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

C Cuello, D Wakefield, N Di Girolamo

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.
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 µl intraperitoneal injection of 150 µg 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 µl) and centrifuged to remove cells and debris, then aliquoted and stored at −70°C until assayed. In a separate experiment, AH was centrifuged, the cells resuspended in endotoxin free saline and stored at −70°C until assayed. Aqueous humour samples were harvested at 24 hours post-LPS injection (n = 6) and from LPS treated rats harvested at 6, 12, 18, 24, 48, 72 hours, and 1 week post-injection. Conditioned AH was 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.

Flow cytometry
Two and three colour immunophenotyping of AH cells was performed using fluorescein isothiocyanate, phycoerythrin, or allophycocyanin.
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 (IgG1κ-FITC, IgG2aκ-Cyc, IgG2bκ-PE, and IgG3κ-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 × 10⁵ cells/sample). Acquisition was measured using a fluorescence activated cell sorter (FacsCan, 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. 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 and 50 ng of recombinant human MMP-2 (Calbiochem Novabiochem, Sydney, Australia) in adjacent
Matrix metalloproteinases in EIU

RESULTS

Histopathology of EIU

Histological examination of rat ocular tissue showed a significant accumulation of leucocytes in the anterior chamber of rats with EIU (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. 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 of EIU. 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, 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 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 (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 suspended 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 1A).

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.

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.36 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.37 In the eye, this proteinase is involved in wound healing with prolonged expression.38 Likewise, MMP-2 expression is persistent throughout the endometrial cycle,39 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.40 MMP-2 displays interstitial collagenolytic activity and is thus capable of denaturing fibrillar collagen.41 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,15 20 34 and angiogenesis,21 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,38 40 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.42

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,43 cytokine,44 and MMP45 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 B46 (a complex only found in neutrophils).

Recently, the role of P and E selectin in the recruitment of inflammatory cells during EIU was evaluated.47 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-6.48 This is relevant, since oestrogen has been shown to prevent LPS induced production of MMP-9 in other models of disease.49

The importance of neutrophil migration and infiltration was highlighted in two recent studies of EIU.50 51 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-GRO52 or anti-IL-8 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-6 and potentiate its chemotactic activity up to 10-fold in an autocrine manner.53

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.54 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.55 This is relevant, since proinflammatory cytokines such as IL-1,56 IL-8,51 TNF-α,56 and the chemokine MCP-1 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.55 56 57 58

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.46 Recently Wallace et al59 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.

Authors’ affiliations
C Cuello, D Wakefield, N Di Girolamo, Inflammation Research Unit, Department of Pathology, University of New South Wales, Sydney, NSW, Australia

REFERENCES
Matrix metalloproteinases in EIU


