The matrix metalloproteinases (MMPs) are a family of over 20 zinc dependent enzymes that cleave various components of the extracellular matrix (ECM). MMPs are associated with a variety of physiological and pathological conditions that involve matrix modelling and remodelling. They are highly expressed in adult reproductive tissues undergoing dramatic reductions in tissue mass, including the endometrium during menstruation and the involution of the breast, uterus, and prostate. MMPs have also been shown to contribute to human fetal development and are present in postnatal tissues. They are also found in the human intervertebral disc. In wound healing, the expression of MMPs is pronounced and depends on the phase of healing. The tissue destruction that occurs in diseases such as rheumatoid arthritis, macular degeneration, periodontitis, and tumour cell invasion are apparently mediated by members of the MMP family. The generation of synthetic inhibitors of MMPs for therapeutic use in these diseases is thus of great interest.

MMPs have been classified into four broad categories based on their substrate specificity and domain organisation. They include collagenases (MMP-1, 8, 13), stromelysins (MMP-3, 10, and 11), gelatinases (MMP-2 and 9), and membrane types (MMP-14–17, 24, 25). MMPs are regulated at several levels including transcription, secretion, activation, and inhibition. Regulation by the latest of these mechanisms is via the MMP family. The generation of synthetic inhibitors of MMPs and TIMPs as new therapeutic strategies. In the present study, we localised the major MMPs (1, 2, 3, and 9) involved in destruction and remodelling of collagenous connective tissues, as well as their principal antagonists (TIMPs 1–4) in the human anterior uveal tract.

Fluorochrome staining allowed us to examine the distribution of MMPs and TIMPs in these heavily pigmented tissues, in which conventional immunohistochemistry has been proved to be unreliable.

**MATERIALS AND METHODS**

**Antibodies and reagents**

Mouse anti-human antibodies to individual MMPs (MMP-1, 2, 3, 9) and TIMPs 1–4 were obtained from ICN Biomedicals, Australia. A control mouse IgG, antibody was run in parallel (Sigma, Australia). Goat anti-mouse immunoglobulins conjugated to Alexa 568, (excitation/emission maxima of 573/596 nm) were purchased from Molecular Probes, Eugene, OR, USA.

**Dissection of human iris and ciliary body**

Seven postmortem human eyes from donors (45–80 years of age) were obtained from the Lions Eye Bank (Sydney, Australia) within 24 hours after death. The eyes were fixed in 10% formalin for 24 hours. The anterior uveal tissue, iris, and ciliary body area were dissected under a microscope and embedded in paraffin.

**Immunohistochemistry**

Expression of MMPs and TIMPs in the iris and ciliary body was examined using mouse anti-human MMPs/TIMPs monoclonal antibodies, followed by the addition of a secondary antibody conjugated to the red emitting fluorochrome Alexa 568. This method, which has previously been validated for immunohistochemistry on paraffin sections minimises interference by autofluorescence. Tissue was serially sectioned at 4 µm thickness and placed on aminopropyltriethoxysilane...
coated slides. Sections were deparaffinised in xylene and rehydrated with ethanol. Slides were boiled in antigen retrieval solution (citrate buffer) for 3 minutes in a microwave. This procedure was repeated three times, followed by three washes in phosphate buffered saline (PBS). Sections were incubated in 3% BSA in PBS for 30 minutes to block non-specific binding. They were then incubated with mouse anti-human MMPs and TIMPs Abs (1:150 dilution in 3% BSA) overnight at 4°C. Sections were washed three times and incubated for 1 hour at room temperature with goat anti-mouse-Alexa 568 (10 μg/ml). After further washing, slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) and sealed with clear nail lacquer.

Negative controls included incubation of sections with an isotype matched monoclonal antibody (mouse IgG,) and omission of the primary antibody. As a positive control, human synovial tissue from a patient with rheumatoid arthritis was stained for MMP-1. To facilitate morphological orientation, adjacent sections were stained with haematoxylin and eosin and examined by light microscopy.

**Fluorescent microscopy and grading**

Slides were examined with an Olympus BX60 microscope equipped with a 100 W mercury burner for epifluorescence illumination and wide band interference filters for green excitation/red emission (520–550 nm band pass, 590 nm long pass). For both fluorescence and bright field microscopy, images were captured with a spot cooled colour digital camera (Diagnost Instruments, Sterling Heights, MI, USA). The intensity of immunofluorescent staining in each area of each section was graded as follows: 0: no staining; 1: weak staining; 2: moderate staining; 3: strong staining. Assignment of a grade was based both on the intensity of the staining and on the magnification at which it could be visualised. Grading was performed by two independent observers masked as to the identity of the samples. Data are expressed as medians and interquartile ranges.

**RESULTS**

Staining for MMPs and TIMPs was apparent throughout the anterior uvea, although there was marked variation in the intensity of staining in different regions (Tables 1 and 2). Interestingly, except for TIMP-3, most MMPs and TIMPs showed similar patterns in their distribution. In general, the intensity of staining in the ciliary body was stronger than in the iris. In the ciliary body, the patterns of intensity of staining were non-pigmented epithelium > ciliary muscle > pigmented epithelium > stromal cells for all the antibodies. Whereas staining for MMP-1, 2, 3, 9, and TIMP-1, 2, 4 was mainly in the cytoplasm of both the non-pigmented and pigmented epithelial cells (Fig 1C), staining for TIMP-3 was mostly in the basement membrane of the epithelium (Fig 1D).

In the iris, staining intensity generally followed this pattern: anterior border > anterior epithelium > stromal cells > posterior epithelium for all the antibodies tested (Table 1, Fig 1A, B). Prominent staining of blood vessel walls was also noted. Figure 1A–D shows representative images of fluorescent staining for MMP-1 and TIMP-3 in the iris and ciliary body.

No staining was observed in the negative controls (Fig 1E left). However, there was intensive staining for MMP-1 in human rheumatoid synovial tissue that was used as a positive control (Fig 1E right).

**DISCUSSION**

To our knowledge this is the first report demonstrating the distribution of MMP-1, 2, 3, 9, and TIMPs 1–4 in the normal human iris and ciliary body. Because of the considerable quantity of melanin in the pigment epithelium in the iris and ciliary processes, conventional immunohistochemistry is not suitable for localisation of MMPs and TIMPs in these tissues. This may be one reason for the lack of detailed studies on the localisation of MMPs/TIMPs in the anterior uvea. The use of a red emitting dye successfully minimises the problem of autofluorescence in paraffin sections.

In the present study, we used this immunofluorescence staining technique to overcome the problem, which allowed us to localise MMPs and TIMPs in anterior uvea in detail.

Our results revealed the widespread distribution of MMPs 1–3 and 9 and TIMPs 1–4 in the anterior uvea. Interestingly, the intensity of the staining of MMPs and TIMPs revealed a similar pattern in their distribution. For example, in the iris, the strongest immunoreactivity was found in the anterior border, followed by the anterior epithelium and stromal cells, whereas the posterior epithelium was weakly reactive. The positive correlation in their distribution of MMPs and TIMPs is in agreement with previous reports indicating that the inhibition is stoichiometric—that is, one molecule of TIMP inhibits one molecule of active MMP. Furthermore, this suggests that the balance between MMPs and TIMPs may be important in maintaining homeostasis in the anterior uvea.

Physiological functions of MMPs include remodelling of the extracellular matrix, cell migration, proliferation, and apoptosis. Their function and specific roles may be regulated by their natural occurring inhibitors. Although MMPs and TIMPs have been demonstrated in the eye and have the potential to impact on ocular physiology, relatively little is known about their function. Recent studies have suggested a role in the maintenance of normal aqueous humour outflow, in maintaining vitreous integrity, and on the ageing of the eye.

In ocular disease, cytokines such as TNF-α and IL-1β, adhesion molecules, and growth factors such as fibroblast growth factor may alter the activity of MMPs or lead to an imbalance between MMPs and TIMPs.
Previous reports from our laboratory showed that elevated MMPs (1, 2, 3, 7, 9) were found in necrotising scleritis, pterygial tissue, infiltrating cells (macrophages, T lymphocytes and neutrophils) of patients with uveal inflammation and animals.

**Figure 1** Expression of MMP-1 and TIMP-3 in the iris and ciliary body. Paraffin sections of the anterior uvea were stained for MMP-1 or TIMP-3, and then visualised with fluorochrome-Alexa 568. (A) MMP-1 in the iris: anterior border (ab); iris stroma (is); anterior epithelium (ae); posterior epithelium (poste). (B) TIMP-3 in the iris: anterior border (ab); iris stroma (is); anterior epithelium (ae); posterior epithelium (poste). (C) MMP-1 in the ciliary body: positive staining mainly located in the cytoplasm of the epithelium, non-pigmented epithelium (npe); pigmented epithelium (pe); ciliary body stroma (cs). (D) TIMP-3 in the ciliary body: basement membrane of non-pigmented epithelium (bnpe); basement membrane of pigmented epithelium (bpe); ciliary body stroma (cs). Negative control (E, left): the primary Ab was omitted; positive control (E, right): rheumatoid synovial tissue stained for MMP-1.

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