

# Neutrophil accumulation correlates with type IV collagenase/gelatinase activity in endotoxin induced uveitis

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*Br J Ophthalmol* 2002;**86**:290–295

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Accepted for publication  
6 September 2001

**Background/aim:** Anterior uveitis is a common inflammatory ocular disease characterised by protein accumulation and leucocyte infiltration in the anterior chamber. The aim of this study was to determine the expression of gelatinases in the aqueous humour (AH) and uvea in an animal model of endotoxin induced uveitis (EIU).

**Methods:** EIU was established in Lewis rats following an intraperitoneal injection of lipopolysaccharide (LPS). AH and ocular tissue were obtained from control animals and those with EIU over a 1 week time course and the samples analysed immunohistochemically and by gelatin zymography.

**Results:** Matrix metalloproteinase (MMP) 2 and 9 levels were elevated in rat AH over a 1 week time course. MMP-2 and MMP-9 levels peaked at the time of maximum uveal inflammation, before returning to baseline levels as the inflammation subsided. MMP-9 was detected in the latent and functionally active form. Total protein extracted from inflamed rat uveal tissue displayed no significant gelatinolytic modulation throughout the time course of EIU. Anterior chamber neutrophils and ciliary body epithelial cells were the most abundant source of the gelatinases.

**Conclusion:** This study has revealed a correlation between infiltrating neutrophils and the presence of elevated gelatinases in EIU. The results suggest that these proteolytically active enzymes may be important mediators of the inflammatory response and contribute to matrix remodelling observed in uveitis. Furthermore, the excess production of MMPs may be a mechanism by which leucocytes, such as neutrophils, gain access to uveal tissue and AH. Therapeutic strategies aimed at reducing MMP activity may be of some benefit in the treatment of uveitis.

Uveitis is a common, potentially sight threatening disease characterised by inflammation of the anterior chamber, protein exudation, and matrix remodelling.<sup>1</sup> Endotoxin induced uveitis (EIU) is an animal model of acute uveitis initiated by intraperitoneal or footpad injection of Gram negative bacterial wall derived lipopolysaccharide (LPS).<sup>2</sup> Although EIU is self limiting and usually resolves by 72 hours, various aspects of this disease mimic the acute phase of human anterior uveitis. Studies of animal models have characterised the leucocyte infiltrate,<sup>3</sup> inflammatory mediators, such as cytokines and chemokines,<sup>4–9</sup> as well as connective tissue degrading enzymes, such as the matrix metalloproteinases (MMPs) in the aqueous humour (AH).<sup>10</sup>

MMPs are a family of neutral endopeptidases with the capacity to denature most components of the extracellular matrix. These enzymes have been implicated in physiological and pathological conditions such as wound healing,<sup>11</sup> embryological development, tumour invasion, and metastasis,<sup>12</sup> and joint destruction.<sup>13</sup> Studies from our laboratory have identified these proteinases in ocular diseases such as uveitis,<sup>10</sup> scleritis,<sup>14</sup> and pterygia,<sup>15–16</sup> while others have detected MMPs in diseased corneas<sup>17</sup> and in proliferative diabetic retinopathy.<sup>18</sup>

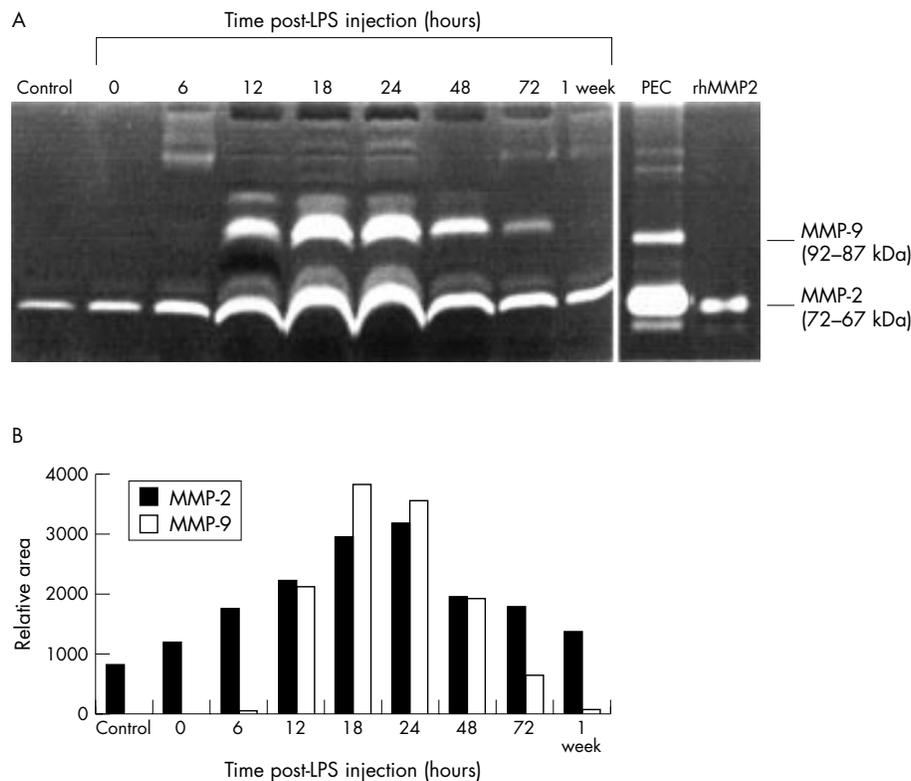
MMPs are regulated at multiple levels, including transcriptional modulation by cytokines and growth factors, post-translational processing of the latent enzyme by serine proteases or other MMPs, and enzyme inhibition. The latter is accomplished by naturally occurring tissue inhibitors of MMPs (TIMPs) of which four have been cloned and characterised in humans.<sup>19</sup> MMPs are grouped according to substrate specificity, and include the collagenases, gelatinases, stromelysins, and the membrane associated MMPs (MT-MMPs). Traditionally, the gelatinases (gelatinase A/MMP-2

and gelatinase B/MMP-9) have received considerable attention, as their activity is directed predominantly against basement membrane collagen type IV, and are thus thought to play a key part in leucocyte extravasation, cell migration,<sup>20</sup> and neovascularisation.<sup>21</sup>

MMP-9 is a major product of activated monocytes,<sup>22</sup> produced by T cells and induced by T cell activation.<sup>20–23</sup> This proteinase is also a storage product, found in specific neutrophil granules, that is released upon activation.<sup>24</sup> MMP-9 has been detected in excess in body fluids such as in the synovial fluid from patients with rheumatoid arthritis,<sup>13</sup> in diseased cerebrospinal fluid,<sup>25</sup> in the tear film of patients with pterygia,<sup>16</sup> in plasma from patients with cancer,<sup>12</sup> in the vitreous of patients with diabetic retinopathy,<sup>18</sup> and in the AH of patients with uveitis.<sup>9</sup> While MMP-9 is an inducible protein, few cell types have been shown to produce inducible levels of MMP-2,<sup>26</sup> perhaps because of significant differences in the promoter sequence between the two genes.<sup>27</sup> MMP-2 has also been detected in normal AH<sup>28</sup> and found at increased levels in the serum of patients with cancer.<sup>12</sup>

The aim of this study was (i) to assess leucocyte accumulation in uveal tissue and AH, and to determine whether this correlated with MMP activity in an animal model of uveitis, and (ii) to determine the cellular source of these enzymes by analysing ocular tissue specimens from diseased animals.

**Abbreviations:** AH, aqueous humour; EAU, experimental autoimmune uveoretinitis; EIU, endotoxin induced uveitis; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; MT-MMPs, membrane associated MMPs; PBS, phosphate buffered saline; TIMPs, tissue inhibitors of MMPs



**Figure 1** Aqueous humour MMP-2 and MMP-9 in EIU. (A) AH from saline injected control animals harvested at 24 hours post-LPS injection (lane 1) and from LPS treated rats harvested at 0, 6, 12, 18, 24, 48, 72 hours, and 1 week post-injection (lanes 2-9 respectively), were analysed by gelatin substrate zymography. Conditioned media derived from PMA stimulated pterygium epithelial cells (lane 10), and recombinant human MMP-2 (lane 11) served as positive controls. Each time point represents pooled AH derived from six animals. Similar results were obtained in two other zymograms. (B) The lytic bands generated by the MMP activity were analysed semiquantitatively.

## MATERIALS AND METHODS

### Animals

Female Lewis rats (6-8 weeks of age) weighing approximately 200 g were used in this study. Animals were purchased and maintained at the animal breeding and holding unit (ABHU) of the University of New South Wales, Sydney. For this study, all animal handling and experimental procedures were in accordance with the ARVO statement for the use of animals in ophthalmic and vision research and the University of New South Wales animal ethics committee.

### Induction of EIU

Rats ( $n = 54$ ) were given a single 50  $\mu$ l intraperitoneal injection of 150  $\mu$ g of lipopolysaccharide (LPS) (*Salmonella typhimurium*, Sigma, Sydney, Australia) dissolved in endotoxin free saline. Control animals ( $n = 6$ ) were injected with saline alone and sacrificed 24 hours later. Experimental animals were sacrificed at varying times (0, 6, 12, 18, 24, 48, 72 hours, and 1 week) post-LPS injection ( $n = 6$ /time point). Additional animals ( $n = 108$ ) were required in order to perform flow cytometric analysis of AH derived cells (see below).

### Aqueous humour and ocular tissue preparation

Eyes were enucleated ( $n = 12$ /group), the AH was pooled (150-250  $\mu$ l) and centrifuged to remove cells and debris, then aliquoted and stored at  $-70^{\circ}\text{C}$  until assayed. In a separate experiment, AH was centrifuged, the cells resuspended in sterile phosphate buffered saline (PBS) then counted and characterised by flow cytometry (see below). Some eyes were snap frozen for protein extraction, or placed in 10% formalin, paraffin embedded and sectioned for immunohistological investigation. Sections from eyes at each time point were routinely stained with haematoxylin and eosin and assessed histologically.

### Total protein extraction

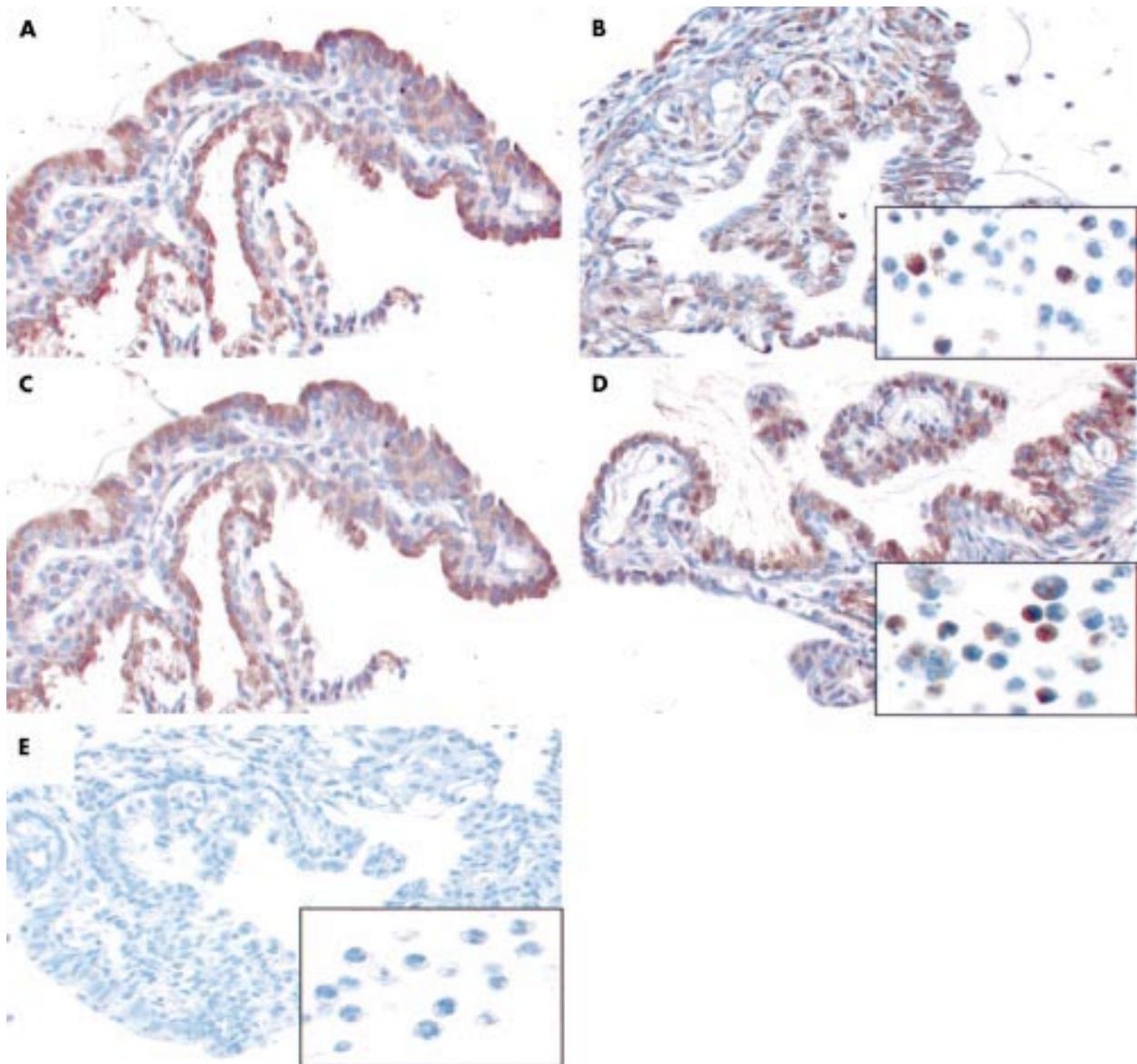
Total protein was extracted from pooled ( $n = 3$ /time point) fresh frozen eyes. The iris and ciliary body were carefully dissected and homogenised in RIPA buffer (250  $\mu$ l; 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS in PBS) in the presence of 10 000 U/ml aprotinin (7.5  $\mu$ l) and 1 mg/ml phenyl methyl sulphonyl fluoride (2.5  $\mu$ l; PMSF, Sigma). Samples were left on ice for 30-60 minutes, centrifuged for 20 minutes at 15 000  $g$  at  $4^{\circ}\text{C}$  and the supernatants stored at  $-70^{\circ}\text{C}$  until used in biochemical assays.

### Immunohistochemistry

Formalin fixed, paraffin embedded serial tissue sections (4  $\mu$ m) were proteolytically digested with Proteinase K (Sigma), quenched for endogenous peroxidase, then incubated with the appropriate blocking serum as described previously.<sup>14</sup> Tissue sections were incubated with rat anti-MMP-2 and anti-MMP-9 (BioSource Int, Camarillo, CA, USA) antibodies (Abs) overnight at  $4^{\circ}\text{C}$ . Sections were extensively washed in TRIS buffered saline ( $10\times$  TBS: 250 mM TRIS base and 250 mM TRIS-HCl, 8.5% NaCl, pH 7.6) and a 1:200 dilution of a goat anti-rabbit biotinylated secondary Ab (Vector Laboratories, Burlingame, CA, USA) applied for 20 minutes at room temperature. Tissue sections were again washed with TBS, then incubated with HRP conjugated streptavidin (Dako Corp, Carpinteria, CA, USA) for 1 hour at room temperature, and the substrate 3-amino-9-ethyl carbazole (Sigma) applied for 5 minutes. Tissue sections were counterstained with haematoxylin. Specificity was verified by incubating sections with no primary Ab. Positive immunoreactivity was observed as red cytoplasmic staining.

### Flow cytometric analysis

Two and three colour immunophenotyping of AH cells was performed using fluorescein isothiocyanate, phycoerythrin, or

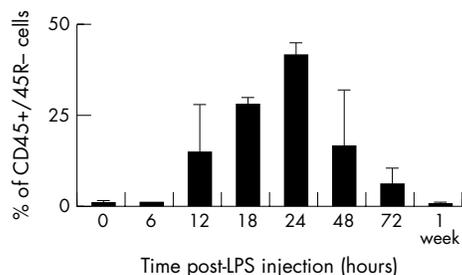


**Figure 2** Localisation of MMP-2 and MMP-9 in rat ocular tissue. Rat eyes were enucleated, the AH collected, and ocular tissue formalin fixed and paraffin embedded for immunohistochemical analysis of MMP-2 (A and B) and MMP-9 (C and D). Positive immunoreactivity is denoted by red cytoplasmic staining with haematoxylin establishing the nuclear counterstain. Insets (B) and (D) demonstrate the specific staining of some remaining anterior chamber neutrophils for the respective Abs. Micrographs (A) and (C) represent ocular tissue derived from 6 hours post-LPS, while specimens in (B), (D), and (E) were obtained 24 hours post-LPS treatment. Tissue sections incubated in the absence of a primary Ab demonstrated no reactivity (E and inset). These results are representative of all the ocular tissue specimens analysed. Original micrograph magnification  $\times 400$ , and insets  $\times 600$ .

cytochrome C conjugated mouse monoclonal Abs specific for rat cell surface markers (Pharmingen International, USA). The low cell numbers limited our analysis to the following: CD45 (leucocyte common antigen, OX-1), CD45R (B cells, HIS24), CD3 (T cells, G4.18), CD4 (Th cells, OX-35), and CD8a (Tc cells, OX-8). Isotype control Abs were used in parallel (IgG<sub>1k</sub>-FITC, IgG<sub>2ak</sub>-Cyc, IgG<sub>2bk</sub>-PE, and IgG<sub>3k</sub>-PE, Pharmingen International). Pooled AH ( $n = 6$ /group) was made up to 10 ml with PBS and spun at 500 *g* for 10 minutes. Aqueous cells were counted and distributed equally for surface labelling ( $1-2 \times 10^5$  cells/sample). Acquisition was measured using a fluorescence activated cell sorter (Facsan, Becton Dickinson) and analysed using the CELLQUEST program (Becton Dickinson Immunocytometry Systems, USA). Each flow cytometric assay consisted of 10 000 scattering events. Instrument settings and gates were optimised using cells stained with each Ab and by excluding dead cells and aggregates.

#### Gelatin substrate zymography

Zymography was performed as previously described.<sup>14-16</sup> In brief, aliquots of AH or total protein extracts were thawed, diluted in non-reducing sample buffer (10% SDS, 4% sucrose, 0.25M TRIS-HCl, pH 6.8, with 0.1% bromophenol blue), and loaded without boiling onto 10% SDS-PAGE gels containing 1 mg/ml gelatin (Sigma). After electrophoresis, the gels were rinsed twice for 30 minutes each in 2.5% Triton X-100 (Sigma), incubated overnight at 37°C in substrate buffer, stained with Coomassie Blue R-250 (Bio Rad, Sydney, Australia), then destained to expose any gelatinolytic activity. A low range molecular weight protein ladder (Bio Rad) was run in adjacent lanes. MMP identity/activity was verified by running a sample of conditioned media derived from phorbol myristate acetate (PMA, Sigma) stimulated pterygium epithelial cells<sup>15</sup> and 50 ng of recombinant human MMP-2 (Calbiochem Novabiochem, Sydney, Australia) in adjacent



**Figure 3** Flow cytometric analysis of aqueous humour neutrophils. Pooled AH from six rat eyes was centrifuged and the cell pellet analysed by flow to determine neutrophil numbers over a 1 week time course post-LPS injection. Neutrophils peaked at 24 hours post-LPS, then gradually returned to basal levels by 1 week post-treatment. The results are expressed as the mean percentage of the total leucocytes counted (SD) of duplicate samples tested.

lanes. The addition of ethylenediaminetetra-acetic acid (EDTA: 10 mM final) and 1,10-phenanthroline (1 mM final) (Sigma) completely abolished all the gelatinolytic activity (data not shown). MMP-2 and MMP-9 activity was semiquantitated by analysing the lytic bands with the GEL DOC 2000 and the QUANTITY ONE program (Bio Rad).

## RESULTS

### Histopathology of EIU

Histological examination of rat ocular tissue showed a significant accumulation of leucocytes in the anterior chamber of rats with EIU<sup>3</sup> (data not shown). Animals sacrificed immediately after injection (T = 0 hours) demonstrated no inflammatory cells, while at 12 hours post-LPS cells began to accumulate, and at 24–48 hours post-LPS, vast numbers of cells were present. At these time points (representing peak inflammation), infiltrating cells consisted predominantly of neutrophils, with some macrophages and lymphocytes.<sup>3</sup> By 1 week post-LPS, inflammation had resolved, and very few cells were detected in ocular tissue and AH. Saline injected control eyes displayed minimal or no inflammation (micrographs not shown).

### Aqueous humour gelatinase activity

Pooled AH samples from animals treated with LPS were analysed by zymography to determine the relative amount and species of gelatinases present over a 1 week time course (Fig 1). MMP-9 activity was not detected in control saline injected animals (Fig 1A, lane 1), nor was it detected at 0 or 6 hours post-LPS. Proteolytic activity for this enzyme was observed at 12 hours, peaked at 18–24 hours, was faintly visible at 72 hours, and was absent by 1 week post-LPS treatment (Fig 1A). Interestingly, both the latent (92 kDa) and the active (87 kDa)

form of this enzyme was detected between 18–24 hours post-LPS, suggesting the presence of natural MMP activators during peak inflammation.

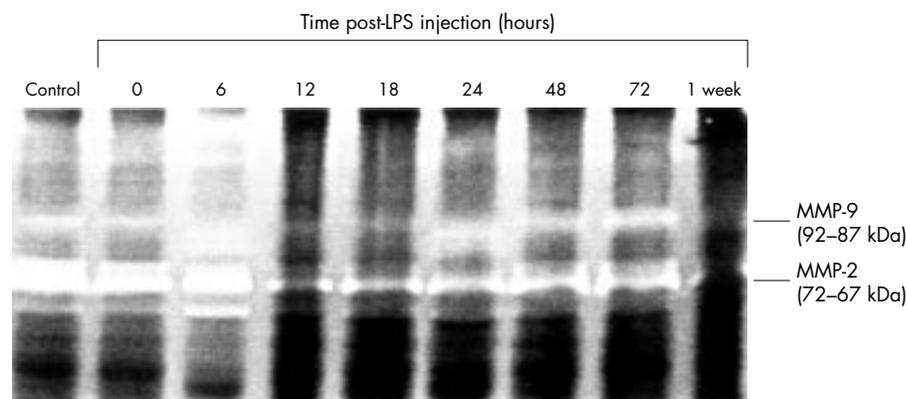
In contrast, MMP-2 was constitutively expressed as it was detected in saline injected control animals. This enzyme was upregulated as early as 6 hours post-LPS, reached maximum levels at 18–24 hours, then returned to baseline levels by 1 week post-LPS. While both the active and the zymogen form of MMP-9 was observed, MMP-2 was predominantly found in the latent configuration (72 kDa). MMP-2 was elevated 3.9-fold when compared to control AH harvested at the same time (24 hours) post-treatment (Fig 1B). The molecular weight of both enzyme species, the co-migration with recombinant human MMP-2 (Fig 1A, lane 11), and the previously characterised gelatinases from PMA stimulated pterygium epithelial cells<sup>10</sup> (Fig 1A, lane 10), and the absence of gelatin digestion in the presence of EDTA and 1, 10-phenanthroline (data not shown), provide strong supporting evidence that these two gelatinolytic species corresponded to MMP-2 and MMP-9.

### Localisation of MMP-2 and MMP-9 in EIU

Rat ocular tissue was sectioned and analysed immunohistochemically to determine the expression of MMP-2 and MMP-9. MMP-2 immunoreactivity was present at both 6 hours (Fig 2A) and 24 hours (Fig 2B) post-LPS, predominantly in the ciliary epithelium. Interestingly, intense staining for this enzyme was restricted to superficial epithelial cells that were in direct contact with the AH. Reactivity for this proteinase was also associated with some remaining AH neutrophils (Fig 2B, inset). Similarly, MMP-9 expression was observed in ciliary epithelial cells and detected at both early (Fig 2C) and later (Fig 2D) time points post-LPS. Intense staining was also displayed by most AH neutrophils (Fig 2D, inset). Ocular tissue derived from saline injected control animals displayed similar staining for MMP-2 and MMP-9, in the ciliary epithelium (data not shown). However, rarely were there any infiltrating neutrophils present in the AH (micrographs not shown). No immunoreactivity was found when the primary Ab was omitted (Fig 2E).

### Neutrophil accumulation in the aqueous humour of EIU

AH samples were pooled from animals with EIU over a 1 week time course, centrifuged, and the cell pellets resuspended and analysed by flow cytometry (Fig 3). Neutrophils, as determined morphologically, and by the number of CD45+/CD45R- cells, steadily increased at 12 hours (14.7% (SD 13.2)), peaked at 24 hours (41.6% (3.3%)), then decreased at 48–72 hours post-LPS. At 1 week post-LPS treatment, neutrophils were sparse. This pattern of neutrophil accumulation precisely coincided with the presence and absence of gelatinase activity (particularly gelatinase B) from rat AH (Fig 1).



**Figure 4** Gelatinolytic activity in rat ocular tissue extracts. After administering LPS, total protein was extracted from pooled rat iris and ciliary body (n = 3) to determine the gelatinolytic activity by zymography. Although MMP-2 and MMP-9 activities were identified in tissue extracts, both proteinases remained relatively constant throughout the time course of EIU.

### Gelatinase levels in rat ocular tissue

The abundant immunoreactivity for MMP-2 and MMP-9 in diseased rat ocular tissue suggested that the majority of the proteolytic activity in the AH may be derived from the ciliary epithelium and other resident cells, and not neutrophils. When the iris and ciliary body were removed from rat eyes and total protein extracted for zymographic analysis, both gelatinase species were detected in the tissue lysates over the EIU time course. However, the activity of these enzymes remained at a reasonably constant level (Fig 4). These results suggest that AH, rather than uveal tissue sampling, may more accurately reflect pathophysiological events in uveitis.

### DISCUSSION

The results of the present study demonstrate the progressive induction and depression of two structurally and functionally related MMPs in the AH of rats with EIU. Both MMP-2 and MMP-9 were maximally induced between 18–24 hours following LPS administration. Furthermore, the active form of MMP-9 was identified by gelatin zymography. MMP-2 was present in the AH of control animals and at early time points post-LPS treatment, suggesting continuous basal synthesis of this protease, as would be expected from a house-keeping-like gene.<sup>26</sup> While MMP-9 was not detected early post-LPS administration, it was induced at later time points.

MMP-2 appears to be an important enzyme particularly in normal physiological processes, such as in the maturing embryo and in the developing eye.<sup>29</sup> In the eye, this proteinase is involved in wound healing with prolonged expression.<sup>30</sup> Likewise, MMP-2 expression is persistent throughout the endometrial cycle,<sup>31</sup> indicating its importance in the extensive tissue remodelling that is associated with the reproductive cycle. Its role in disease pathology is just as critical, where studies have identified the constitutive expression of MMP-2 in normal prostate, with greater expression in prostatic intraepithelial neoplasia, and maximal expression in prostatic adenocarcinoma.<sup>32</sup> MMP-2 displays interstitial collagenolytic activity and is thus capable of denaturing fibrillar collagen.<sup>33</sup> The apparent constitutive expression of this enzyme in the ciliary body suggests it may play an important part in the maintenance and turnover of matrix in this tissue. While in an inflammation setting, the preferential and pronounced activity against basement membrane type IV collagen makes both MMP-2 and MMP-9 likely to be involved in leucocyte migration, extravasation,<sup>15 20 34</sup> and angiogenesis,<sup>21</sup> processes commonly observed in uveitis.

Having determined the gelatinolytic activity in the AH of rats with EIU, immunohistochemistry was performed to determine the cellular source of both gelatinases. Our studies identified ciliary epithelial cells and AH infiltrating neutrophils as the predominant source of these enzymes. Although previous studies have detected MMP-9 in neutrophils,<sup>24 35</sup> to our knowledge the expression of these two MMPs by the ciliary epithelium has not been previously observed. Furthermore, intense staining for both gelatinases was associated with the more peripheral ciliary epithelial cells. Direct contact with AH (known to contain inflammatory mediators), may activate these cells, resulting in increased MMP production. Studies in rheumatoid arthritis have demonstrated a similar pattern of cellular activity and of differential MMP expression by synovial fluid bathed synovial lining cells.<sup>36</sup>

Neutrophil accumulation (Fig 3) correlated closely with increased gelatinolytic activity in AH samples (Fig 1), suggesting that these AH enzymes were predominantly secreted by infiltrating polymorphonuclear leucocytes. Evidence to support this hypothesis is presented in Figure 4, where tissue levels of MMP-2 and MMP-9 were not significantly modified over the 1 week time course of EIU. This is of considerable interest, since it implies that analysing

tissue specimens may not always reflect the stage of disease. Analysis of AH may be a more reliable gauge for estimating inflammation, as total leucocyte numbers, chemokine,<sup>37</sup> cytokine,<sup>38</sup> and MMP<sup>10 38</sup> levels are easily estimated. Furthermore, the presence of a high molecular weight (~130 kDa) gelatinolytic activity in the non-reduced SDS-PAGE zymogram (Fig 1A), is likely to represent lipocalin/progelatinase B<sup>39</sup> (a complex only found in neutrophils).

Recently, the role of P and E selectin in the recruitment of inflammatory cells during EIU was evaluated.<sup>40</sup> This study demonstrated the importance of these molecules in the initial phase of cellular infiltration into the iris and ciliary body, as inhibition of both adhesion molecules abrogated leucocyte accumulation in the AH. A similar investigation has shown the protective effects of oestrogen against cellular infiltration in EIU, probably by reducing the expression of E-selectin and IL-6.<sup>41</sup> This is relevant, since oestrogen has been shown to prevent LPS induced production of MMP-9 in other models of disease.<sup>42</sup>

The importance of neutrophil migration and infiltration was highlighted in two recent studies of EIU.<sup>7 8</sup> Both investigations demonstrated increased expression of two potent neutrophil chemoattractants (GRO and IL-8) that localised predominantly to the ciliary epithelial cells. Animals treated with anti-GRO<sup>7</sup> or anti-IL-8<sup>8</sup> Ab resulted in the suppression of AH neutrophils by 80% and 66% respectively. This is not surprising, since neutrophil derived MMP-9 can cleave several amino acid residues from the amino terminus of IL-8 and potentiate its chemotactic activity up to 10-fold in an autocrine manner.<sup>43</sup>

Our data are in general agreement with other studies, which have shown that MMP-9 is not usually found in the AH of normal subjects, although it is abundant in the AH of patients with active uveitis.<sup>38</sup> The same study concluded that MMP-2 and MMP-9 levels significantly correlated with disease activity and cytokine levels, although the cellular source was not disclosed. Other studies have demonstrated an increased secretion of MMP-9 by neutrophils in response to endotoxin and cytokines, such as tumour necrosis factor, interleukin-8, and granulocyte colony stimulating factor.<sup>28</sup> This is relevant as proinflammatory cytokines such as IL-1,<sup>37</sup> IL-6,<sup>6</sup> IL-8,<sup>7 8</sup> TNF- $\alpha$ ,<sup>44</sup> and the chemokine MCP-1<sup>7</sup> have been detected in the AH of patients and experimental animals with uveitis. Such cytokines and chemokines are known to be potent modulators of MMPs.<sup>15 33 45 46</sup>

The functional significance of MMPs in immune disorders, such as uveitis, should not be underestimated, as recent work has shown that MMP-9 deficient mice are resistant to experimental autoimmune encephalomyelitis.<sup>47</sup> Recently Wallace *et al*<sup>48</sup> demonstrated the increased expression of neutrophil collagenase (MMP-8), matrilysin (MMP-7), and macrophage metalloelastase (MMP-12) mRNA in experimental autoimmune uveoretinitis (EAU). Furthermore, the disease onset in this animal model was delayed by the administration of a synthetic peptide inhibitor of MMPs (BB1101). These previous findings and our current data strongly implicate the gelatinases and other MMPs in the pathogenesis of uveitis.

### ACKNOWLEDGEMENTS

Grant support: National Health and Medical Research Council of Australia.

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