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Intermediate Filaments in Oral Neoplasia. I. Oral Cancer and Epithelial Dysplasia

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The major value of intermediate filaments (IFs) in biological and applied research lies in their high order of cell and tissue specificity. This is particularly well illustrated in keratin (K) expression in various oral epithelia. Although the original class of IF is usually conserved in tissues after neoplastic transformation, epithelia show a tendency to shift their pattern of keratin expression in a manner which, while not predictable with precision, may sometimes be of diagnostic or prognostic significance. This review compares the keratins in normal oral epithelia, which show a mainly site-dependent expression, with those in squamous cell carcinoma. Key changes in the latter are the presence of simple epithelial keratins, K8 and K18 (occasional K7), reduced expression of differentiation-linked keratins (K1, K10, K4 and K13) and a tendency for down-regulation of primary keratins, K5 and K14. Moderate and severe dysplasias also tend to exhibit K8 and K18 with concomitant disordered expression of differentiation-linked keratins. There are reports of similar changes after neoplastic transformation in other mucosal sites and skin. Before this information can be applied diagnostically in immunocytochemical studies, the anti-keratin antibodies must be fully characterised and their interaction with the relevant tissue, both frozen and conventionally processed, should be evaluated.
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INTRODUCTION

THE CYTOSKELETON of eukaryotic cells consists of three main groups of filament proteins, 5 nm diameter microfilaments, 25 nm microtubules and 7–10 nm intermediate filaments (IFs), as observed in the electron microscope. Unlike the other cytoskeletal proteins, actin and tubulin, IFs show a considerable degree of tissue specificity [1–6]. In this review, we will examine some fundamental features of the IF system and its expression in normal oral mucosa, explore the changes which occur in malignancy and dysplasia, then consider some potential diagnostic implications of these findings. In a further review we will examine the changes in IF expression associated with neoplasia in other oral tissues.

CLASSES, STRUCTURE AND FUNCTIONS OF INTERMEDIATE FILAMENTS

Based on immunological and biochemical properties and patterns of tissue distribution, six major classes of intermediate filaments have been defined [2, 7–10] (Table 1). These include acidic keratins (type I), basic keratins (type II), vimentin, desmin and glial fibrillary acidic proteins (type III), the neurofilament triplet (type IV), nuclear lamins (type V), and nestin (type VI). The two keratin (K) subfamilies contain the largest number of proteins (Table 2), some 20 separate gene products in all [1, 11]. Type I and type II keratins are distinguished by their different isoelectric points, antigenic determinants and two-dimensional tryptic peptic maps [1, 5, 12–15]. Results from nucleic acid hybridisation also support the division of keratins into the two subfamilies [16]. A major concept to have emerged from these studies is that the expression of keratins is tightly linked to epithelial differentiation, a given histological category of normal epithelium being reflected to a large extent by the composition and pattern of its keratins. A straightforward, numerical classification of keratins [1] has stood the test of time and is best appreciated in the context of tissue distribution (Table 2).

Keratin filaments are assembled from heterodimer subunits composed of a type I and type II protein, each encoded by its own gene. Although many keratins may be present in a given epithelial cell, types I and II are present in equimolar amounts [5]. The expression of keratin pairs in normal epithelia has been shown to operate in a consistent manner. Thus, K7, K8 and K18 are found among simple epithelia whilst K5 and K14 are present in basal cells of stratified and pseudostratified epithelia. In stratified epithelia, different forms of keratinisation are associated with the addition of secondary, or differentiation-specific, keratins, K1 and K10 in cornified epithelia such as epidermis, K4 and K13 in non-cornified epithelia such as oesophagus and K3 and K12 uniquely in corneal epithelium. Other keratins of relevance include K6 and K16 which are present in oral mucosal epithelia but not in normal epidermis from most bodily sites [17]. K19 has a number of unique features. It is expressed in many simple

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Table 1. Classes and distribution of intermediate filaments

Type	Name	Number of protein chains	Distribution	Size (kD)
I	Acidic keratins	~16	All epithelia	40–64
II	Neutral basic keratins	~15	All epithelia	50–70
III	Vimentin	1	Mesenchymal cells	53
	Desmin	1	Myogenic cells	52
	Glial fibrillary acidic protein	1	Glial cells and astrocytes	51
IV	Neurofilaments	~4	Most neurones	57–150
V	Lamins	~4	Nuclear lamina	60–70
VI	Nestin	1	Neuroepithelial stem cells	?

Table 2. Classes and distribution of keratin filaments

Size (kD)	Type II	Distribution in epithelia	Type I	Size (kD)
			K9	64
68	K1	Cornified stratified	K10	56.5
65.5	K2		K11	56
63	K3	Cornea	K12	55
59	K4	Non-cornified stratified	K13	54
58	**K5**	Basal cells	**K14**	50
			K15, K17	50, 56
		Basal (non-cornified)	K19	40
56	K6	Fast turnover stratified	K16	48
54	K7	Simple epithelial cells	K19	40
52.5	**K8**		**K18**	45
			K20	47

Primary keratins of stratified and simple epithelia are indicated by bold text.

epithelia [18, 19] as well as in basal cells of non-cornified, stratified epithelia [20].

Some insight into the function of IFs can be gained from a consideration of their molecular structure. A fundamental three domain structure (head, rod and tail) is shared by all IFs (Fig. 1). The single feature distinguishing an IF protein chain from all other proteins is its central α-helical rod domain of about 310 amino acid residues that has been remarkably well conserved in its size, secondary structure and, to a great extent, in its amino acid sequence. The non-helical amino- and carboxy-terminal domains reveal considerable length and sequence differences between different IF proteins [21]. Heterodimers of types I and II keratins form stable filaments, confirmed when the properties of artificial homo- and heterodimers were compared [5, 15, 22, 23].

Postulated functions for IFs have included the organisation and maintenance of cell shape and possibly signal transduction [24]. Immunofluorescence staining with monoclonal antibodies (Mabs) to keratins in epithelial cells have revealed an intracellular cytoplasmic network which appears to be attached to the cell membrane via desmosomes and thus, through this linkage to other cells, to be part of an intercellular structural complex conferring mechanical stability on epithelia [25]. This interpretation has been developed by two lines of recent research, one on the discovery of a link between keratin gene mutations in certain clinical disorders of epidermis and the other on the IF-associated protein, desmoplakin. Keratin gene mutations found in epidermolysis bullosa simplex [26–29] and in epidermolytic hyperkeratosis [30, 31] provide support for the view that the keratin filament network contributes to the shape of the epithelial cells and structural stability of the epidermis as a whole. In addition, mutations in desmoplakin can disrupt not only the keratin and vimentin filaments in cells expressing desmosomes [32], but the IF network around the nucleus [33], findings which demonstrate that IFs may function in nucleo-cytoplasmic interactions.

ORAL MUCOSAL EPITHELIA

Initial studies on the keratin composition of normal oral mucosal epithelia involved immunoblotting [34], supplemented by immunohistochemistry with Mabs to several groups of keratin proteins [35]. The use of Mabs to individual keratin proteins has enabled a more precise mapping of keratin distribution in different oral mucosal sites [36, 37].

Cornified epithelia of the attached gingiva and hard palate contain the primary pair K5 and K14 in basal and sometimes in suprabasal cells; the secondary, differentiation-specific pair, K1 and K10, is consistently present above the level of the rete processes [36, 37]. This pattern of keratin expression is similar to that found in epidermis except that, additionally, K6 and K16 are located suprabasally in oral epithelial [36]. These proteins are regarded as indicators of high cell turnover and are induced in epidermis during wound healing and in hyperproliferative disorders [38]. Their presence in oral cornified epithelium is in line with its generally high turnover rate. Simple epithelial keratins K7, K8 and K18, and also K19, are

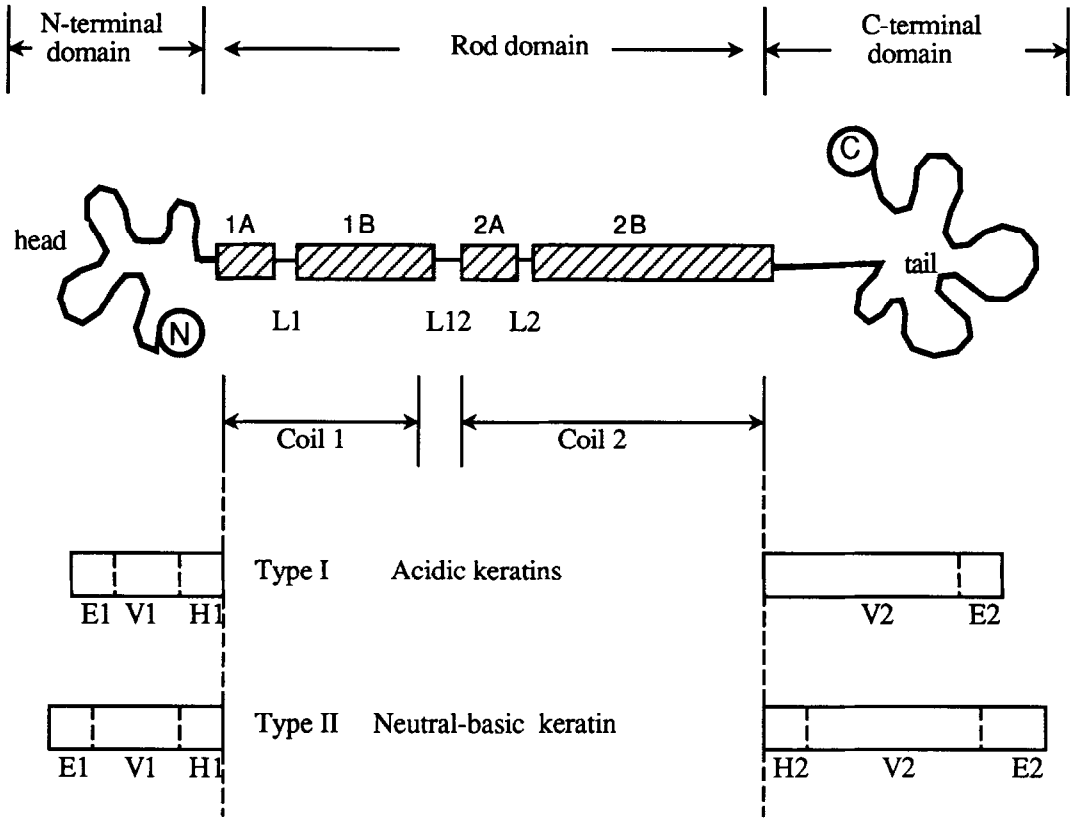

Fig. 1. The main features of IF protein structure. The common structure of IFs includes rod, head and tail domains. The rod domain consists of four α-helical units designated 1A, 1B, 2A and 2B interrupted by three 'linkers': L1, L12 and L2. The end domains may also be divided into subdomains based on homologous (H), variable (V) and end (E) sequences [3]. N = amino- and C = carboxy- at the head and tail domains, respectively.

present in Merkel cells [39] notably in gingiva and hard palate [36, 40–43].

Non-cornified epithelia of the alveolar and buccal mucosa and mucosa from the floor of mouth and ventral and lateral surfaces of the tongue express the same primary keratin pair of K5 and K14 as in cornified epithelium. Cells of the differentiation compartment, generally all suprabasal cells, express K4, K13, K6 and K16. In addition, K19 is distributed heterogeneously in non-cornified epithelium, predominantly in basal cells but occasionally in suprabasal cells [36, 42, 44, 45]. This combination of keratin expression is detected similarly in oesophageal epithelium [13].

Although it is well accepted that K1 and K10 are markers of cornification in stratified epithelia, and that K4 and K13 are markers of non-cornification, one should be aware that small sub-populations of suprabasal cells in cornified epithelia, such as hard palate and attached gingiva, express K4 and K13; also, in predominantly non-cornified epithelia such as in buccal mucosa, similar small cell groups express K1 and K10. The presence of these 'inappropriate' sub-populations appears to be due to normal variation rather than to minor pathological change [36, 40, 46].

The epithelia of the dorsal surface of the tongue show mixed patterns of keratin expression. In filiform and fungiform papillae, keratin expression is similar to that of cornified epithelia elsewhere, whereas interpapillary epithelium expresses the keratins of non-cornified epithelia. In addition, K19 and simple epithelial keratins K8 and K18 have been detected in taste buds related to sensory cells [37, 47]. The complex pattern of keratin expression of the tongue appears to be tightly linked to the specific functional requirements of this masticatory and gustatory mucosa.

Studies on gingival epithelia using keratin Mabs have revealed sharp anatomical differences. Attached gingival epithelium shows the same pattern of keratin synthesis as that of other oral cornified epithelia whilst the sulcular epithelium expresses non-cornification markers K4 and K13 [46, 48, 49]. Junctional epithelium has a keratin composition which is unique amongst stratified epithelia, a combination of K5, K14 and K19 being expressed uniformly in all cell layers frequently in the absence of secondary keratins [48, 49]. The distinct K19 expression has been used in this context to detect junctional epithelium following regeneration [50].

Thus, in normal oral epithelia, the keratin profile with its high order of site specificity may indicate adaptation to the different regional and functional requirements and the above studies have provided a baseline against which to assess changes in the IF system accompanying neoplastic transformation.

HUMAN ORAL SQUAMOUS CELL CARCINOMAS (SCC) AND DYSPLASIA

Some early biochemical investigations into keratin proteins in SCCs indicated a wider range of keratin expression than would be expected from the pattern shown by their normal parent epithelium [51–56]. In more recent studies, Mabs have been used to localise individual keratins in small subpopulations not detectable by biochemical means. Here we focus on

altered keratin expression in oral SCCs but as indicated later, similar trends have been observed in SCCs from other sites.

Oral SSC

Two main trends have been reported in oral SCCs with respect to keratin expression. The first is a reduction in secondary keratins (K1 and K10; K4 and K13). In well differentiated SCCs, these keratins are generally conserved [43, 57–62] being localised to prickle cells and cells at the centre of some tumour islands. Such central cells correspond to the cornified and non-cornified compartments of normal stratified epithelia and are most commonly seen to be parakeratinised in haematoxylin and eosin stained sections. Unlike normal oral epithelia, neoplastic cells often co-express both sets of differentiation keratins (K1 and K10; K4 and K13) [43, 57, 60]. In less well differentiated SCCs fewer clusters of epithelial cells contain these keratins.

A second feature of altered keratin expression in SCC is the anomalous expression of simple epithelial keratins, notably K18, slightly less frequently K8 and sometimes K7. K8 and K18 have been detected to an extent, in both frequency and intensity, which appears to vary inversely with the degree of differentiation [43, 57–59, 61, 62]. The strongest expression has been found in poorly differentiated tumours which are known to have a worse prognosis [63]. The synthesis of K7, K8 and K18 in a SCC therefore may have a bearing on the behaviour of the malignancy, a concept further supported by the detection of stronger expression of these keratins in lymph node metastases from oral SCCs than in their concurrent primary neoplasms [43] (Shirlaw *et al.*, manuscript in preparation). Linkage of K8 and K18 to behaviour of SCCs is nevertheless far from established, a recent study showing that expression of these keratins is localised at the invasive front, irrespective of histological grading [64]. Keratin 7, which in normal simple epithelium is expressed similarly to K8 and K18 [18, 19], is less frequently detected in head and neck squamous cell carcinomas, an indication that in malignancy this keratin behaves differently, its role remaining to be fully investigated.

A further reported change in keratin composition in SCCs is a raised level of K19 as detected by SDS-electrophoretic and immunoblotting analysis [58, 61]. This finding has not been supported by immuno-cytochemical studies, the amount and distribution of K19 varying markedly from tumour to tumour. Oral SCCs may be uniformly positive, uniformly negative or heterogeneous, regardless of the degree of differentiation [43, 57]. The exact site of origin, in particular whether the tumour arose from an originally cornified or non-cornified site, may have a bearing but this factor remains to be investigated, together with any possible influence of the original cornification pattern on tumour behaviour.

Despite the presence of additional keratins in malignant neoplasms, from the early studies it was apparent that the expression of primary keratins in malignancy was conserved [1] thus potentially facilitating the distinction between adenocarcinomas and SCCs in the case of anaplastic tumours. This concept may have to be modified in time as a number of reports have emerged which indicate a possible loss or reduction in K5 and/or K14 in poorly differentiated oral SCC [59, 61, 65–67]. Interpretation of these results should be guarded, however, as the techniques or specimens used (SDS gel electrophoresis and immunoblotting or immunocytochemistry on formalin-fixed, paraffin-embedded tissues with antibodies, some of which stained several keratins) are not optimal for the demonstration of these keratins in tissues. Gel electrophoresis is likely to be insufficiently sensitive and conventionally processed specimens may have altered K5 and K14 which are very conformation-sensitive [68, 69]. This apparent loss of primary keratins in poorly differentiated SCCs could be an important aspect of altered keratin expression in malignancy and, if substantiated, may have diagnostic implications.

Thus, examination of the keratin profile of oral SCC reveals that the differentiation of oral mucosal epithelia can be modulated to express an altered pattern of keratins during the development of malignancy. A question which then arises is whether the antecedents of these changes can be detected in dysplastic epithelium.

Oral epithelial dysplasia

Dysplastic changes in oral epithelium are considered to be linked to premalignancy in a proportion of cases, particularly if the changes are severe [70]. Some characteristics of dysplastic epithelium can be interpreted as manifestations of disordered differentiation. Thus, it might be expected that disturbances of keratin expression might accompany dysplasia.

A number of studies have been undertaken to investigate keratin expression in oral epithelial dysplasia by both immunocytochemical and biochemical means and some consistent features have emerged. Expanded expression of K5 and K14 beyond the basal layer to include a variable proportion of the total epithelial thickness has been demonstrated by immunocytochemistry [60, 65, 66]. This is in line with the frequent finding of 'basal cell hyperplasia' in epithelial dysplasia and with the reduced expression of differentiation keratins, K1 and K10 or K4 and K13 [43, 57, 60, 61, 66]. These studies also indicate a tendency for the greatest loss of differentiation keratins where dysplasia is most severe. Expression of K1 and K10 is usually spatially separate from that of K4 and K13 but in malignancy and dysplasia the two pairs may be co-expressed in the same groups of cells [43]. This may be explained by the frequency with which dysplastic epithelium in normally non-cornified sites undergoes parakeratinisation. A similar phenomenon has been described in lingual epithelium from a case of dyskeratosis congenita, a premalignant condition [71].

Several reports describe an expanded expression of 'fast cell turnover' keratins, K6 and K16, in dysplastic epithelium [57] or in clinical 'leukoplakia' regardless of the presence of dysplasia [58, 61], phenomena possibly associated with a higher cell turnover rate of dysplastic epithelium compared to normal [72]. Interestingly, expression of this keratin pair appears reduced in invasive SCC [57, 58, 61].

Simple epithelial keratins, K8 and K18, have been detected in some samples of 'leukoplakia' especially from non-cornified sites [61], and in areas of severe dysplasia or microinvasion [43]. These keratins were localised mainly to the deeper layer. In a recent study (Shirlaw *et al.*, manuscript in preparation), simple epithelial keratin expression, especially that of K18, correlated strongly with severity of dysplasia, all severely dysplastic cases being positive.

There are controversial reports of K19 expression in oral dysplastic epithelium. One study suggested that suprabasal expression of this keratin could be a marker of premalignant change [73] but another group has reported variable ex-

pression of K19, usually confined to the basal and parabasal layers in dysplasia, a similar distribution to that in non-cornified epithelia [43]. An immunoblotting study has shown that there is significant synthesis of K19 protein in leukoplakia, whether or not dysplastic change is present [61]. The fact that expression of K19 is widespread not only in normal non-cornified epithelium but in inflamed gingiva [41, 46] and in human papilloma virus (HPV) infected oral epithelia [74] suggests that the status of K19 as a 'marker' of premalignancy should be reevaluated.

In summary, there is evidence in dysplasia of changes in keratin expression involving basal, suprabasal, 'fast cell turnover' and simple epithelial keratins, this last group containing the most likely candidates to aid the prediction of malignancy. However, it is generally accepted that most individual components which make up the dysplastic epithelial phenotype correlate poorly, if at all, with eventual malignant transformation [75] and further studies are necessary to clarify this issue.

Non-oral SCC

Keratin expression in non-oral SCCs has been examined with essentially similar results to those described above [64]. In epidermal SCCs simple epithelial keratins are expressed more extensively in poorly differentiated than in more highly differentiated tumours [76]. Cervical SCCs express simple epithelial keratins and K19 to a greater degree than either oral or epidermal SCCs but in cervical dysplasia the keratin changes are essentially similar to those reported in oral mucosa [77–79].

DIAGNOSTIC IMPLICATIONS

There are several levels at which an understanding of IF expression has spread into the diagnostic field. The most widely used application has been to help clarify the tissue of origin in anaplastic tumours [80, 81] where diagnosis based solely on histological criteria can lead to error and inappropriate treatment [82]. Some IF classes are sufficiently conserved in anaplasia to play a role as tissue-specific markers (e.g. keratins, desmin) although vimentin is too widely expressed to be diagnostically useful for many neoplasms and may also be co-expressed with keratins in high grade carcinomas [83–85]. Some antibodies to IFs now have a place amongst the most commonly used antibodies in diagnostic immunocytochemistry [86, 87].

Amongst the ideal attributes of an antikeratin antibody for use in diagnostic immunocytochemistry are that it should recognise representatives of all keratin pairs, that it should recognise keratin epitopes in both frozen and conventionally processed material and that it should not cross-react with other cytoplasmic constituents. Unfortunately, no antibody has all these properties. Several multivalent antikeratin antibodies work satisfactorily in frozen sections but those which can be used successfully in paraffin sections tend to stain only a limited number of keratins [25]. These, which include antibodies to simple epithelial keratins, rely for their usefulness on the abnormal expression of simple epithelial keratin in SCCs (as described above) as well as in adenocarcinomas.

A number of investigators have made reference to the value of primary keratin expression when making a diagnostic distinction between adenocarcinoma and squamous cell carcinoma, a problem with poorly differentiated carcinomas when traditional staining methods only are used. Some reports have indicated that the immunocytochemical detection of K8 and K18 (but not K5 or K14) serves to identify an adenocarcinoma whereas expression of K5 and K14 can be used as identifiers of cells of basal or keratinocyte origin [43, 88]. There are possible sources of error however. Reduced expression of primary keratins and the production of simple epithelial keratins by some SCCs (see section on SCCs and dysplasia above) may simulate a 'simple' epithelial phenotype and thus, at least in theory, lead to over diagnosis of adenocarcinomas. In practice, other criteria such as mucin production and EMA staining are used to help differentiate between these two groups of tumours. Thus, complete reliance on keratins as markers of histogenesis in malignant neoplasms should not be encouraged.

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