TRANSITION OF NORMAL ORAL MUCOSA TO SQUAMOUS CELL CARCINOMA INVOLVES INDUCTION OF αvß6 INTEGRIN.

by

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ABSTRACT

Integrins are a family of cell surface receptors that mediate cell-matrix adhesion. They are believed to be essential molecules involved in tumour cell invasion and metastasis. It has been shown previously that the αvß6 integrin, a fibronectin and tenascin receptor, is not expressed in normal tissues, but may be associated with malignancy. In this study, we have used immunohistochemical localization technique to examine the expression and distribution of αvß6 integrin in frozen sections of oral specimens representing 40 leukoplakia patients, 11 squamous cell carcinomas (SCCs), 5 hyperplasia, 8 lichen planus, 2 mucosal wounds, 3 periodontal pocket samples and 11 normal subjects. Integrins αv, ß1, ß3, ß4, ß5, ß6, fibronectin, and tenascin were localized using specific antibodies. The expression of ß1 integrins was consistent throughout the basal layer, and that of the ß4 at the cell surface facing the basement membrane of all tissues. The integrins ß3 and ß5 were both absent from all normal and leukoplakia tissue specimens. The integrin αvß6 was highly expressed throughout the whole lesion of 90% of the SCCs but it was not present in any of the normal specimens. It was also expressed in 25% of the leukoplakia specimens, and 90% of the lichen planus samples, but in none of the tissues with hyperplasia only. In some leukoplakia sections, the expression of αvß6 integrin was only seen in a few cells at the tips of the rete ridges, whereas in others a range of basal cells expressed this integrin. Fibronectin and tenascin were both present in the connective tissue underneath the epithelium of all the sections, and their expression was similar in both αvß6 positive and αvß6 negative tissues. Based on these findings and earlier results, we speculate that the expression of αvß6 integrin in leukoplakia lesions could play a role in their malignant transformation and particularly in tumour cell migration and invasion in fibronectin-rich matrix. Moreover, in lichen planus lesions, the induction of this integrin may be caused by cytokines associated with inflammation that are specific for lichen. This study proposes that the presence of αvß6 integrin in premalignant tissues could be used as an adjunct in tumour diagnostics.

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INTRODUCTION

Integrins are a family of cell surface receptors that mediate cell-cell and cell-extracellular matrix adhesion in various cell types including epithelial keratinocytes (Watt and Jones, 1993; Larjava et al., 1996). These receptors are heterodimeric transmembrane glycoproteins composed of an alpha (α) and beta (ß) subunit. Each α subunit can form dimers with several ß subunits and vice-versa. More than 20 different α and 8 ß-subunits are currently known. These subunits can variously combine to form more than 22 different cell surface receptors that have distinct ligand binding specificities.

Normal skin and mucosal epithelium express multiple integrins. In normal epithelium, α6ß4 integrin binds to laminin-5 of the anchoring filaments and serves as an integral component of the hemidesmosome (Stepp et al., 1990; Sonnenberg et al., 1991). α2ß1 and α3ß1 integrins are localized in basal epithelial cells. They are known to be involved in cell-cell binding and binding of various collagen types and laminin-5, respectively (Staquet et al., 1990, Carter et al., 1990). Basal keratinocytes do not normally express αvß3 and αvß6 integrins (Haapasalmi et al., 1995; Breuss et al., 1995). αvß5 has been detected from the areas of normal buccal mucosa (Adams and Watt, 1991). On the other hand, it has been also reported to be absent from normal oral mucosa (Larjava et al., 1993). αvß6 is exclusively epithelial integrin that has been shown to bind to fibronectin and tenascin (Sheppard et al., 1990; Prieto et al., 1993). Its expression is restricted to only a few locations in healthy adult tissues in humans (Breuss et al., 1993). Expression of αvß6 integrin is, however, induced during wound healing and in squamous cell carcinoma (Haapasalmi et al., 1996; Breuss et al., 1995). It is not known at which stage of transformation of oral epithelial cells to squamous cell carcinoma the expression of αvß6 integrin begins. Epithelial cells that are in the process of malignant transformation can be found in some of the oral leukoplakia lesions. 2.2-17.5% of oral leukoplakia have been reported to transform to SCC (Gupta et al., 1980; Silverman et al., 1984). We investigated, therefore, whether epithelial cells in oral leukoplakia express αvß6 integrin and whether this change could be associated with the malignant transformation.

Chapter One

REVIEW OF THE LITERATURE

Oral Mucosa

The term mucous membrane is used to describe the body cavities that are in contact with the external environment (Cate, 1994). In the oral cavity, this lining is referred to as oral mucous membrane or oral mucosa. Anatomically, oral mucosa is situated between skin and intestinal mucosa and shows some properties of each.

Oral mucosa is comprised of two major tissue components. The uppermost layer is a stratified squamous epithelium which is referred to as *oral epithelium* and is analogous to the epidermis of skin, and the underlying layer is a connective tissue layer that is referred to as *lamina propria* or corium which resembles the dermis of skin. The junction between the oral epithelium and lamina propria is very corrugated. It is filled with the upward projection of connective tissue called the *connective tissue papillae,* and downward folds of epithelium called *rete ridges* or *pegs* [Fig. 1]. At the interface between the epithelium and connective tissue there is a layer of basement membrane.

Oral epithelium

This tissue is the primary barrier between the oral environment and the deeper tissue layers. Embryonically, oral epithelium has been thought to be derived from both ectoderm and endoderm (Bhaskar, 1980). The precise boundary between the two tissues is not defined, but it is believed that the epithelium of the structures that develop in the branchial arches (tongue, pharynx, larynx) is endodermal in origin, whereas the epithelium covering the palates, cheeks, and gingiva is ectodermal. This epithelium is a stratified squamous epithelium consisting of cells that are tightly attached to each other. It maintains its structure by intense mitotic divisions of the cells in the deeper layer and their migration to the surface where they replace the cells that are shed. There are

some nerves that pass into the oral epithelium, but it is devoid of blood vessels. It is suggested that the oral epithelial cells could be considered to consist of two functional groups (Cate, 1994):

1. Those whose function is to divide and provide new cells. These are referred to as *progenitor population.* These cells are located at the basal layer of the thinner epithelia (floor of the mouth), and in the lower two or three layers of thicker epithelia (Cate, 1994). A small number of progenitor cells are considered to represent stem cells and their function is to produce basal cells and retain the proliferative potential of the tissue (Cate, 1994). A larger portion of progenitor population are those which increase the number of cells available for subsequent maturation, and are thus termed transit amplifying cells. These cells are different in that they have a restricted division potential. Their ability to divide will be determined by external influences. If an external demand is made for more cell production, the stem cells will be the first ones to be influenced. However, an increase in the number of stem cells, will ultimately raise the number of transit amplifying cells. Despite all the homeostasis mechanisms in the body, a permanent damage in the cell's life cycle may lead to malignant changes (Leigh et al., 1994).

Stem cells are the most adhesive cells of the keratinocytes and known to express the highest level of ß1 integrins, immediately followed by the transit amplifying cells (Regezi and Sciubba, 1993). It has been reported that keratinocytes with characteristics of stem cells can be isolated from cultured human epidermis on the basis of high surface expression of ß1 integrins (Jones and Watt, 1993). This finding enables the investigators to find out more about the stem cells and the transit amplifying cells and their possible molecular markers.

2. The second group contains cells that continually undergo differentiation or maturation to form a protective surface layer. These are referred to as *maturing population.* In general, these cells follow two main patterns: keratinization and non-keratinization.

Watt et al., (1993), have shown that the terminal differentiation of human epidermal keratinocytes can be inhibited by the proportion of ß1 heterodimers occupied by ligands. For instance, fibronectin will inhibit the terminal differentiation through binding to α5ß1 integrin (Watt

et al., 1993).

One name that is often given to an epithelial cell because of its content of keratin filament is keratinocyte. The keratinized epithelium is found in the hard palate, gingiva, and in some regions of specialized mucosa on the dorsum of the tongue [Fig. 2]. It is inflexible, tough, and resistant to abrasion.

The first cell layer of keratinized oral epithelium is referred to as basal layer or *stratum basale*. The cells in this layer are adjacent to the basement membrane and are the least differentiated among the oral epithelial cells. They are cuboidal or columnar in shape and are the site of most cell divisions. Above the basal layer are a few rows of larger ovoid cells that are referred to as prickle-cell layer or *stratum spinosum.* These cells contain membrane-coating granules in their upper part. When prepared for histological examinations, these cells shrink away from each other and remain in contact only at desmosomes. The next layer, the granular layer or *stratum granulosum,* is intensely basophilic. This layer consists of flattened cells which contain keratohyalin granules. These granules are basophilic particles and are irregular in shape. They are about 0.5 to 1 μm in size and are thought to be synthesized by the ribosomes that can be seen surrounding them (Cate, 1994). It is also thought that they form the matrix in which filaments of the keratinized layer are embedded or aggregated (Cate, 1994). The uppermost layer (surface layer) is composed of extremely flattened and dehydrated cells termed squames. This is the keratinized layer or *stratum corneum* in which all organelles have been lost. It stains bright pink with eosin and does not contain any nuclei.

About 10% of the cell population in the oral epithelium are non-keratinocytes [Fig. 3]. These are a group of cells that do not participate in the process of maturation seen in oral epithelia. They represent a variety of cell types such as melanocytes, Langerhans' cells, Merkel's cells. All of these cells, except for Merkel's cells, lack desmosomal attachments to adjacent cells, so that during the histological preparation the cytoplasm shrinks around the nucleus to produce a clear halo. That is the reason that some histological sections of oral epithelium contain cells that differ in

appearance from other epithelial cells in having a clear halo around their nuclei. The non-keratinized epithelium is mostly found on the lining mucosa of the oral cavity, which is present on the lips, cheeks, alveolar mucosa, soft palate, underside of the tongue, and floor of the mouth. In many areas, this type of epithelium is generally thicker than the keratinized epithelium. The stratum basale and stratum spinosum resemble those described for keratinized epithelium, although the cells in non-keratinized epithelium are somewhat larger and intercellular prickles are less distinguished. The granular layer does not exist, therefore the third cell layer in this epithelium type is referred to as intermediate layer or *stratum intermedium.* These cells are slightly flattened and contain a lot of glycogen. Finally, the uppermost layer is the superficial layer or *stratum superficiale.* This layer contains slightly flattened cells with scattered filaments and glycogen which do not stain intensely with eosin as does the surface of keratinized epithelium. Very few organelles are present in stratum superficiale, but the nuclei persist.

In some areas, oral epithelium becomes parakeratinized. In these cases, the cells retain pyknotic and condensed nuclei and other partially lysed cell organelles until they desquamate [Fig. 4].

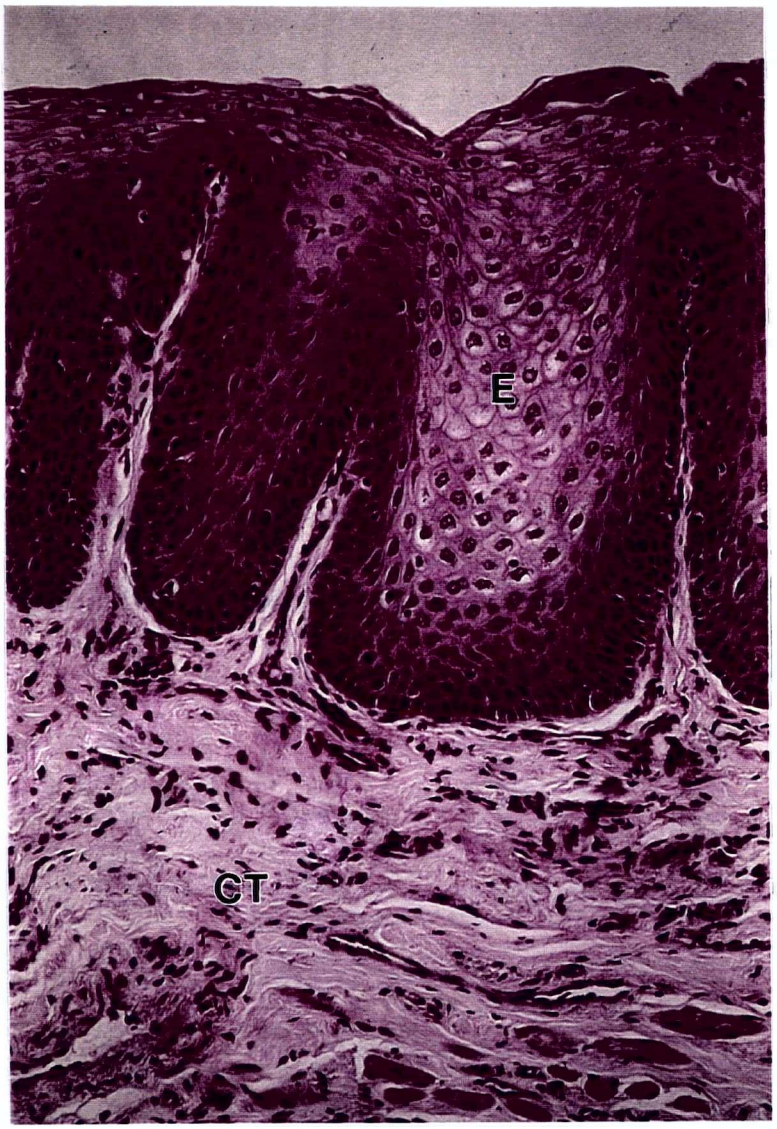


Figure 1. Oral mucosa

E=Epithelium; CT=Connective tissue

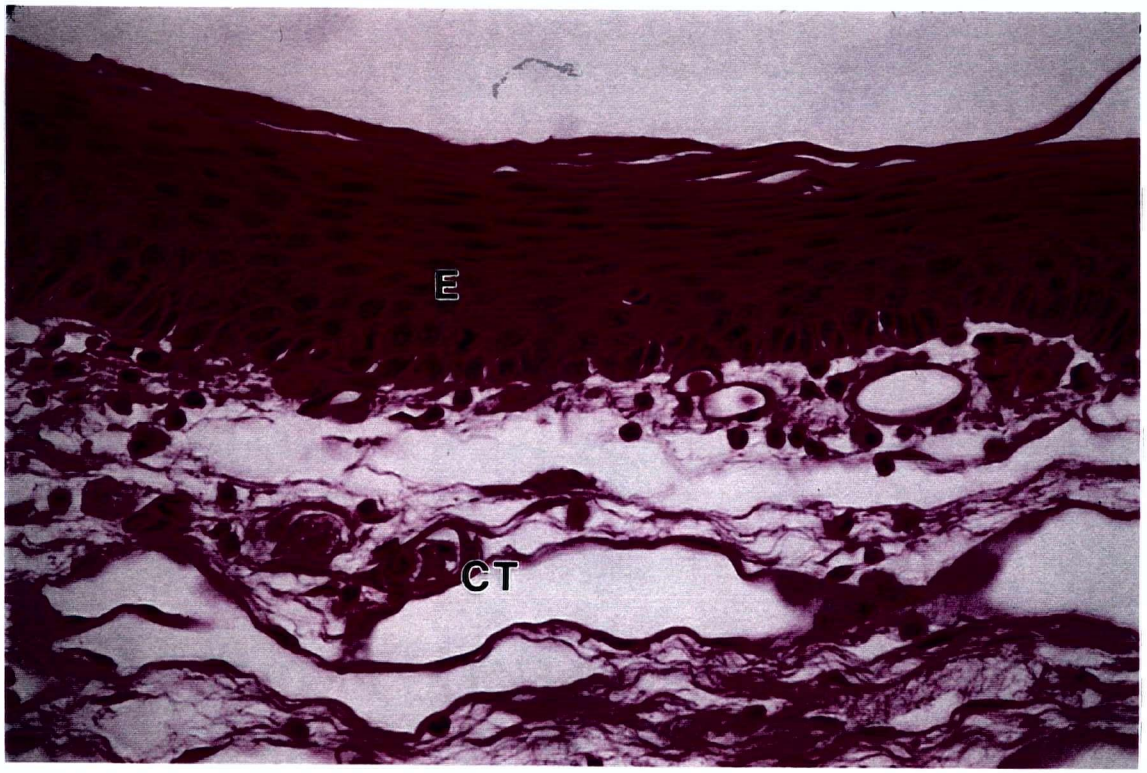


Figure 2. Keratinized oral epithelium; E=Epithelium, CT=Connective tissue

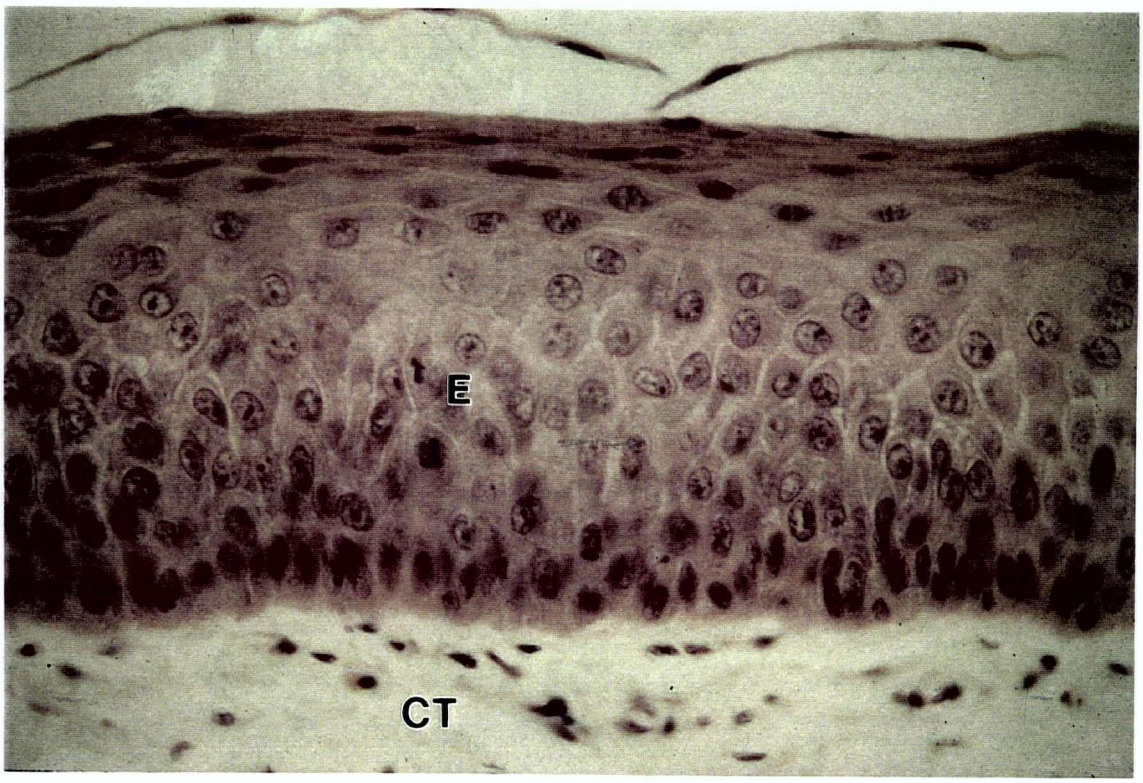


Figure 3. Non-keratinized oral epithelium; E=Epithelium, CT=Connective tissue

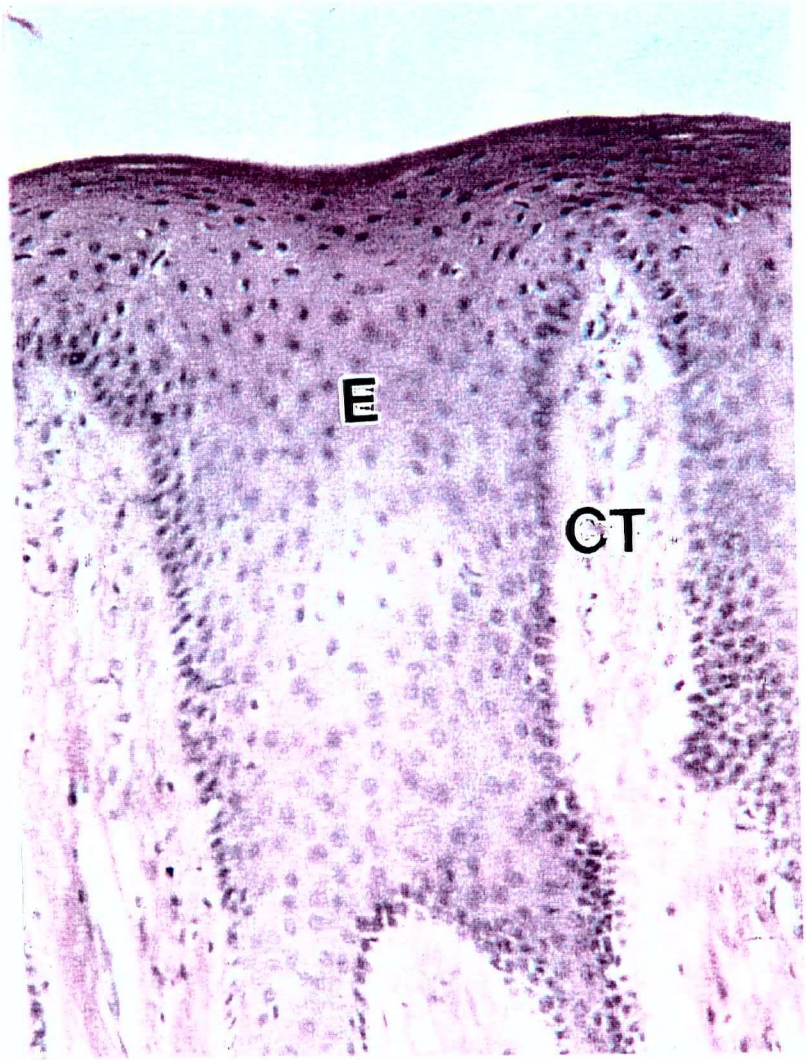


Figure 4. Para-keratinized oral epithelium

E=Epithelium, CT=Connective tissue

Oral Leukoplakia

Leukoplakia is believed to be the most common pre-malignant oral mucosal lesion (Sciubba, 1995). Leukoplakic reaction indicates that the chance for developing malignancy in a certain individual, at a certain site, is greater than usual. The term leukoplakia is derived from the Greek 'leucos' meaning "white" and 'plakos' meaning "plaque" (Welsh, 1955). In general, leukoplakia is defined as a white patch or plaque that cannot be rubbed off and cannot be characterized clinically or pathologically as any other disease (Sciubba 1995; WHO, 1984; Lucas, 1984). Some leukoplakia lesions appear to be associated with tobacco chewing and smoking (WHO, 1978). Some lesions are believed to be associated with some of the etiological factors that are discussed in connection with oral carcinoma (Lucas, 1984). Lesions of leukoplakia are not confined to the oral cavity. They are common on the male and female external genitalia, and have been reported on the mucous surfaces of the renal pelvis, the bladder, the urethra, the vagina, the anus, the auditory canals and the nostrils (Welsh, 1955; Shklar, 1984).

Histopathology

Histologically, the two most common changes observed in the epithelium of leukoplakic lesions are hyperkeratosis and a thickening of epithelium (Lucas, 1984). Several factors such as inflammation, hyperplasia, hyperkeratosis, atypia, acanthosis and dysplasia may influence the change in the morphology of the leukoplakic oral mucosa. As a whole, leukoplakia is a general term containing all the above mentioned phrases which are often associated with the epithelial changes. By definition, the term hyperplasia directly accounts for an increase of thickness of the epithelium due to proliferation of cells in all epithelial layers (Bánóczy, 1982). However, in atypia, individual cells in those mucosal lesions that may in time become malignant will be changed. In hyperkeratosis, either the orthokeratotic or parakeratotic layer will be increased in thickness abnormally (Bánóczy, 1982). When the cells of the stratum spinosum increase in number, the epithelial change is referred to as acanthosis. Finally, dysplasia is a collective term for

all disorders of epithelial cell differentiation except invasive and carcinoma in situ. The diagnosis of dysplasia is verified histologically if two or more of the following signs are present simultaneously. Epithelial dysplasia is graded respectively as mild, moderately severe or severe depending on the presence of either two, two to four, or five or more of the following signs (Listed by Bánóczy, 1982):

"1. Abnormal stratification of the epithelium
2. Basal cell hyperplasia
3. Drop-shaped rete ridges
4. Increase of mitotic activity
5. Altered polarity of basal cells
6. Increase of the nuclear cytoplasmic ratio
7. Nuclear polymorphism
8. Nuclear and nucleolar hyperchromatism
9. Increase of nucleolar size
10. Keratinization of single cells or groups of cell in the stratum spinosum
11. Loss of intercellular junctions"

In addition to these signs, there are other methods that help pathologists recognize dysplastic lesions. For instance, silver-binding nucleolar organizing regions (AgNOR) in normal oral buccal mucosa epithelium, oral leukoplakia, and oral squamous cell carcinoma have been studied (Chattopadhyay et al., 1994). Apparently, the mean AgNOR count per nucleus increased from healthy mucosa to leukoplakia to SCC. Tissues showing dysplasia in leukoplakia and SCC cases showed higher counts, wider scatter, and smaller size of AgNOR dots in the nuclei. The study seems to suggest that this method has some potential in distinguishing between dysplastic and non-dysplastic leukoplakia.

In leukoplakic lesions, there are also changes seen in the connective tissue portion of the oral mucosa. For instance, inflammation is often associated with leukoplakia, and is characterized by the presence of scattered or aggregated chronic inflammatory cells, predominantly lymphocytes, in the subepithelial connective tissue [Fig. 5].

Approximately 10-15 percent of all cases of leukoplakia eventually develop into epidermoid carcinomas if left untreated. Many years ago, Sturgis and Lund (1935) studied 312 cases of leukoplakia and found that 12 percent of the lesions developed into carcinoma. Not long after that, Shafer and Waldron (1961) reviewed 332 tissue specimens of clinical oral white lesions and found hyperkeratosis or parakeratosis with acanthosis in 190 cases; hyperkeratosis, parakeratosis, and acanthosis with focal atypia in 26 cases; carcinoma-in-situ in 6 cases; and invasive carcinoma in 27 cases. In another study of 723 patients who had oral leukoplakia in India, Mehta and colleagues (1969) found epithelial dysplasia in 10.7 percent. Bánóczy and Csiba (1976), in a histological study of 500 cases of leukoplakia, offered a definition and criteria for evaluation of dysplasia. 9.6 percent revealed the presence of epidermoid carcinoma, and 24 percent showed some evidence of epithelial dysplasia.

Not all leukoplakia will develop into cancer. However, it is desirable to identify biomarkers for detection of those premalignant lesions that will progress. Several investigators have undertaken methods to identify markers which would predict the malignant transformation of oral leukoplakia lesions. For instance, Mutirangural et al. (1996) have recently reported elevated activity of telomerase, a ribonucleoprotein complex, in leukoplakic lesions of the head and neck area. According to their data, telomerase activity is detectable in about 38.5% of these premalignant lesions, 83.3% of which belongs to dysplasia. This may mean that the telomerase activity is detectable at the late stages of carcinogenesis, but before the actual transformation to carcinoma, and could be used as a marker for detection of the head and neck carcinoma. In addition, overexpression of p53 protein has been noted in some leukoplakia tissue sections and many oral SCCs (Shintani et al., 1995; Girod et al., 1995). These results demonstrate that p53

overexpression may have an important role in the early stages of oral tumorigenesis. Moreover, Girod et al., (1994) showed that p53 protein was highly expressed in premalignant lesions and suggest that the increase in the number of p53-positive specimens was correlated with dysplasia and loss of differentiation in these lesions. Various studies suggest that the overexpression of p53 was generally observed in tumours obtained from heavy smokers and drinkers (Langdon and Partridge, 1992; Kaur et al., 1994; Wood et al., 1994).

Clinical manifestation

The use of the term leukoplakia can be misleading. Lesions showing histological changes that are usually diagnosed as pre-malignant, are frequently pink, red or mottled (Regezi and Sciubba, 1993). Then again, lesions that appear merely as white patches, with perhaps a minimal degree of ulceration, and that could well be called leukoplakia, may be found in microscopic examination to be definite invasive carcinoma. In the widest sense, the term leukoplakia may be restricted to those lesions that cannot be characterized clinically as any other disease. Recently, an international group of epidemiologist, clinicians and pathologists with experience of working on oral white lesions and attempting to apply the previously published definitions in their clinical practice participated in a symposium devoted to the further development of oral white lesion (Axéll et al., 1996). They have given an etiological description for leukoplakia as well as a clinical description. The etiological description identified two categories of leukoplakia: those of unknown etiology (idiopathic) and those associated with or thought to result from, the use of tobacco (tobacco associated). Whitish patches or plaques for which a local cause other than the use of tobacco could be identified were to be listed according to the known cause and not be designated as leukoplakia. Their clinical description sub-divided leukoplakia into a homogenous type and three sub-types of non-homogenous leukoplakia (nodular, exophytic, and erythroleukoplakias). Overall, these investigators have defined leukoplakia as "a predominantly white lesion of the oral mucosa that cannot be characterized as any other definable lesion; some oral leukoplakia will

transform into cancer".

The leukoplakic lesions may occur at any site in the oral mucous membrane. The tongue, used to be the most common site for leukoplakia (Robinson and Miller, 1983) [Fig. 6]. Today, the mandibular and buccal mucosa account for half of all the cases of leukoplakia (Regezi and Sciubba, 1993). Some leukoplakic lesions are quite small and circumscribed, others can be extensive, involving large areas of mucosa. Not all leukoplakic lesions are uniformly white; some are whitish-yellow or grey and others are mottled or "speckled" and fall under the nodular type category. In the homogeneous type, the surface of the plaque may be smooth or wrinkled. Smooth lesions sometimes show small cracks or fissures (Lucas, 1984; Pindborg, 1980).

Clinical features of oral leukoplakia may be variable and primarily depend upon the degree of surface keratinization (Pindborg, 1980). Initial, early lesions may appear as granular red and grey areas. More advanced lesions are raised and appear white, often with erythematous margins. Severe long standing involvement may consist of thick, leathery white plaques, often verrucous in areas. Unfortunately, there is not a good correlation between severity of clinical features and histological evidence of dysplasia. For that reason, it would be very useful to invent a test which aids the pathologists to identify which leukoplakic lesions will eventually become malignant.

The relationship of leukoplakia to carcinoma is not in doubt; malignancy does follow in a proportion of cases. What is not known, however, is the extent to which this occurs, both with regard to the incidence of coexisting leukoplakia in patients with carcinoma and to the incidence of leukoplakic lesions that subsequently become carcinomatous.

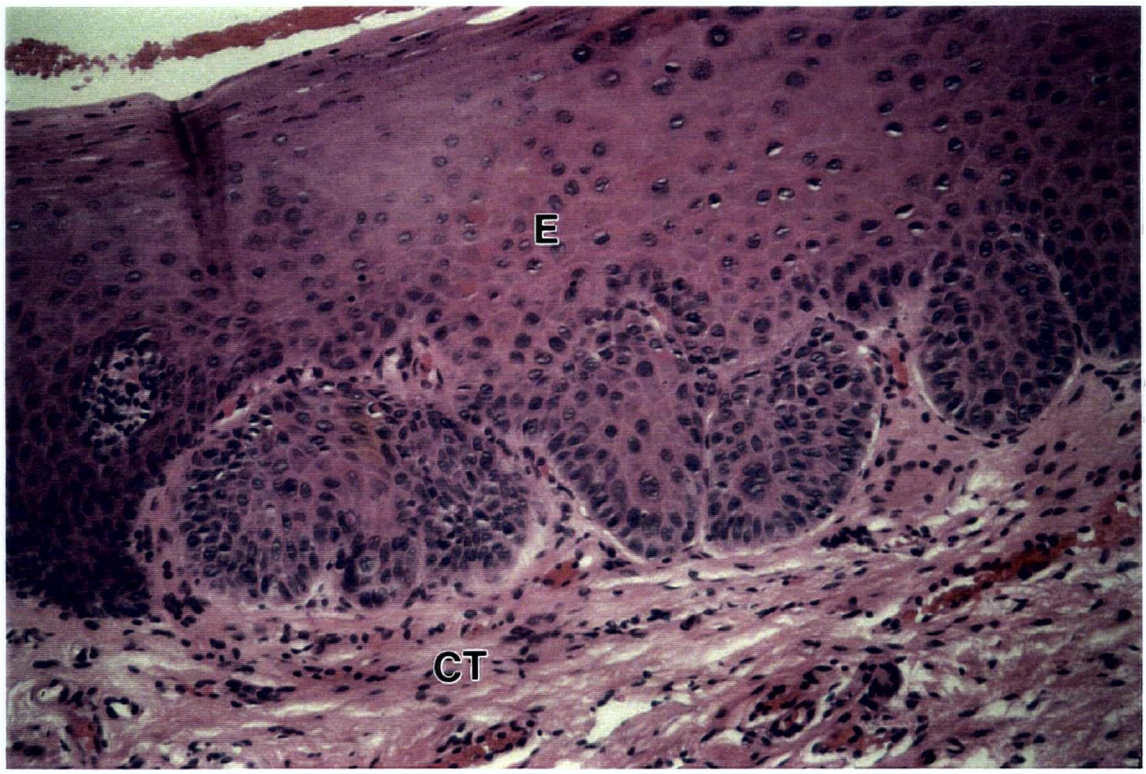


Figure 5. Severe dysplasia; E=Epithelium, CT=Connective tissue

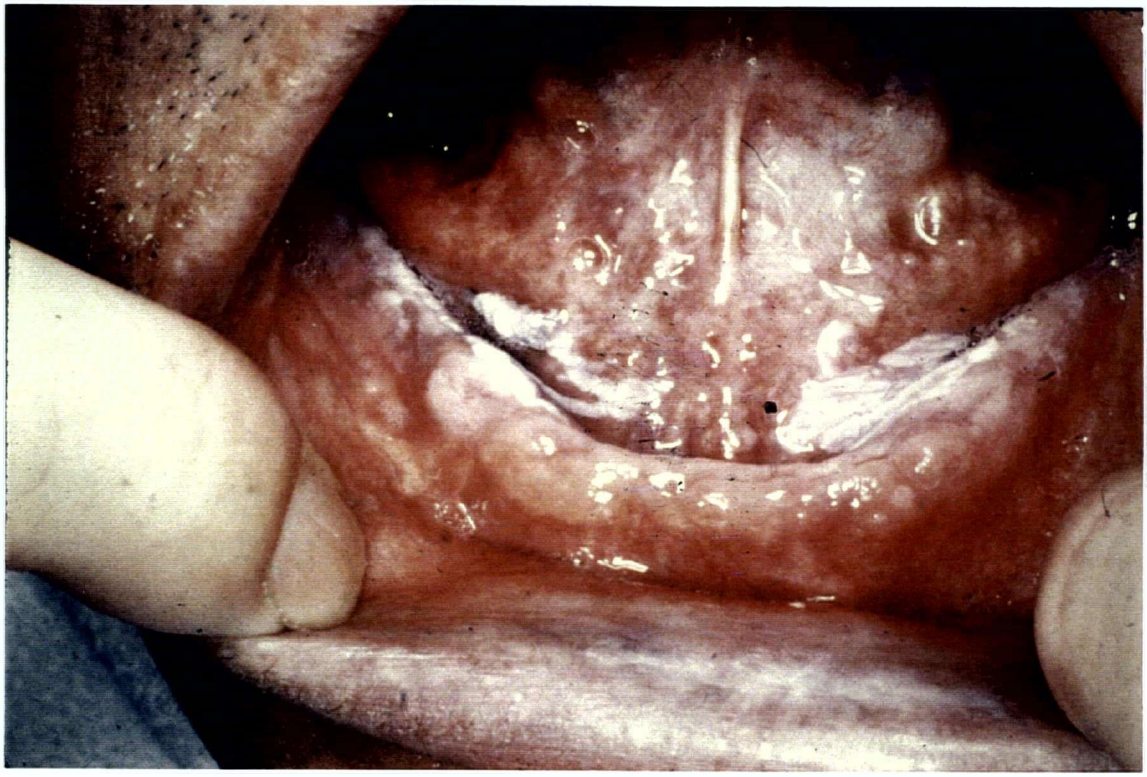


Figure 6. Dysplasia of tongue

Oral Lichen Planus

Of all the diseases which may manifest themselves in the oral cavity, and which bear a more or less close resemblance to leukoplakia, lichen planus deserves first consideration. Oral lichen planus may involve the buccal mucosa, the tongue, the lips, the hard palate, the gingiva and the anterior pillars. In general, lichen planus is a common mucosal condition with an estimated prevalence of 0.02% to 1.2% in the widespread population (Duffey et al., 1996). Some investigators believe that both men and women are affected at roughly the same ratio, and children are rarely affected (Regezi and Sciubba, 1993), whereas, more recent studies report that the majority of affected patients are women in their mid-life with no specific ethnic inclination (Duffey et al., 1996). The severity of the lesion is often associated with the stress level of the patients.

One of the factors that is associated with the development of lichen planus in oral mucosa is believed to be an imbalance in cytokines/cytokine-receptors and their antagonists (Feliciani et al., 1996). Cytokines are polypeptide growth factors produced by most nucleated cells in the body, including epithelial cells (Feliciani et al., 1996). They provide a cell to cell communication system between adjacent cells, between cells at distant sites, and intercellular effects. Epithelial cells in chronically inflamed oral mucosa produce a variety of cytokines (Feliciani et al., 1996). While it is unlikely that epithelial cell activation alone is the initial event in lichen planus, it is likely that pro-inflammatory cytokines released by epithelial cells contribute to the pathogenesis (Feliciani et al., 1996).

Some investigators have shown that oral lichenoid lesions can be caused by allergic reactions to a number of factors, such as mercury in amalgam fillings (Pang and Freeman, 1995). It is thus believed that the amalgam replacement will result in the rapid recovering or improvement of the lesions. If, however, the lesions transform to carcinoma, it is either because the mercury allergy is a secondary factor, or because the malignant transformation has started to form before the removal of the mercury.

Although lichen planus is believed to represent a delayed hypersensitivity reaction, in most

cases no readily identifiable allergenic substances has been discovered (Eversole, 1994).

Histopathology

Microscopically, the oral lesions of lichen planus are similar to the skin lesions. In mucosal lesions, the epithelium is often atrophic. The rete ridges may show a 'saw tooth' appearance and the basal cells frequently degenerate (Lucas, 1984). There is well marked chronic inflammatory infiltration in the lamina propria, forming a differentiated band confined to the most superficial portion of the subepithelial connective tissue (Lucas, 1994) [Fig. 7 A and B]. This band like infiltrate characteristically adjoins densely to the basal layer of the epithelium and thus obscure it. The epithelium may be separated from the lamina propria in some areas (Lucas, 1984).

In general, oral lichen planus is characterized by lymphocytic mucositis, basal cell lysis, and lymphocyte transmigration into the epithelial compartment (Pindborg, 1980). Practically, all of the infiltrating cells are T lymphocytes, and plasma cells are very rarely seen (Regezi and Sciubba, 1993). However, Langerhans cells and macrophages process the epithelial antigens and provide the T lymphocytes with their antigenic information, thus stimulating proliferation of T cells (Regezi and Sciubba, 1993). These activated T cells, T8 lymphocytes, consequently become cytotoxic for basal keratinocytes (Regezi and Sciubba, 1993).

Immunohistological studies have demonstrated fibrin accumulation in almost all cases of lichen planus (Laskaris et al., 1981; Pihlman et al., 1985). However, this finding is not believed to be specifically diagnostic since identical deposits were observed in other disease types such as Lupus erythmatosis (Laskaris et al., 1982). Fibrin deposition in the vessel walls and lumen of lichen planus tissue specimens may reveal the autoimmune nature of this disease (Mashkilleison et al., 1990). One theory proposes that the "basic cause of clinical manifestation of lichen planus is disordered microcirculatory system of the buccal mucosa, resultant from deposition of the immune complexes and fibrin in the vessel walls and lumen" (Mashkilleison et al., 1990).

Haapalainen et al. (1995), have reported a prominent amount of the collagen type VII in

striated patterns associated with the connective tissue component of the oral lichen planus lesions. The abnormal localization of collagen type VII has been similar but less dramatic in dermal lichen planus regions. In normal mucosa, this collagen has been restricted to the basement membrane zone, and the expression pattern is continuous. However, alteration in these areas have been observed. Smoller and Glusac (1994) have reported the disruption of type VII collagen in the lamina lucida regions of the basement membrane zone in those lesions of lichen planus resulting in blisters.

Clinical manifestation

Lesions of lichen planus are small, whitish or grayish, smooth, velvety, slightly projecting, grouped or isolated, rounded papules having a slight inflammatory halo (Welsh, 1955). "They may be arranged in annular, linear, or spiderweb patterns, or they may unite and form silver-white plaques with a reticulated, filigree-like appearance" (Welsh, 1955). A large proportion of oral lichen planus are asymptomatic, but occasionally, the patients complain of a dry or burning sensation and pain (Strassburg and Knolle, 1994). Specific types of lichen planus have been identified. The most common type is the reticular type which is mostly seen in the area of the buccal mucosa (Regezi and Sciubba, 1993). The reticular types are characterized by the presence of keratotic striae, Wickham's striae, in a symmetric annular pattern (Strassburg and Knolle, 1994) [Fig. 8].

Another type of lichen planus is known as the plaque form which is clinically very similar to the lesions of leukoplakia (Strassburg and Knolle, 1994). However, the distribution pattern of this type, unlike leukoplakia, is multi-focal. These plaques may appear smooth or somewhat irregular and their primary site is the dorsum of the tongue (Welsh, 1955). The other types of the lichen planus are the atrophic form, the erosive form, and the bullous form, with the first variant being the most symptomatic and the latter being the least common type (Regezi and Sciubba, 1993).

Although clinical and histological diagnostic criteria for leukoplakia and lichen are well-established, differentiation of the two conditions may still be problematical to both the clinicians and histopathologists. However, lesions of lichen planus do not become so thick or so white as leukoplakia (Regezi and Sciubba, 1993). They are more likely to become lace-like, bluish-white, and get surrounded by satellite lesions. Lesions of leukoplakia do not assume the reticulated pattern of lichen planus, nor do they appear in such symmetrical distributions (Strassburg and Knolle, 1994). Finally, histopathological studies have revealed that in lichen planus both acanthosis and hyperkeratosis are present, but not as abundantly as in leukoplakia (Regezi and Sciubba, 1993). The infiltrate is more dense, and more intimately associated with the epidermis in lichen planus, than is the infiltrate in leukoplakia, and is primarily lymphocytes as opposed to heterogeneous inflammation in leukoplakia.

Opinions have been divergent as to whether or not oral lichen planus is precancerous. Some reports have suggested a high incidence of oral squamous cell carcinoma in oral lichen planus patients and have implicated lichen planus as a premalignant lesion (Duffey et al., 1996). Others believe that in most instances the appearance of carcinoma in the oral cavity of patients who have pre-existing lichen planus is coincidental (Shklar, 1984, Krutchoff et al., 1978), many investigators have reported that malignant transformation occurs in 1-10 percent of the cases (Fulling, 1973; Pindborg, 1980).

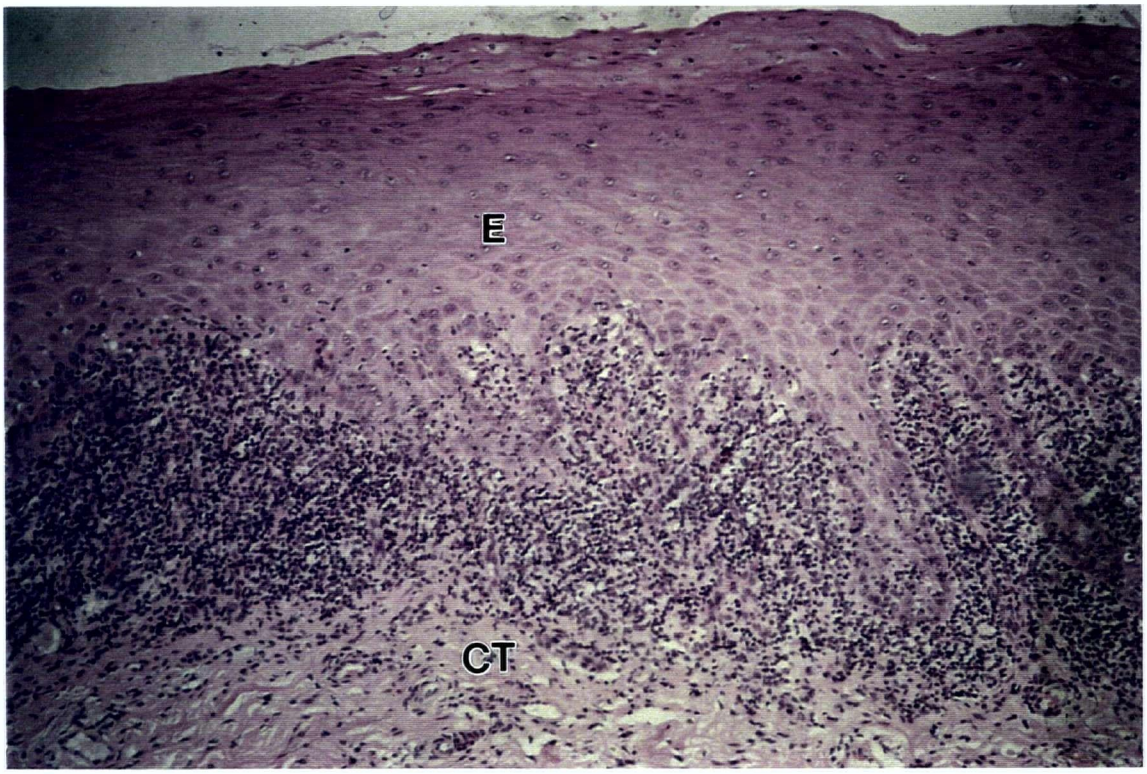


Figure 7A. Oral lichen planus; E=Epithelium, CT=Connective tissue

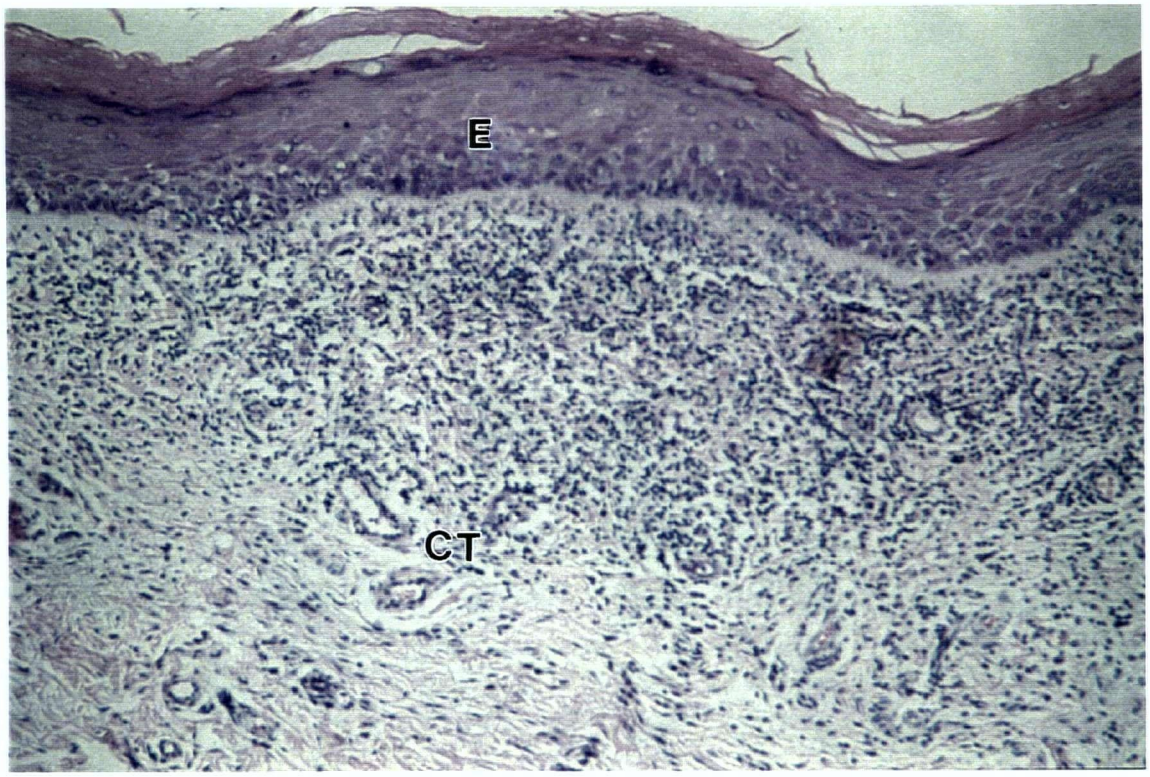


Figure 7B. Oral lichen planus; E=Epithelium, CT=Connective tissue

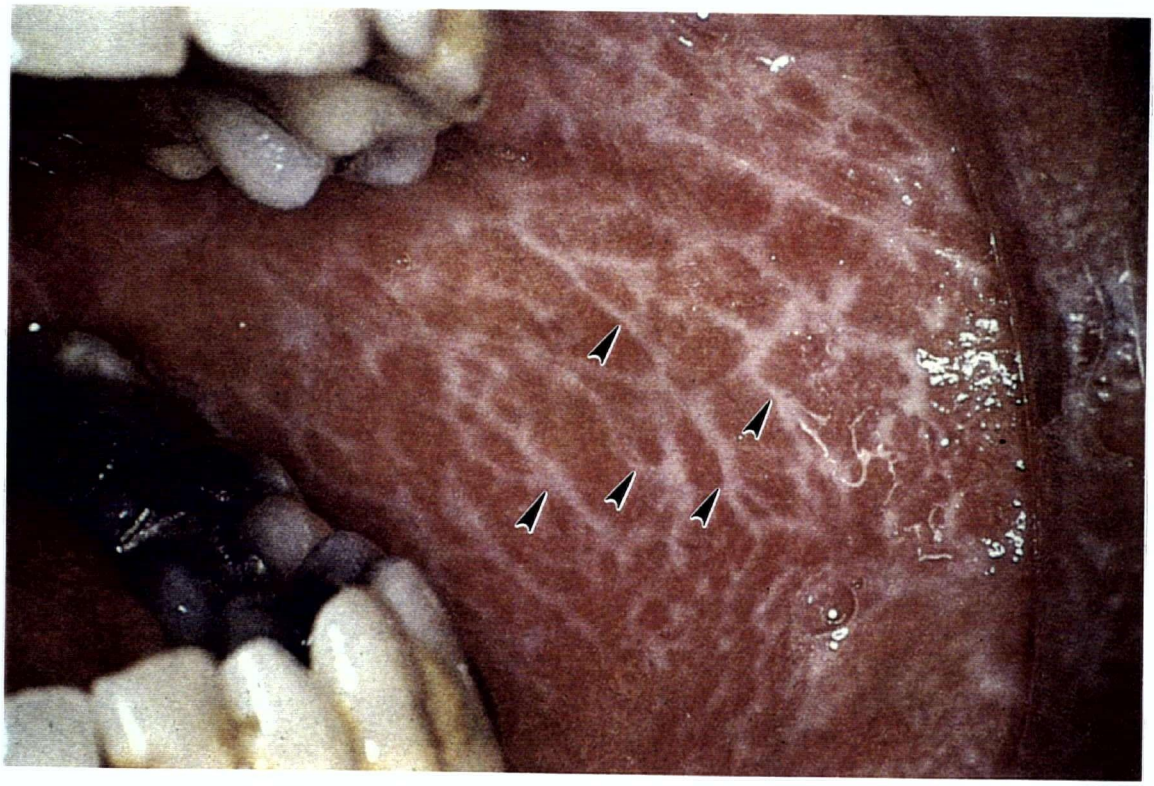


Figure 8. Wickham's Striae (arrow heads)

Oral Squamous Cell Carcinoma

Cancer is the second most common cause of mortality in the world today. More than half of the new cases of cancer recorded in the world occur in developing countries where 70-80% of the world population lives (Parkins et al., 1988). The American Cancer Society has estimated that more than one million new cancers (excluding skin) will be diagnosed each year (Heng and Rossi, 1995). Of these, approximately 2.5% are cancer of the oral cavity and pharynx. Oral squamous cell carcinoma accounts for approximately 9 out of 10 cases of oral malignancy, which are frequently preceded by various precancerous lesions. Oral squamous cell carcinoma (SCC) comprises a significant fraction (5%) of all the malignancies among the world population (American Cancer Society, 1988) and more than 40% of all malignancies in South Asia (Daftary et al., 1991; Sankaranarayanan, 1990). Although it forms a small proportion of all malignancies in Europe and North America, the incidence is rising, especially among young adults (Johnson and Warnakilasuriya, 1993; La Vecchia et al., 1992). It is responsible for over 9000 deaths in the U.S.A. every year (Marshal et al., 1992). Males over 50 years of age used to be more affected than their female counterparts of the same age, with the ratio of about 3:1 (Robinson and Miller, 1983). However, the ratio has shifted to 2:1, due to an increase in the number of female smokers and their longer life expectancy (Regezi and Sciubba, 1993). Despite the advances in surgery, radiation therapy, and chemotherapy, a large group of patients die with the disease still localized to the regional area (Oliver et al., 1996).

Tobacco chewing and smoking, alcohol, nutritional deficiencies, and dietary habits have been implicated in the etiology of the oral cancer (De Stefani et al., 1990; Blot et al., 1988; Jayant and Deo, 1986). Poor oral hygiene increases the risk of oral cancer, although this effect is much smaller than those of cigarette smoking and alcohol consumption.

Histopathology

Most oral SCCs are moderately or well differentiated (Cawson et al., 1994). There is epithelial proliferation, and invasion to the underlying tissue by strands and islands of dysplastic cells. The differentiation degree corresponds to the histological and clinical appearance of the carcinogenic tissues, and contains poor, moderate, and well differentiated classifications. By definition, poorly differentiated carcinomas are the tumours that are bigger in diameter and may expand beyond the primary region. However, in the case of the tumours of well differentiated carcinomas the diameter may not be as big and the changes in the morphology of the oral mucosa are minor (Rapidis et al., 1976). In some cases of oral SCC, the subepithelial connective tissue becomes infiltrated heavily with inflammatory cells, and therefore, creates a large circular laminated hyaline structure surrounded by epithelial cells. This is referred to as *epithelial pearl* [Fig. 9].

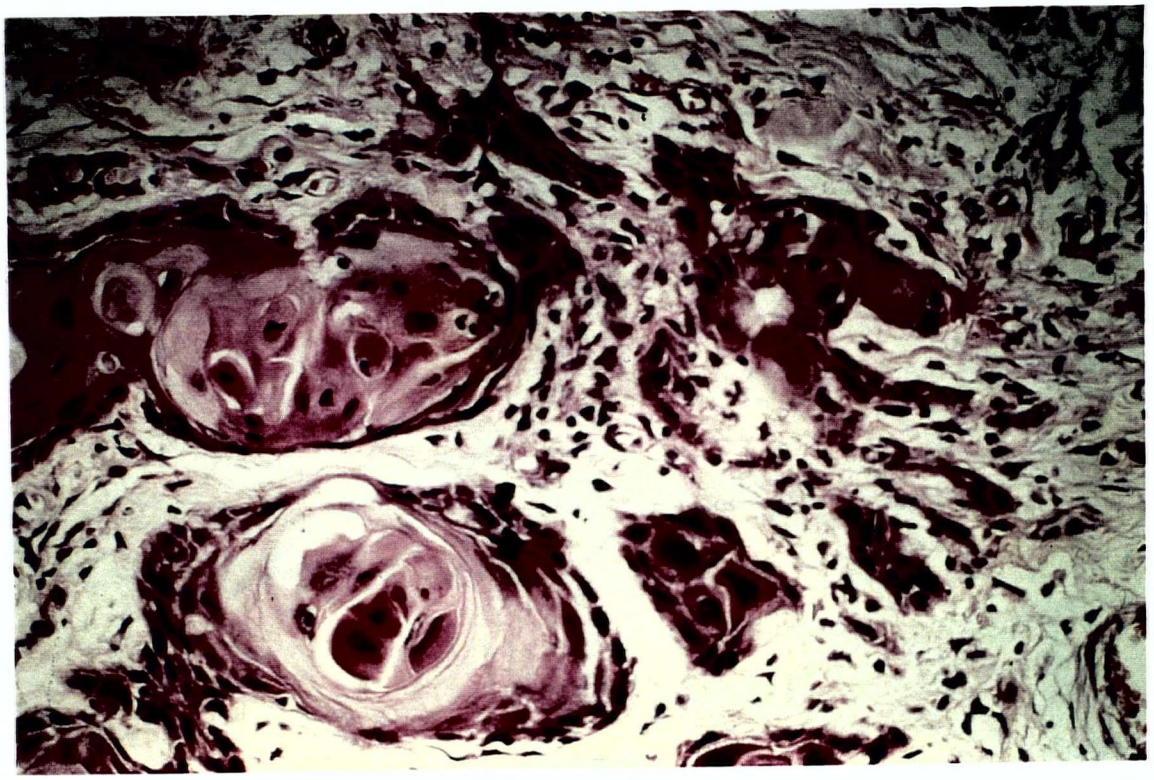


Figure 9. Oral SCC

In less differentiated tumours, cell nests may be absent, although there may be individual cell keratinization and obvious prickle cells. In poorly differentiated tumours, there may be no evidence of keratinization, and neoplastic cells show extreme degrees of hyperchromatism with many mitosis and giant tumour cells. Tumours excite a variable response in the stroma. In many cases, there is florid lymphocyte and plasma cell infiltration. In others, the inflammatory response is minimal, but there is fibroblastic proliferation of the surrounding stroma to the tumour. The epithelial pearls may rupture, resulting in a foreign body-type giant cell reaction to the keratin. The tumour spreads into the underlying muscle, nerves, glandular tissue, and ultimately bone. It invades the lymphatics and spreads by permeation to the regional lymph nodes.

There are several explanations as to how cancer arises. There are two gene classes that play significant roles in cancer development. In their normal state, they are responsible for standard growth sequence of cells. Proto-oncogenes and tumour suppressor genes encourage and inhibit such growth respectively (Weinberg, 1996). When mutated, proto-oncogenes are activated and do not follow their usual signals, thus become carcinogenic. Inactivation of tumor suppressor genes, on the contrary, enhance carcinogenesis. When mutated, these two genes together facilitate the proliferation and migration of cells to the nearby tissues, sometimes forming masses at distant sites in the body. Malignant transformation is characterized by disruption of cytoskeletal organization, changes in adhesion dependent responses, and decrease in cell adhesion (Ben-Ze'ev et al., 1997).

Alteration in the expression of p53 gene has been reported in various types of carcinomas. p53 (wild type) is a tumour-suppressor gene which halts abnormal growth in normal cells. When a mutation of this gene occurs it allows a cancer to grow. It has been shown that p53 protein is overexpressed during the early stages of oral SCC development, but no relation has been demonstrated between this overexpression and clinicopathological factors, such as tumour size, or its histological differentiation stage (Shintani et al., 1995). However, Girod et al., (1995) have shown that the overexpression of this gene is related to degree of differentiation and stage of the

tumour. It is believed that the tumour suppressor gene p53 is mostly detected in very high amounts in the tumour biopsies that are taken from heavy smokers and drinkers (Langdon and Partridge, 1992; Kaur et al., 1994).

In some cases of cancer, the metastasis follows. Generally, most normal cells in the body stay in the tissue to which they belong. Cancer cells, however, evade the controls that keep normal cells in place. These cells, contrary to normal cells, are anchorage independent, thus escape growth control, avoid apoptosis and will spread (Ruoslahti et al., 1996). It has been indicated that extracellular matrix is significantly involved in the invasion and metastasis of oral squamous cell carcinoma since the immunohistochemical examination of a variety of these extracellular matrices [fibronectin (FN), tenascin (TN), laminin (LM)] has shown a close association between them and the invasive oral squamous cell carcinoma (Shinohara et al., 1996). However, the mechanism behind metastasis and invasion is yet to be investigated.

Clinical manifestation

The clinical appearance of small, early oral SCC may vary from a white, thickened, or verrucous lesion to a velvety plaque or a chronic painless ulcer. However, it appears red in colour more often than white, along with rolled margins. More than 90% of oral SCCs are found to be moderately or well differentiated tumours. Metastasis generally occurs in about 80% of the lesions to submandibular or cervical lymph nodes (Murthi et al., 1986).

In western countries, the most common intraoral site for SCC is the lateral border of tongue (Kitamura et al., 1992). In this case, the tumour invades the surrounding tissue and the tongue becomes progressively fixed and immobile so that speech and swallowing becomes difficult, and discomfort and pain become major symptoms. The spread to regional lymph nodes depends on the location of the tumour and may be bilateral. The second most common site in these countries are floor of the mouth, gingiva and buccal mucosa. On the other hand, in South-East Asian countries, there is site tendency for buccal mucosa because of tobacco chewing habits [Fig 10]. This is

followed by lateral border of tongue, floor of the mouth, and gingiva (Sankaranarayanan, 1990).

Carcinoma of the lower lip is the most common site of extra-oral cancer (Cawson et al., 1994). However, lip mucosa is anatomically and biologically different from the rest of the oral mucosa and surrounding skin. It is particularly susceptible to actinic damage, which appears to be the major cause of carcinoma at this site. The majority of SCCs arise on the vermilion border of the lower lip, and it mostly affects the middle-age white males who are very much exposed to sun light or drying winds (Cawson et al., 1994). SCC of the lip can be successfully treated by surgery or radiation therapy (Fongione et al., 1994).

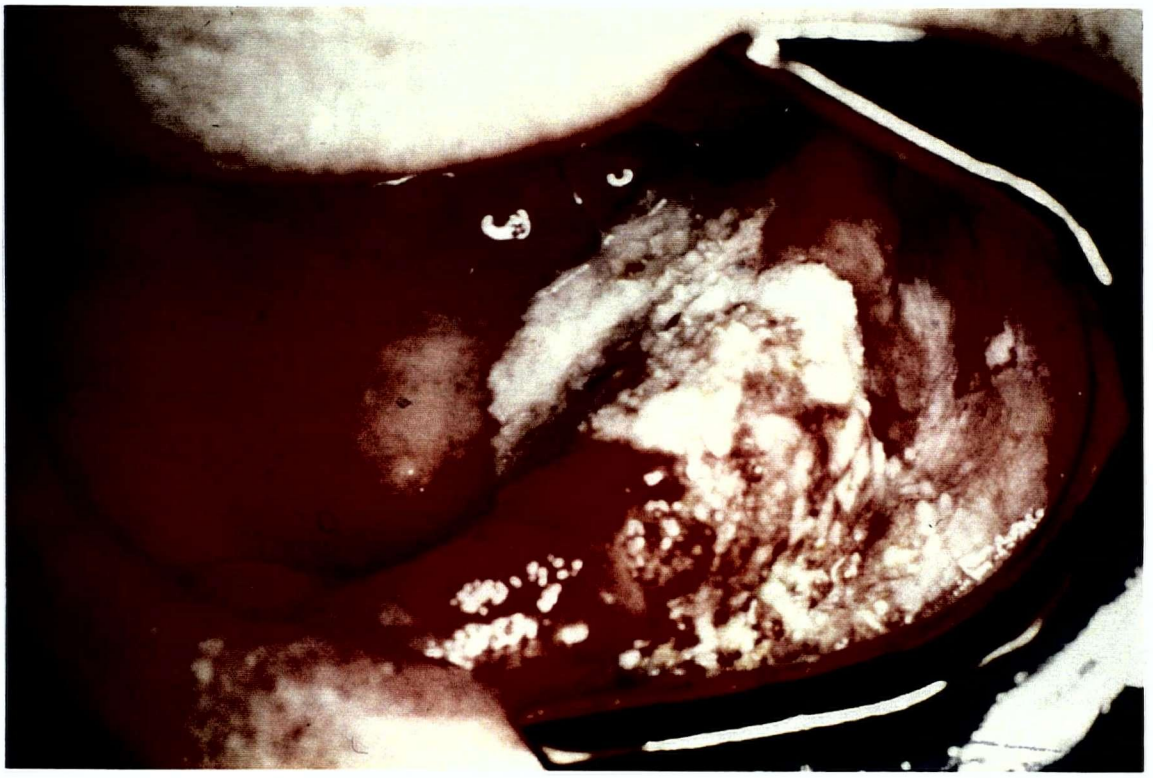


Figure 10. Oral SCC

Integrins

The name integrin was introduced to signify the presumed role of these proteins in integrating the intracellular cytoskeleton with the extracellular matrix. Since the initial identification of the integrin family, rapid progress has been made in characterizing the structure of the family members and in identifying the structural motifs involved in ligand binding.

Integrin structure and function

Integrins are a family of cell surface receptors that mediate cell-cell adhesion and also adhesion to extracellular matrix including epithelial keratinocytes (Giancotti and Mainiero, 1994; Heino, 1993; Hynes, 1992; Watt and Jones, 1993; Larjava et al., 1996). These receptors are heterodimeric complexes of an alpha (α) and beta (ß) subunit. α and ß integrin subunits are produced independently in the endoplasmic reticulum. Each α subunit can form dimers with several ß subunits and vice-versa. At least 20 different α and 8 ß-subunits are currently known. These subunits can variously combine to form more than 22 different cell surface receptors that have distinct ligand binding specificities. They are transmembrane glycoproteins which form a functional receptor mediating cell-cell or cell-matrix adhesion, migration and invasion (Hynes, 1992). Some integrin heterodimers can only recognize one ligand, but most integrins can bind to many different ligands. For instance, fibronectin is the only ligand for α5ß1 (Carter et al., 1990; Adams and Watt, 1990), whereas α3ß1 has three known ligands: collagen, laminin, and fibronectin (Carter et al., 1991; Carter et al., 1990, Staquet et al., 1990). Ligand specificity varies depending on the cell type and functional state, and a single cell can have several integrin receptors for the same ligand. The ligand binding site of integrins is formed by physical association of the NH_2-terminal extracellular sequence of both α and ß subunits. Ligand binding causes receptor clustering that then leads to cytoskeletal organization and intracellular signalling (Miyamoto et al., 1995). The ligand binding affinity of an integrin can be modulated by activation of α and ß chains by different agents, including divalent cations (Gailit and Ruoslahti, 1988).

As it is mentioned before, integrins mainly function in mediating adhesion of cell to extracellular matrix. These surface receptors are also responsible for ligand binding specificities and then each convey distinct signals to the interior of the cells upon binding to their ligands. For instance, ß1 integrin receptor subunits are shown to be concentrated in cell-cell contacts of keratinocytes in vivo and function in the maintenance of cell-cell interactions of human keratinocytes (Larjava et al., 1990).

Integrins have also been shown to allow cells to sense the chemical characteristics of the surrounding matrix and to respond to that (Adams and Watt, 1993). Integrin mediated signalling has been implicated in keratinocytes differentiation (Adams and Watt, 1989), as well as formation of polarized epithelium in some developing organs like kidney (Sorokin et al., 1990). Aside from their biological importance to fundamental cellular processes, the medical importance of the integrins is rapidly being realized as well. Integrins have been found to play a role in platelet aggregation, immune functions, tissue repairs, and tumour invasion. Some diseases are already known to be caused by mutations in integrin genes (Ruoslahti, 1991; Sheppard 1996).

Epithelial integrins and their ligands

Members of integrin family are expressed in virtually every cell of most multicellular organism, however their role in health and disease has remained obscure (Huang et al., 1996). Epithelial cells in adult tissues are generally stationary cells, but these cells nevertheless express several different integrins (Sheppard, 1996), which perform a number of in vivo functions that require unique interactions with the extracellular matrix. Because the local concentration of integrin ligands is altered by injury, inflammation, and remodelling, signals initiated through integrins are likely to play important roles in the responses of epithelial cells to each of these processes (Sheppard, 1996).

Surface epithelia all have the capacity to repair areas of alteration. This repair process involves at least three functional changes in the epithelial cells themselves: cell spreading, migration

and proliferation. Each of these precesses require the participation of integrins. Although specialized epithelia may express unique integrin repertoire, the pattern of integrin expression is remarkably similar in most surface epithelia. The structural and functional definition of epithelial integrins can contribute to the unveiling of the complex mechanisms that regulate adhesion in epithelial cells. A critical property of epithelial cells is their capacity to proliferate and/or migrate short distances as a response to breaks in the continuity of epithelial sheets, or as part of physiological regeneration. To properly perform theses tasks, changes in adhesive properties must be coordinated with respect to external cues and to growth stages (Quaranta, 1990).

Normal skin and mucosal epithelium express multiple integrins. The distribution pattern of several integrins in various tissue types is summarized Table 1.

Table 1. Expression of different integrin subunits in normal and wounded human epithelia

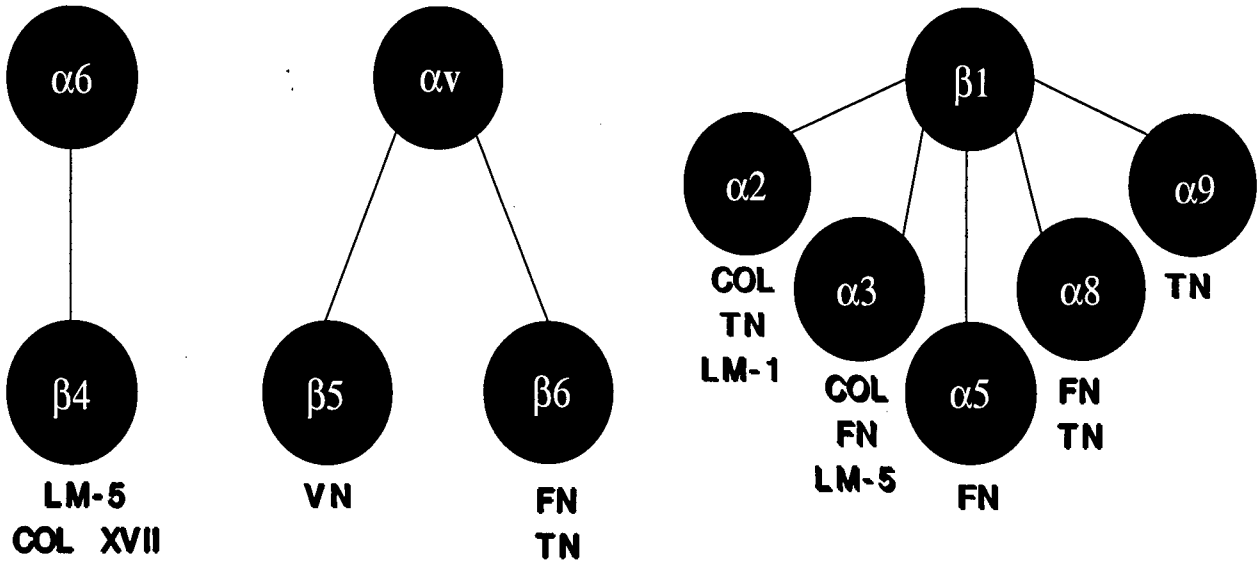
Integrin	Organ Expressing Each Integrin	Reference
ß1	Normal gingival epithelium Normal epidermis Normal cervical epithelium Normal breast epithelium Normal colon mucosa Normal embryonic stem cells	Larjava et al., 1992; 1993 Peltonen et al., 1989; Savoia et al., 1993 Hughes et al., 1994 Koukoulis et al., 1991; Jones et al., 1992; Pignatelli et al., 1992 Koretz et al., 1991; Nigam et al., 1993 Bagutti et al., 1996
ß3	Endothelial cells of normal mammary tissues	Pignatelli et al., 1992
ß4	Normal gingival epithelium Normal epidermis Normal cervical epithelium Normal breast epithelium	Larjava et al., 1992; 1993 Peltonen et al., 1989; Rossen et al., 1994; Savoia et al., 1993 Hughes et al., 1994 Koukoulis et al., 1991; Jones et al., 1992; Pignatelli et al., 1992
ß5	Normal buccal mucosa	Jones et al.,1997
ß6	keratinocytes of human incisional wounds	Larjava et al., '93; Haapasalmi et al., '96
α2	Normal gingival epithelium Normal epidermis Normal cervical epithelium Normal breast epithelium Normal colon mucosa Normal embryonic stem cells	Larjava et al., 1993 Peltonen et al., 1989; Savoia et al., 1993 Hughes et al., 1994 Koukoulis et al., 1991; Jones et al., 1992; Pignatelli et al., 1992 Koretz et al., 1991; Nigam et al., 1993 Bagutti et al., 1996
α3	Normal gingival epithelium Normal epidermis Normal cervical epithelium Normal breast epithelium	Larjava et al., 1993 Peltonen et al., 1989; Savoia et al., 1993 Hughes et al., 1994 Koukoulis et al., 1991; Jones et al., 1992; Pignatelli et al., 1992
α5	Migrating keratinocytes of human incisional wounds	Larjava et al., 1993; Haapasalmi et al., 1996
α6	Normal gingival epithelium Normal epidermis Normal cervical epithelium Normal breast epithelium Normal colon mucosa Normal embryonic stem cells	Hormia et al., 1992; Larjava et al., 1993 Peltonen et al., 1989; Rossen et al., 1994; Savoia et al., 1993 Hughes et al., 1994 Koukoulis et al., 1991; Jones et al., 1992; Pignatelli et al., 1992 Koretz et al., 1991; Nigam et al., 1993 Bagutti et al., 1996
αv	Normal buccal mucosa Endothelial cells of normal mammary tissues Migrating keratinocytes of human incisional wounds	Jones et al., 1997 Pignatelli et al., 1992 Larjava et al., 1993; Haapasalmi et al., 1996

Briefly, ß1, α2, and α6 integrin subunits have been shown to be strongly expressed by the basal layer of normal cervical epithelium (Hughes et al., 1994), and by normal breast epithelium (Koukoulis et al., 1991; Jones et al., 1992; Pignatelli et al., 1992). Their expression have been shown to be homogeneous throughout the normal colon mucosa (Koretz et al., 1991; Nigam et al., 1993), as well as in normal embryonic stem cells (Bagutti et al., 1996). However, the knockout of ß1 in these embryonic cells has been shown to diminish the expression of a few integrins including α2, α6, and ß1 itself which severely impairs the ECM's assembly in these mice (Bagutti et al., 1996). In normal epidermis, both α6 and ß4 subunits are expressed by basal cells and the expression is polarized against the basement membrane (Rossen et al., 1994; Savoia et al., 1993). However, in the case of ß4, the staining was restricted mainly to the basal aspect of these cells, and not at all in the endothelial cells, emphasizing its epithelial specificity. In colonic epithelium, αv, ß3, and ß5 have been reported to be absent (Nigam et al., 1993). αvß3 is however, strongly expressed by the endothelial cells of normal mammary tissues, but the expression appears weak on the breast epithelium (Pignatelli et al., 1992).

There are a few integrins that generally seem to be absent from normal epithelium. For instance, α4, α5, αv and ß3 integrins are often absent from most normal epithelial cells including the myoepithelial cells of normal breast (Koukoulis et al., 1991), normal oral mucosa (Larjava et al., 1993; Kosmehl et al.,1995), and normal renal mucosa (Korhonen et al., 1992). However, α5 and αv were both expressed in migrating keratinocytes of normal human incisional wounds (Larjava et al., 1993; Haapasalmi et al., 1996).

Integrin receptors are always expressed in their heterodimeric forms. Figure 11 has been modified from previous studies (Larjava et al., 1996), to summarize the heterodimers of αv, ß1, and ß4 families along with their corresponding ligands.

FIGURE 11. Expression of various integrin heterodimers and their ligands in human keratinocytes.



Please refer to "ABBREVIATION", Chapter Seven on page 73.

α2ß1, α3ß1, and α6ß4 are receptors for matrix proteins known to be present in epithelial basement membrane (i.e. Col-IV, and LM-5), and are found in the basal layer of human epidermis (Peltonen et al., 1989; Savoia et al., 1993). However, the spatial pattern of constitutive expression of each of these integrins is distinct. The pattern of expression of α6ß4, localization of the basal surface of basal cells, fits best with what one would expect for a protein whose principal function is the maintenance of adhesion to the basement membrane (Stepp et al., 1990). In epidermis and gingiva, α6ß4 has been localized to basal cell layer facing the basement membrane (Kajiji et al., 1990; Hormia et al., 1992; Larjava et al., 1993). α3ß1 integrin binds to laminin-5 (also known as epiligrin, nicein, or kalinin) (Carter et al., 1991; Adams and Watt, 1991), but it has also been reported to bind to fibronectin and collagen (Staquet et al., 1990; Carter et al., 1990). α3ß1 integrin is normally concentrated at the lateral surface, but this receptor may also be expressed at lower levels at the basal surfaces of cells throughout the epithelium. α2ß1 integrin may interact with the basement membrane components. It has been shown to mediate the adhesion of keratinocytes to collagen types I and IV in vitro (Staquet et al., 1990; Carter et al., 1990). This integrin is also involved in adhesion to laminin-1 (Carter et al., 1990; Adams and Watt 1991). Both α2ß1 and α3ß1 integrins can be found in basal epithelial cells of skin and oral epithelial cells and may play a role in cell-cell adhesion (Larjava et al., 1996). In fact, ß1 integrin may be able to mediate the cell-cell adhesion of resting keratinocytes by either binding to unknown ligands or through integrin-integrin interaction (Carter et al., 1990; Larjava et al., 1990).

Normal cells deposit fibronectin, laminin, collagen, and other extracellular matrix components around themselves as a network of insoluble protein (Ruoslahti, 1991). Fibronectin is a component of the extracellular matrix and plasma. It functions in cell adhesion, differentiation and migration. The main fibronectin receptor is α5ß1 which is expressed in many other cell types in vitro such as epidermal keratinocytes (Singer et al., 1988). Basal keratinocytes express other integrins such as α8ß1 and α9ß1 (Yokosaki et al., 1994). α8ß1 integrin is also expressed in smooth muscle cells, lung myofibroblasts and nerve cells, in which it binds vitronectin and

tenascin. α9ß1 integrin is considered to be the main receptor for tenascin, however, it may also be detected in the cells that do not synthesize tenascin such as basal keratinocytes (Yokosaki et al., 1994). αvß6 integrin is another known receptor for fibronectin and is shown to be absent from most normal cell lines and tissues. However, this integrin has been shown to be remarkably induced during a later stage of wound healing (Larjava et al., 1996). Fibronectin can stimulate growth of colon cancer cells cultured within 3-dimensional collagen matrices (Agrez, 1989). Recently, it has been shown that αvß6 integrin, a fibronectin-binding receptor, enhances growth of colon cancer cells in-vitro and in-vivo (Agrez et al., 1994).

The αv integrin can be associated with multiple ß-subunits and can bind to a variety of matrix proteins depending on its associated ß-subunit (Ruoslahti, 1991). αvß6 is exclusively an epithelial integrin and its expression is restricted to only a few locations in healthy adult tissues in humans (Breuss et al., 1993). It has been previously described to be expressed in culture epithelial cells (Breuss et al., 1993), including epidermal keratinocytes (Haapasalmi et al., 1996). Airway epithelial cells express αvß6 integrin. In vitro, this integrin is simply expressed as a result of placing airway epithelial cells in primary cultures. High levels of ß6-mRNA are only found in two very specialized epithelial cell type: a portion of the kidney tubule epithelium, termed macula densa, and the endometrial epithelium of secretory phase uterus (Breuss et al., 1993). Lower ß6-mRNA levels have been detected in epithelium of salivary gland ducts, gall bladder, and epididymis, but not in skin or lung (Breuss et al., 1993).

αv subunit has been shown to be absent from normal keratinocytes of oral mucosa (Haapasalmi et al., 1996; Larjava et al., 1993; Breuss et al., 1993). However, Jones et al. (1997) have recently reported the presence of αv subunit in normal buccal mucosa. They have also localized the αvß5 integrin in these tissues. It was expressed in the tissues of buccal mucosa but the staining pattern was weak compared to αv subunit (Jones et al., 1997).

In vivo, αvß6 integrin is expressed diffusely on all cell types in the airway epithelium of patients with a number of inflammatory lung disease (Breuss et al., 1995). To examine the role

αvß6 integrin plays in vivo more extensively, Huang et al. (1996), have generated mice lacking ß6 integrin expression using homologous recombination in embryonic stem cells. These mice develop and reproduce normally, but develop functionally significant infiltration of their skin and lungs with inflammatory cells. Thus αvß6 may be involved in the modulation of local factors that are accountable for cell activation or proliferation of inflammatory cells such as lymphocytes (Huang et al., 1996).

Fibronectin and Tenascin

Fibronectin is a component of extracellular matrix and is produced by several cell types including epithelial cells, connective tissue cells and blood cells (Uitto, 1991). Fibronectin is coded by a single gene and its primary structure normally contains three types of repeating homologous sequences. Thus this protein exists in at least 20 different isoforms. Fibronectin is the main ligand for α5ß1 integrin which was originally characterized in extracellular matrix contacts of fibroblasts (Singer et al., 1988), however, it is also a receptor for αvß6 integrin. Fibronectin functions in cell adhesion, migration, embryonic differentiation, defense and repair (Hynes 1983; Ruoslahti, 1988). In addition, it may have a role in proliferation and synthetic activity of cells.

In addition to fibronectin, αvß6 integrin binds to tenascin (Prieto et al., 1993; Yokosaki et al., 1996). Tenascin is an extracellular matrix protein which is expressed during development and wound healing (Erickson and Bourdon, 1989). It has been independently found in many different types of tissues such as dense connective tissues, embryonic brain tissues, and mammary carcinomas. Tenascin exists in different isoforms and its multi-domain structure suggests possible multiple but independent functions of this matrix protein (Spring et al., 1989; Saga et al., 1992). However, knock-out of the tenascin gene results in a developmentally normal mouse (Saga et al., 1992).

It has been reported that in highly invasive primary tumours, the expression of fibronectin and tenascin in the tumour stroma at the same site was markedly increased when compared with

normal tissues, whereas the expression of laminin and collagen-IV in the basement membrane along the tumour-stroma borderline were significantly decreased (Harada et al., 1994). In peritumour stroma in metastatic lymph nodes, the expression pattern of laminin, collagen-IV, fibronectin and tenascin was similar to that in highly invasive primary tumours (Harada et al., 1994).

Integrins and malignancy

For years, it has been thought that the process of invasion and metastasis, which in most cases are the characteristics of malignancy, are associated with the cell's altered adhesive properties (Yamada, 1992). The fact that integrins play an important part in these alterations is also recognized (Ruoslahti, 1991). Therefore, the cellular functions of these surface receptors have been extensively studied. Tumour cell metastasis normally interferes with the adhesion process to the neighboring cells at the primary tumour sites. It involves invasion into blood vessels and extravasation to distant sites. This process involves making and breaking contacts with extracellular matrix at these sites, and therefore may cause some changes in the expression of integrins in the tumour cells (Ben-Ze'ev et al., 1997). Table 2 briefly summarizes some of these changes.

Most integrins of the oral mucosa have been reported to be expressed at the basal and the suprabasal layer of the epithelium. Epithelial cell's interactions with basement membrane are important in maintaining tissue architecture and function. The anatomical and functional relationships between epithelial cells and their basement membrane are clearly altered in malignancy (Hughes et al., 1994). Most integrins seem to be downregulated in malignant tissues, but there are a few that are up-regulated. α2 and α3 integrins are shown to be highly expressed by all cells in invasive squamous cell carcinoma of cervix (Hughes et al., 1994). In lung carcinoma, α1 integrin expression is reduced (Suzuki et al., 1993), whereas αv and ß6 integrins are upregulated (Breuss et al., 1995). αvß6 integrin has been detected in oral squamous cell carcinoma as well and is

believed to be associated with epithelial tumour migration (Breuss et al., 1995; Jones et al., 1997). Furthermore, in renal cell carcinomas, the emergence of the αv subunit, and disturbance of α6ß1 integrin-mediated cell-basement membrane interaction, were reported to correlate with increasing histological grades in these cells (Korhonen et al., 1992).

Table 2. Expression of different integrins in malignant human epithelia

Integrin	Expression of Integrins	References
ß1	Down-regulation in skin SCC	Peltonen et al., 1989
	Down-regulation in colon carcinoma	Koretz et al., 1991; Pignatelli et al., 1990; Nigam et al., 1993
	Down-regulation in breast carcinoma	Koukoulis et al., 1991; Pignatelli et al., 1991
ß4	Loss of polarity in skin SCC	Rossen et al., 1994
	Loss of polarity in oral SCC	Downer et al., 1993
	Down-regulation in breast carcinoma	Koukoulis et al., 1991; Pignatelli et al., 1992; Jones et al., 1992; Natali et al., 1992
ß6	Up-regulation in lung carcinoma	Breuss et al., 1995
	Up-regulation in oral SCC	Breuss et al., 1995; Jones et al., 1997
α1	Down-regulation in breast carcinoma	Koukoulis et al., 1991; Pignatelli et al., 1991
	Down-regulation in lung carcinoma	Suzuki et al., 1993
α2	Down-regulation in colon carcinoma	Koretz et al., 1991; Pignatelli et al., 1990; Nigam et al., 1993
	Down-regulation in breast carcinoma	Koukoulis et al., 1991; Pignatelli et al., 1991
	Up-regulation in cervix SCC	Hughes et al., 1994
α3	Focal loss in colon and skin carcinoma	Jones et al., 1992; Peltonen et al., 1989
	Down-regulation in breast carcinoma	Koukoulis et al., 1991; Pignatelli et al., 1991
	Up-regulation in cervix SCC	Hughes et al., 1994
α6	Loss of polarity in skin SCC	Rossen et al., 1994
	Loss of polarity in oral SCC	Downer et al., 1993
	Down-regulation in breast carcinoma	Koukoulis et al., 1991; Pignatelli et al., 1992; Jones et al., 1992; Natali et al., 1992
	Down-regulation in bladder carcinoma	Liebert et al., 1994
	Down-regulation in prostate carcinoma	Knox et al., 1994
	Up-regulation in pancreatic carcinoma	Weinel et al., 1992
	Up-regulation in hepatocellular carcinoma	Volpes et al., 1993
	Up-regulation in cervical carcinoma	Hughes et al., 1994
αv	Up-regulation in lung carcinoma	Breuss et al., 1995
	Up-regulation in oral SCC	Breuss et al., 1995; Jones et al., 1997

Downregulation of ß1 in squamous cell carcinoma of the skin (Peltonen et al., 1989), in colon carcinoma (Koretz et al., 1991; Pignatelli et al., 1990; Nigam et al., 1993), and in breast carcinoma (Koukoulis et al., 1991; Pignatelli et al., 1991) has been reported. The expression of other epithelial integrins may also change due to alteration in physiological or morphological state of the tissue. For instance, in squamous cell carcinoma of the skin, loss of polarity of α6 and ß4 integrins has been reported (Rossen et al., 1994); however, the intensity of the latter was increased. Reduced expression of α6 integrin has also been shown in bladder carcinoma (Liebert et al., 1994), and prostate carcinoma (Knox et al., 1994). However, the expression of this integrin seems to increase in pancreatic carcinoma (Weinel et al., 1992), hepatocellular carcinoma (Volpes et al., 1993), and in cervical carcinoma (Hughes et al., 1994). Downer et al., (1993) and Sugiyama et al., (1993) have reported the loss of polarization of α6ß4 integrin in oral squamous cell carcinoma, which coincides with the loss of basement membrane components. α6 integrin expression has been shown to be increased in well-differentiated squamous cell carcinoma of the oral cavity, but decreased in the invasive areas of poorly differentiated oral squamous cell carcinoma (Kosmehl et al., 1995). Moreover, Korhonen et al., (1992) have studied the integrin expression in renal tumours of various grades and concluded that α6ß1 integrin is highly expressed in tumours of grade I, but, downregulated in the grade II and III tumours. Other studies have reported the reduced expression of α6 and ß4 integrins in breast carcinoma (Koukoulis et al., 1991; Pignatelli et al., 1992; Jones et al., 1992; Natali et al., 1993). The latter finding was contradictory with the earlier results of Falcioni et al. (1986), which showed that breast tumour progression is associated with upregulation of α6ß4 integrin. Similarly, Koukoulis et al., (1993), claim that α6 integrin expression is downregulated in colon carcinoma, which is inconsistent with the findings of Koretz et al., (1991) who have reported no difference in expression of this integrin between normal and carcinogenic colon tissues.

In general, there are quantitative changes of integrin expression in oral squamous cell carcinomas and other types of cancers which usually correlate with carcinoma differentiation and

proliferative activity. However, there is no single change in integrin expression or function that is characteristic of all or most malignant tissues. Rather, different normal tissues have their characteristic expression repertoires, and the integrin profile of their malignant counterparts usually resemble those of the original tissues, but have been partially altered upon transformation (Miettinen et al., 1993).

Chapter Two

AIM OF THE STUDY

The specific aims of this study were:

1. To examine the expression of αvß6 integrin subunit in normal, leukoplakic, lichen planus and malignant oral mucosal epithelium.
2. To compare αvß6 expression to that of other epithelial integrins, (ß1, ß3, ß4, ß5), in the same tissue specimens.
3. To investigate the relation between integrin expression pattern and the malignant transformation of leukoplakic and lichen planus lesions.

The hypothesis is that the expression of αvß6 integrin in leukoplakia lesions could play a role in their malignant transformation and particularly in tumour cell migration and invasion in fibronectin-rich matrix. Furthermore, the presence of this integrin in premalignant lesions may be used as a marker in cancer diagnosis.

Chapter Three

MATERIALS AND METHODS

Tissues

Biopsies were taken from the area of oral cancer and oral leukoplakia, from 40 patients of BC Cancer Agency, Canada. Also, 6 normal specimens, 11 sections of oral cancer (5 frozen and 6 paraffin), and 8 oral lichen planus specimens were received in collaboration with the University of Oulu, Oulu, Finland. Normal oral tissues (5), periodontal pocket samples (3) and hyperplastic oral mucosa biopsies (5) were obtained during intraoral surgical procedures necessary for treatment at the University of British Columbia, Canada. The non-leukoplakic hyperplastic lesions were either idiopathic (1), drug-induced, e.g. amlodipine bensylate (1), or caused by irritation by dentures (3). 3-day and 7-day-old human mucosal wound specimens were obtained from a collection stored in the laboratory (Larjava et al., 1993).

Patients Charts

The follow-up data of all the leukoplakia patients were collected from Cancer Agency, where the original tissue specimens were gathered from. Patients were followed regularly (at least once a year) by their dentists after the original biopsy. The most recent follow-up information for each patient (1 to 4 years after the diagnosis) was collected from 30 of the 40 patients. The results obtained from 10 patients had to be eliminated from the study due to either poor condition of the tissue samples or unavailability of the follow-up charts.

Antibodies

Monoclonal antibody to the ß1 integrin (Mab 13; 1.1:300) subunit was a generous gift of Kenneth Yamada, NIDR/NIH, and that to the αvß6 integrin complex (E7P6; 1:10) was a kind gift of Dr. Dean Sheppard of Lung Biology Center, University of San Francisco. {The words αvß6 and ß6 have been alternately used throughout the whole experiment and they both refer to αvß6

integrin}. Monoclonal antibody to αv integrin (L230; 1:10) (Houghton et al., 1982) was purified from cell culture supernatant of hybridoma cells grown in our laboratory, and the antibodies against ß4 integrin (AA3; monoclonal, 1:300), fibronectin (polyclonal, 1:500), and tenascin (polyclonal, 1:500) were purchased from Gibco BRL, Gaithersburg, MD, U.S.A. The monoclonal antibodies against αvß3 integrin complex (Mab 1976; 1:500), and αvß5 integrin complex (Mab 1961; 1:100) were purchased from Chemicon, Temecula, CA, U.S.A. The affinity-purified rhodamine-conjugated secondary antibodies, both monoclonal and polyclonal (1:50); were purchased from Boehringer-Mannheim Biochemicals, Indianapolis, IN. Vectastain ABC kit (mouse IgG and rabbit IgG) and DAB enhancing solution were all obtained from Vector Laboratories Inc., Burlingame, CA.

Grading and Diagnostic Methods

The intensity of the stainings was graded visually using a +/- scale. Specimens were classified as follows: (-) no staining was seen; (-/+) some sections were positive, and some negative, [see table 3]; (+) suggests there are some positive cells; (++) means intense staining is observed in the basal cell layer; and (+++) determines strong antibody staining is detected in basal cell layer, sometimes suprabasal cells and/or connective tissue [FN, TN]. **(ND)** indicates that the tissue specimens are not stained with this particular antibody, therefore the result is *not determined.*

The progression/improvement of the disease in the long term follow-up part of the study was done by a dentist who was blind to all the staining results. He examined all the clinical and pathological data of each patient and compared the condition of the affected areas from the time of the biopsy to the last two recent visits of the patients. Patients were followed at least once a year. However, since the first set of biopsies were taken in 1993, the follow-ups (the last two visits) ranged from 8 months to 4 years among all the patients. If the disease had worsen (either bigger in size, invasion, metastasis, or transformation to SCC), it was recorded as a disease progression. If none of the above conditions was applicable, then the disease was recorded as either no change,

improved, or resolved accordingly. These results were then used to calculate the sensitivity and specificity of αvß6 integrin staining as a possible prognostic test.

Immunohistochemistry

Immunofluorescence

Fresh tissue blocks were taken from oral mucosa and mounted in OCT compound, snap frozen in liquid nitrogen and cut in a cryostat. Frozen sections 5 µm thick were placed on glass slides (2-3 on each slide) which were treated with acetone containing 3-aminopropyl-triethoxy-silane (Tespa; Sigma Chemical Co., St. Louis, MO, U.S.A.), and fixed briefly in chilled acetone (-20°C). Immunolocalization of integrins was performed as described previously (Larjava et al., 1993). Briefly, sections were washed with phosphate buffer saline (PBS/BSA, 1mg/ml) and incubated with optimally diluted primary antibodies in 0.1% PBS/BSA in a humid chamber over night. After washing with PBS/BSA 4 times for 10 minutes each time, sections were further incubated for 60 minutes in affinity-purified rhodamine-conjugated secondary antibodies diluted in 0.1% PBS/BSA. Sections were then washed with PBS/BSA once and mounted using Krazy Glue (Borden Company LTD.). All tissue specimens were stained with most antibodies (Table 3). Negative controls using non-immune antibody or secondary antibody alone were run with each batch of slides. Samples were examined using a Zeiss Axioskop 20 fluorescence microscope, and photographed using an MC 80 Zeiss microscope camera.

Immunoperoxidase

This part of the experiment was performed on all the αvß6 positive specimens to confirm the specificity of the immunofluorescence technique. A few paraffin sections were also stained using this method, however, due to limited number of antibodies effective on paraffin sections, only αv and ß1 antibodies were used to stain these specimens (10). Sections were cut and fixed

for fluorescence staining. Before staining, sections were treated with 0.3% hydrogen peroxide to quench endogenous peroxidase activity, washed in PBS/BSA and stained using ABC kit. All incubations were carried out in a humid chamber at room temperature. After a brief washing in PBS/BSA, the presence of antigen was visualized by staining with DAB (diaminobenzidine) under a light microscope for one minute. The purpose of this step was to avoid non-specific staining of the tissue sections with DAB. Therefore, as soon as the staining of the antigen layer appeared under the microscope, the reaction was stopped in tap water. The stained sections were then incubated with sodium bicarbonate (0.05M, PH=9.6) for 10 minutes immediately followed by incubation of DAB enhancer for 10 seconds. The DAB enhancer is performed to intensify the expression of antigen stainings. Sections were then incubated and counterstained with hematoxylin for one minute. They were then washed in tap water for a few minutes and then mounted using Entellan super glue. Appropriate positive and negative controls were run in parallel with each batch of slides. The positive controls were 7-day-old wounds and the negative ones were incubations of the same sections with the secondary antibody alone.

Antigen retrieval methods were performed using several techniques (Zolotukhim et al., 1996). However, the staining results did not seem to be vary from the ones that were done without this technique.

Chapter Four

RESULTS

Immunohistochemical staining of normal tissues

The integrins of ß1 and ß4 families were present in all normal tissues. ß1 integrins were localized at the periphery of the basal cells and in the connective tissue and endothelial cells [Fig. 12A]. ß4 integrin was localized at the basal surface of the basal keratinocytes [Fig. 12B]. All the normal specimens that were stained with antibodies against either αvß6 or αv integrin were negative [Fig. 13A and B]. Fibronectin and tenascin were both present in the connective tissue, especially at the area close to the basement membrane zone (not shown). None of the normal section were stained with ß3 or ß5 integrins which confirms our earlier findings (not shown).

Immunohistochemical staining of leukoplakia tissues

Our collection of leukoplakia, lichen planus, SCC and control specimens is presented in the tables 3 and 4. The leukoplakia tissue specimens were histologically graded (dysplasia, hyperplasia, hyperkeratosis, inflammation, etc.) by two different pathologists independently. Not all the frozen tissues were in good conditions. Some of the tissue sections (10) were not representative of the original H&E biopsies, therefore were eliminated by the pathologists. Expression of ß1 and ß4 integrins resembled that of normal tissues in most specimens [Fig. 14 A and B]. In some specimens, the expression of ß1 was found in several cell layers but often appeared somewhat reduced in the intensity [Fig. 15]. 25% of all the leukoplakia specimens expressed αvß6 integrin. The majority of the αvß6 positive leukoplakia samples were associated with hyperplasia, or dysplasia (Table 4). The expression was in most cases confined to the basal keratinocytes at the tip of the rete ridges. No or very little suprabasal expression was observed. Localization using antibodies to αv or αvß6 complex resulted in similar distribution pattern [Fig. 16 A and B]. Epithelial cells of inflammatory, drug-induced or idiopathic hyperplasia or chronic

inflammatory lesions (periodontitis) did not express αvß6 integrin [Fig. 17]. None of the leukoplakia sections expressed ß5 or ß3 integrin [Fig. 18 A and B]. Both fibronectin and tenascin were expressed underneath the oral epithelium of the leukoplakic tissues, at the area near the basement membrane zone [Fig. 19 A and B].

Immunohistochemical staining of lichen planus tissues

The staining pattern of lichen specimens with antibodies against ß1 and ß4 integrin was similar to that of the normal tissues [Fig. 20 A and B]. However, in some of tissue specimens, the staining was not continuous throughout the whole basal layer, and patchy losses could be observed. The area at the basal layer or the basement membrane zone which did not express ß1 and ß4 integrins usually coincide with the positively stained areas for αvß6 integrin. The staining pattern of ß6 paralleled that of αv, however, the staining for αv integrin appeared to be relatively stronger [Fig 21 A and B]. ß3 and ß5 integrins were both absent from all the lichen planus specimens [Fig. 21 C and D]. αvß6 integrin was very strongly present at area of rete ridges of 85% of all the lichen sections [Fig. 20 C]. The staining with αvß6 integrin was consistent throughout the whole length of the rete ridges [Fig. 20 C].

Immunohistochemical staining of cancer tissues

Several cell layers of SCC lesions expressed ß1 and ß4 integrins [Fig. 22A and B]. The staining pattern of these two integrins in the malignant tissues was more predominant and observed in more cell layers than that in the normal tissues. Antibodies to αv and αvß6 integrins were also strongly present when localized in the specimens with SCC [Fig. 23A and B]. Tumour cells in several cell layers appeared to express these integrins. 80% of the sections were positive for the expression of αvß6 integrin, and 90% were positive when stained with αv integrin antibody only. This data includes the SCC paraffin sections as well (6).

Immunohistochemical staining of epithelial wound

Three and seven day old wounds were stained with antibodies to αv and αvß6 integrins as positive controls. None of the 3-day-old wound specimens expressed either one of the integrins, whereas, in 7-day-old wounds, both αv and αvß6 reacted with the areas of the wound where it was healing [Fig. 24 A]. The control staining was performed on the same tissues using the secondary antibodies alone [Fig 24 B]. All these control specimens were negative.

Table 3. Integrin expression in pre-malignant and malignant oral mucosa.

	Integrin Staining Intensity								
	n	ß1	ß3	ß4	ß5	ß6	αv	FN	TN
Leukoplakia	30								
• Dysplasia	22								
-Mild	15	++	-	++	-	-/+	-/+	+++	+++
-Moderate	6	++	-	++	-	-/+	-/+	+++	+++
-Severe	1	++	-	++	-	-/+	-/+	+++	+++
• Hyperplasia (with/without Dp)	11	++	-	++	-	-/+	-/+	+++	+++
• Hyperkeratosis (with/without Dp)	6	++	-	++	-	-/+	-/+	+++	+++
Lichen Planus	8								
• All types	8	++	-	++	-	+	+	ND	ND
SCC	11								
• Grade I	6	++	ND	++	ND	++	++	ND	ND
• Grade II	3	++	ND	++	ND	++	++	ND	ND
• Grade III	2	++	ND	++	ND	++	++	ND	ND
Control	22								
• Normal	11	+++	-	+++	-	-	-	+++	+++
• 7-Day-old Wound	3	++	ND	++	ND	++	++	+++	+++
• Non-Leukoplakic hyperplasia	5	++	-	++	-	-	-	ND	ND
• Non-Leukoplakic chronic inflammation	3	++	-	++	-	-	-	ND	ND

(-) No staining; (-+) Some specimens positive, some negative, [see table IV]; (+) Positive cells; (++) Intense staining in basal cell layer; (+++) Strong staining in basal cell layer, sometimes suprabasal cells and/or connective tissue [FN, TN]; (**ND**) Not determined.

Table 4. Immunolocalization of normal oral mucosa, leukoplakia, hyperplasia and SCC using αv and αvß6 integrin.

#	DISEASE	αv	β6
1.	At	**1/1**	0/1
2.	Cinf	0/1	0/1
3.	Dp	**2/7**	**2/7**
4.	Dp, Cinf	0/3	0/3
5.	Dp, Hk	0/4	0/4
6.	Dp. Hp	**2/5**	**2/5**
7.	Dp, Hp, Hk, VC	**1/1**	**1/1**
8.	Dp, Pk	0/1	0/1
9.	Hk	**1/1**	**1/1**
10.	Hp	**3/4**	**3/4**
11.	Pk	**1/1**	**1/1**
I	Normal	0/11	0/11
II	Hp	0/5	0/5
III	7-day-old Wound	**3/3**	**3/3**
IV	Lichen planus	**7/8**	**7/8**
V	SCC	**10/11**	**4/5**

Please refer to "ABBREVIATION", Chapter Seven on page 73.

Leukoplakia specimens (1-15) were graded based on histological criteria. Numbers on the last two columns indicate the number of positive/total specimens examined in each case.

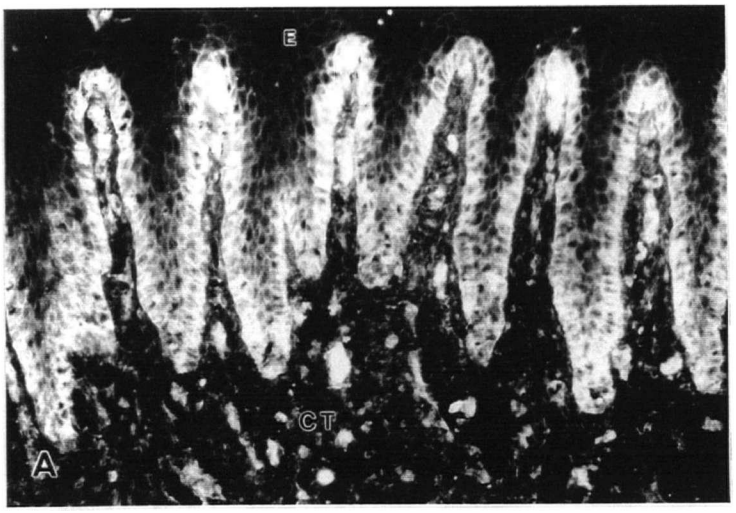


Figure 12 A. Localization of ß1 integrin in normal tissues

E=Epithelium, CT=Connective tissue

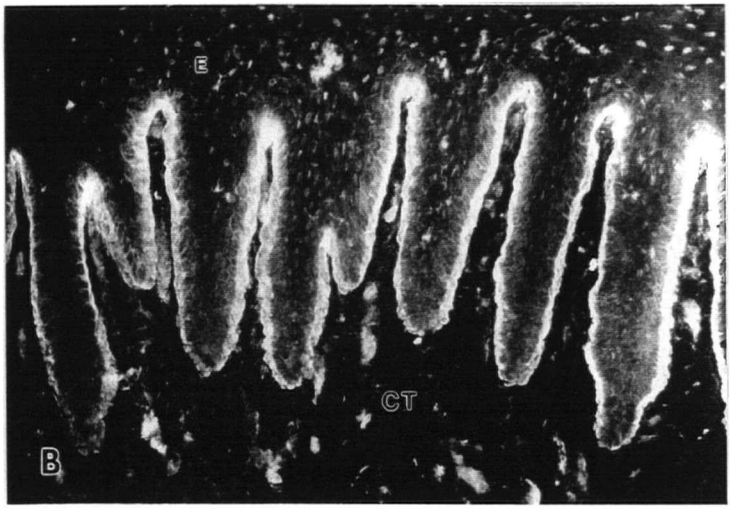


Figure 12 B. Localization of ß4 integrin in normal tissues

E=Epithelium, CT=Connective tissue

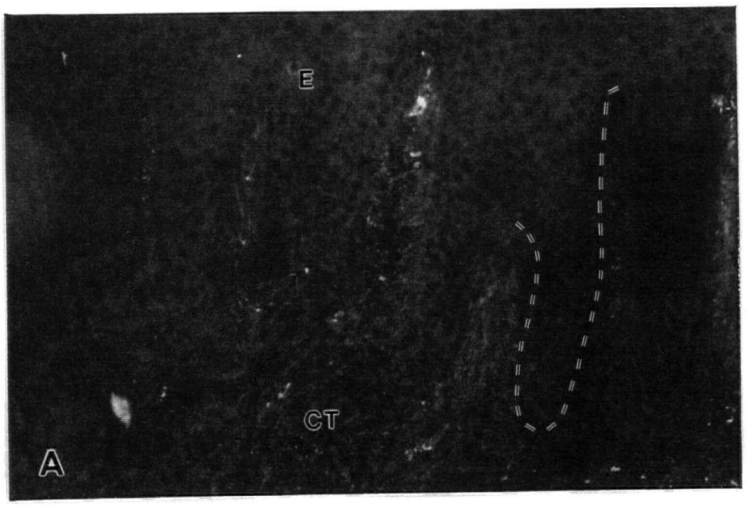


Figure 13 A. Localization of ß6 integrin in normal tissues

E=Epithelium, CT=Connective tissue

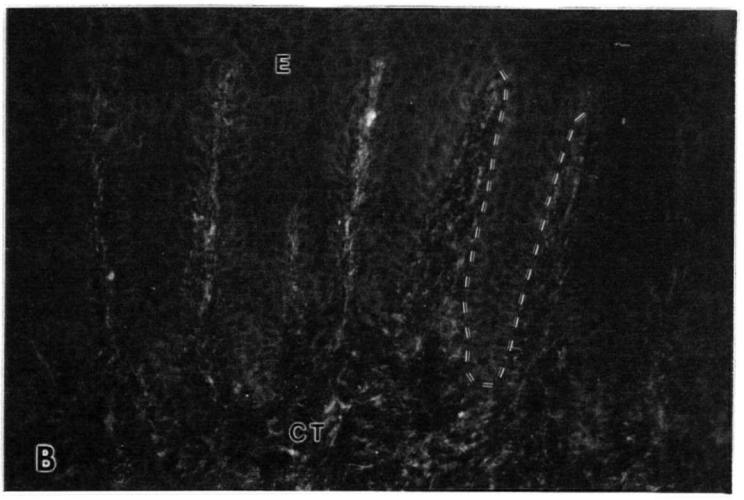


Figure 13 B. Localization of αv integrin in normal tissues

E=Epithelium, CT=Connective tissue

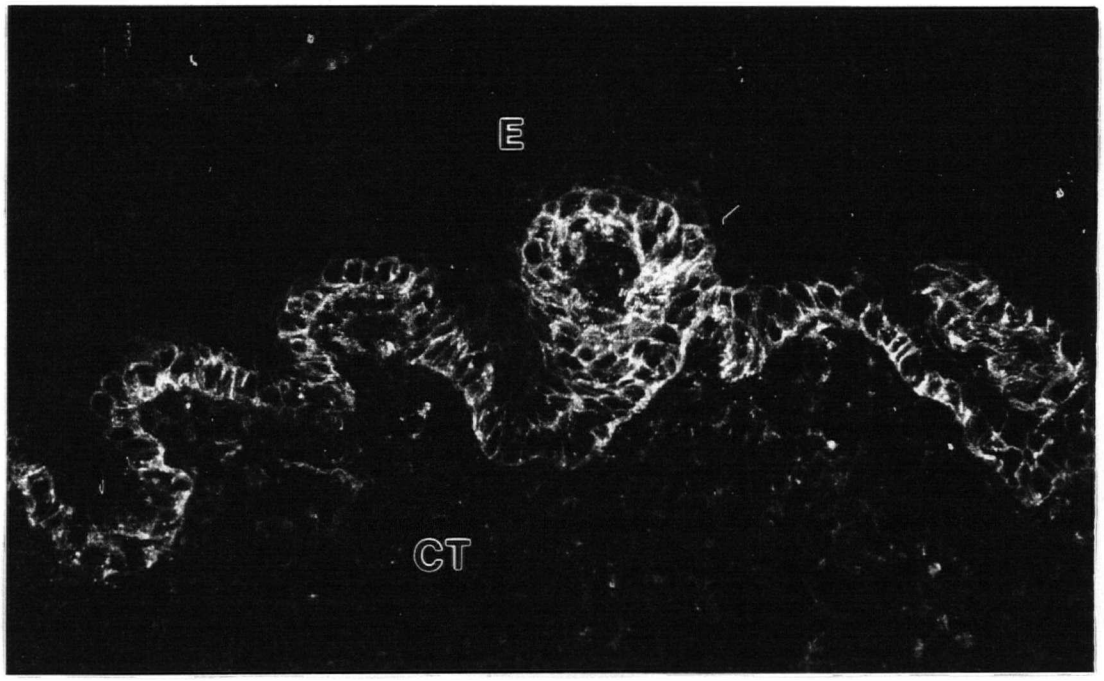


Figure 14 A. Localization of ß1 integrin in leukoplakic tissues E=Epithelium, CT=Connective tissue

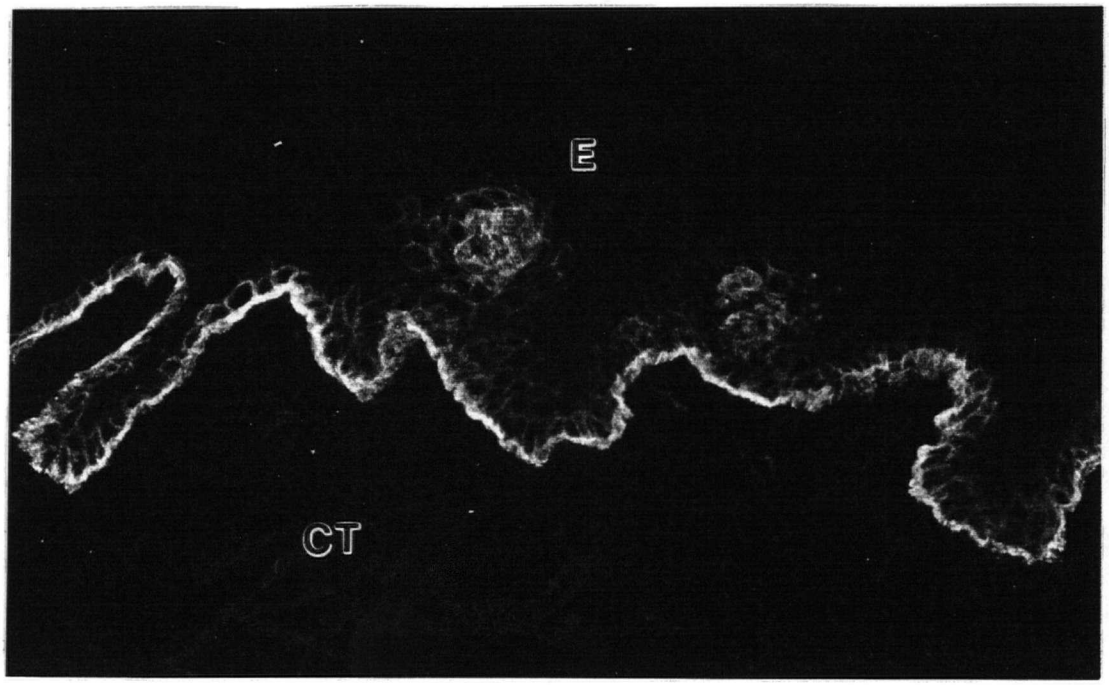


Figure 14 B. Localization of ß4 integrin in leukoplakic tissues E=Epithelium, CT=Connective tissue

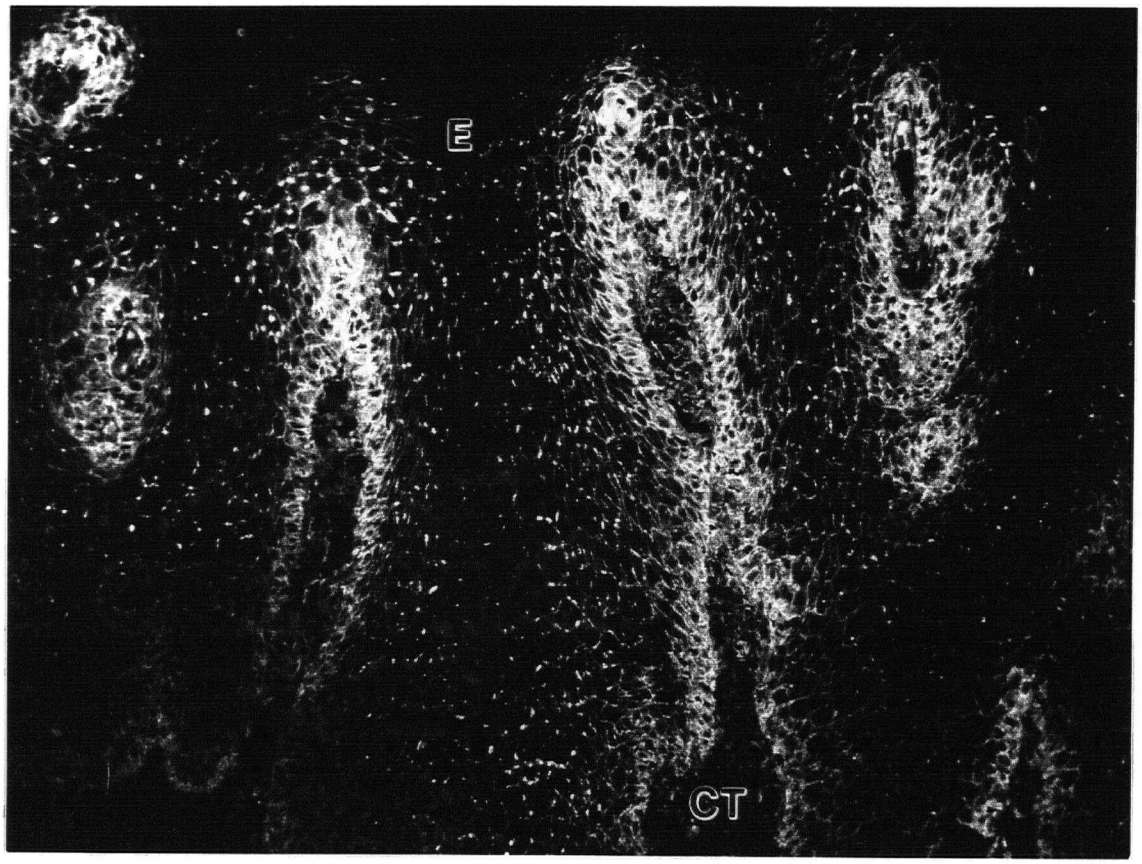


Figure 15. Localization of ß1 integrin in leukoplakic tissues

E=Epithelium, CT=Connective tissue

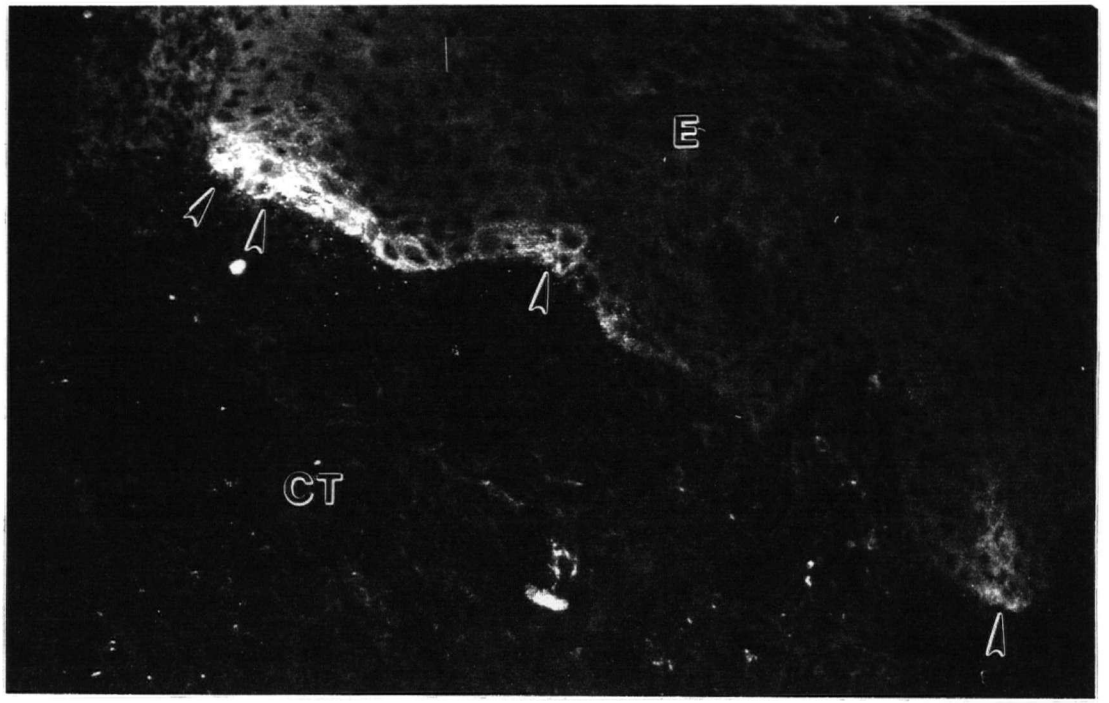


Figure 16 A. Localization of ß6 integrin in leukoplakic tissues E=Epithelium, CT=Connective tissue

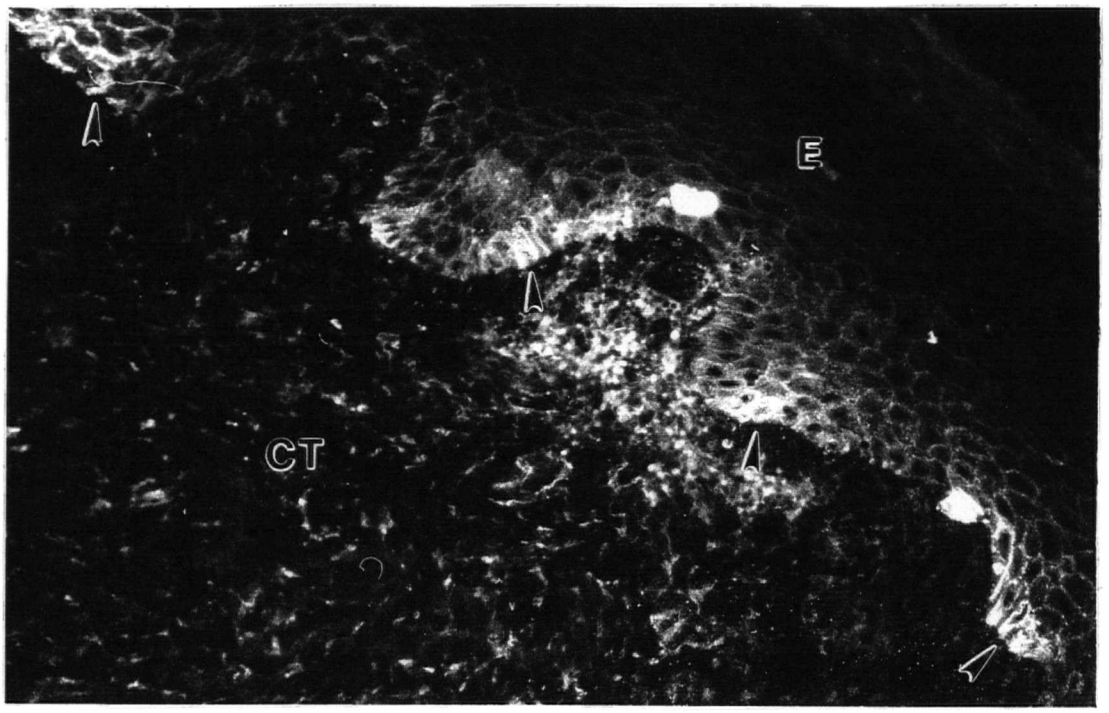


Figure 16 B. Localization of αv integrin in leukoplakic tissues E=Epithelium, CT=Connective tissue

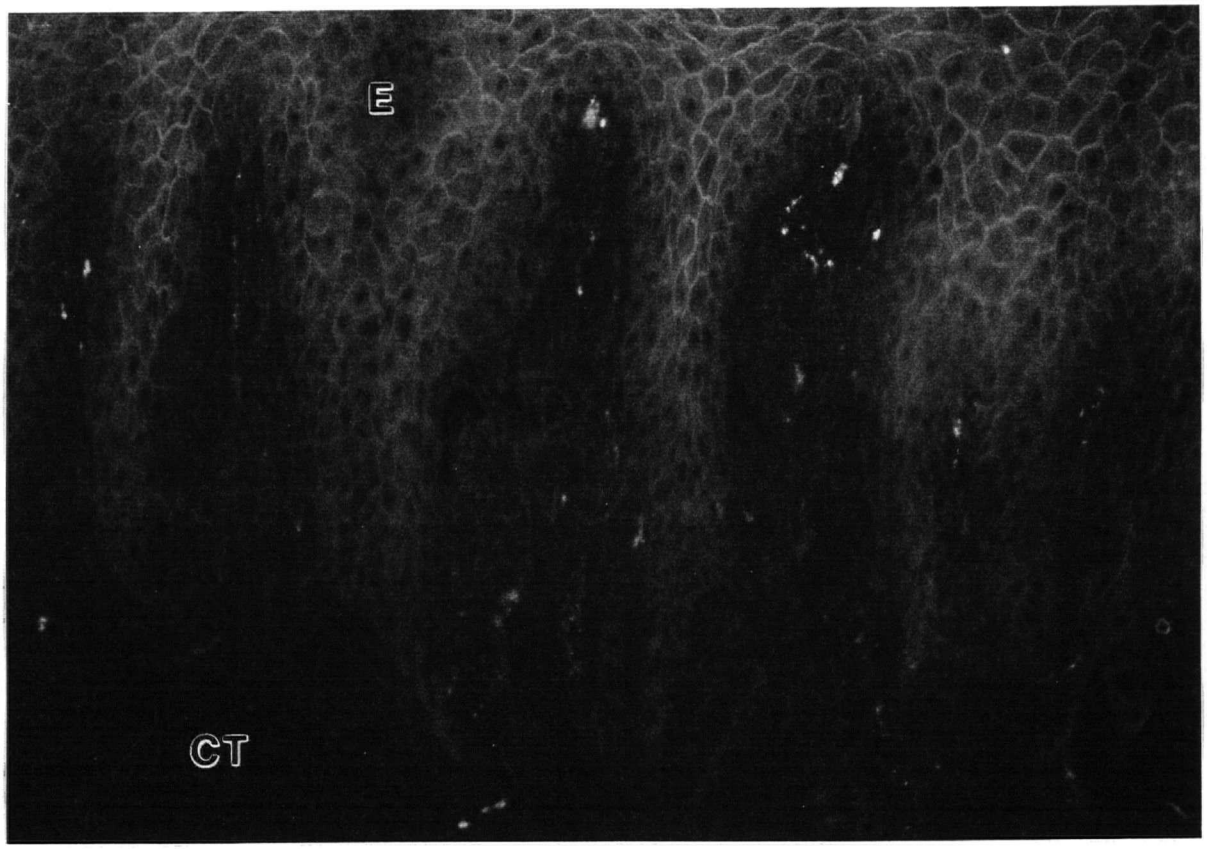


Figure 17. Localization of αvß6 integrin in non-leukoplakic hyperplasia E=Epithelium, CT=Connective tissue

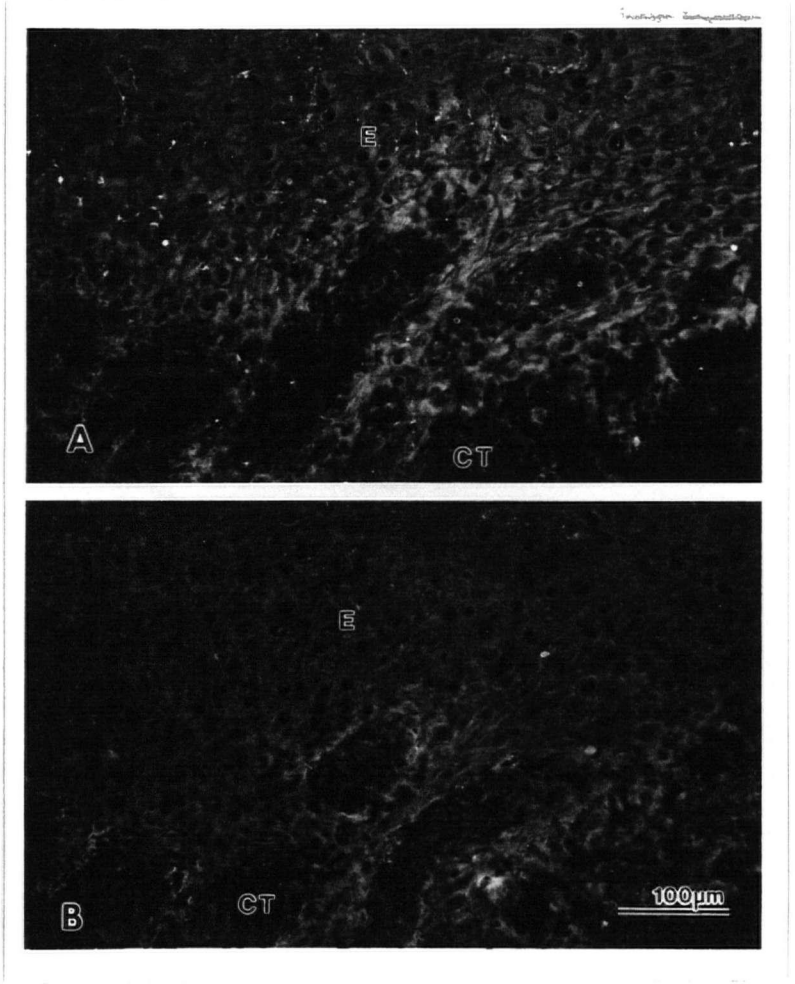


Figure 18.

A. Localization of ß3 integrin in leukoplakic tissues

B. Localization of ß5 integrin in leukoplakic tissues

E=Epithelium, CT=Connective tissue

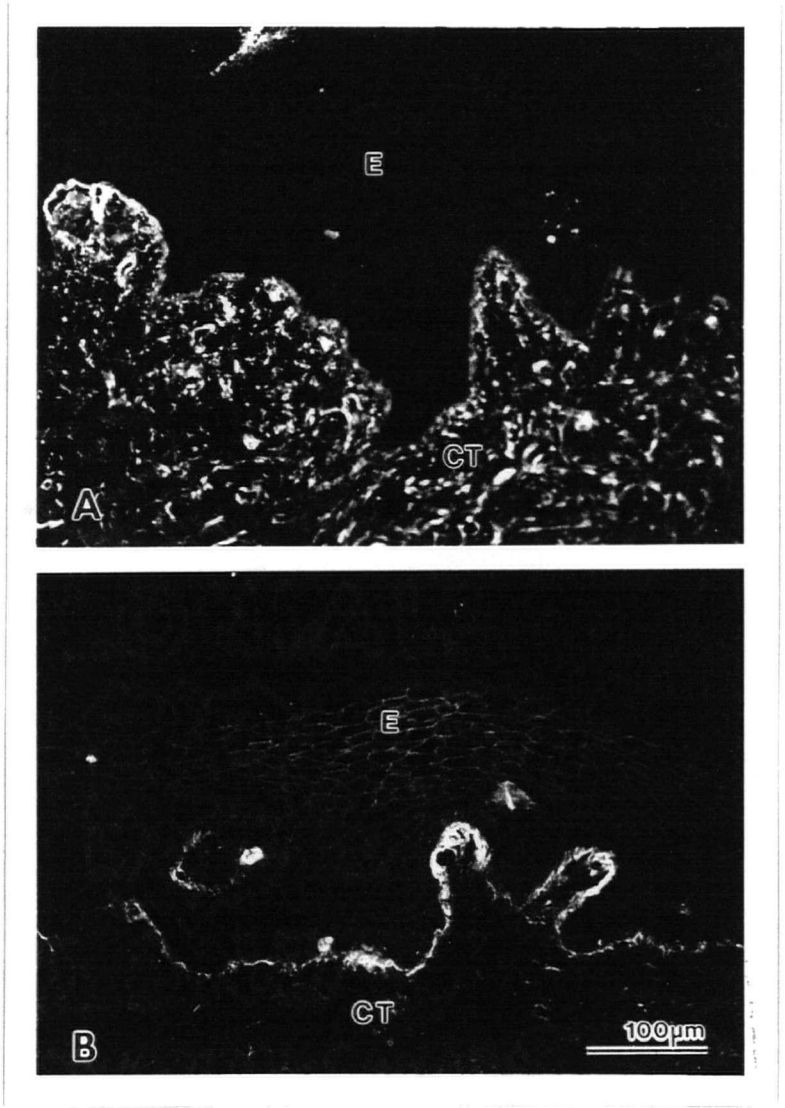


Figure 19.

A. Localization of FN in leukoplakic tissues

B. Localization of TN in leukoplakic tissues

E=Epithelium, CT=Connective tissue

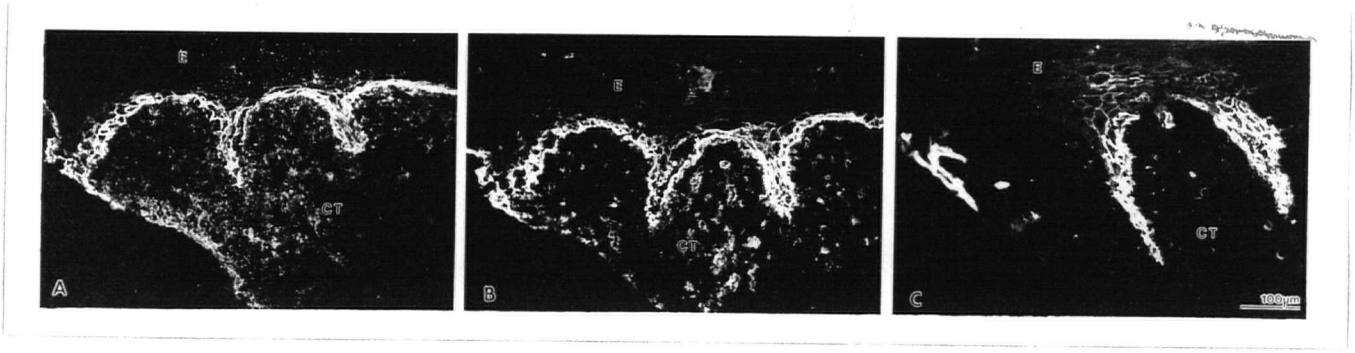


Figure 20. Localization of (A) ß1, (B) ß4, and (C) ß6 in lichen planus tissue E=Epithelium, CT=Connective tissue

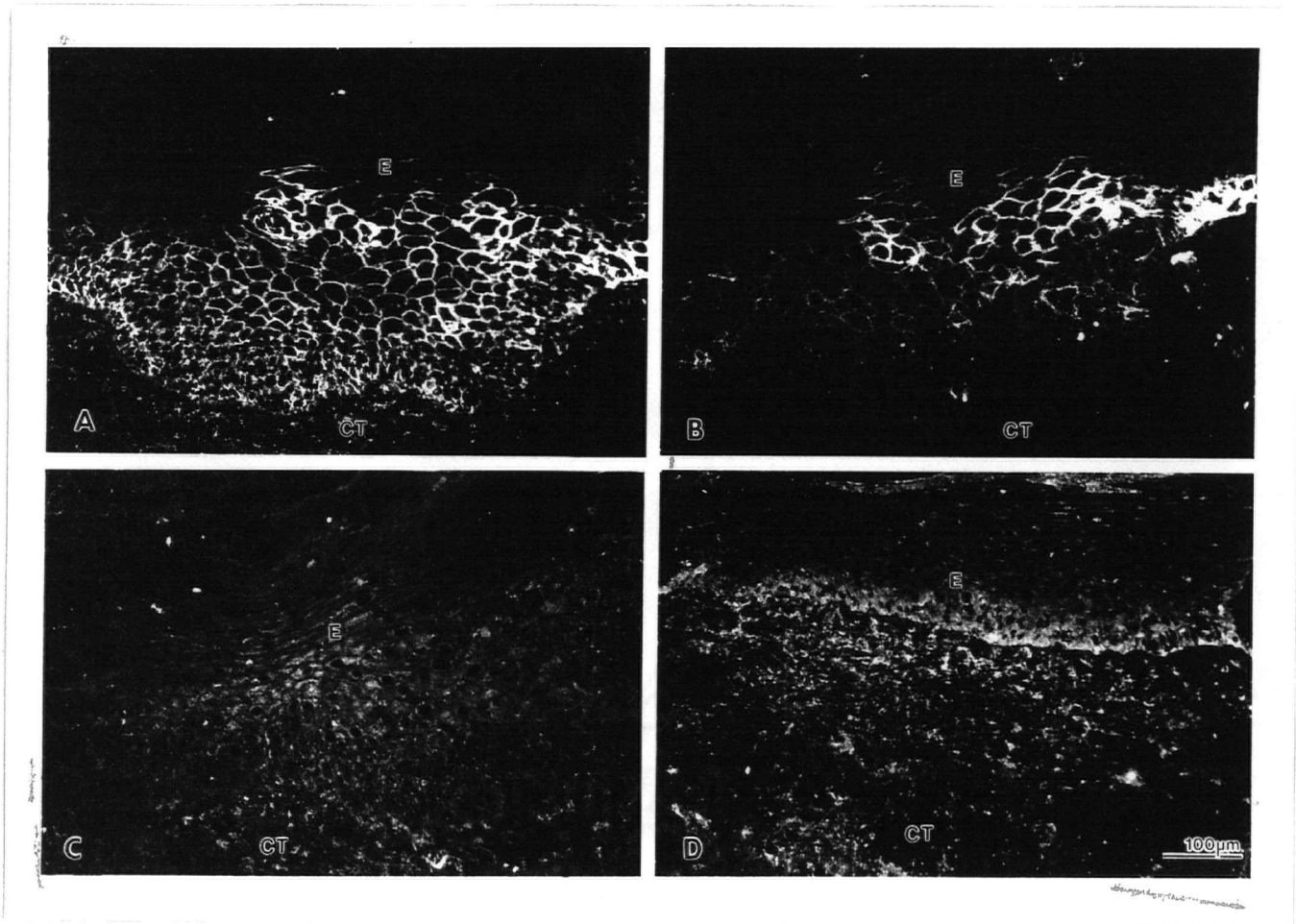


Figure 21. Localization of (A) αv, (B) ß6, (C) ß3, (D) ß5 in lichen planus tissues E=Epithelium, CT=Connective tissue

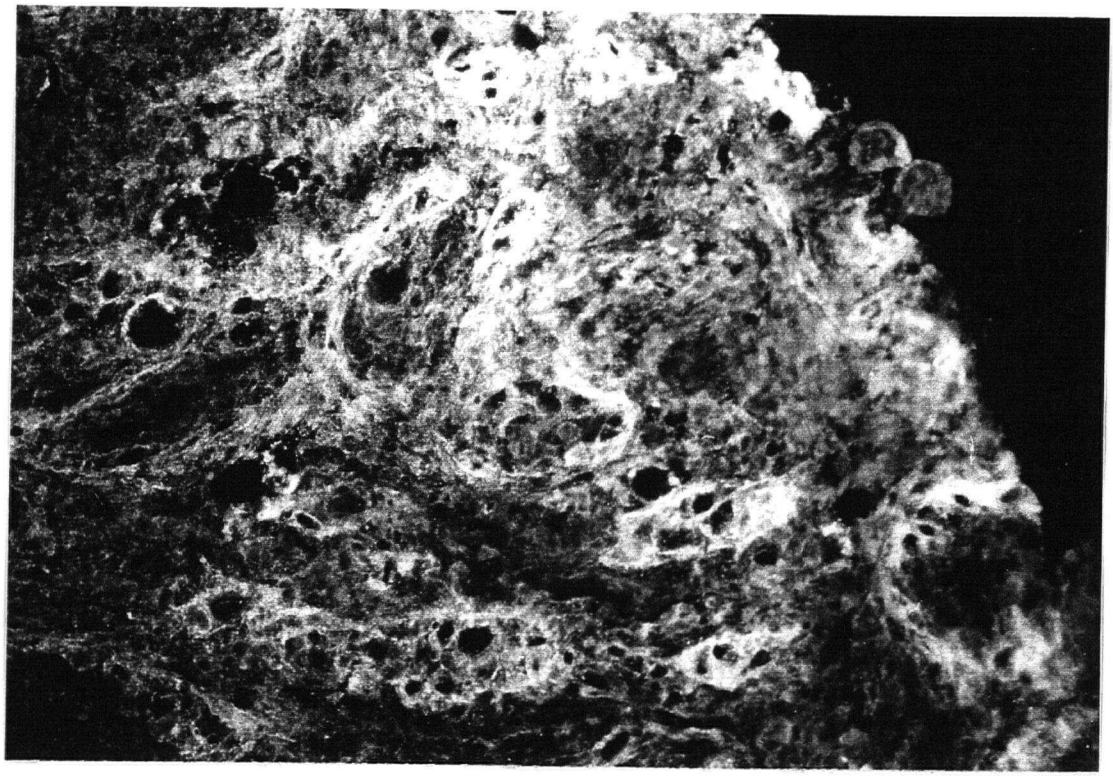


Figure 22A. Localization of ß1 integrin in Oral SCC

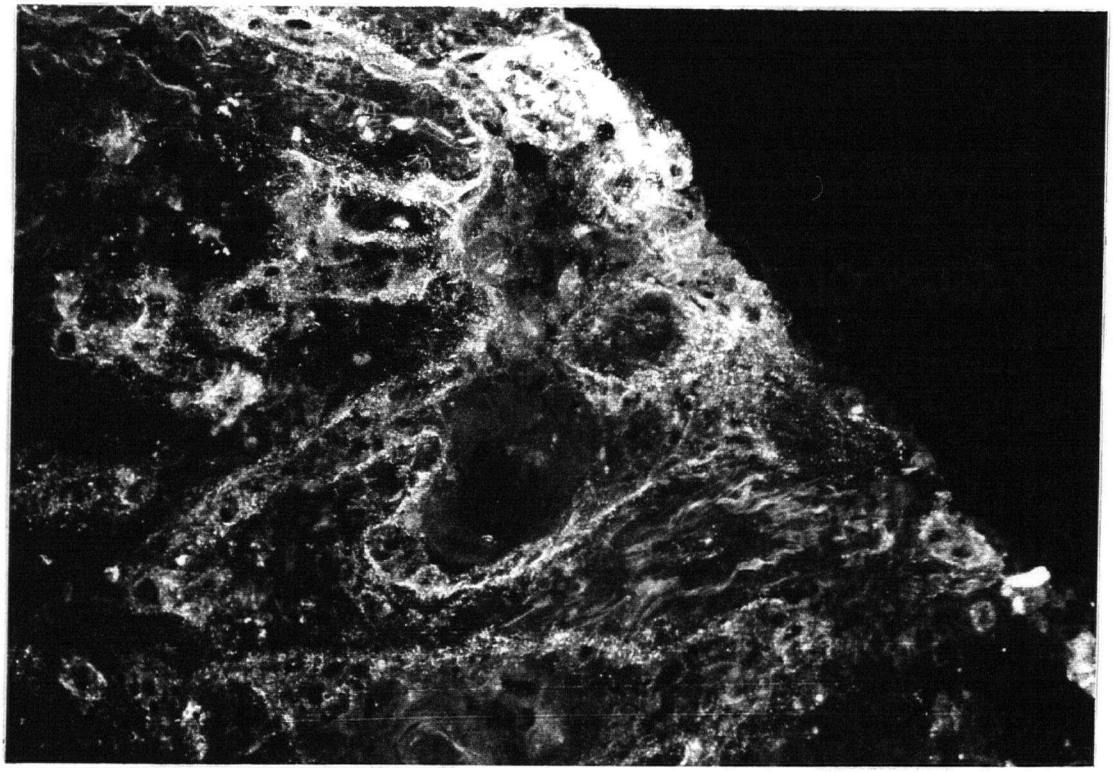


Figure 22 B. Localization of ß4 integrin in Oral SCC

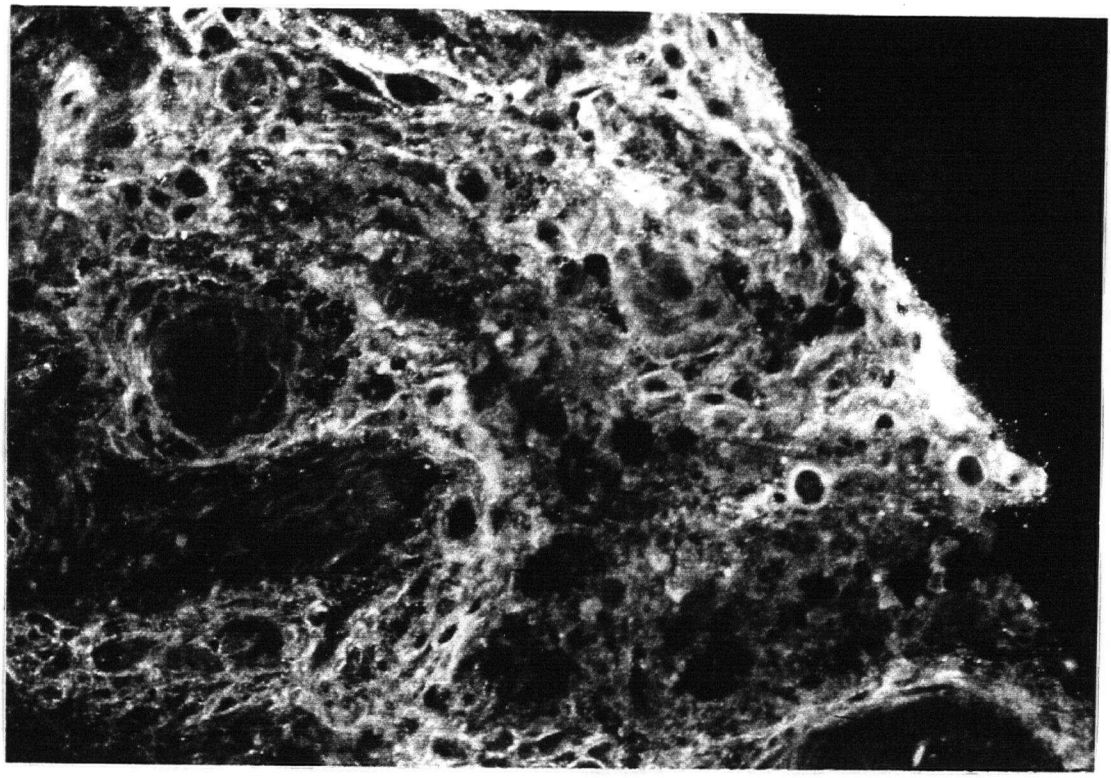


Figure 23 A. Localization of αv integrin in Oral SCC

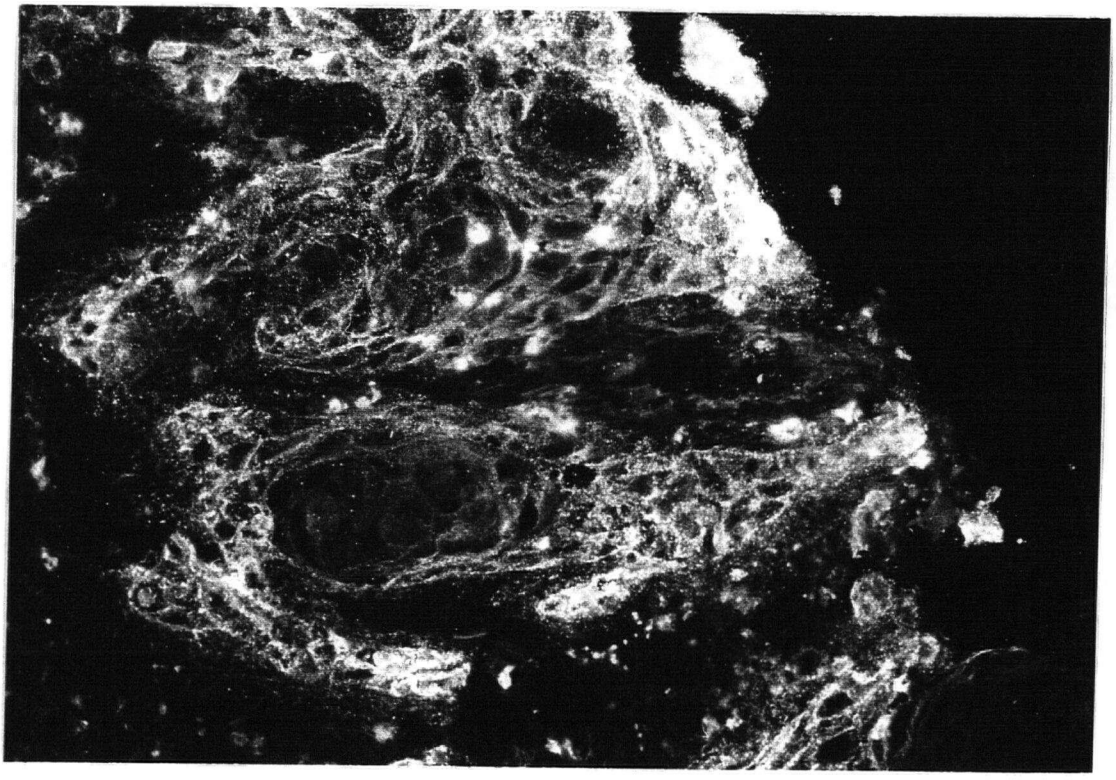


Figure 23 B. Localization of ß6 integrin in Oral SCC

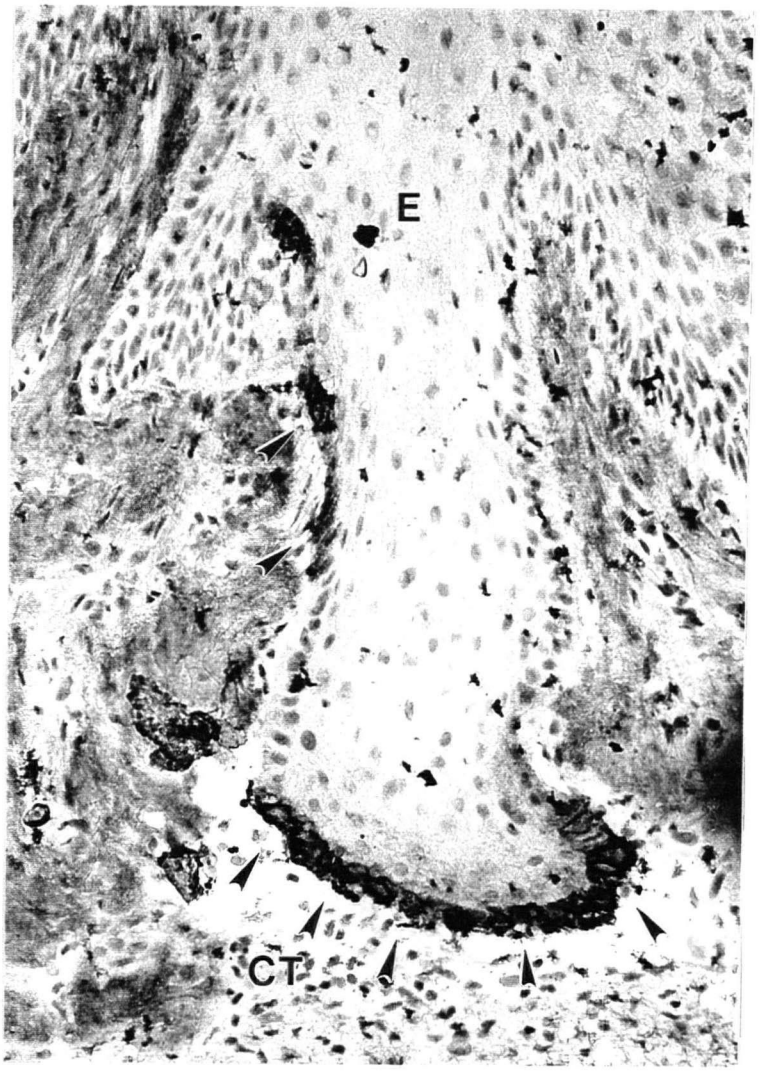


Figure 24 A. Localization of ß6 integrin in 7-day-old wound
Arrow heads point to the wound area on the epithelium
E=Epithelium, CT=Connective tissue

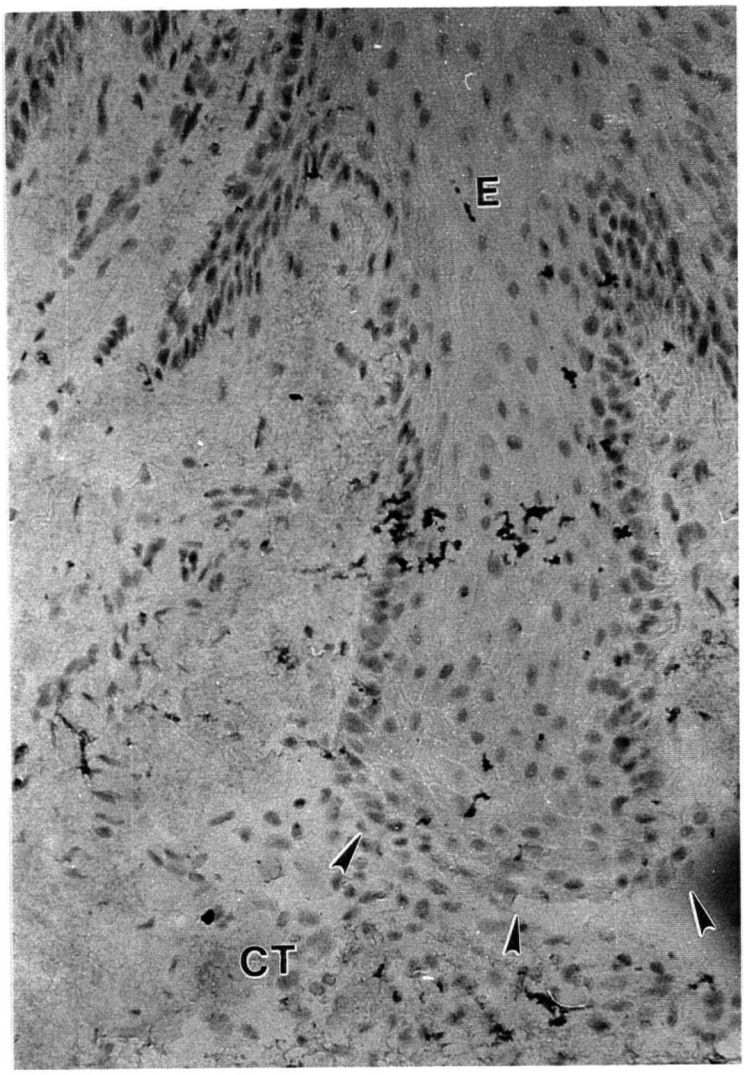


Figure 24B. 7-day wound (2^{o} antibody only)

Arrow heads point to the wound area on the epithelium

E=Epithelium, CT=Connective tissue

Follow-up data of the leukoplakia patients

The charts of all the leukoplakia patients were reviewed and their status in one year or more after their biopsies were taken was studied. The date of the original biopsy and that of the last follow-up visit of the patient were recorded. It ranged from 8 months to four years with the average time of two years. The following formulas (Beck, 1995) measure the sensitivity, specificity, positive predictive value and the negative predictive value.

Table 5 shows the data of the patients with the disease whose tissues were stained with αvß6 integrin antibody.

Table 5. Number of αvß6 integrin positive/negative specimens versus the disease progression

	Disease Progression		
	Yes	No	Total
αvß6 Positive	6 **(A)**	4 **(B)**	10 **(A+B)**
αvß6 Negative	0 **(C)**	18 **(D)**	18 **(C+D)**
Total	6 **(A+C)**	22 **(B+D)**	28

Of the 6 tissue specimens that were positive for αvß6, 3 were moderate dysplasia, 2 were mild dysplasia and 1 was atypia. For the diagnosis of the remaining tissues, refer to table 4.

Sensitivity= A/A+C ⇒ 6/6= 100%

Specificity= D/B+D ⇒ 18/22= 82%

Positive Predictive Value= A/A+B ⇒ 6/10= 60%

Negative Predictive Value= D/C+D ⇒ 18/18= 100%

The above calculation can be interpreted as follows:

If the positive expression of αvß6 integrin is considered as a prognostic test, the proportion of people with the progressed disease is identified 100% of the times by this test. It means that the tissues of all the patients whose diseases has deteriorated will express αvß6 integrin. This was determined by calculating the sensitivity. Likewise, when the disease improves or does not change, the test will be correct 82% of the times and that is defined by the specificity formula.

The positive predictive value illustrates that in 60% of the people whose biopsies expressed αvß6 the disease has progressed. However, the negative predictive value demonstrates that none of the patients with the negative diagnostic tests have an active disease.

Chapter Five

DISCUSSION

Tumour metastasis is a complex multiple process that includes cell adhesion as an important pre-requisite. Reduced cell adhesion and aggressive malignant phenotype might be related to down-regulation of the integrin receptors. The integrins provide a molecular basis for the mechanisms underlying tumour progression. They appear to be important determinants of aggressive malignant phenotype. Therefore, measuring the integrins may be of considerable value as a predictor of an axillary disease and may allow the tailoring of axillary surgery to suit the individual needs of each patient.

Using integrin expressions as a diagnostic tool might introduce some conflicting results. For instance, some studies (Friedrichs et al., 1995) show that high expression level of α6 integrin in human breast carcinoma is correlated with reduced survivors, whereas another study (Gui et al., 1995) shows the down regulation of this integrin in breast tumours.

Different patterns of integrin expression in tumours have been reported. In the development of malignant melanomas, the upregulation of integrins is directly correlated with the metastatic potentials (Damjanovich et al., 1992). Epithelial derived tumours appear to be different. Downer et al., (1993) have reported the focal or extensive loss of BM components and of α6ß4 integrin in oral squamous cell carcinoma. Our studies provide evidence that the expression of ß4 integrin in SCC tissues is either unchanged or up-regulated when compared with normal tissues. However, the stage of the disease and the site it occurs could considerably alter the expression pattern of integrins and perhaps this could be an explanation for the contradictory findings. Staining for ß1 integrins in oral SCC and leukoplakia tissue specimens reveals that these integrins are often over-expressed and can sometimes be detected in suprabasal layer in addition to basal layer. However their intensity is weaker in some cases. Other studies have reported the down-regulation of ß1 in different areas such as in breast carcinoma (Koukoulis et al., 1991; Pignatelli et

al., 1991; Gui et al., 1995), in colon carcinoma (Koretz et al., 1991; Pignatelli et al., 1990; Nigam et al., 1993), in skin SCC (Peltonen et al., 1989), and in inflamed oral tissues (Haapasalmi et al., 1995). In this study the expression pattern of both ß1 and ß4 integrins in oral leukoplakia tissue specimens was similar to that in normal tissues, however, in a few cases, the intensity of the staining was weaker than usual. The considerable variation of staining pattern of ß1 and ß4 integrins in tumours, and the fact that they are present in normal tissues make it difficult to use their localization in tumour diagnosis. However, this does not rule out the importance of these receptors in tumour cell behaviour. For example, LM-5 which is highly expressed in SCC and metastasis could serve as a migratory ligand for these integrins. The presence of integrins in the cell surface does not guarantee that the integrin is in an active form (Ruoslahti, 1991). In addition, the migratory behaviour of cells depends on both the amount of the matrix and the numbers of cell surface integrins (Palecek et al., 1997). Low-affinity binding to FN through αvß6 integrin may be more beneficial for tumour cell migration than binding via α5ß1 integrin that mediates high-affinity binding.

We have observed induction of αvß6 integrin in the areas of some oral leukoplakia and majority of SCCs. αvß6 integrin expression is restricted to epithelia and is up-regulated in parallel with morphogenetic events, tumorigenesis, inflammatory response, and epithelial repair (Breuss et al., 1995). It has been detected from infants' kidneys, lung and skin, but mostly undetectable in normal adult kidney, lung and skin (Breuss et al., 1995). This integrin is believed to play a major role during re-epithelialization of human wounds (Haapasalmi et al., 1996). Wounds in knock-out mice, however, appear to heal normally (Huang et al., 1996). It has previously been shown that ß6 integrin is strongly expressed in the specimens of squamous cell carcinoma derived from the oral cavity (Breuss et al., 1995). It is also believed that this integrin is expressed in the proximal airway epithelium of some (but not all) cigarette smokers who develop lung cancer (Liebert et al., 1994). ß6 integrin is also highly expressed in 50% of colon cancer cell lines (Agrez et al., 1996). During wound healing, αvß6 integrin is up-regulated in later stages of wound healing. Its

distribution is circumferential over the basal cells and occasionally around suprabasal cells (Clark et al., 1996). It appears that αvß6 integrin is not necessary for epidermal migration but rather may provide proliferation signals to the healing epidermis from the underlying tenascin and fibronectin-rich matrix (Haapasalmi et al., 1996). It is also possible that αvß6 integrin expressing epithelial cells have a phenotype that participates in epithelial-connective tissue cross-talk favoring the matrix deposition by the fibroblasts. The expression of αvß6 integrin in leukoplakic lesions was investigated in this study. 25% of these specimens expressed αvß6 integrin. The majority of these αvß6 integrin positive tissue specimens were associated with dysplastic and hyperplastic lesions. It appears that αvß6 integrin has the capacity to contribute to cellular proliferation (Weinacker et al., 1995). However, none the idiopathic or drug-induced or inflammatory hyperplasia expressed this integrin suggesting that cell proliferation alone cannot be counted for the induction of αvß6 integrin. Moreover, the majority of the SCCs and lichen planus tissues and all the 7-day-old wounds expressed αvß6 integrin. One explanation for these results could be that during many physiological and pathological conditions, such as the ones named above, the modification of the cell behavior is partly mediated by cytokines and different growth factors. As mentioned above, αvß6 integrin positive keratinocytes which have different phenotypes may express different cytokines. These cytokines may change the cellular integrin pattern by autocrine control and also promote inflammation and modulate matrix production of cells during wound healing, lichen planus and malignant transformation. Interestingly, αvß6 integrin has recently been found to be also associated with the modulation of epithelial inflammation (Huang et al., 1996). In general, epithelial cells play a significant role in alteration of local inflammatory responses by secretion of a number of inflammatory cytokines (Sheppard, 1996). For instance, a number of the environmental stimuli can induce the epithelia of the conducting airways in the lung to secrete cytokines involved in the proliferation and local survival (Sheppard, 1996). TGFß1 significantly increases the surface expression of αvß6 integrin in cultured keratinocytes (Zambruno et al., 1995; Haapasalmi et al., manuscript). This cytokine, however, does not change the surface expression

of any of ß1 or α6ß4 integrins (Haapasalmi et al., manuscript). Epithelial cells in SCCs and lichen planus lesions are likely to secrete different types of cytokines. It may not be likely that the activation of epithelial cells alone in these pathological conditions is the initial event in these diseases; however, it is believed that the cytokines that are secreted by these epithelial cells contribute to the pathogenesis (Feliciani et al., 1996). In fact, the epithelium from lichen planus lesions secretes a variety of cytokines including interleukin-1ß (IL-1ß) and tumour necrosis factor-α (TNF-α) at the levels that are ten to twenty times greater than those in normal gingiva and two to three folds greater when compared with keratinocytes of the chronically inflamed gingiva (Yamamoto and Osaki, 1995). The over-production of these cytokines in oral lichen lesions could be an explanation for the induction of αvß6 integrin in these lesions. In SCCs, the level of IL-3 has been reported to be elevated (Yamamoto et al., 1993). However, it is thought that the secretion of this cytokine may also be stimulated by IL-1ß and TNF-α (Yamamoto et al., 1993). It is not known, however, whether IL-1ß, TNFα or TGFα are able to induce de novo expression of αvß6 integrin.

Many investigators believe that tumour marker diagnosis is not important for detection of cancer (Kobayashi and Kawakubo, 1994). However, a number of different diagnostic markers for cancer have been proposed. For instance, Jensen et al., (1982) have reported that angiogenesis induced by normal human breast tissue may be used as a probable marker for precancer. Classification of dysplasia into three categories has been proposed to be a successful tool for cancer diagnosis (Morson, 1985). Recently, the disaccharide Gal-GalNAc has been introduced by Yang and Shamsuddin, (1996), as a bio-marker of colon carcinogenesis. Based on our findings, we propose that the induction of αvß6 integrin in leukoplakic tissue specimens could be potentially used as a diagnostic marker for cancer.

We found that the expression of αvß6 integrin may serve as an adjunct tool in predicting malignant transformation of oral leukoplakia. High αvß6 integrin expression seems to be associated with malignant areas of the oral mucosa. Further studies with a much larger sample size

are required, however, in order to discover more about the relation between induction of αvß6 integrin and the transformation of oral leukoplakic lesions.

Chapter Six

SUMMARY & CONCLUSION

Integrins mediate cell-cell adhesion and also adhesion to extracellular matrix including epithelial keratinocytes (Giancotti and Mainiero, 1994; Heino, 1993; Hynes, 1992; Watt and Jones, 1993; Larjava et al., 1996). The αvß6 is exclusively an epithelial integrin and its expression is restricted to only a few locations in healthy adult tissues in humans (Breuss et al., 1993). High levels of ß6-mRNA are only found in two very specialized epithelial cell types: a portion of the kidney tubule epithelium, termed macula densa, and the endometrial epithelium of secretory phase uterus (Breuss et al., 1993). Lower ß6-mRNA levels have been detected in epithelium of salivary gland ducts, gall bladder, and epididymis, but not in skin or lung (Breuss et al., 1993). Cultured epithelial cells, however, express αvß6 integrin (Breuss et al., 1993; Haapasalmi et al., 1996).

During wound healing, αvß6 integrin is up-regulated in keratinocytes facing the granulation tissues matrix (Haapasalmi et al., 1996; Clark et al., 1996). It has been proposed that αvß6 integrin could function in conveying signals between the healing epidermis and the underlying tenascin/fibronectin-rich matrix (Haapasalmi et al., 1996). αvß6 integrin is expressed diffusely on all cell types in the airway epithelium of patients with a number of inflammatory lung diseases (Breuss et al., 1995). Knock-out mice lacking ß6 gene develop significant infiltration of inflammatory cells in their skin and lungs (Huang et al., 1996) suggesting that αvß6 integrin could participate in regulation of epithelial driven inflammation. Chronic inflammation alone, however, does not appear to be associated with induced αvß6 integrin expression.

In this study, the αvß6 integrin was absent from all the normal epithelial tissues, but highly expressed in the tumour islands of most of the squamous cell carcinomas (SCCs) confirming previously published results (Breuss et al., 1995). It is believed that αvß6 integrin is expressed in the proximal airway epithelium of some (but not all) cigarette smokers who develop lung cancer

(Breuss et al., 1995). Recently, it has also been shown that αvß6 integrin enhances growth of colon cancer cells in-vitro and in-vivo (Agrez et al., 1994). We demonstrate in this study that αvß6 integrin is also expressed in epithelial cells of some oral leukoplakia. It is not known, however, whether these lesions become transformed to SCC or whether this change is transitional and reflects a wound healing-type of reaction. High αvß6 integrin expression is seen in malignant areas of the oral mucosa suggesting that αvß6 integrin is used by the tumor cells for their invasion in the fibronectin/tenascin rich matrix. Based on these findings and earlier results, we speculate that the expression of αvß6 integrin in leukoplakia lesions could provide the cells with the necessary receptor for invasion during their malignant transformation. The expression of αvß6 integrin may be, therefore, necessary but not sufficient for malignant transformation. αvß6 integrin is the only integrin that is induced in SCC. Loss of ß1 and ß4 integrins is common in SCC although the expression of ß4 has reported to be increased in some studies (Rossen et al., 1994; Savoia et al., 1993). Epithelial cells in leukoplakia expressed ß1 and ß4 integrins concomitantly with αvß6 integrin indicating that there is no selective down-regulation of any particular integrin that is normally expressed to accommodate αvß6 integrin expression.

In this study, the αvß6 integrin was absent from all the normal epithelial tissues, but highly expressed in the tumour islands of most of the squamous cell carcinomas (SCCs). It was also expressed in either the basal layer or the tip of the rete ridges of some of the leukoplakia epithelia. The positive expression of αvß6 integrin was used to explore its putative role as a diagnostic test. The charts of the patients were reviewed and their status within one to three years after the biopsies were taken were evaluated. The tissues of all the patients whose disease have progressed expressed αvß6 integrin, suggesting the test was 100% sensitive. However, not all the biopsies expressing αvß6 integrin came from the patients whose diseases have progressed. Therefore, the positive predictive value was shown to be 60%. On the other hand, the specificity of the test was 82% meaning when the disease has improved or resolved the test is correct 82% of the times. In addition, all the αvß6 negative patient were among the ones whose disease improved or resolved,

therefore the negative predictive value of the test was 100% accurate. More specimens have to be examined before any definitive conclusion can be drawn on the relationship between induction of αvß6 integrin and prediction of malignant transformation.

Chapter Seven

ABBREVIATIONS

AT=Atypia

Cinf=Chronic Inflammation

COL XVII=Collagen type XVII

COL=Collagen

CT=Connective Tissues

DP=Dysplasia

E=Epithelium

FN=Fibronectin

HK=Hyperkeratosis

HP=Hyperplasia

KERAT=Keratinized

LM-1=Laminin type 1

LM-5=Laminin type 5

LM=Laminin

PK=Parakeratosis

SCC=Squamous Cell Carcinoma

TN=Tenascin

ULC=Ulcer

VC=Verrucous Carcinoma

VN=Vitronectin

PBS=Phosphate Buffer Saline

BSA=Albumin, Bovine Serum

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