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Expression and distribution of MMPs and TIMPs in human uveal melanoma☆

K. Lai [a], R.M. Conway [a], R. Crouch [b], M.J. Jager [c], M.C. Madigan [a,*]

[a] Save Sight Institute & Discipline of Ophthalmology, University of Sydney, Sydney, NSW, Australia
[b] Anatomical Pathology, Prince of Wales Hospital, Randwick, NSW, Australia
[c] Department of Ophthalmology, Leiden University Medical Center, Leiden, The Netherlands

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ABSTRACT

Matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) are involved in tumour invasion, metastasis and angiogenesis, and have been implicated as progression markers in uveal melanoma, although their topographical expression has not been fully described. In this study we compared the distribution and specificity of several classes of MMPs (MMP-1, -2, -9, -19, and MT1-MMP) and physiological MMP inhibitors (TIMP-2 and -3) in different regions of the tumour microenvironment and adjacent choroid in a series of primary uveal melanomas. Paraffin sections of untreated uveal melanomas ($n = 18$, 3/18 spindle; 11/18 mixed, and 4/18 epithelioid) were examined for MMP-1 (collagenase 1), MMP-2 and MMP-9 (gelatinases A and B), MT1-MMP (membrane-type 1-MMP), MMP-19, TIMP-2 and TIMP-3 (tissue inhibitors of MMPs), using indirect peroxidase immunohistochemistry. The distribution and intensity of immunolabelling was graded semi-quantitatively (0–3) by 2 independent observers. Non-parametric analyses were used to test for associations between tumour cell type, and the average grade of MMP or TIMP expression. Immunostaining for MMP-1, -9, -19 and MT1-MMP was ≥Grade 2 in more than 70% of specimens, and a heterogeneous pattern of MMP-1, -9, MT1-MMP and TIMP-3 expression was observed. At the tumour-scleral interface (TSI), melanoma cells had a flattened morphology and a much reduced MMP and TIMP expression, with a high expression in tumour areas adjacent to the TSI. Tumour vasculature and stromal cells strongly expressed MMP-2. We also observed heterogeneous immunostaining of the vasculature by MMP-1, -9, MT1-MMP and TIMP-2 antibodies, and of the extravascular matrix by MMP-9 antibody. The distinct immunostaining patterns observed for MMPs and TIMPs within uveal melanoma are consistent with their involvement in tumour growth and angiogenesis. In particular, the heterogeneous expression within regions of the tumours, and the localized expression in vasculature and stromal cells emphasises the importance of the tumour microenvironment in the pathogenesis of uveal melanoma (and other tumours).

1. Introduction

Uveal melanoma is the most common primary adult eye cancer, with an average incidence of 8 per million per year [USA (Seddon et al., 1994), Australia (Vajdic et al., 2003)]. Clinically evident metastases, predominantly in the liver, may present after a prolonged disease-free interval, do not respond well to treatment, and are often fatal within 12–15 months following presentation (Gambrelle et al., 2007; Gragoudas et al., 1991).

Matrix metalloproteinases (MMPs) and Tissue Inhibitors of MMPs (TIMPs) are well established to be involved in the growth, invasion and metastases of tumours (reviewed Nagase et al., 2006). MMPs are a family of zinc-dependent proteinases involved in degrading extracellular matrix (ECM) components including collagen, laminin and proteoglycans. According to their primary structure, substrate preference and localization, MMPs are generally classified into collagenases (MMP-1, -8 and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3 and -10), matrilysins (MMP-7, -11 and -26), membrane-type MMPs (MMP-14, -15, -16, -17, -24 and -25) and other MMPs (MMP-12, -19, -20, -21, -23, -27 and -28) (Nagase et al., 2006; Visse and Nagase, 2003). MMPs play an important role in physiological tissue remodeling in embryonic development, angiogenesis and wound healing, and facilitate tumour cell invasion and metastasis via ECM degradation, cytokine and growth factor activation, modulation of cell–cell adhesion and promotion of angiogenesis (Handsley and Edwards, 2005; Stamenkovic, 2000). TIMPs can regulate or inhibit MMP activity and are involved in cell invasion, metastases and

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* Corresponding author. Save Sight Institute, GPO Box 4337, Sydney, NSW 2001, Australia. Tel.: +61 2 9382 7283; fax: +61 2 9382 7318.
E-mail address: michele@eye.usyd.edu.au (M.C. Madigan).

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angiogenesis (Chirco et al., 2006; Lambert et al., 2004). Furthermore, they are implicated in promotion as well as in inhibition of cell growth and apoptosis (Chirco et al., 2006; Lambert et al., 2004).

In uveal melanoma, expression of (latent) MMP-2 and -9 has been associated with a higher incidence of metastatic disease (El-Shabrawi et al., 2001; Rao et al., 2004; Vaisanen et al., 1999). More aggressive epithelioid choroidal melanomas or epithelioid regions of mixed tumours have been found to predominantly express latent MMP-9 (El-Shabrawi et al., 2001). A small series of mixed morphology uveal melanomas were found to express MMP-2, -9, -13 and MT1-MMP (Laver, 2002). A significant role for MMPs in uveal melanoma metastases has been suggested by a study showing that patients with tumours that expressed TIMP-1 and -2, the main physiological inhibitors of MMP-2 and -9, had a better survival rate (El-Shabrawi et al., 2001). Gene microarray studies recently showed downregulated TIMP-3 mRNA expression in Class 2 uveal melanomas with a poor prognosis (Singh et al., 2007), and no expression of TIMP-3 was found in 6/10 uveal melanomas with monosomy 3 (associated with metastases and poor prognosis) (Nareyeck et al., 2005). Furthermore, a 5-fold decrease in TIMP-3 gene expression regulated by DNA methylation has been reported in metastatic compared to primary uveal melanoma cells, thus identifying TIMP-3 as a progression marker (van der Velden et al., 2003). However, a conclusive association between TIMP-3 mRNA and protein expression in primary uveal melanomas and tumour histopathology (cell type and presence of PAS+ loops and networks) has not been shown to date (Nareyeck et al., 2005; van der Velden et al., 2003).

The topographical expression of MMPs and TIMPs, including regional differences in the distribution of immunolabelling of tumour cells, stroma and the vasculature has not been fully described in uveal melanoma. To better understand the dynamics of the tumour microenvironment, we compared the distribution and specificity of several classes of MMPs (MMP-1, -2, -9, -19 and MT1-MMP) and physiological inhibitors of MMPs (TIMP-2 and -3) in uveal melanomas and adjacent choroid.

2. Materials and methods

2.1. Specimens

This study was performed under the tenets of the Declaration of Helsinki and with approval from the Human Ethics Committee University of Sydney. Eyes from patients with untreated uveal melanoma were enucleated, fixed in 10% neutral buffered formalin (NBF) and paraffin embedded ($n = 18$; patient's age range: 41–85 years). Normal human eyes ($n = 5$; donor age range 39–80 years) provided by the Lions NSW Eye Bank with consent and ethical approval, were fixed in 10% NBF and paraffin embedded, and used as controls. Uveal melanoma and normal eyes were stored and processed according to the same protocol. Paraffin sections were cut at 8 μm and collected on Super-Frost Plus (Menzel-Glaser, Saarbruckener, Germany) slides.

2.2. Tumour histopathology: Hematoxylin & Eosin (H&E) and Periodic Acid Schiff (PAS)

Sections were dewaxed in xylenes, rehydrated through a series of alcohols to water and stained with Mayer's hematoxylin and eosin, dehydrated through alcohols and xylenes, mounted in DePeX (BDH Pty Ltd, Merck, Kilsyth, Australia) and coverslipped, before being examined with a Leica light microscope. From H&E staining, melanomas were graded as spindle ($n = 3$), mixed ($n = 11$) or epithelioid ($n = 4$). Extracellular matrix (ECM) patterns were examined in a separate series of sections stained with Periodic Acid Schiff (PAS) using standard protocols. Briefly, sections were dewaxed and rehydrated prior to incubation in 0.5% periodic acid solution, followed by staining in Schiff's reagent, and rinsing in freshly prepared 0.4% sodium metabisulphite until the pink reaction product reached the desired density. After a further rinse in water, sections were dehydrated through alcohols and xylenes, mounted in DePeX and coverslipped.

2.3. Immunohistochemistry

Sections were dewaxed, rehydrated and rinsed in 0.1 M phosphate buffer with 0.9% sodium chloride (PBS, pH 7.4). For antigen retrieval, sections were incubated in 0.01 M citrate buffer (pH 6) at 80 °C for 15 min, cooled to 40 °C, and rinsed in PBS. This was followed by immersion in 5% H_2O_2 for 5 min to inhibit endogenous peroxidase activity, then rinsing in PBS. Sections were incubated at room temperature (RT) in either 10% normal sheep or normal donkey serum for 20 min followed by incubation for 72 h at 4 °C in primary antibody (Table 1). After rinsing in PBS, sections were incubated in the appropriate biotinylated secondary antibody (Table 1) for 1 h at RT, rinsed in PBS, followed by 1 h at RT in ExtrAvidin peroxidase (1:200, Sigma Aust. Pty Ltd, Sydney Australia). Antibody binding was visualised using Vector NovaRED substrate (Vector Labs Pty Ltd, Burlingame, CA, USA). Sections were counterstained in hematoxylin for 10–15 s, dehydrated through alcohols and xylenes, and coverslipped in DePeX. Negative controls were incubated in appropriate non-specific immunoglobulins or IgG antibody (Table 1).

2.4. Grading of immunostaining

The distribution and intensity of specific immunostaining for each MMP and TIMP was graded in masked fashion by 2 independent observers using a semi-quantitative scale where 0 = none, 1 = weak (low intensity staining of tumour cells regardless of the percentage of positive cells, and medium intensity staining of ≤20% cells); 2 = moderate (medium intensity staining of >20% tumour cells, high intensity staining of ≤20% tumour cells), and 3 = strong (high intensity staining of >20% tumour cells). Differences in grading were resolved by joint review and if necessary, consultation with a third independent observer. The grading of the two observers was significantly correlated ($P < 0.05$; Kendall's rank correlation). Non-parametric linear regression analysis was used to test for an association between cell type (1 = spindle; 2 = mixed or 3 = epithelioid) and immunolabelling for each MMP or TIMP (Grades 0–3). We also assessed the distribution and intensity of MMPs and TIMPs in normal human eyes.

Table 1
Antibodies

Primary and secondary antibodies	Species	Manufacturer[a]	Dilution
MMP-1 monoclonal (latent/active)	Mouse	Calbiochem	2.5 μg/ml
MMP-2 monoclonal (latent/active)	Mouse	NeoMarkers	4 μg/ml
MMP-9 polyclonal (latent/active)	Rabbit	NeoMarkers	2 μg/ml
MT1-MMP monoclonal (latent/active)	Mouse	Calbiochem	4 μg/ml
MMP-19 polyclonal (latent/active)	Rabbit	Calbiochem	0.5 μg/ml
TIMP-2 monoclonal (latent/active)	Mouse	NeoMarkers	1:50
TIMP-3 monoclonal	Mouse	Calbiochem	2 μg/ml
Non-specific IgG1 monoclonal	Mouse	Dako	1:100
Rabbit immunoglobulins (Ig)	Rabbit	Dako	0.5, 2 μg/ml
Biotinylated anti-mouse Ig	Sheep	GE Healthcare	1:100
Biotinylated anti-rabbit Ig	Donkey	GE Healthcare	1:100

[a] Calbiochem Merck Pty, Kilsyth, Australia; NeoMarkers, Fremont CA USA; Dako Aust. Pty Ltd, Botany, Australia; GE Healthcare Bio-Sciences Pty Ltd, Rydalmere, Australia.