7.4 buffer. Each section was placed in 0.6% hydrogen peroxide in 80% methanol in order to quench endogenous peroxidase. After rinsing in Tris buffer, the sections were incubated in 1% bovine serum albumin (BSA) Tris as a blocking agent followed by immersion of one section in mouse Mab to c-*myc* oncoprotein (Clone 9E10, mouse IgG_1, Oncogene Science, Uniondale, New York) diluted 1:20 (5 μg/ml) with Tris–HCl pH 7.4. A second section serving as a negative control was incubated with Mab to human immunoglobulin light chain lambda (DAKO, Carpinteria) diluted at the optimal (1:500) concentration for detection in plasma cells (lambda Ig chains are not identifiable in keratinocytes).

The sections were incubated at 4°C overnight (12 h) followed by Tris–HCL pH 7.4 rinses ×3 on a rotating nutrator and subsequent application of secondary biotinylated goat antimouse antisera at 37°C for 30 min (DAKO universal detection system for mouse monoclonal antibodies, Carpinteria). After three rinses the sections were treated for 30 min at 37°C with peroxidase–antiperoxidase complex. Labelled immunoreactants were then visualised with hydrogen peroxide substrate and diaminobenzidine chromogen (30–45 min incubation). Sections were then evaluated for intracellular location (plasma membrane, cytosol, nucleus) and localisation according to site and epithelial strata. Quantitation of immunoreactant nuclear binding was performed with a fixed rectile grid at 40×. Approximately 200 cells were assessed in three separate fields and the number of labelled cells were counted and expressed in relation to the total number of cells counted.

$$\frac{\text{Number of positive nuclei}}{\text{Total number of cells counted}} \times 100 = \text{label index.}$$

A one way analysis of variance was employed to compare groups. It should be noted that during preliminary studies, some tissues failed to stain for c-*myc* oncoprotein, including both benign keratoses and dysplasias. This failure for all samples to stain uniformly can probably be attributed to excessive heat during the processing of paraffin embedded tissues, particularly when paraffin or slides are subjected to heat in excess of 60°C.

RESULTS

The immunocytochemical labelling with antibody to c-*myc* was consistently localised to nuclei, although sporadic cytoplasmic labelling was occasionally observed (Table 1, Fig. 2). Among the benign keratoses, nuclear labelling was confined to the basal cell layer in most instances; however in 3 cases, labelling of spinous cell nuclei was observed despite a lack of cytological atypia (Fig. 3a). Nuclear labelling was also more pronounced in fields with submucosal inflammatory cell infiltration, a feature that accounted for the wider variance observed among HPK lesions as compared to HOK leukoplakias. Among the dysplasia, carcinoma *in situ* and carcinoma cases, nuclear labelling was consistently found in all strata that

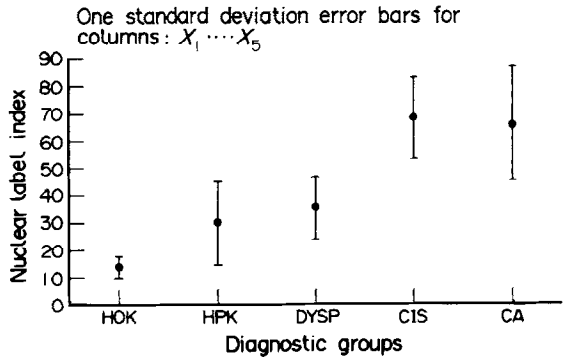

Fig. 2. Quantitative comparison of c-*myc* nuclear labelling among the five groups. HOK = hyperorthokeratosis, HPK = hyperparakeratosis, DYSP = mild to moderate dysplasia, CIS = severe dysplasia/carcinoma *in situ*, CA = invasive squamous cell carcinoma.

Table 1. *One Factor ANOVA-Repeated Measures for* $X_1 \ldots X_5$

Source	df	Sum of squares	Mean square	F-test	*P* values
Between subjects	9	1654.02	183.78	0.242	0.9857
Within subjects	40	30371.6	759.29		
Treatments	4	22668.32	5667.08	26.484	0.0001
Residual	36	7703.28	213.98		
Total	49	32025.62			

Reliability estimates for—all treatments: −3.132; single treatment: −0.179.

Group	Means	S.D.	S.E.
HOK	13.7	3.773	1.193
HPK	30.1	15.409	4.873
DYSP	35.3	11.557	3.655
CIS	68.3	14.922	4.719
CA	66.3	20.78	6.571

Analysis of variance for c-*myc* nuclear labelling among the study groups disclosing a high level of statistical significance ($P<0.0001$). The lower table delineates the mean, standard deviation and standard error of the mean among the five diagnostic categories. HOK = hyperorthokeratosis, HPK = hyperparakeratosis, DYSP = mild dysplasia, CIS = carcinoma-*in situ*/severe dysplasia, CA = invasive squamous cancer.

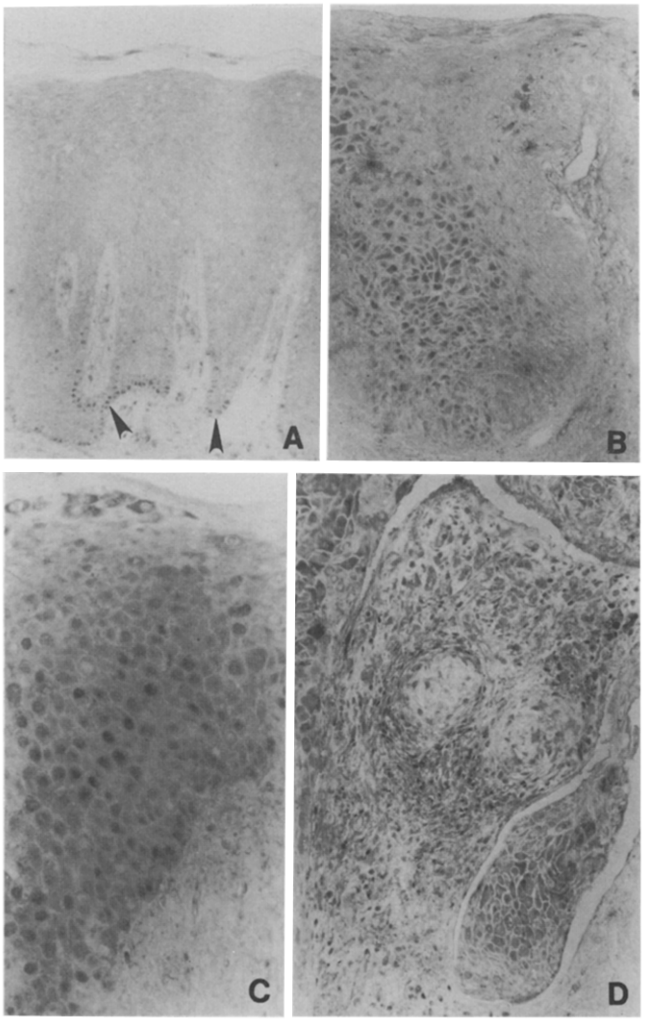

Fig. 3. Immunohistochemical labelling with Mab to c-*myc* oncoprotein. (a) Hyperorthokeratosis, only basal cells label (arrowheads) (40×). (b) Moderate dysplasia, spinous layer cells are positive (100×). (c) Carcinoma *in situ*, nuclei in all strata are decorated (160×). (d) Invasive carcinoma both nuclear and cytoplasmic labelling are evident (40×). Immunoperoxidase without any counterstain.

harboured atypical cells and the degree of labelling increased with advancing gradations of atypia (Fig. 3b, c, d).

A statistical difference was found between HOK and all other groups, between HPK and CIS, CA, and between DYSP and CIS, CA. There was no significant difference in nuclear counts between HPK and DYSP; however, despite the lack of statistical significance, DYSP cases showed suprabasilar staining whereas HPK cases exhibited predominantly basal layer nuclear labelling. As mentioned previously, in foci of submucosal inflammation, the basal layer nuclear count was significantly higher than in non-inflamed regions of HPK or HOK. No significant difference in nuclear labelling could be identified between CIS and CA. The large variance among the CA cases is a reflection of cellular differentiation. Well differentiated tumours with keratin pearl formation exhibited lower nuclear counts than tumours with solid neoplastic nests lacking any keratin differentiation and most positively stained nuclei were located on the peripheral cell zones of the tumour islands. In the less differentiated lesions, over 80% of the cell population possessed c-*myc* oncoprotein.

DISCUSSION

During the process of malignant neoplasia, cells lose their capacity to control the cell cycle; they dedifferentiate and enter a proliferative state. This process, while not clearly understood, involves alterations in a group of cell cycle control proteins including the cyclins, *cdc*2 encoded proteins and *p*34 [30, 31]. These cell division control elements are probably governed by various transactivating proteins that serve to drive the cycle (oncogene products, growth factors, internal signalling proteins) or bring it to a halt (antioncogenes). C-*myc*, among other proteins, has been shown to stimulate cell division and evidence has been put forward to suggest that upregulation of this oncoprotein during the transition from G_0 to S phase may involve transactivation of other genes which encode proteins that impact the cell cycle regulator proteins [32].

Numerous studies have indicated that c-*myc* RNA and oncoprotein are overexpressed in a variety of malignant neoplasms [10–29]. Although c-*myc* has been shown to be overexpressed in head and neck squamous cell carcinoma, no previous studies have focused on the early stages of oral carcinogenesis [21–29]. The results of this study indicate that c-*myc* oncoprotein is expressed in the basal cell layer of oral

leukoplakias that exhibit hyperortho- and hyperparakeratosis without cytological atypia. Parakeratotic lesions were found to have a significantly increased number of labelled basal cell nuclei as compared to orthokeratinised lesions. It was noted, however, that the parakeratinised lesions in this sample had a greater incidence of submucosal mononuclear cell infiltrates than the orthokeratinised lesions. It is well known that imflammatory responses in oral mucosa are associated with epithelial hyperplasia (i.e. sulcular epithelium in periodontitis, cyst lining epithelium in apical periodontal cysts, marginal epithelium adjacent to healing ulcers). It is possible that the higher prevalence of basilar labelling in hyperparakeratotic lesions is a reflection of cytokine or mitogenic factor secretion from subepithelial leucocytes.

Although the benign keratoses exhibited labelling restricted to basal cells, three instances showed sporadic labelling of lower spinous layer cells. These cells lacked cytological atypia. This study was not longitudinal in design and, therefore the significance of this finding cannot be ascertained. It is noteworthy that approximately 6% of benign leukoplakias will evolve into carcinoma over a 5–7 year follow-up period [1, 3]. In this context it would be important to know whether c-*myc* labelling of spinous layer cells might prove to be a presage for potential malignant transformation. Longitudinal studies are needed to explore the predictive value of oncoprotein expression in benign oral leukoplakias.

c-*myc* Expression in hyperorthokeratosis was significantly less than in mild to moderately dysplastic lesions whereas no quantitative difference was noted between hyperparakeratotic and dysplastic epithelia. Significantly, while no quantitative differences were noted, nuclear labelling extended into the lower spinous layer in all instances of dysplasia, corresponding to foci with cytological atypia. Labelling of spinous cell nuclei was significantly increased in severely dysplastic lesions/carcinoma *in situ*. When CIS group lesions were compared with invasive carcinoma, no significant differences were observed; in both instances nuclear labelling exceeded 65%. These data would indicate that the proliferative potential of both CIS and invasive carcinoma epithelial cells as assessed by c-*myc* labelling is comparable. This would imply that once cells exhibit top to bottom atypia, their full malignant potential has been reached. The higher degree of variance observed in the carcinoma cases is a relection of differentiation in the tumour cell populations. Poorly differentiated tumours exhibited much higher levels of nuclear positivity than did well differentiated lesions, a feature also noted by Butt and coworkers [29]. While not specifically assessed in this investigation, this finding would indicate that the correlation between histological grade of malignancy and the degree of c-*myc* expression may be correlated with behaviour. Nevertheless, the use of c-*myc* labelling to predict behaviour along invasive carcinomas would not be expected to offer any advantage over routine histopathological assessment.

The appearance of c-*myc* oncoprotein in squamous cell carcinoma has been considered to be a late event and perhaps not as important as other transforming events in neoplastic transformation [9, 21]. In contrast, in this immunohistochemical study, we have demonstrated that c-*myc* is expressed at the very inception of cell transformation as assessed histopathologically. Whether or not the oncoprotein has undergone any mutational changes during the process of neoplastic transformation cannot be determined from this investigation. It is conccivable that detection of c-*myc* in more dysplastic lesions reflects retention of an altered form of the oncoprotein or, alternatively, perhaps its half-life is considerably prolonged owing to perturbations in catabolic processes. Lastly, the appearance of c-*myc* in more advanced stages of cytological atypia could simply represent an increase in transcriptional activity not necessarily coupled with progressive cell transformation. Conversely, this oncogene product could have important implications in the initial phases of malignant transformation in oral precancer.

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