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Intermediate Filaments: the Prognostic Marker for Conjunctival Melanomas

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Abstract

Intermediate filaments (IFs) are highly regulated and conserved during cell transformation and tumor development. The co-expression of keratin 8, 18 and vimentin has been shown to be related to recurrence and metastasis in both cutaneous and uveal melanomas. This thesis provides the first immunohistochemical evidence that the co-expression of keratin 8, 18 and vimentin is present in one third of conjunctival melanomas. The results show that conjunctival melanomas co-expressing IFs are mixed cell tumors with diffuse growth patterns. It is also shown that melanoma cells co-expressing IFs are mainly located around the peripheral or marginal area of the tumors. In addition, this thesis indicates that the initial tumor thickness significantly increases in conjunctival melanomas co-expressing IFs. Therefore, it is demonstrated that the co-expression of IFs plays an important role in influencing the malignant progression of conjunctival melanomas, and the co-expression of IFs can be used as a prognostic marker for conjunctival melanomas.

Résumé

Les filaments intermédiaires (FIs) sont régularisés et préservés pendant le processus de transformation cellulaire et pendant le développement de tumeurs. Il a été démontré que la co-expression des kératines 8 et 18 de même que de la vimentine est intimement liée à la récurrence et à la formation de métastases des mélanomes de la peau et de l'uvée. Pour la première fois, cette thèse a établi grâce à des analyses immunohistochimiques qu'un tiers des mélanomes de la conjonctive contiennent à la fois les kératines 8, 18 et la vimentine. Les résultats démontrent que les mélanomes de la conjonctive renfermant ces FIs sont de différents types cellulaires et affichent un modèle de croissance diffuse. Ce travail indique également que les cellules des mélanomes renfermant ces deux types de FIs sont principalement localisés en marge des tumeurs. De plus, cette thèse prouve que l'épaisseur initiale de la tumeur augmente de manière significative pour les mélanomes conjonctivaux contenant les FIs. Par conséquent, il est démontré que la co-expression des FIs peut influencer de manière importante la progression maligne des mélanomes de la conjonctive et que cette même co-expression pourrait être utilisée comme outil de pronostic pour ce type de mélanome.

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Chapter 1: Introduction

1.1 Justification

Conjunctival melanomas are rare, unilateral ocular malignancies typically affecting Caucasian people past middle age. Because of the rarity of conjunctival melanomas, they remain one of the most dreaded and unpredictable ocular tumors and one of the most debated topics of ocular oncology.

Although both conjunctival melanomas and uveal melanomas are ocular tumors that are embryologically derived from neural crest cells, they show different biological behaviors. Conjunctival melanoma cells reside in the epithelium. When they metastasize, melanoma cells have to penetrate the epithelial basement membrane to contact the mesenchyme of the substantia propria, and then malignant melanoma cells disseminate through lymphatics to regional lymph nodes. In contrast, uveal melanoma cells, which are located in the mesenchymal compartment of the choroid, ciliary body, or iris, do not require a breach of the basement membrane to initiate contact with other mesenchymal elements. Since the interior of the eye lacks lymphatics, uveal melanoma cells always disseminate through blood vessels going first to the liver. The prognosis of conjunctival melanomas is related to the tumor thickness, and tumor-infiltrating lymphocytes are associated with a favorable prognosis. However, the prognosis of uveal melanomas is related to the tumor base, and tumor-infiltrating lymphocytes bring an unfavorable prognosis in uveal melanomas.

Although the conjunctiva belongs to the mucosal system, characteristics of conjunctival melanomas are very similar to their cutaneous counterparts. The natural history of this neoplasm has not been clearly established. Some conjunctival melanomas never recur after local excision, some often recur over varying time

intervals, and some have already metastasized when first diagnosed. To date, we do not know which factors might influence the different prognosis for conjunctival melanomas. According to the study of conjunctival melanomas in Sweden 1969-91 (Seregard & Kock, 1992), 71% of conjunctival melanomas were associated with primary acquired melanosis (PAM) with atypia, 17% arose from conjunctival nevi, and 12% occurred *de novo*.

How could these different types of proliferating melanocytes account for their different prognosis? What happens when normal melanocytes transform to malignant melanoma cells? What is the prognostic indicator for conjunctival melanomas? In order to answer these questions, much research has been done in the last decade. The study by Burnier *et al* (1999) has proved that when conjunctival melanocytes transform into malignant melanoma cells, their morphological appearances are obviously different. In addition to these changes, other studies (Seregard, 1993; Langmann *et al*, 1993) showed that during the melanocyte transformation the expression of genes such as Ras oncogene, and the activation of proliferation-associated antigens such as proliferating cell nuclear antigen (PCNA) may be selectively increased.

Recently, more exciting studies of intermediate filaments (IFs) have shown a correlation between the recurrence and metastasis of carcinomas and melanomas (Raymond & Leong, 1989; Zarbo *et al*, 1990; Hendrix *et al*, 1992; Fuchs *et al*, 1992; Chu *et al*, 1996; Hendrix *et al*, 1998; Morilla-Grassa A *et al*, 2000). Generally, the immunohistochemical diagnosis of melanomas depends on a few unique antigens present in melanoma cells, which include vimentin, not keratins. However, in

carcinomas, the diagnosis includes keratins, not vimentin. When keratin 8, 18 and vimentin are co-expressed in tumor cells, they show the more malignant phenotype. Studies suggested that the co-expression of keratins and vimentin might affect the signaling pathway in tumor cells to influence their prognosis. In this case, tumorigenic signals stimulate multiple cell surface receptors on tumor cells, further activate the E26 transformation-specific oncogenes (Ets) via Ras oncogenes. Then Ets activate the AP-1 transcription family. Furthermore, transcription factors act like a cascade to promote keratin 8, 18 expression. Growing evidence (Seftor *et al*, 1992; Juliano & Haskill, 1993; Malik & Parsons 1996) indicates that transmembrane integrins, which are a family of cell-surface proteins that mediate cell-substratum and cell-cell adhesion, act as link proteins that interact with IFs and the extracellular matrix (ECM). While IFs increase in the tumor cell plasma, they not only mediate cell shape and spreading but also act as signal transducers, which relay information from the ECM to the cell nucleus through integrins. The co-expression of keratins and vimentin may change the integrin profile, and increase the invasive and metastasitic activity of melanoma cells (Oshima *et al*, 1996; Hendrix *et al*, 1996).

Until now, there has been no study to indicate if there is a correlation between the increased co-expression of IFs and the worse prognosis of conjunctival melanomas. If the co-expression of keratins and vimentin could be shown to correlate with malignant transformation and metastasis in conjunctival melanomas, it could help to elucidate the natural history of conjunctival melanomas as well as help in the early diagnosis and prompt therapeutic management of conjunctival melanomas. In

addition, it could establish the foundation for the future of immunotherapy and gene therapy in conjunctival melanomas.

1.2 Hypothesis

The following hypothesis was formed based on previous concepts:

When normal, nevocytic or PAM melanocytes transform into malignant melanoma cells, there may be an increase in the co-expression of keratin 8, 18 and vimentin. In simple terms, the greater the co-expression of these markers on melanoma cells, the worse the prognosis may be for the patient.

1.3 Objectives

To test this hypothesis, the following objectives were established:

1. In order to demonstrate that increased expression of intermediate filaments is associated with melanocytic transformation, the co-expression of keratin 8, 18 and vimentin will be investigated in conjunctival melanomas, conjunctival nevi, and primary acquired melanosis.

2. In order to determine whether the co-expression of intermediate filaments in conjunctival melanomas correlates with worse prognosis, an association between the co-expression of keratin 8, 18 and vimentin and the initial tumor thickness of conjunctival melanomas will be investigated.

1.4 Literature Review

1.4a Anatomic characteristics of the conjunctiva

The conjunctiva is a mucous membrane that covers the posterior surface of eye lids and the anterior surface of the globe, with the exception of the cornea. It is subdivided into the palpebral, fornical, and bulbar regions. Histologically, it contains two or more layers of stratified columnar epithelium, except at the limbus and the palpebral margins where stratified squamous epithelium is present. Conjunctival melanocytes are mainly located in the conjunctival epithelium, at or close to the epithelial basement membrane. The substantia propria layer is composed of fibrovascular connective tissue of varying density and thickness. Lymphatic channels are present in all parts of the conjunctival stroma, and extend much closer to the epithelium. These channels drain medially to the submandibular lymph nodes and laterally to the preauticular lymph nodes (McLean *et al*, 1994; Spencer & Zimmernan, 1996; Seregard, 1998).

1.4b Origin of conjunctival melanocytes

Conjunctival melanomas arise from conjunctival melanocytes (Liesegang & Campbell, 1980; Folberg *et al*, 1985; Farber *et al*, 1998). During embryogenesis, melanocytes migrate from the neural crest to reach the mucous membrane of the conjunctiva, where they reside in the superficial or deep layer of the epithelium. There are three types of melanocytes. Dendritic melanocytes are intraepithelial cells that lie along the basal epithelial layer above the basement membrane. They are called dendritic melanocytes because they have racemose dendrites that carry melanin granules to adjacent epithelial cells. They give rise to benign epithelial

melanosis and acquired melanosis. The second type – nevocytes include intraepithelial nests of oval cells (Type A), sheets of oval to cuboidal cells (Type B), and even fibroblast-like cells in the sub-mucosa (Type C). All of them give rise to different conjunctival nevi. Finally, the deep or fusiform melanocytes form the third type, which reside in the sub-epithelium, give rise to the nevus of Ota, melanosis oculi, and the blue nevus. All these three types of melanocytes, in addition to forming benign melanocytic lesions of the conjunctiva, have the potential for transforming into malignant melanomas. This is especially true with dendritic melanocytes. In general, all melanocytes are capable of producing melanin. Sometimes they appear non-pigmented clinically or histologically because their melanosomes are either too few, too small, or too minimally melanized to be detected (McLean *et al*, 1994). In this case, conjunctival melanomas are difficult to differentiate from tumors of the conjunctival epithelium.

1.4c Characteristics of melanocytic lesions

1.4c.i Conjunctival nevi

Conjunctival nevi are congenital and benign melanocytic tumors. They typically appear as solitary, well-circumscribed, and pigmented or non-pigmented lesions (Plate 1.1a), which mainly locate at the limbus, but may occur at other sites of the conjunctiva. However, conjunctival nevi located in the palpebrae or fornices are extremely rare. These lesions may be flat or elevated. They may be present at birth, but are usually not detected until after childhood or early adolescence when these lesions acquire pigmentation (Gerner *et al*, 1996; Grin *et al*, 1998). We should keep

in mind that 30% of conjunctival nevi may remain clinically amelanotic in adulthood (Henkind, 1978; Jakobiec *et al*, 1989).

Histologically, there are mainly three types of conjunctival nevi according to nevocytic locations (McLean *et al*, 1994). If nevocytes are only located in the epithelium, this lesion is called the junctional nevus. If they reside in both the epithelium and sub-epithelium, it is called the compound nevus (Plate 1.1b). If they are only located in the sub-epithelium, it is called the subepithelial nevus. Clinically, most conjunctival nevi are compound or subepithelial, and only in the young are pure junctional nevi found.

1.4c. ii Primary acquired melanosis

Primary acquired melanosis (PAM) is a unilateral neoplastic melanocytic proliferation lesion of the conjunctiva. It typically appears as a flat, diffuse, multi-centric, pigmented lesion in the conjunctiva (Plate 1.2a). It may affect any area of the conjunctiva but more often the limbus. PAM occurs predominantly in Caucasian people after middle age (Folberg *et al*, 1985; Jakobiec *et al*, 1989; Gloor & Alexandrakis, 1995).

Histologically, it has been divided into two types – PAM without atypia and PAM with atypia. PAM without atypia is characterized by the epithelial hyper-pigmentation without melanocytic hyperplasia, or by increased melanocytes at the epithelial junction area without cytological atypia. PAM with atypia reveals more marked and atypical melanocytic hyperplasia, including the enlargement of melanocytes containing larger nuclei and prominent nucleoli (Plate 1.2b). There are

four types of atypical melanocytes in the conjunctiva: 1) small polyhedral cells 2) spindle cells 3) large melanocytes (dendrites), and 4) round epithelioid cells. These atypical cells may grow along the basal layer, or present as patterns of intra-epithelial nests, or individually invade into the epithelium (pagetoid growth). PAM with atypia is the most common precursor of the conjunctival melanoma (Seregard, 1998). PAM without atypia does not generally progress to malignancy (Folberg *et al*, 1985). It is interesting that the incidence of PAM with atypia progressing to the melanoma is estimated in different ways. If PAM with atypia is only composed of basal proliferating melanocytes, the risk of developing into the conjunctival melanoma is 22% (Jakobiec *et al*, 1989). If PAM with atypia is composed of prominent atypical epithelial cells, the risk of progressing to melanoma is 75% (Folberg *et al*, 1985). If melanocytes invade the epithelium in a pagetoid fashion or replace the epithelium in PAM with atypia, the risk of melanoma developing is 90% (Mclean *et al*, 1994).

1.4c. iii Conjunctival melanomas

Conjunctival melanomas may present in all conjunctival areas (McLean *et al*, 1994; Seregard, 1998). They may be derived from PAM, or nevi, or *de novo*, and with variable pigmentation. For these reasons, any change in a pigmented lesion of the conjunctiva, particularly growth with increasing elevation, should be suspected of being a malignant melanoma (Farber *et al*, 1998; Grin *et al*, 1998). There are two readily recognizable growth patterns in conjunctival melanomas. (1). Melanomas with nodular growth patterns appear as solitarily elevated lesions, clinically and pathologically lacking the aggressive behavior. (2). Melanomas with diffuse growth

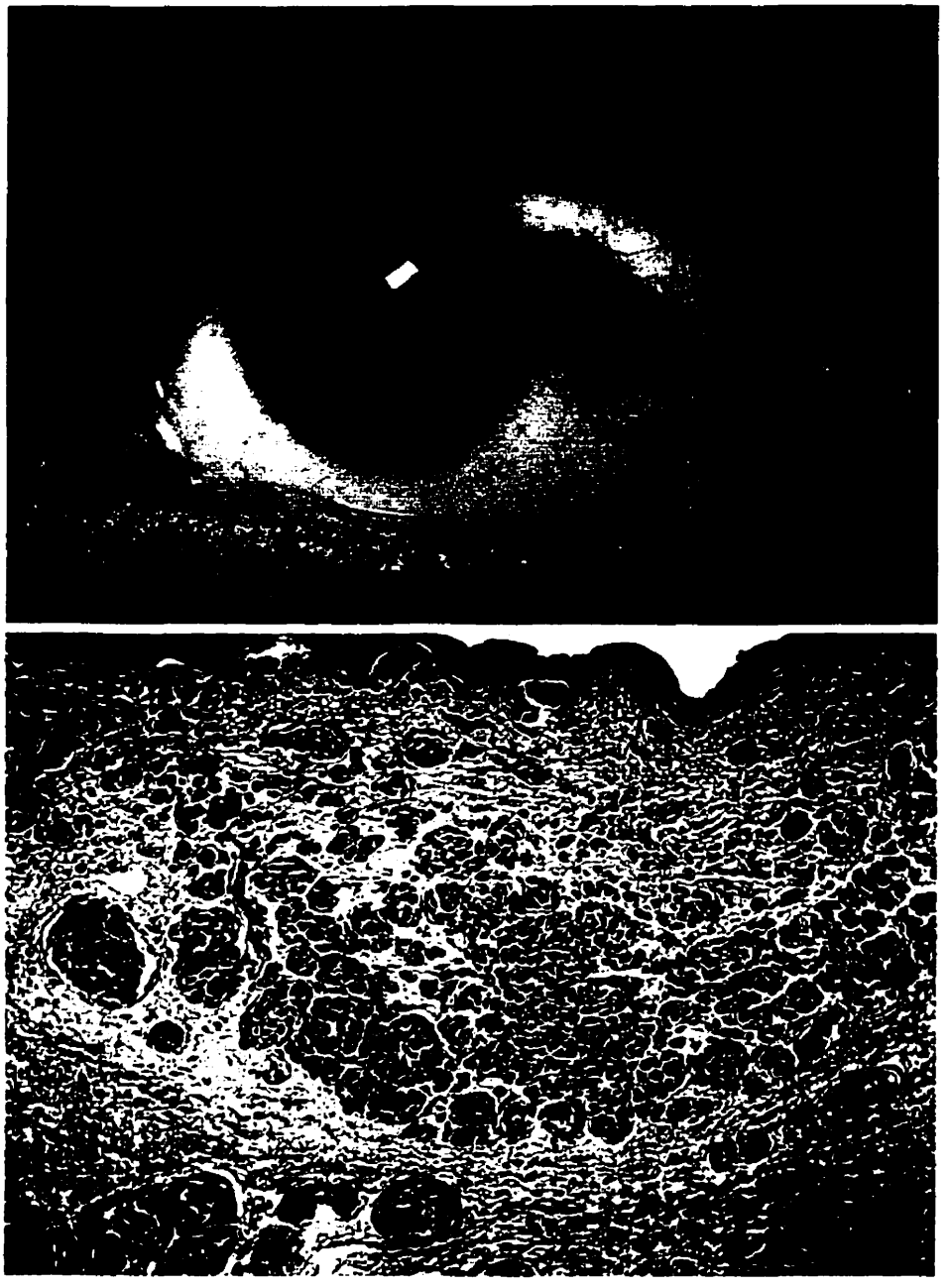

Plate 1.1 A solitary, well-circumscribed, pigmented conjunctival nevus(a). The conjunctival compound nevus (H&E) shows that nevocytes are located in both the intra-epithelium and the substantia propria of the conjunctiva (b).

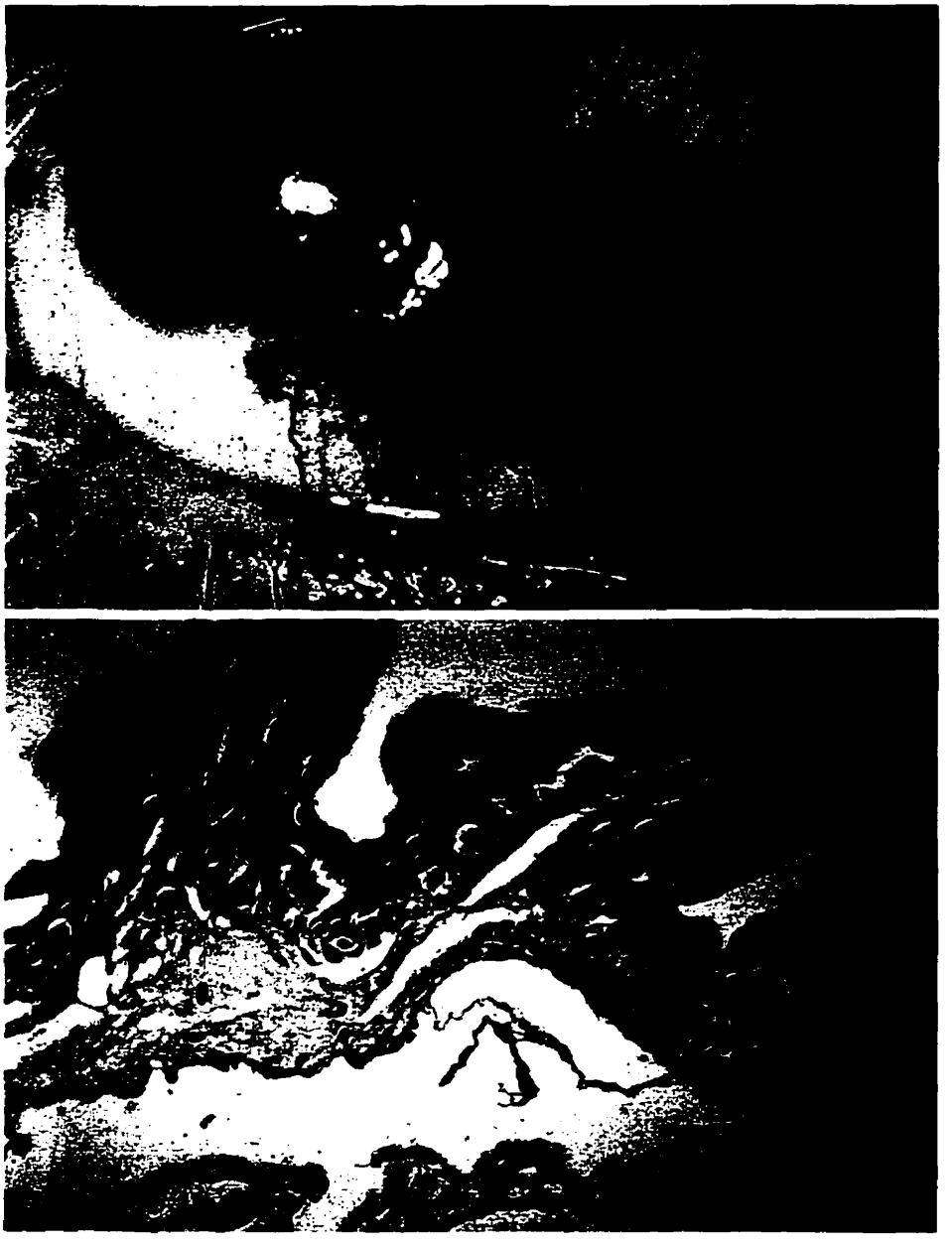

Plate 1.2 A diffuse, multicentric, pigmented primary acquired melanosis (PAM) (a). PAM with mild atypia (H&E) shows that enlarged and atypical melanocytes disseminate throughout the intra-epithelium of the conjunctiva (b).

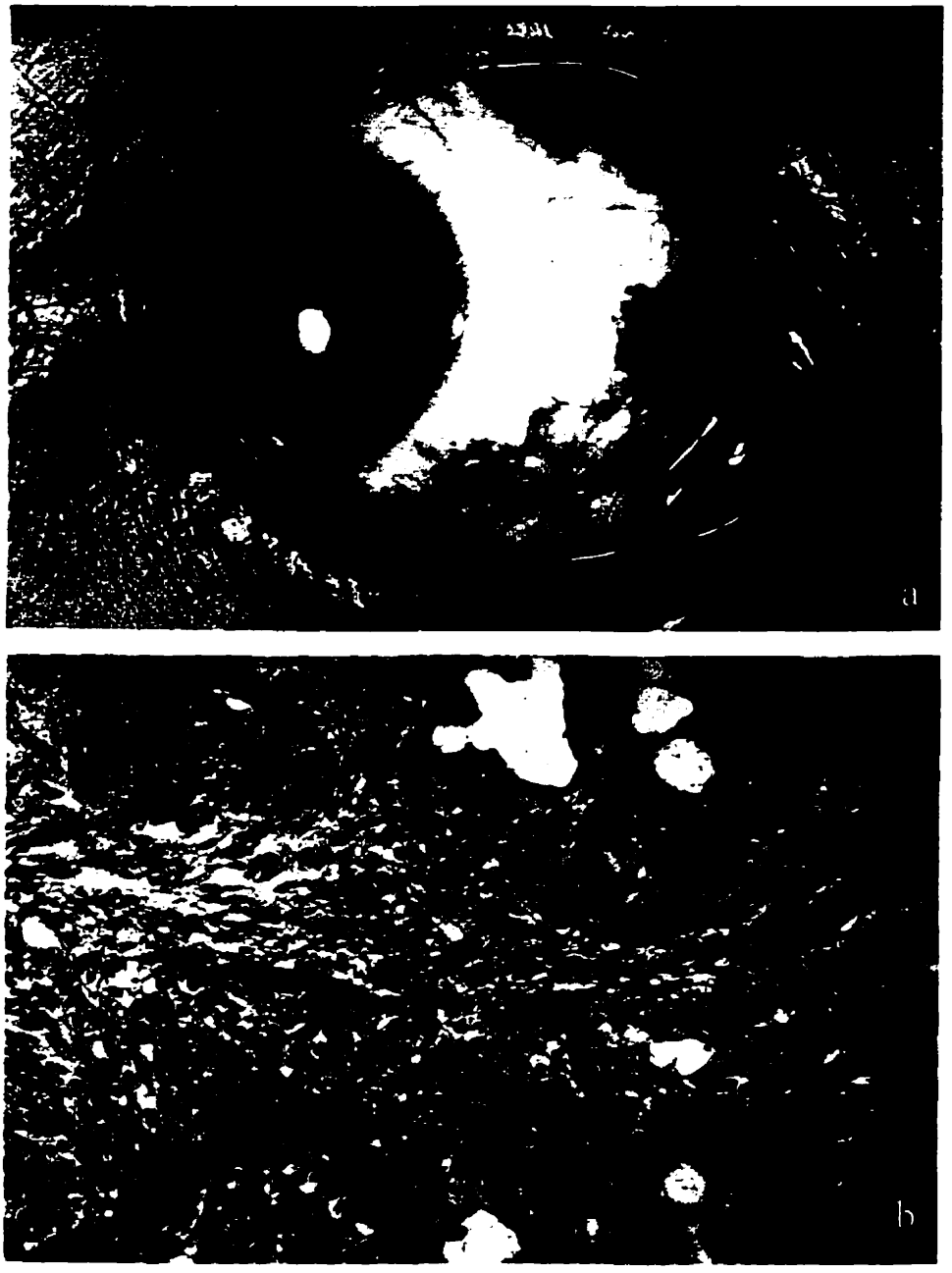

Plate 1.3 A dark-pigmented conjunctival melanoma with diffuse growth pattern (a). The mixed cell conjunctival melanoma (H&E) shows that some large malignant melanoma cells with large nuclei, prominent nucleoli, clumping of the chromatin, and the mititic activity (b).

patterns (Plate 1.3a) exhibit radial or horizontal lesions, having the invasion behavior.

Histologically, malignant melanoma cells invade from the epithelium into the substantia propria of the conjunctiva. The malignant cells present features such as large melanocytes with large or plemorphic nuclei, prominent nucleoli, clumping of the chromatin, and increased mitotic activity (Plate 1.3b). There are four cell types in conjunctival melanomas, which include epithelial cell type, polyhedral cell type, spindle cell type, and mixed cell type (McLean *et al*, 1994; Grin *et al*, 1998). Frequently, conjunctival melanomas contain a combination of cell types – the mixed cell type (Plate 1.3b).

1.4d Melanomas of the conjunctiva

1.4d.i Incidence

Conjunctival melanomas are rare ocular tumors, which comprise only 2% of all ocular malignancies, or make up less than 3% of excised biopsies of conjunctival lesions (McCartney *et al*, 1995; Seregard, 1998). The annual average age-adjusted incidence rate for conjunctival melanomas is estimated to be 0.012 per 100,000 persons (Scotto *et al*, 1976). Furthermore, the Swedish national survey from 1969 through mid-1991 reported an annual incidence rate of 0.024 cases per 100, 000 inhabitants (Seregard *et al*, 1992). Recently, Chang *et al* (1998) reviewed the incidence of melanomas (84,836 cases) between 1985 through 1994 in United States. They concluded that ocular melanomas accounted for 5.3% of all kinds of

melanomas (4522 cases). Conjunctival melanomas accounted for 4.8% of ocular melanomas (216 cases).

1.4d. ii Distribution

- ***Gender***

According to the population based data reviewed by Chang *et al* (1998), different kinds of melanomas had different distributions by gender. These data revealed a higher percentage of females (63.5%) than males with mucosal melanomas, due to tumors arising in the female genital tract. Most studies showed that conjunctival melanomas were distributed equally in both genders (Liesegang & Campbell 1980; De Potter *et al,* 1993; Chang *et al,* 1998). However, a study by Lommatzsch *et al* (1990) found that there was a clear preponderance of female patients (64.2%) in conjunctival melanomas. Another study of a large series of 256 cases of conjunctival melanomas (Paridaens *et al*, 1994) confirmed that 40.2% of cases (103 cases) were males and 59.8% of cases (153 cases) were females.

- ***Age***

Overall, most conjunctival melanomas occur after 50 years of age. Few, if any cases, appear to arise in children or adolescents (McDonnell *et al,* 1989; Paridaens *et al,* 1994; Seregard, 1998). Paridaens *et al* (1994) found there was a tendency towards higher mortality in the older age group (>69 years). On the contrary, De Potter *et al* (1993) stated that the metastasis was not statistically related to the age of patients.

- ***Ethnic origin***

Conjunctival melanomas, like melanomas in other sites, are predominantly in fair-skinned Caucasian people (more than 90%), and rarely in Asian or African people (Chang *et al,* 1998; Seregard, 1998; Singh *et al*, 1998).

1.4d. iii Risk influence factors

The etiology of conjunctival melanoma remains to be elucidated. However, many studies showed that there are several risk factors influencing the incidence of conjunctival melanomas.

- ***Ultraviolet radiation***

Massive data now clearly indicate that high doses of ultraviolet radiation (UVR) may cause skin melanomas (Boyle *et al,* 1995; Elwood, 1996). UVR is thought to induce mutation in the N-ras gene, which then converts these genes into active oncogenes (Bos, 1989). Since most conjunctival melanomas appear to arise from the UVR-exposed bulbar surface, it would be tempting to conclude that UVR may also induce malignant melanocytic transformation in conjunctival sites. But there are no compelling data to suggest that UVR is a causative agent in the formation of conjunctival melanomas. A study by El-Shabrawi *et al* (1999) could not detect point mutations of the N-ras gene in their conjunctival melanoma samples. This study did not support UV-exposure as being causative in the genesis of conjunctival melanomas. However, they suggested that even though no direct involvement of UVR in the development of conjunctival melanomas could be detected, there existed

some evidence that sunlight might play a role in profoundly influencing the host-tumor immune interaction during early stages of melanoma growth, giving rise to the development of conjunctival melanomas.

- ***Hormonal influence***

It has been suggested that pregnancy may promote the biological behavior of malignant melanomas (Grin *et al,* 1996), but the data have been conflicting. Some single case reports indicated that the tumor progression of cutaneous and conjunctival melanoma may occur during pregnancy (Jay, 1965; Feffeira *et al,* 1998). Paridanes *et al* (1991) found that in a group of 15 women with conjunctival melanomas, 40% of cases were shown to have estrogen receptor positiveness, and in the control group (normal conjunctival tissue specimens of gender matched patients) the estrogen receptor was negative. In contrast, a study by Foss *et al* (1995) showed that most of conjunctival melanomas stained strongly for heat-shock protein 27 but none of melanomas showed positive nuclear staining for either the estrogen or the progesterone receptor. However, Dunean and associates (1994) in a study of cutaneous melanomas called attention to the fact that estrogen may have non-receptor-mediated effects and that hormone levels still could affect melanocytes by other means. Clearly, the role of hormones in conjunctival melanomas is unknown.

- ***Genetics***

Progression from normal melanocyte to metastatic melanoma is a multistep, multigenetic process. The complex series of progressive events involve melanoma cell attachment to matrix components and localized proteolysis of the ECM,

followed by migration through the basement membrane and movement into the circulation or lymphatics, invasion of the surrounding tissue, and finally proliferation in the new micro-environment (Smith *et al*, 1996). It is clear that progression of melanomas involves numerous genetic alterations.

Cytogenetics Cytogenetic and functional studies have identified at least six chromosomal regions that presumably harbor mutations important in the development and/or progression of cutaneous melanomas and in melanoma cell lines. These changes map to chromosome areas 1p, 6q, 7q, 9q, 10q and 11q. Yet, only one melanoma-predisposing gene, which is the mutated cyclin-dependent kinase (cdk) inhibitor, mapping to chromosome 9p21, has been confirmed in clinical studies so far (Welch & Goldberg, 1997). Uveal melanomas are usually sporadic in the absence of obvious genetic predisposing factors (Singh *et al*, 1996). In the study of White *et al* (1998), they found that in the large posterior uveal melanomas the presence of cytogenetic abnormality of chromosome 3 and 8 was associated with a poor outcome, and an abnormality of chromosome 6 appeared to have a protective effect. Cytogenetic studies also suggest that chromosomal abnormalities differ between uveal melanomas and conjunctival melanomas (Char, 1989). To date, there is no recognized chromosomal abnormality implicated in the tumorigenesis of conjunctival melanomas (Seregard, 1998).

Molecular genetics Oncogene research has been prolific over the past 10 years. The study of oncogenes has considerably advanced our understanding of the molecular mechanisms leading to cancer. There are two types of molecular tumor markers, which include both dominantly acting transforming genes and tumor

suppressor genes. The dominant transforming genes, collectively called "oncogenes", are altered forms of normal cellular genes called "proto-oncogenes." Proto-oncogenes are highly conserved in evolution, and their products are important regulators of normal cell growth and differentiation. The oncogenes, on the other hand, are not found in normal cells, but are generated by the activation of their corresponding proto-oncogenes during tumor development (Wallis & Macdonald, 1999). The oncogene activation occurs by amplification, or transposition, or point mutation. Their products have often been shown to be important components of intracellular signaling pathways that regulate cell proliferation in response to growth factors stimulation. These products include growth factors, growth factor receptors, signal transducers, and transcription factors. Oncogene functions also involve the direct control of the cell cycle and inhibition of apoptosis.

Ras oncogene Ras oncogene is a membrane associated GTPase protein and is a crucial regulator of cell shape, motility and growth, downstream from growth factor receptors. The Ras signalling system is particularly important with respect to malignant transformation. It contains molecules with important oncogenic roles in human tumorigenesis, which activate a series of kinases and ultimately lead to phosphorylation of nuclear transcription factors therefore altering gene expression (Wallis & Macdonald, 1999). Langmann *et al* (1993) evaluated the expression of the Ras oncogene in uveal, iris, and conjunctival melanomas. They found that in the case of metastatic melanomas, including metastatic conjunctival melanomas, marked expression of Ras p21 and pan Ras oncogenes was obvious. All other melanomas showed a moderate expression pattern for these oncogenes.

P53 oncogene P53 oncogene is a tumor suppressor gene and a critical controller of the normal growth of cells. Normal p53 is a sequence specific DNA binding protein that functions as a transcription factor. It acts as a "guardian of the genome" by preventing the proliferation of cells with damaged DNA by shutting down the cell cycle clock until the DNA damage is corrected, or alternatively it will initiate cell death through apoptosis. More than 50% of human tumors were found to express mutated p53 (Lecine *et al*, 1991; Hollstein *et al*, 1991; Darnton 1998). A signal case report by Tucker *et al* (1994) suggested that p53 gene alteration was recognized in PAM with atypia that later progressed to the conjunctival melanoma. However, Seregard (1996) was convinced that p53 alterations were not significant events in the development of conjunctival melanomas. The absence of p53 expression in PAM and minimal staining of conjunctival melanomas did not correlate with the cell growth, suggesting that alterations of p53 were uncommon, and may only be late events in conjunctival melanomas.

Cytokine genes Conjunctival melanomas, which have a relatively good prognosis as compared to other melanomas, have been investigated morphologically and pathologically. Recently, a case study by Abe *et al* (1998) examined the expression of several cytokine genes in conjunctival melanoma and other pigmented cells of the eye. It found that potent inhibitors of tumor cell growth such as interleukin (IL) 2, IL 4, IL 6 and γ-interferon (IFN) were expressed in conjunctival melanoma cells, and not in other pigmented cells of the eye. The basic fibroblast growth factor gene, which has also been known to stimulate melanoma cell growth, was not expressed in the conjunctival melanoma, and it showed +/- or weak

expression in the pigmented cells of the eye and in the conjunctival melanosis. Although only limited cytokine gene expression was examined, these results may suggest an influence of these cytokines on the growth of conjunctival melanomas.

1.4d.iv Metastasis and mortality

The incidence of local recurrence of conjunctival melanomas ranges from approximately 18 to 56% (De Potter *et al,* 1993). In 1994, the study by Paridaens *et al* indicated that the 5-year survival of conjunctival melanomas was 82.9% and 10-year 69.3%. Recently, Chang *et al* (1998) analyzed the National Cancer Data Base (NCDB) in the United States on cutaneous and noncutaneous melanomas between 1985 through 1994. They showed that the 5-year mortality of conjunctival melanomas was 16.5%, which was the best prognosis compared with other melanomas. From these data we can recognize that even though conjunctival melanomas are rare, they are still potentially life threatening tumors.

Conjunctival melanomas share with cutaneous melanomas a definite tendency to invade the lymphatic system and spread initially to the regional lymph nodes (Mclean *et al*, 1994; Sepencer & Zimmerman, 1996). The conjunctiva is richly supplied with lymphatic channels, some of which are situated very superficially. Thus, even minimally invasive, conjunctival melanomas have the potential for reaching the lymphatic circulation. Once tumor cells have gained access to the lymphatic system, they may also acquire the potential for hematogenous

dissemination through vessels within the lymphatic system. Eventually metastases from conjunctival melanomas may present in most sites of the body.

There are four major factors influencing the recurrence and metastasis of conjunctival melanomas (Crawford, 1980; Jakobiec, 1980; Folberg *et al*, 1985; Fuchs *et al*, 1989; Lommatzsch *et al*, 1990; De Potter *et al*, 1993; Paridaens *et al*, 1994). They include: 1) the location of the tumor, which involves the palpebral, fornices, polica, caruncle, and lid margins; 2) the initial thickness of the tumor, whether it is more than 1~4 mm; 3) tumor cell types, which involve both the epithelial cell type and the mixed cell type; 4) the multicentric feature of the tumor. Interestingly, the study by De Potter *et al* (1993) found that the only risk factor statistically associated with local recurrence and distant metastasis of conjunctival melanomas was the primary method of tumor management. They suggested that a carefully planned wide excision of the tumor and supplemental cryotherapy were beneficial in reducing the risk. So far the prediction of the metastatic behavior of conjunctival melanomas remains difficult.

1.4e Immunohistochemistry profile

Although most conjunctival melanomas are easily identified through the examination of hematoxylin and eosin (H&E) stained slides by light microscopy, there are some that may be difficult to differentiate from pigmented or non-pigmented carcinomas of the conjunctiva. The increased availability of numerous antibodies reacting with formalin-resistant epitopes has made immunohistochemistry

a routine procedure in the evaluation of melanocytic tumors (Wollina *et al,* 1991; Ruiter & Brőcker, 1993; Bhan, 1995; Jensen *et al,* 1997).

At present, melanocytic differentiation markers are mostly used in clinical diagnosis and research fields. A differentiation marker should have a preference for all melanocytic lesions and either not be detectable or rarely be detectable in normal cell types, or their corresponding tumors. Although all types of melanocytic lesions may show the expression of differentiation antigens, this may be only focal or may even be lost because of progressive cellular heterogeneity in advanced primary and metastatic melanomas. This phenomenon may hamper the reliability of immunohistochemistry in the diagnostic process.

1.4e.i S-100 protein

The S-100 protein is an acidic, calcium–binding protein that was isolated from bovine brain extract. It consists of α-α, α-β, and β-β, subunits, respectively. S-100 protein is not only distributed abundantly in the nervous system of vertebrates but also in several non-neural cells such as melanocytes, Langerhans cells, chondrocytes, and myoepithelial cells. The presence of S-100 protein can be demonstrated in practically all types of melanocytic lesions, including amelanotic melanomas and their metastases (Cochran *et al,* 1982; Nakajima *et al,* 1982; Stefansson *et al,* 1982; Cochran *et al,* 1993; Blessing *et al,* 1998). Polyclonal and monoclonal antibodies against S-100 protein are effective on paraffin sections (Loeffel *et al,* 1985; Fujita *et al,* 1991). The staining pattern is both intracytoplasmic and intranuclear. Most

studies show an intense diffuse staining. S-100 protein is very sensitive for both nevocytes and melanoma cells. Therefore, it cannot differentiate benign from malignant melanomas. Its high sensitivity, which was positive in 82.6 - 100 % of conjunctival melanomas (McDonnell *et al*, 1991; Steuhl *et al*, 1993), paired to a low specificity. Because S-100 protein is detectable in a variety of benign melanocytic lesions and other tissue tumors, such as peripheral nerve tumors, this phenomenon limits its value in the tumor diagnosis.

1.4e.ii HMB-45

HMB-45-defined antigen resides in melanosomes before melanin deposition in the cell cytoplasm (Bacchi *et al*, 1996). HMB-45 is a mouse monoclonal antibody against a whole cell extract of a human melanoma (Gown *et al*, 1986). Over the years, it has been demonstrated that it is a highly sensitive and specific reagent for the identification of melanomas (Ordõñez *et al*, 1988; Burnier *et al*, 1991; Zimmer *et al*, 1991). HMB-45 immunoreactivity is seen in normal fetal and neonatal melanocytes and not in adult resting melanocytes, whereas it is detectable in junctional nevi and not in intradermal nevi (Bacchi *et al*, 1996). The staining pattern is intracytoplasmic and often heterogenous. Steuhl *et al* (1993) found that more than 95% of conjunctival melanomas expressed HMB-45 marker. At the site of tumor invasion, infiltrating cells showed increased HMB-45 reactivity. Unfortunately, unlike cutaneous intradermal nevi, conjunctival subepithelial nevi contain HMB-45 positive cells. Glasgow *et al* (1990), McDonnell *et al* (1991), and Steuhl *et al* (1993)

found that HMB-45 antibody did not appear useful in distinguishing between different conjunctival melanocytic lesions.

1.4f Prognostic studies

As tumors grow, qualitative changes occur in a process termed tumor progression. During progression, subsets of cells develop the ability to metastasize (Welch & Tomasovic SP, 1985; Miller & Heppner, 1990). Survival time for patients is directly related to the rapidity of the metastatic process. In order to retard melanoma-related mortality, it is essential to lower or eradicate the metastatic process. Therefore, it is necessary to increase our knowledge of mechanisms underlying metastasis and to identify reliable progression parameters for use as prognostic markers in conjunctival melanomas.

1.4f.i Melanocyte proliferation

Proliferation is one of the most fundamental biological processes because of its role in normal growth and maintenance of tissue homeostasis as well as in tumor expansion. The assessment of proliferation has become popular in histopathology as a means of predicting the behavior of tumors - that is, their likelihood of local recurrence, their metastatic potential, and the role of growth in metastasis (Diest *et al*, 1998). However, Farber (1996) doubted these theories and argued that cell proliferation is not always a major risk factor for cancer. The following facts support his opinion. Stomach carcinomas with atrophic rather than hypertrophic gastritis

have a greater risk of malignancy. Many carcinogens of quite different chemical compositions are clear-cut inhibitors of cell proliferation in various organs and tissues, implying that cell proliferation is a minor component of carcinogenesis.

The proliferating activity of a tumor has been assessed by various methods, which include mitotic counts, incorporation of the tritiated thymidine or bromodeoxyuridine, flow cytometric DNA analysis, and detection of the proliferation-associated antigen by immunohistochemistry. The proliferating activity in malignant melanomas has been found to correlate well with the tumor thickness, mitotic counts, and progression. The study of cell proliferation has advanced considerably owing to the identification of non-histone nuclear proteins such as Ki-67 and proliferating cell nuclear antigen (PCNA) within the nuclei of dividing tumor cells. By defining tumor proliferation by Ki-67 antigen and PCNA expression, it is possible to identify those melanocytic lesions which carry a high risk of progression to malignant melanomas (Smolle *et al*, 1989; Dervan *et al*, 1992; Stone *et al*, 1996; Karlsson *et al*, 1996).

Seregard (1993) investigated expression of PCNA in 20 specimens of conjunctival melanoma. He found that patients who subsequently died of metastatic disease had significantly higher PCNA counts than patients with a survival of at least five years without clinical signs of metastatic disease. Therefore, PCNA may be used as a prognostic indicator in conjunctival melanomas. In two other studies Seregard (1996) and Chowers *et al* (1998) assessed the proliferation activity of PAM with atypia compared with PAM without atypia. Results showed that PAM with

atypia had significantly higher Ki-67 and PCNA positive cell counts than PAM without atypia.

1.4f.ii The initial tumor thickness

The association of the depth of invasion of an invasive cutaneous melanoma with increased risk of metastasis is a fundamental observation. Consequently, the same statement was found to be true in prognostic studies of conjunctival melanomas. Silvers and co-workers (1978) recognized that none of the patients with conjunctival melanomas less than 1.8 mm thick died of metastastic melanomas. Jakobiec (1980) commented that the tumor thickness was the most important prognosticator for conjunctival melanomas. Folberg et al (1985) confirmed that the thickness of the patient's thickest conjunctival melanoma was inversely correlated with the survival for patients who had melanomas with PAM, and all lesions measuring more than 0.8 mm thick were at high risk for the development of metastases. Lommatzsch et al (1990) also confirmed the rate of metastases of conjunctival melanomas larger than 2 mm thick was 31.4% and significantly higher than that of the group with smaller tumors, with 11.1%. A large series study of conjunctival melanomas by Paridaens et al (1994) found that the initial tumor thickness of the tumor was significantly associated with survival. However, the study identified a significant interaction between tumor thickness and tumor location on patient survival.

In conclusion, there appears to be a continuous worsening of the prognosis with increasing tumor thickness, but no clear threshold to determine the prognosis has been established.

1.4f.iii Intermediate filaments

Recently, a series of exciting studies of intermediate filaments (IFs) in tumor cells have shown a correlation with recurrence and metastases in malignant melanomas and carcinomas. Methods used in these studies included: 1) the immunohistochemical analysis for detecting IF antigen expression in tumor cells; 2) an *in vitro* invasion assay for examining the invasion potential of tumor cells, 3) a semi-qualitative Western blot analysis for demonstrating IF protein; 4) the Northern blot analysis to indicate the relative amount of IF mRNA expression; 5) the use of keratin 8, 18 antisense inhibitor in the highly metastatic tumor cell lines, or transfected by keratin 18 cDNA in the lower metastatic tumor cell lines to observe their effects on invasive ability. These data, combined with changing views of IFs within the cancer literatures of the last decade, underscore the evolving role of IFs as dynamic cytoskeletal structures involved in cell cycle signaling, cellular differentiation, and pathogenesis.

IFs in eukaryotic cells constitute a considerable part of cytoskeleton proteins in addition to actin filaments and microtubules (Lazarides, 1980; Geiger, 1987; Steinert & Roop, 1988). IFs derive their name from their diameter (10 nm), as compared to actin filaments (6 nm) and microtubules (23 nm). The structure of IFs is rod-like,

and comprised of trimer subparticles (Osborn & Weber, 1982; Skalli & Goldman, 1991; Fuchs, 1994). IF proteins, which include the nuclear lamins, sometimes share as little as 20% sequence identity. The approximately 50 members of the IF super-family exhibit cell-type-specific and often complex patterns of expression. Immunological and biochemical criteria allow us to distinguish at least 6 different types of IFs (Moll *et al*, 1982; Hendrix, 1996), which include 1) keratins that are characteristic of epithelial cells; 2) vimentin filaments that occur in mesenchymally derived cells, astrocytes, sertoli cells, vascular smooth muscle cells, and many cultured cell lines; 3) desmin filaments that are typical of most types of myogenic cells; 4) neurofilaments that are typical of neuronal cells; 5) glial filaments that are typical of astrocytes; 6) nestin that was newly discovered in melanoma. During the cell transformation and tumor development, the cell type specificity of IFs is largely conserved, and classification of tumors by their specific type of IFs has recently become very valuable in clinical histodiagnosis (Steiner & Liem, 1990; Oshima, 1992; McLean & Lane, 1995; Klymkowsky, 1995).

- ***Keratin 8 and 18***

Keratins are the largest and most complex group of IF proteins. They are further divided into type I keratins, which are basic and include K1 - K8, as well as type II, which are acidic and include K9 - K20. Keratins are expressed as heteropolymer pairs consisting of specific type I and type II proteins. Their expression is highly regulated during the embryonic development and cellular differentiation; for example, keratin 8 and 18 are first expressed in almost all of simple epithelial cells

during embryogenesis. When cells differentiate, they stop making the simple epithelial keratins, and start to express other keratins (Klymkowsky, 1995; Hendrix *et al*, 1996). This more primitive phenotype is re-expressed during the development of malignant neoplasms in an analogous fashion to the retrograde-expression of other oncofetal antigens, which is referred to as the "dedifferentiated" phenotype. For example, carcinomas may not continue to express keratins presented in the tissue of origin and, further, may produce additional keratins such as keratin 8 and 18 that are absent in the normal tissues. The immunohistochemical demonstration of keratins in these so-called "dedifferentiated" neoplasms has become the current standard in differential diagnosis of carcinomas versus amelanotic melanomas (Miettinen *et al*, 1985; Zarbo *et al*, 1990).

- ***Vimentin***

Vimentin belongs to type III of IF proteins, and is expressed as a homopolymeric intermediate filament. It is most widely expressed by most mesenchymal cells and a variety of transformed cell lines and tumors (Osborn & Weber, 1983). During embryogenesis, mesenchymal cells initially appear to express vimentin, followed by the appearance of differentiation-specific IFs and later by the disappearance of vimentin in a series of steps leading to cellular differentiation (Dahl, 1981; Raymond & Leong, 1989; Klymkowsky, 1995). Vimentin is also found to be expressed by normal melanocytes and malignant melanoma cells. The immunohistochemical diagnosis of melanomas depends upon a few unique antigens present in the membrane and cytoplasm of melanocytes, which include vimentin. Huszar *et al*

(1983), Caselitz *et al* (1983), and Gown *et al* (1985) found that all cutaneous melanomas were vimentin positive and keratin negative. They confirmed that vimentin could serve as a useful differentiation marker in determining the origin of various cells and tissues.

- ***Co-expression of IFs***

Most major cell types *in situ* contain only a single IF protein type (Osborn & Weber, 1982). The expression of vimentin is traditionally regarded to be a marker of cells of mesenchymal origin. On the other hand, keratin is regarded as a marker of cells of epithelial origin. The co-expression of keratin and vimentin appears to be a rare event *in situ* but may occur in certain instances, *e.g.*, in some cells of the human parotid gland (Caselitz *et al*, 1981). Lane *et al* (1983) reported that the co-expression of keratins and vimentin was found *in situ,* in the parietal endoderm of the mouse embryo at 8.5-13.5 days old. Ramaekers *et al* (1983) described co-expression of keratins and vimentin in metastatic human carcinoma cells present in ascites and pleural fluids. They suggested that the presence or absence of an additional vimentin might influence mitotic as well as motile activity of tumor cells. In 1989, Raymand and Leong found that there was a positive relationship between vimentin expression in infiltrating ductal carcinoma and high rates of tumor growth. The expression of vimentin in carcinomas was potentially a predictor of the aggressive tumor cell behavior.

The same situation seems to occur in malignant melanomas. In 1990, Zarbo *et al* performed the one- and two-dimensional western blot analysis and an immunohistochemical survey of 100 cutaneous melanomas, and found anomalous keratin expression occurred only in metastatic or recurrent melanomas. This study raised an interesting question of possible association with melanoma progression. Furthermore, other studies (Hendrix *et al,* 1992; Fuchs *et al,* 1992; Ben-Izhak *et al,* 1994; Chu *et al,* 1996; Katagata & Knodo, 1997) showed that the co-expression of keratin 8, 18 and vimentin in human cutaneous or uveal melanomas was associated with increased invasion and metastatic potential. Hendrix's group (1998) confirmed that human uveal melanoma cells which predominantly co-expressed keratin 8, 18 and vimentin showed 6-fold more invasion through ECM as compared with uveal melanoma cells expressing vimentin only, and were 8- to 13-fold more invasive than normal uveal melanocytes. When they treated the melanoma cells with antisense olignonucleotides to keratin 8, 18, these cells were predominantly vimentin-positive and keratin-negative, and showed a significant decrease in migratory ability. These findings provided the justification for additional studies of the association between the co-expression of keratins and vimentin and metastases of uveal melanomas.

The persistent expression of keratin 8, 18 in melanoma cells may reflect the activation of intracellular signaling pathways (Oshima *et al,* 1990; Wasylyk *et al,* 1993; Pankov *et al,* 1994; Werner *et al,* 1995; Kodandapani *et al,* 1996; Oshima *et al,* 1996).

The cellular transformation results in the altered transcription of a number of cellular genes, some of which are likely important for tumor growth, progression,

invasion, and metastasis. Tumorigenic signals, which could come from neighboring cells or ECM, stimulate multiple cell surface receptors that include epithelial growth factor receptor (EGFR), neu-oncogene, and integrins. These receptors further activate the Ras signaling pathway that activates the E26 transformation-specific oncogene (Ets) family of transcription factors through phosphorylation. Ets transcription factors have two functions to influence the production of keratins, one of which acts through the intron enhancer of K18. and the other activates the AP-1 transcription family through Jun and Fos. These transcription factors act like a cascade to promote the expression of keratin 8, 18.

The co-expression of keratins and vimentin by carcinoma or melanoma cells is advantageous for migratory and invasive functions, due to unique interactions between cell surface receptors, cytoskeleton proteins, and ECM. Growing evidence has shown that IFs influence the invasion and metastasis of tumor cells through the family of integrins (Ingber, 1991; Hynes, 1992; Seftor *et al*, 1992; Juliano & Haskill, 1993; Albelda, 1993; Qian *et al*, 1994; Hendrix *et al*, 1996; Danen *et al*, 1998). The integrin-mediated signaling may have important effects on cell adhesion and migration. Integrins are transmembrane glycoproteins comprised of heterodimeric α and β subunits, which are widely distributed on many cell types, linking the internal cytoskeletal network of cells with the extracellular environment. Because of the complexity of the integrin family, it has been useful to group integrins according to their cell-binding activity. They can be segregated into one of three groups: those that function as cell-cell adhesion molecules, those that bind primarily to major constituents of the basement membrane, and those that bind primarily to ECM

proteins. They play a critical role in the key steps of metastasis: adhesion, migration and invasion (Akiyama *et al*, 1990; Albelda *et al*, 1990; Ruoslahti, 1992).

Metastasis is a complex, yet well co-ordinated, process that can be defined by a series of integrated events: cell attachment to ECM, proteolytic dissolution of the matrix, and movement of cells through the digested barrier (Liotta, 1986). The transformation of melanocytes into malignant melanoma cells results in changes to their repertoire of cell surface markers, most notably increased expression of some integrin subunits, such as $\alpha v\beta 3$, $\alpha 4\beta 1$ (Albelda *et al*, 1990; Li *et al*, 1998; Danen *et al*, 1998). Integrins have two key functions. One is the internal regulation of the cellular affinity (inside to out), and another is the external modulation of the cellular behavior by ECM (outside to in). Tail parts of integrins are located in the cytoplasm connecting with cytoskeletal proteins. In this case, IF proteins not only mediate the cell shape and spreading but also act as signal transducers, which transmit signals from ECM to the cell nucleus through the integrins, to further regulate gene expression as well as modulate cellular functions. Therefore, it has become clear that there is a versatile and complex array of interactions, modulations, and signaling events in which integrins play a central role during the tumor cell growth, invasion, and metastasis.

Chapter 2: Materials and Methods

2.1 Materials

2.1a Tissue specimens

A compilation was made of 54 melanocytic lesions of the conjunctiva, spanning a 14-year period, from 1985 to 1999. These specimens were all formalin-fixed, and paraffin-embedded, and included 20 cases of conjunctival nevus, 19 cases of PAM, and 15 cases of conjunctival melanoma. All cases were obtained from the Ophthalmic Pathology Registry of McGill University.

2.1b Primary antibodies

In this study, primary antibodies against melanocytic differentiation antigens – S-100 protein and HMB-45, were used to further confirm the diagnosis of all cases. Primary antibodies against intermediate filament antigens – keratin 8, 18 and vimentin, were used to confirm the hypothesis of this thesis. Their applications are summarized in Table 2.1.

2.1c Biotinylated secondary antibodies

The biotinylated secondary antibodies serve as an efficient "link" between a primary antibody and a complex substrate labeled with enzymes. According to different primary antibodies, the "link" antibody should be chosen appropriately. If the primary antibody was a monoclonal antibody from mouse, the "link" antibody should be the biotinylated rabbit anti-mouse immunoglobulins. In contrast, if the primary antibody was a polyclonal antibody from rabbit, the "link" antibody should be the biotinylated swine anti-rabbit immunoglobulins. Their applications are summarized in Table 2.2.

Table 2.1 Applied primary antibodies

Reagent	Clonality	Source	Supplier	Dilution
Anti-S-100 protein	polyclonal	rabbit	DAKO, Z311	1:300
HBM-45	monoclonal	mouse	DAKO, M634	1:50
Anti-vimentin (V9)	monoclonal	mouse	DAKO, M725	1:30
Anti-keratin 8, 18 (Cam 5.2)	monoclonal	mouse	Becton-Dickinson, 349205	1:10

Table 2.2 Applied biotinylated secondary antibodies

Reagent	Immunogen	Source	Supplier	Dilution
Swine anti-rabbit	immunoglobulins	rabbit	DAKO, E353	1:500
Rabbit anti-mouse	immunoglobulins	mouse	DAKO, E354	1:200

2.2 Methods

2.2a Tissue processing

After local surgical removal tissues were routinely formalin-fixed (10% formalin for 24 hours), and paraffin-embedded. Paraffin sections were cut at 5 μm, mounted on commercially provided silanized slides (Surgipath, Snowcoat, X-tra), and dried overnight at 37°C, or at 60°C for at least 60 minutes (min) for the immunostaining.

2.2b Hematoxylin and eosin (H&E) staining

Sections of conjunctival melanocytic lesions were cut at 5 μm thickness from routinely processed paraffin blocks, and stained with the standard staining procedure of hematoxylin and eosin (H&E) for visual assessment and measurement of the initial tumor thickness.

2.2c Immunohistochemistry staining

Immunohistochemistry staining was manually carried out by the avidin-biotin complex (ABC) method, including heat-induced antigen retrieval and enzymatic antigen retrieval procedures (Pinkus *et al*, 1985; Lan *et al*, 1995; Shi *et al*, 1995; Hazelbag *et al*, 1995; Fan *et al*, 1997). The ABC method belongs to a modification of indirect enzyme-linked immunohistochemistry resulting from the application of the high affinity of avidin for biotin to link reactants in multistep staining procedures. Avidin, an eggwhite 68-kD glycoprotein, has four binding sites for the vitamin biotin. Generally, biotin has been covalently conjugated with the secondary

antibodies as the link antibody. Open sites on avidin from the avidin-biotin-horseradish peroxidase (ABC) complex bind to the biotin on the link antibody. Furthermore, upon oxidation, added chromogens become colored products to indicate the immune reaction in tissue cells. The strong affinity of avidin for biotin makes the ABC method highly sensitive. With this method, excellent results can be achieved on formalin-fixed, paraffin-embedded specimens.

Briefly, slides were deparafinized in xylene, rehydated through 100% ethanol, and treated with 0.3% H_2O_2 in methanol for 30 min at room temperature (RT) in order to block endogenous peroxidase activity. The heat induced epitope retrieval (HIER) procedure was carried out in 0.75M Tris base (pH 10) in a 100°C water bath for 30 min. Except for vimentin, the other antigens required the use of 0.1% trypsin (pH 7.8) digestion at 37°C for 10 min to reveal the specific epitopes. All immunoreagents were diluted with 0.05M Tris-buffered saline (TBS, pH 7.6) containing 2% (w/v) bovine serum albumin (BSA). Between every step, the slides were washed for two 5-min changes in TBS. All procedures were executed at RT (~20°C). The incubation with primary antibodies was carried out in a moist chamber for 1 hour (hr) at 37°C. The slides were then incubated with the appropriate biotinylated secondary antibody, and further with the streptavidin and biotinylated horseradish peroxidase reagents (StreptABComplex/HRP, DAKO, K377) in a moist chamber at 37°C for 30 min, respectively. Subsequently, slides were incubated with 3-amino-9-ethylcarbazole (AEC) chromogen (DAKO, K3469, ready-to-use) in a moist chamber for 5-10 min. While monitoring the resulting red stain microscopically, the reaction was stopped, immersed in cold tap water,

counterstained with Gill II hematoxylin (Surgipath), and mounted with aqueous mounting medium (DAKO, S3025).

Negative control slides were treated in an identical manner except that the primary antibodies were omitted.

Positive control slides consisted of stock tumors or normal tissues known to contain the determinant of interest. They included the optic nerve tissue for S-100 protein, skin melanoma for HMB-45, conjunctival epithelium and conjunctival cyst epithelium as internal positive controls for keratin 8, 18, and conjunctival blood vessels as internal positive controls for vimentin.

2.2d Evaluation of staining results

Staining results were evaluated by two investigators independently. When scoring results diverged, the agreement was reached through discussion.

2.2d.i Classification of melanocyte lesions

The diagnosis of melanocyte lesions of the conjunctiva was confirmed by examination of H&E slides under the microscope using standard histopathologic criteria.

2.2d.ii Grading of the immunostaining

The degree of immunostaining was evaluated for all melanocytic lesions. It was defined on a two-score grade: - for negative, + for positive. The positive patterns were further scored as focal or diffuse. Positive staining was considered diffuse if

most melanocytes (approximately more than 80%) reacted with a particular antibody, and focal if staining occurred in only a small number (approximately less than 20%) of melanocyts in the sample.

2.2e Measurement of the initial tumor thickness

In order to determine the correlation between the co-expression of IFs and the prognosis in conjunctival melanomas, we used the initial tumor thickness as the parameter. The initial tumor thickness was measured only for conjunctival melanomas. The quantitative technique used was identical to that used by Silvers and co-workers (1978), which was a modification of the technique of Breslow (1970, 1975) for measuring the thickness of cutaneous melanomas. By means of an ocular micrometer, calibrated to the working microscope, the maximal thickness of the tumor was measured on the H&E stained slide from the surface of the epithelium of the conjunctiva to the tumor's deepest level of invasion.

Chapter 3: Results

3.1 Patient characteristics

3.1a Conjunctival nevi

There were 20 cases of conjunctival nevus, including 17 compound nevi (85%) and 3 subepithelial nevi (15%). In total cases, there were 7 males (35%) and 13 females (65%), whose age range was from 7 to 82 years old, with the mean age of 44.20 ± 21.60 (standard deviation, SD) years old. These characteristics are summarized in Table 3.1.

3.1b Primary acquired melanosis

There were 19 cases of PAM, including 8 PAM with atypia (42.11%) and 11 PAM without atypia (57.89%). Among all cases, there were 8 males (42.11%) and 11 females (57.89%), whose age range was from 29 to 85 years old, with the mean age of 54.22 ± 18.01 (SD) years old. These characteristics are summarized in Table 3.2.

3.1c Conjunctival melanomas

There were 15 cases of conjunctival melanoma, including 13 mixed cell types (86.67%), 1 small polyhedral cell type (6.67%), and 1 epithelial cell type (6.67%). Among all cases, there were only 2 cases with nodular growth patterns, and the rest were all with diffuse growth patterns. There were 8 males (53.33%) and 7 females (46.67%), whose age range was from 37 to 86 years old, with the mean age of 62.40 ± 18.55 (SD) years old. These characteristics are summarized in Table 3.3.

Table 3.1. Patient characteristics for conjunctival nevi

No. of patients	20
Gender (M / F)	7 / 13
Age (yrs ± SD*)	44.20 ± 21.60
Type (Compound / Subepithelial)	17 / 3

*Data presented as the mean ± standard deviation (SD)

Table 3.2. Patient characteristics for primary acquired melanosis

No. of patients	19
Gender (M / F)	8 / 11
Age (yrs ± SD)	54.22 ± 18.01
Type (with atypia / without atypia)	8 / 11

Table 3.3. Patient characteristics for conjunctival melanomas

No. of patients	15
Gender (M / F)	8 / 7
Age (yrs ± SD)	62.40 ± 18.55
Type (M / P / E)*	13 / 1 / 1
Pattern (D/ N)†	13 / 2

* M mixed cell conjunctival melanomas; P polyhedral cell conjunctival melanomas; E epithelial cell conjunctival melanomas. † D diffuse growth patterns; N nodular growth patterns.

3.2 Immunostaining characteristics

Levels of the expression of S-100 protein, HMB-45, vimentin, and keratin 8, 18 varied among the 54 melanocytic lesions of the conjunctiva. No immunostaining was seen in the negative control sections.

3.2a Conjunctival nevi

Compound nevi in 17 cases of compound nevus, 16 cases (94.12%) were S-100 protein positive, which included 15 diffuse and 1 focal staining pattern, whereas only 1 case (5.88%) presented a negative reaction for S-100 protein. There were 13 cases (76.47%) of HMB-45 positive staining, which included 10 focal and 3 diffuse staining patterns. Four cases (23.53%) were negative for the HMB-45 marker. All cases (100%) presented vimentin positive reactions, which included 15 diffuse and 2 focal staining patterns. Interestingly, 3 compound nevi (17.65%) showed keratin 8, 18 focal positive reactions, and the rest (82.35%) were negative for keratins.

Subepithelial nevi all 3 cases of the subepithelial nevus presented diffuse positive staining for both S-100 protein (100%) and vimentin (100%), whereas they were all negative for both HMB-45 (100%) and keratin 8, 18 (100%).

3.2b Primary acquired melanosis

PAM with atypia in the 8 cases of PAM with atypia, 7 cases (87.5%) presented positive reactions for S-100 protein, which included 1 diffuse and 6 focal staining patterns, whereas only 1 case (12.5%) presented a negative reaction for S-

100 protein. All 8 cases (100%) presented HMB-45 positive reactions, including 6 diffuse and 2 focal staining patterns, and all cases (100%) were positive for vimentin, including 2 diffuse and 6 focal staining patterns. In anti-keratin 8, 18 staining of slides, we found that the whole epithelium of the conjunctiva, with the exception of the stratified squamous component, showed diffuse positive staining for Cam 5.2 monoclonal antibody (Plate 3.1). Since we could not determine whether melanocytes in PAM were positive or negative for keratin expression, we excluded immunostaining results of keratin 8, 18 in all cases of PAM.

PAM without atypia in 11 cases of PAM without atypia, there were 10 cases (90.91%) of focal positive reaction for S-100 protein, and only 1 case (9.09%) presented a negative reaction for S-100 protein. 5 cases (45.45%) showed HMB-45 focal positive reactions, and the rest (54.55%) were HMB-45 negative. For vimentin staining, 9 cases (81.82%) presented positive reactions, which included 4 diffuse and 5 focal staining patterns, and 2 cases (18.18%) showed negative reactions.

3.2c Conjunctival melanomas

Mixed cell melanomas all 13 cases (100%) of mixed cell conjunctival melanomas presented diffuse positive staining for S-100 protein. They also presented positive reactions for HMB-45 (100%), which included 11 diffuse and 2 focal staining patterns. All cases (100%) presented positive reactions for vimentin, which included 12 diffuse and 1 focal staining patterns. In keratin 8, 18 staining, 5 cases (38.46%) showed focal positive reactions, and the rest (61.54%) presented negative staining for keratin.

Polyhedral cell melanoma there was only 1 case of polyhedral cell melanoma. It showed diffuse positive reactions for both S-100 protein and vimentin, as well as negative reactions for both HMB-45 and keratin 8, 18.

Epithelial cell melanoma there was only 1 case of epithelial cell melanoma. It presented diffuse positive reactions for S-100 protein, HMB-45, and vimentin, as well as a negative reaction for keratin 8, 18.

All the above characteristics of immunostaining are summarized in Table 3.4.

3.3 Characteristics of immuno-reactions of antibodies

Anti-S-100 protein in this study, anti-S-100 protein antibody was very sensitive but lacked specificity. Almost all of benign and malignant melanocytic lesions were positive for S-100 protein.

HMB-45 in this study, HMB-45 antibody had more specificity but less sensitivity. Almost all of conjunctival melanomas and all PAM with atypia were positive for HMB-45. Although 76% of compound nevi were positive, most positive nevocytes were focally located around the junction areas. All 3 subepithelial nevi were negative for HMB-45.

Anti-vimentin the anti-vimentin antibody seemed to be the most sensitive for melanocytic lesions of the conjunctiva. Except for 18% of PAM without atypia that were negative, all the rest of lesions including nevi, PAM, and melanomas showed positive reactions for vimentin.

Anti-keratin 8, 18 there were some interesting phenomena in keratin staining. 1). 38% of mixed cell conjunctival melanomas showed focal or zonal positive staining (For all kinds of conjunctival melanomas it was 33.33%). 2). 18% of compound nevi also showed focal positive staining (For all kinds of nevus it was 15%). 3). Since the epithelium (except the part of squamous epithelium) of the conjunctiva was also positive for keratin 8, 18, we could not distinguish epithelial cells from melanocytes in PAM.

These characteristics of the percentage of positive immuno-reactions are summarized in Table 3.5.

Table 3.4. Immunostaining characteristics

Diagnosis	S-100		HMB-45		Vimentin		Keratins	
	+	-	+	-	+	-	+	-
Nevi								
Com.	15d*	1	3d	4	16d	0	0d	14
	1f*		10f		1f		3f	
Sub.	3d	0	0	3	3d	0	0	3
PAM								
W/o	0d	1	0d	6	4d	2	∝	
	10f		5f		5f			
W/a	1d	1	6d	0	2d	0	∝	
	6f		2f		6f			
Melanomas								
M.	13d	0	10d	1	12d	0	0d	8
	0f		2f		1f		5f	
P.	1d	0	0	1	1d	0	0	1
E.	1d	0	1d	0	1d	0	0	1

* d diffuse staining; f focal staining. ∝ non-specific diffuse staining seen in all specimens. *Com.* compound nevi; *Sub.* subepithelial nevi; *W/o* PAM without atypia; *W/a* PAM with atypia; *M.* mixed cell conjunctival melanomas; *P.* polyhedral cell conjunctival melanomas; *E.* epithelial cell conjunctival melanomas.

Table 3.5. Characteristics of the percentage of positive immuno-reactions

	Nevi		PAM		Melanomas		
	Com.	**Sub.**	**W/a**	**W/o**	**M.**	**P.**	**E.**
S-100	94	100	88	91	100	100	100
HMB-45	76	0	100	45	100	0	100
Vimentin	100	100	100	82	100	100	100
Keratins	18	0	∝	∝	38	0	0

∝ non-specific diffuse staining seen in all specimens.

3.4 Characteristics of the co-expression of IFs

3.4a In conjunctival melanomas

Among all of the 15 cases of conjunctival melanomas, there were 5 cases (33.33%) that co-expressed both keratin 8, 18 and vimentin. These cases were all mixed cell types (Plate 3.2a, 3.3a) with diffuse growth patterns. The immunostaining of keratin 8, 18 presented zonal or focal positive distribution, in which keratin positive cells were mainly located around the peripheral area of tumors (Plate 3.2e, 3.3d) or the marginal area of tumor nests (Plate 3.3e,f). Two cases of conjunctival melanomas with nodular growth patterns both showed keratin negativity (Plate 3.4e) even though they were both big tumors with diameters of 5 and 14 mm, and all mixed cell types (Plate 3.4a). These characteristics are summarized in table 3.6.

3.4b In conjunctival nevi

Interestingly, the co-expression of IFs was also found in 3 cases (15.00%) of compound nevi with some large and atypical nevocytes (Plate 3.5a, 3.6a). The age range of these patients was from 7 to 26 years old, and all were female. The positive reactions of the co-expression of IFs were only found in those large and atypical nevocytes, which were mainly located near the junction areas (Plate 3.5e, 3.6e). These characteristics are summarized in Table 3.7.

3.5 The initial tumor thickness of conjunctival melanomas

In this study, the positive immunostaining for keratin 8, 18 all presented in conjunctival melanomas with diffuse growth patterns. In order to get the correct

statistical analysis, the initial tumor thickness was only measured in conjunctival melanomas with diffuse growth patterns, except for 2 nodular conjunctival melanomas. We blindly measured the tumor thickness of these 13 cases without knowing results of the immunostaining of IFs. Results showed the initial tumor thickness of conjunctival melanomas with the co-expression of IFs was obviously much thicker than conjunctival melanomas without the co-expression of IFs. In the group showing co-expression of IFs, the range of the tumor thickness was from 1.31 to 4.25mm, with a mean of 2.35 ± 1.32mm (SD). In the keratin negative group, the range of the tumor thickness was from 0.23 to 1.10mm, with a mean of 0.62 ± 0.24mm (SD). These characteristics are summarized in Table3.8.

Table3.6. Co-expression of IFs in conjunctival melanomas

Diagnosis	Age	Sex	Vimentin	Keratin 8,18
MCM.	37	F	+ diffuse	+ focal
MCM.	57	M	+ diffuse	+ zonal
MCM.	38	F	+ diffuse	+ focal
MCM.	81	M	+ diffuse	+ multi-focal
MCM.	76	F	+ diffuse	+ zonal

MCM. = mixed cell conjunctival melanoma

Table 3.7. Co-expression of IFs in conjunctival nevi

Diagnosis	Age	Sex	Vimentin	Keratin 8,18
Compound nevus	7	F	+ diffuse	+ focal
Compound nevus	13	F	+ diffuse	+ focal
Compound nevus	26	F	+ diffuse	+ focal

Table 3.8. The tumor thickness in conjunctival melanomas

	CKN* (+)	CKN (-)
Range (mm)	1.31 – 4.25	0.23 – 1.10
Mean (mm) ± SD*	2.35 ± 1.32	0.62 ± 0.24

* CKN keratin 8, 18; * SD standard deviation.

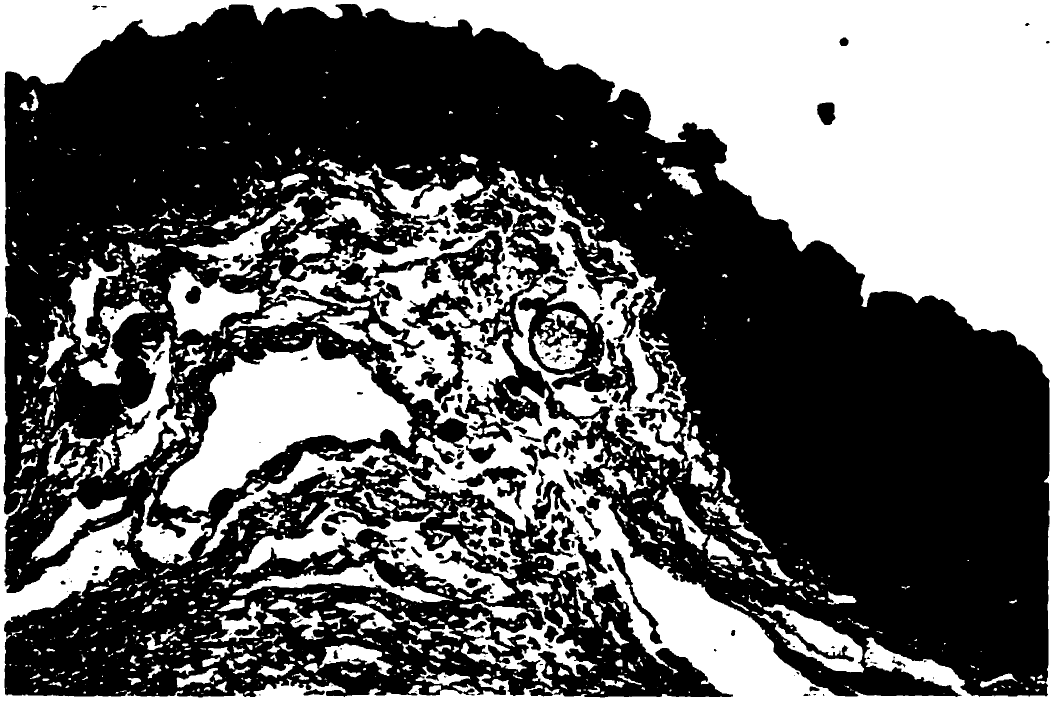

Plate 3.1 Keratin 8, 18 staining in a PAM with atypia. The whole epithelium of the conjunctiva presents diffuse positive staining, in which melanocytes can not be distinguished from epithelial cells.

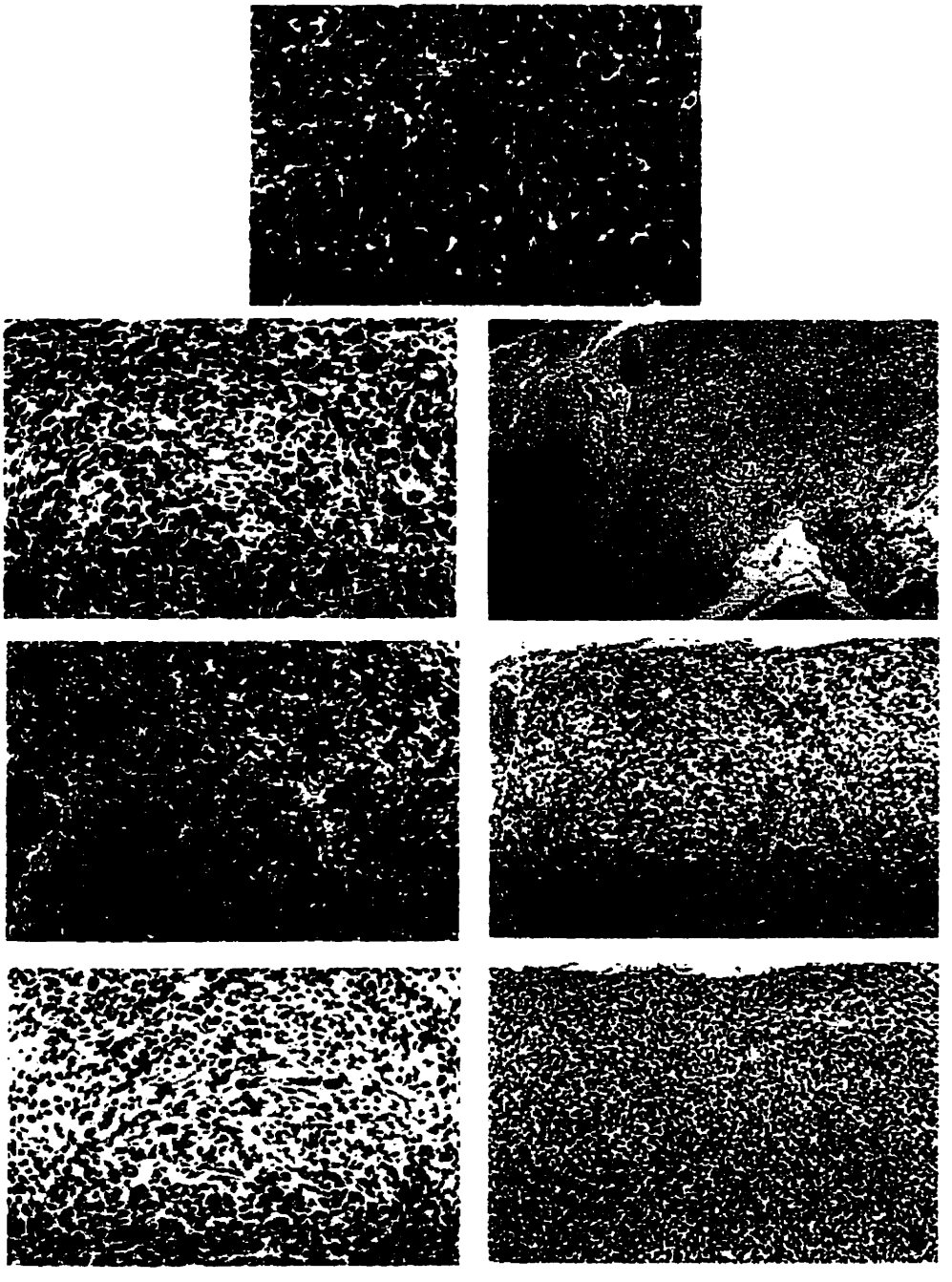

Plate 3.2 A mixed cell type conjunctival melanoma (H&E) (a). The diffuse positive staining of S-100 protein in both the cytoplasm and nucleus (b). HMB-45 staining is predominent in the cytoplasm and heterogenous (c). Diffuse cytoplasmic positive staining of vimentin (d). Keratin 8, 18 positive melanoma cells are mainly located around the peripheral area of the tumor (e). Keratin staining is cytoplasmic (f). Note that there is no staining in the negative control slide (g).

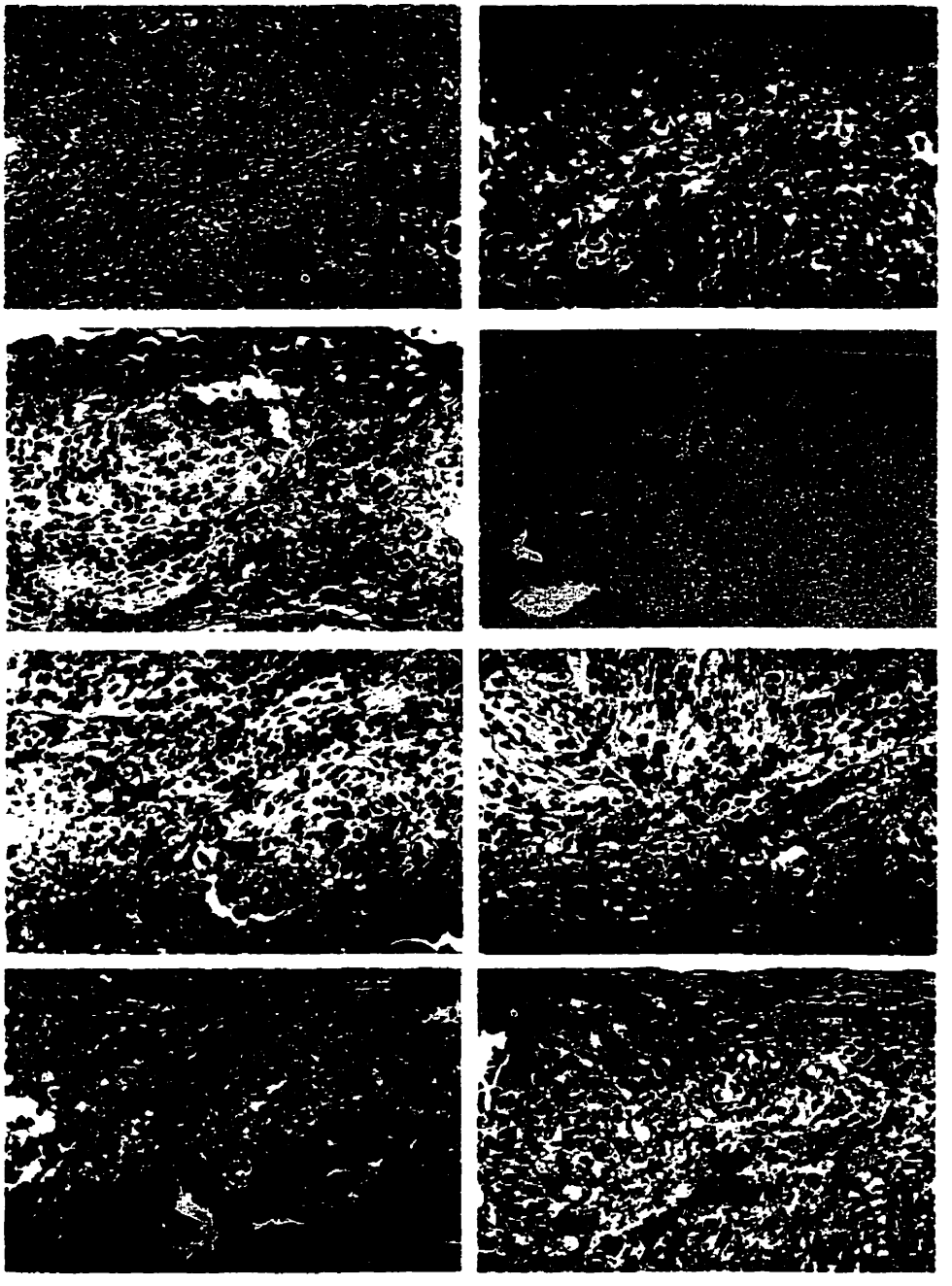

Plate 3.3 A mixed cell type conjunctival melanoma (H&E) (a). S-100 protein staining is diffuse positive (b). HMB-45 staining is heterogenous (c). Keratin 8, 18 positive melanoma cells are located around the peripheral area of the tumor (arrow) (d). Keratin positive tumor cells are located in the marginal area of both left and right tumor nests (arrow) (e & f). Note that same tumor nests present vimentin diffuse positive staining including keratin positive tumor cells (arrow) (g & h).

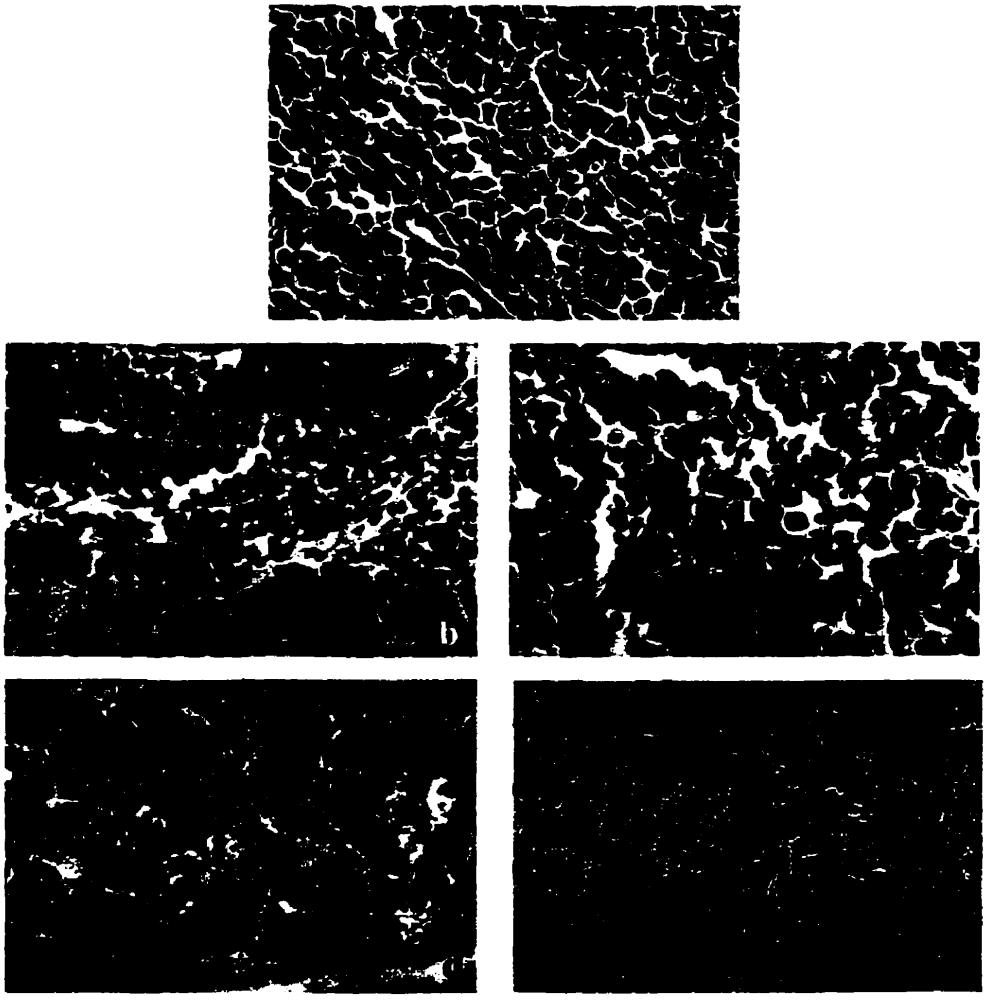

Plate 3.4 A mixed cell type conjunctival melanoma (H&E) (a). The diffuse positive staining of S-100 protein (b), HMB-45 (c) and vimentin (d). Non-staining presents in the keratin 8, 18 slide (e).

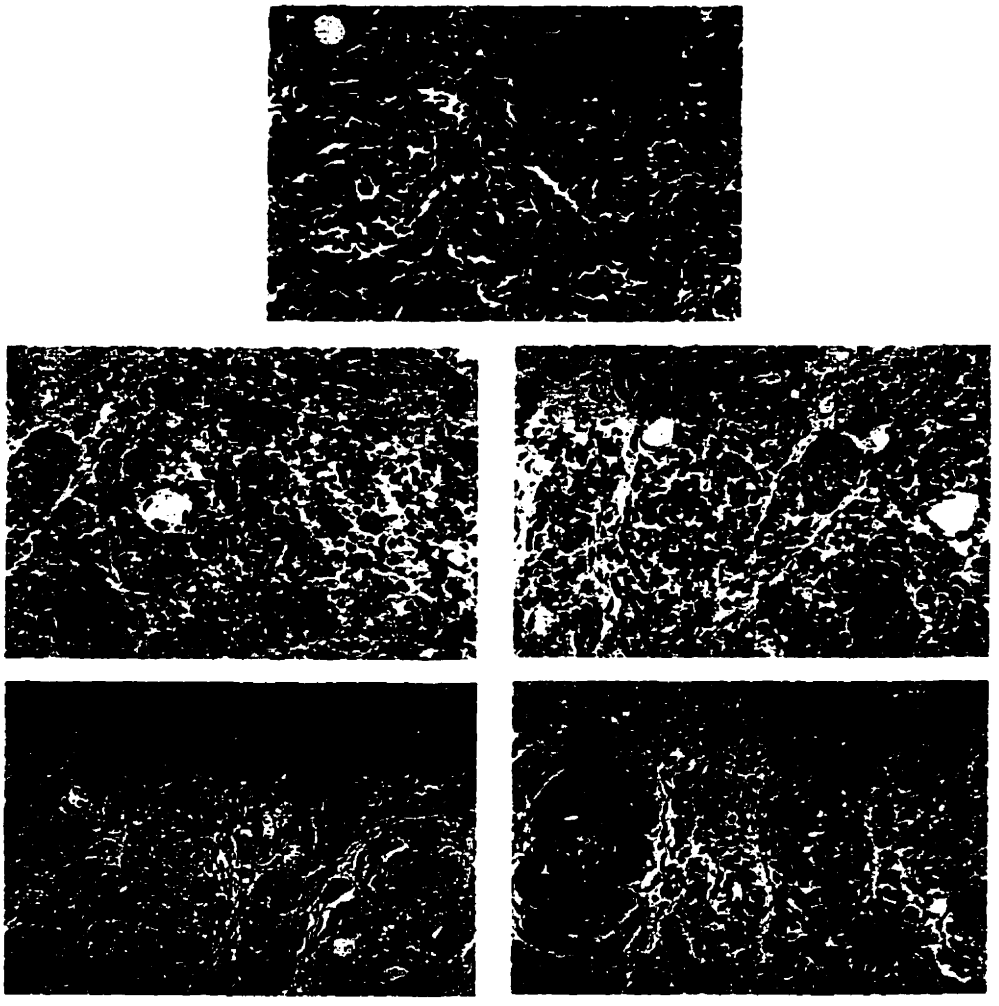

Plate 3.5 A conjunctival compound nevus with some large, atypical nevocytes (H&E) (a). All immunostaining for S-100 protein (b), HMB-45 (c) and vimentin (d) are all diffuse positive. Cysts that show keratin 8, 18 strong positive staining (arrow) (e) act as the internal positive control. Note that some nevocytes are obviously keratin positive.

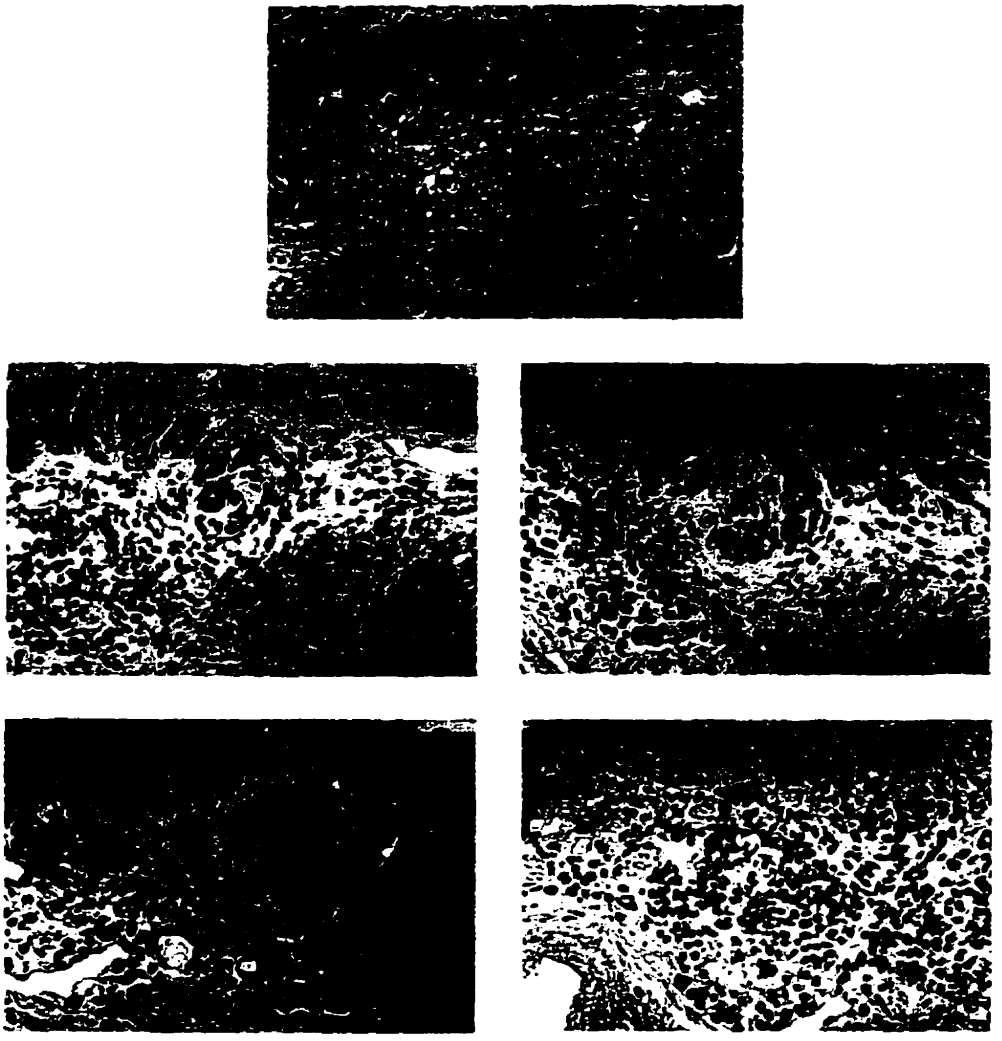

Plate 3.6 A conjunctival compound nevus with some large atypical nevocytes in the junctional area (H&E) (a). S-100 protein staining is diffuse positive (b). HMB-45 shows heterogenously positive staining (c). Vimentin staining is also diffuse positive (d). Note that only those atypical nevocytes located in the junction area present keratin 8, 18 focal positive staining (e).

Chapter 4: Discussion

4.1 Introductory remarks

Keratins and vimentin are both IFs, which are considered principal components of the cytoskeleton of mammalian cells. Although earlier studies emphasized the use of these cell type-specific markers in tumor differentiation and pathology, recent studies have confirmed that the co-expression of keratin 8, 18 and vimentin is associated with recurrence and metastasis in cutaneous and uveal melanomas (Hendrix *et al*, 1992; Chu *et al*, 1996; Hendrix *et al*, 1998). The co-transfection of a poorly invasive melanoma cell line with cDNAs for keratin 8, 18 resulted in increased cytoskeletal interactions at focal contacts with ECM involving integrin cell signaling events, which contribute to a more active migratory or invasive behavior (Chu *et al*, 1996). The transient knockout of keratin 8, 18 in melanoma cells which co-expressed IFs results in a significant decrease in the migratory ability – similar to levels achieved by cells positive only for vimentin (Hendrix *et al*, 1998). Therefore, the expression of keratin 8, 18 by melanoma cells correlates with an invasive phenotype.

The expression of IFs is highly regulated during embryonic development and cellular differentiation. Immature embryonic cells may co-express keratin 8, 18 and vimentin. The co-expression of keratin 8, 18 and vimentin are lost at the later stage of cellular differentiation (Lane *et al*, 1983). The co-expression of IFs by tumor cells may be attributed to the reversion of such cells to an embryonic pattern, and thus be in accordance with the theory of "dedifferentiation" proposed for such malignancies. Otherwise, cells that co-express both keratins and vimentin are regarded as "interconverted" in that they display both mesenchymal and epithelial phenotype markers. The exact function of IFs in general, especially the significance of the co-

expression of IFs observed in melanomas, remains enigmatic. IFs appear to connect to the nuclear surface and extend throughout the cytoplasm terminating at the plasma membrane. Such associations provide a continuous link that may have important implications for the organization of the cytoplasm, intracellular or intercellular communication, and perhaps information transport into and out of the nucleus. Many studies suggested that signals from ECM are transferred via integrins to the cytoskeletal proteins, which further act as transducers to the cell nucleus. This mechanism may control cell growth, behavior, and differentiation. This thesis is the first to explore the presence of the co-expression of IFs in conjunctival melanomas and to correlate the co-expression of IFs with the prognosis for conjunctival melanomas.

4.2 Co-expression of IFs in conjunctival melanomas

The first objective of this thesis was to investigate and detect the co-expression of IFs in conjunctival melanomas, nevi and PAM. This study first indicated the co-expression of keratin 8, 18 and vimentin in one third of conjunctival melanomas. The data demonstrated that vimentin is indeed a constant cytoskeletal feature of all melanomas, but keratin can also be found. In this study the incidence of the co-expression of IFs in all types of conjunctival melanoma was 33.33%. In the positive group co-expressing IFs, keratin 8, 18 staining showed zonal or focal positive patterns, and vimentin staining showed diffuse positive patterns. This study showed that the frequency of positive keratin immune reactivity in formalin-fixed and paraffin-embedded tissue specimens was markedly different from the study of Zarbo *et al* (2%, 1990) and Ben-Izhak *et al* (9.7%, 1994). It is possible that the epitope

demasking techniques that we used – light heat and trypsin digestion may have revealed epitopes missed in the earlier studies. Formalin is by far the most commonly used cross-link fixative for immunohistochemistry, but it has a profound damaging effect on primary, secondary, and tertiary protein structure, which is necessary for the preservation of morphology and for the site specific immobilization of soluble antigens (Larsson, 1993). Epitope retrieval methods have revolutionized the field of immunohistochemistry. With epitope retrieval, many monoclonal antibodies that were restricted in their use to fresh-frozen material can now be used on archival paraffin-embedded material. The mechanism of epitope retrieval by either enzymatic digestion or heating is not clearly understood. Proposed mechanisms of heat epitope retrieval include denaturing of binding proteins and breaking of formaldehyde – induced covalent bonds (Boon & Kok, 1994; Zhen *et al*, 1997). Enzymatic epitope retrieval is thought to act by enzymatic digestion of surface binding proteins, thereby exposing the masked antigenic site (Cattoretti *et al*, 1993; Battifora *et al*, 1995). The results of immunohistochemistry staining in this study showed the benefits of epitope retrieval using the common anti-IF antibodies. Enzymatic epitope retrieval was only used with anti-keratin antibody since enzyme treatment can destroy vimentin reactivity, whereas heating retrieval was used with both anti-keratin and anti-vimentin antibodies. The staining results were clearly shown in this thesis.

Analyzing characteristics of the co-expression of IFs, keratin positive melanoma cells were mainly located near the peripheral area of tumors or the marginal area of tumor nests with zonal or focal positive patterns. Maybe this phenomenon suggests

that melanoma cells with the co-expression of IFs have a more aggressive behavior, more easily losing contact with adjacent cells, and more easily invading the surrounding normal tissues. Ramackers *et al* (1983) noted that the co-expression of both keratins and vimentin by cancer cells presented in ascites and pleural fluids, whereas solid tumors expressed only keratins. The study by Miettinen & Franssila (1989) also found that the lymph node metastasis of melanomas showed a population of keratin-positive highly elongated cells with dendrite-like processes. These cells often were especially numerous at the marginal area of metastatic infiltrates. Hendrix *et al* (1998) revealed that uveal melanoma cells that predominantly co-expressed keratin and vimentin were 6-fold more invasive through ECM, compared with cells expressing vimentin only. These studies have proved that the co-expression of IFs in tumors may influence the motile or invasive activity of tumor cells and may have an effect on growth rate and aggressive behavior of metastastic tumor cells.

4.3 Co-expression of IFs in conjunctival nevi

In this study, the co-expression of IFs was surprisingly found in 3 cases of conjunctival compound nevus, a non-malignant condition. Benign melanocytic nevi are the most common pigmented or non-pigmented lesions of the conjunctiva. Although conjunctival nevi occasionally give rise to conjunctival melanomas, this appears to be rare. It is clear that there is a wide spectrum of "benign but atypical" melanocytic proliferating lesions. Some of the variation of melanocytic lesions might simply be explained by the anatomic location, the type of lesion, the age of patients, and external factors. However, there still remains a group of melanocytic

lesions with atypical junction proliferation, which are not only difficult to classify, but also are difficult to assess in terms of their natural history as to whether they are precursors of melanomas. These 3 cases of conjunctival compound nevi all showed diffuse positive staining for vimentin and focal positive staining for keratin 8, 18. The keratin positive nevocytes were mainly located around the junction areas of the conjunctival nevi, and most of them showed a larger and more atypical appearance. Interestingly, these 3 cases were all younger females leading one to speculate whether the co-expression of IFs in conjunctival nevi was affected by estrogens, or was only a phenomenon of nevocytic proliferation, or just showed a transient "interconverted" situation between epithelial cells and nevocytes. We so far do not know the answers to these speculations. Further studies should be carried out to resolve these questions.

4.4 Co-expression of IFs in primary acquired melanosis

This study found that the immunohistochemistry staining was not valuable for the study of the co-expression of IFs in PAM. First of all, there was no difference in vimentin staining of proliferating melanocytes between PAM with atypia and PAM without atypia. Secondly, for keratin 8, 18 staining, all cases of PAM, including those with or without atypia, were impossible to distinguish from each other since the whole epithelium of the conjunctiva (except the part of stratified squamous epithelium) showed strong diffuse positive staining. It means that we could not distinguish melanocytes from epithelial cells in the conjunctiva with Cam 5.2 monoclonal antibody for keratin 8, 18. According to these results, immunohistochemistry staining of IFs is not a useful marker for proliferating

melanocytes in PAM. However, in this study, HMB-45 staining showed that 100% of PAM with atypia were postive, whereas only 45% of PAM without atypia were focally positive. In this regard, HMB-45 is more suitable for studying melanocytic proliferation in PAM.

4.5 Co-expression of IFs related with worse prognosis

Whether the co-expression of IFs could be a prognostic marker for malignant tumor cells still remains in question. The group of Hendrix (1992; 1998) confirmed that the co-expression of IFs in cutaneous and uveal melanomas acted as a predictive marker. However, Fuchs *et al* (1992) doubted if the co-expression of IFs was useful for determining the prognosis in uveal melanomas. They revealed that although keratin 8, 18 expression was of diagnostic significance and can denote low levels of epithelial differentiation, keratin expression was not an independent prognostic factor in uveal melanomas, and did not predict whether the tumor would metastasize. Since conjunctival melanomas are very rare ocular melanocytic tumors, there are only a few limited prognostic studies available. The secondary objective of this thesis was to assess the correlation between the co-expression of IFs and the prognosis of conjunctival melanomas. In this study the co-expression of IFs was compared to the initial tumor thickness, because the thickness of the conjunctival melanoma, as in the skin, is the most important prognostic feature related to malignancy (Mclean *et al*, 1994). Jakobiec (1980) commented that tumor thickness is the "sole sovereign prognosticator in conjunctival melanomas." A large clinicopathological study by Paridaen *et al* (1994) also confirmed the correlation between the tumor thickness and the worse prognosis of conjunctival melanomas.

The result of this study first indicated that the co-expression of IFs was related with a worse prognosis for conjunctival melanomas. First, the initial tumor thickness was significantly different between the keratin 8, 18 positive group and those not expressing this marker. In the former group the mean tumor thickness was 2.35mm with the standard deviation of 1.32mm, and in the latter group it was 0.62 ± 0.25mm. Secondly, all conjunctival melanomas in the keratin positive group were of mixed cell types and with diffuse growth patterns. These characteristics have also been proved to be related to a worse prognosis in conjunctival melanomas (Paridaens *et al*, 1994). According to Lommatzsch *et al* (1990), epithelial cell conjunctival melanomas do not have as great a tendency to progress to malignancy as do epithelial uveal melanomas. In this study there was only 1 case of epithelial cell conjunctival melanoma that was keratin 8, 18 negative. Therefore, it is impossible to determine whether there was an association with malignancy or not. Finally, 2 cases of conjunctival melanoma with nodular growth pattern were keratin 8, 18 negative, despite the fact that they were both big tumors (their diameters were 5 and 17 mm, respectively) and of mixed cell types. Under the microscope, the smaller one had an intact capsule, and the bigger one showed that some parts of the capsule had been broken and some malignant melanoma cells had invaded the soft tissue of the orbit. Surprisingly, even these obviously invasive tumor cells were still keratin negative. These observations could indicate that conjunctival melanomas with nodular growth patterns have a less invasive and malignant phenotype compared with those with diffuse growth patterns.

4.6 Implications of results

Although there have been some reports on prognostic factors for cutaneous melanomas and uveal melanomas, there is little information available on prognostic factors associated with conjunctival melanomas. The evaluation of various prognostic markers as well as the recognition of risk factors for the malignant melanoma development are extremely important in the management of conjunctival melanomas. The management of conjuncitval melanomas is an important factor in preventing recurrence and eventual metastasis of this malignant tumor, including a carefully planned wide excision and supplemental cryotherapy according to the prognostic estimation (De Potter *et al*, 1993). Unlike the dermatologist, the ophthalmologist may not be able to excise a large conjunctival lesion because extensive removal of the conjunctiva may reduce the population of mucus-secreting goblet cells, and further may interfere with corneal wetting. Dry eye conditions often lead to increased infection, corneal ulceration, and painful loss of vision. The fact that the co-expression of IFs correlated with a worse prognosis for conjunctival melanomas may help ophthalmologists manage the treatment and therapy of them. However, whether the co-expression of IFs in conjunctival melanomas can act as an independent prognostic marker still needs to be investigated and confirmed by future studies.

The phenomenon of the co-expression of IFs in the same melanoma cells was shown in this study. It may also be possible to use IF expression as a target for immunotherapy in conjunctival melanomas. For example, by linking a cytotoxic agent to anti-keratin 8, 18 antibody, which will target the conjugate to malignant

melanoma cells co-expressing IFs, it should be possible to selectively damage these more invasive melanoma cells.

4.7 Future studies

There are a number of ideas for further research that arise from the findings of this thesis.

First of all, from the current findings, we have demonstrated that the co-expression of IFs was correlated to negative prognostic parameters for conjunctival melanomas. Therefore, it could be predicted that patients with conjunctival melanomas co-expressing IFs tend to show a greater propensity for local tumor recurrence and distant tumor metastasis. Further studies should investigate the clinical follow-up information on these patients to confirm whether the clinical results show greater progression of conjunctival melanomas with the co-expression of IFs as compared to those without the co-expression of IFs. The future study should attempt to find correlations between the co-expression of IFs and other clinical parameters, such as patient's age and gender, tumor location, and lymphatic invasion, and to confirm whether the co-expression of IFs is an independent prognostic marker for conjunctival melanomas.

Secondly, it has been shown that the co-expression of IFs presented in those malignant melanoma cells mainly located in the tumor peripheral or marginal areas. The future study should use immunohistochemical double staining to detect whether the co-expression of IFs is really present in the same melanoma cells or not. If this

characteristic was proved, it could further provide a target for immunotherapy or genetherapy for conjunctival melanomas.

Finally, the present work was based on formalin-fixed and paraffin-embedded sections and used immunohistochemistry staining. These procedures caused major changes in tumor marker expression and are relatively insensitive in the detection of the expression of IF markers. The future study should repeat these studies using cryostat sections where tumor antigens are not destroyed. It is also necessary to assess the invasive properties of viable conjunctival melanoma cell lines *in vitro*. In addition, RT-PCR based assays could be used to detect IF markers with greater sensitivity.

4.8 Summary and conclusions

In conclusion, this thesis is the first to provide the immunohistochemical evidence that co-expression of keratin 8, 18 and vimentin was present in one third of conjunctival melanomas. It was shown that conjunctival melanomas with the co-expression of IFs were all of mixed cell types with diffuse growth patterns. Furthermore, it was indicated that these melanoma cells with the co-expression of IFs were mainly located around either the peripheral or marginal area of tumors. In addition, this thesis also first demonstrated that the co-expression of IFs in melanoma cells was directly proportional to the initial tumor thickness of conjunctival melanomas. Data further showed the correlation between the co-expression of keratin 8, 18 and vimentin and the worse prognosis in conjunctival melanomas. As

such, these findings indicate that the co-expression of keratin 8, 18 and vimentin can be used as a prognostic marker in conjunctival melanomas.

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