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HELMINTHOLOGY - ORIGINAL PAPER

Matrix metalloproteinase-2 and matrix metalloproteinase-9 in mice with ocular toxocariasis

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Abstract

In ocular toxocariasis, *Toxocara canis*-induced inflammatory reaction can lead to eye destruction and granuloma, which is formed by immune cell infiltration and concurrent extensive remodeling tissue. Herein, the histomorphology of granuloma and proteinase production in the eye of *T. canis*-infected BALB/c mice were investigated. Pathological effects substantially increased after the infection culminated in a severe leukocyte infiltration and granuloma formation from days 4 to 56 post-inoculation. The matrix metalloproteinase (MMP)-2 and MMP-9 activities remarkably increased, compared with those of uninfected control, by gelatin zymography and Western blot analysis in ocular toxocariasis. Granuloma formation had a remarkably positive correlation with MMP-2 and MMP-9 levels. We suggested that *T. canis* larvae and leukocytes infiltrated from blood vessel both migrated into corpus adiposum orbitae. Activated leukocytes secreted MMP-2 and MMP-9, leading to fibronectin degradation. The imbalance of MMP-2/TIMP-2 and MMP-9/TIMP-1 may play a role in inflammatory cell infiltration and extracellular matrix degradation, forming granuloma, in ophthalmological pathogenesis of *T. canis* infection.

Keywords Fibronectin · Granuloma · Metalloproteinase · Ocular · *Toxocara canis*

Introduction

Intraocular inflammations can be characterized histopathologically as purulent or nonpurulent and granulomatous or nongranulomatous, depending on the stage of the disease and the pattern of infiltrating leukocytes. An inflammatory response is a protective mechanism of the host to defend against infections and to promote tissue repair (Krauss and Woodward 1993). Ocular toxocariasis is a zoonotic parasitic infection mainly caused by the intraocular invasion of a third-stage larva of *Toxocara canis*, usually presenting as a severe unilateral intraocular inflammatory response (Duguid 1961). Critical infection leads to invasion of the retina, subsequently forming granuloma either peripherally or in the posterior pole. The granuloma drags the retina and leads to distortions, heterotopia, or macular detachment. Depending on the region of infection in the eye, the patient may have minor visual impairment or blindness (Despommier 2003).

Matrix metalloproteinases (MMPs) have been implicated in ocular diseases such as uveitis (Di Girolamo et al. 1996), scleritis (Di Girolamo et al. 1997), and pterygia (Di Girolamo et al. 2000). MMP-9 participates in extracellular matrix (ECM) remodeling after wounding of the corneal surface and has been associated in the pathogenesis of keratoconus (Galvis et al. 2015) and dry eye (Messmer et al. 2016). In parasitic infection, gelatinases are important for immune cell migration, macrophage recruitment, and effective granuloma formation. Granulomatous fibrosis of rats infected with *Angiostrongylus cantonensis* is strongly associated with MMP-2 and MMP-9 (Hsu et al. 2005). In patients with a solitary cysticercus granuloma, cytokines, MMP-2, and MMP-9 in the cerebrospinal fluid (CSF) and serum are elevated (Lalla et al. 2015). Granulomatous fibrosis results from an imbalance of the normal processes of synthesis and degradation of ECM components. The fibrotic response is an irreversible process characterized by a progressive accumulation of connective tissue proteins (Crouch 1990). Fibronectin is a

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multifunctional, high molecular-weight glycoprotein found in plasma, ECM, and CSF. It is involved in cellular adhesion, cellular migration, and phagocytosis (Ouaissi and Capron 1985). Under normal conditions, fibronectins are present in the CSF at low concentrations. Changes in the levels of this protein in the central nervous system are associated with various pathological conditions (Nasu-Tada et al. 2006).

Toxocara larvae can directly invade the intraocular tissues and induce a granulomatous reaction, but its biochemical characteristics are poorly understood. To investigate whether the proteolytic enzymes could be induced by *T. canis* larvae in eyes, we developed a mouse experimental model to examine the production of MMP-2 and MMP-9 in the ocular fibrosis of *T. canis*-infected rats. Furthermore, we examined gelatinase expression profiles during larval migration and whether the expression would coincide with the histopathology.

Materials and methods

Experimental animals

Male BALB/c mice (specific pathogen-free grade and 5 weeks old) were purchased from the National Laboratory Animal Center, Taipei, Taiwan. The mice were maintained in a 12-h alternating light-and-dark cycle photoperiod and were provided with Purina Laboratory Chow and water ad libitum. The mice were kept in our laboratory for more than 1 week prior to experimental infection. All procedures that involved animal use and care were approved by the Institutional Animal Care and Use Committee of Chung Shan Medical University, Taiwan, and were performed in accordance with the institutional guidelines for animal experiments.

Larval preparation

Hatched *T. canis* larvae were obtained according to Savigny (1975), with slight modification. Briefly, *T. canis* eggs were recovered from the uteri of adult female worms obtained from the gastrointestinal tracts of dogs at autopsy. The eggs were washed and stored in 4% formalin at room temperature (21 °C) for 4 weeks. Following embryonation, the embryonated eggs were incubated in 2% formalin at room temperature (18–23 °C) with weekly gentle agitation for 3–5 weeks. The eggs were washed thrice with phosphate-buffered saline (PBS) to remove the formalin, and the mixture was centrifuged for 10 min at 400×*g*. The supernatant was discarded, and the pellet was stirred in 10 ml 3% sodium hypochlorite for 1 h. After the eggshell lysis, the mixture was again centrifuged for 10 min at 400×*g*. The pellet was washed thrice with PBS and collected for experimental studies.

Animal infection

A total of 72 BALB/c strain male mice were randomly assigned to six groups: control, day 4, day 7, day 14, day 28, and day 56. The mice of experimental groups (day 4, day 7, day 14, day 28, and day 56) were inoculated with 2000 embryonated *T. canis* eggs each through a stomach tube and sacrificed on days 4, 7, 14, 28, and 56 post-inoculation (PI), respectively. The control mice received only water and were sacrificed on day 56 PI. The eyes of all groups were collected for experimental studies. These tissues were processed for histology, zymography, and Western blot.

Histology

The eyes from each mouse were fixed separately in 10% neutral buffered formalin at room temperature for 24 h. The fixed specimens were dehydrated in a graded ethanol series (50, 75, and 100%) and xylene, then embedded in paraffin at 55 °C for 24 h. Several serial sections were incised at 5 μm thickness. Paraffin was removed by heating the sections for 5 min at 65 °C. These sections were dewaxed by washing thrice for 5 min each in xylene, then rehydrated through 100, 95, and 75% ethanol for 5 min each, and finally rinsed with distilled water. After staining with hematoxylin (Muto, Japan) and eosin (Muto, Japan), pathological changes were examined under light microscopy (Leica, Germany).

Hematological examination

Five infected blood samples were collected on days 4, 7, 14, 28, and 56 PI. Five control samples were collected on day 56 PI. Peripheral blood was collected from the tail vein and examined with a cell counter for total leukocyte counts. The differential cell count was assessed with Wright-Giemsa staining (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) in 3 μL/smear.

Gelatin zymography

T. canis-induced production of proteinase in mouse eyes was analyzed by gelatin zymography as described previously (Chiu and Lai 2013). In brief, *T. canis*-infected eyes from mice were individually homogenized in a buffer containing 0.1% Triton X-100, 150 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.5 mM K_2HPO_4. The homogenates were then centrifuged at 12,000×*g* at 4 °C for 10 min to remove debris, and the protein contents of the supernatants were determined with protein assay kits (Bio-Rad, USA) by using bovine serum albumin (BSA) as the standard. An equal volume of sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate (SDS), and 0.05% bromophenol blue) was added to the samples, which contained 30 μg of eye tissue

protein. The protein contents of supernatants were loaded on 7.5% (mass/volume) SDS-polyacrylamide gel electrophoresis that had been co-polymerized with 0.1% gelatin (Sigma, USA). Stacking gels contained 4% (mass/volume) polyacrylamide, without gelatin substrate. Electrophoresis was performed in running buffer (25 mM Tris, 250 mM glycine, 1% SDS) at room temperature at 110 V for 1 h. The gel was washed twice at room temperature for 30 min each in 2.5% Triton X-100 and then washed again twice with ddH_2O for 10 min each. The gel was incubated in reaction buffer (50 mM Tris-HCl, pH 7.5, containing 200 mM NaCl, 10 mM $CaCl_2$, 0.02% Brij-35, and 0.01% NaN_3) at 37 °C for 18 h. The gel

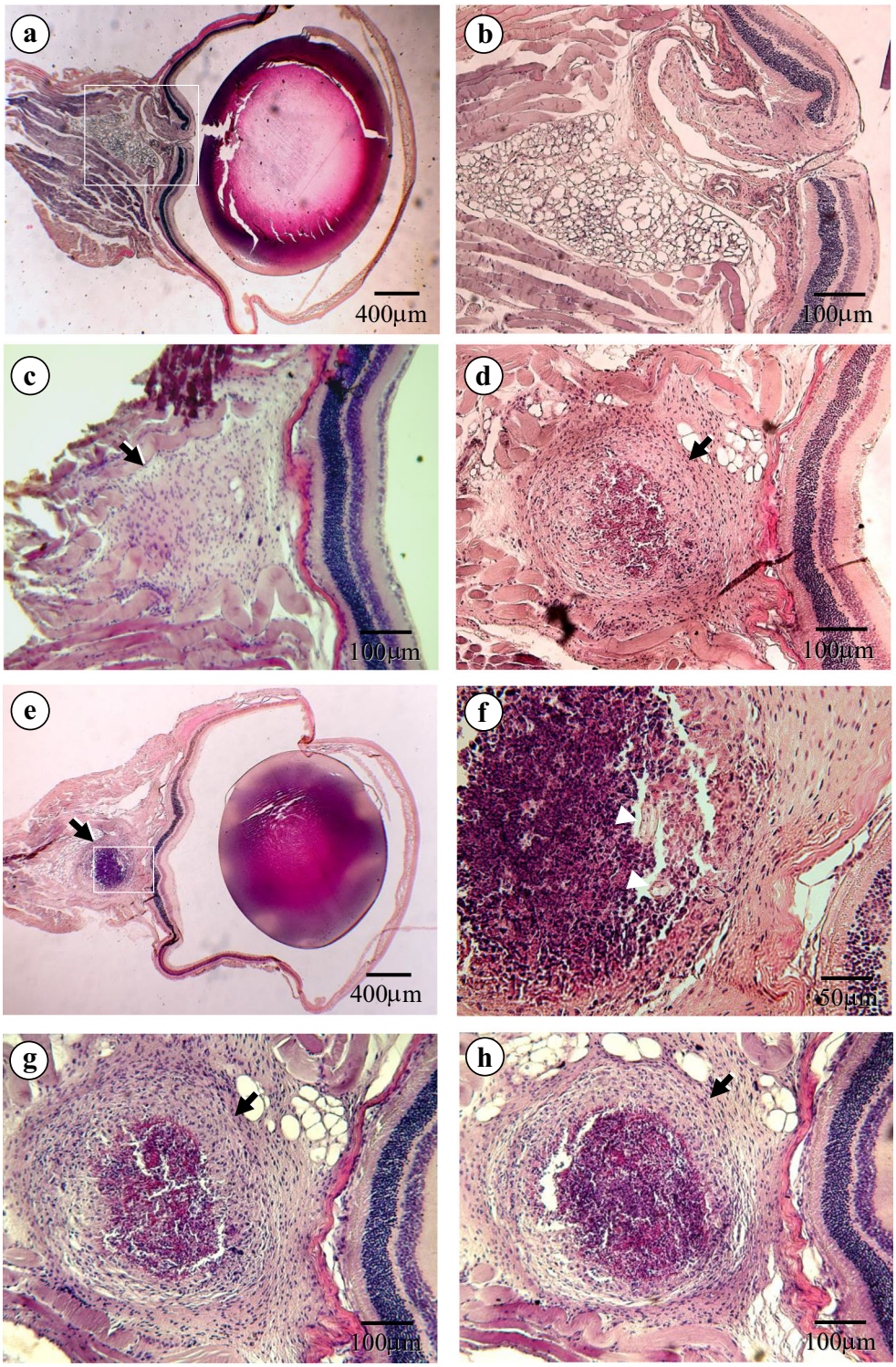

Fig. 1 Histopathological changes in the corpus adiposum orbitae of eye. **a** Eye of uninfected control. **b** Enlargement of corpus adiposum orbitae, shown in the rectangle of Fig. 1a. **c** A granuloma-like structure (arrow) in corpus adiposum orbitae on day 4 post-inoculation (PI). **d** A diffuse chronic granulomatous inflammation (arrow) on day 7 PI. **e** The granuloma (arrow) was formatted in corpus adiposum orbitae on day 14 PI. **f** Enlargement of the portion, shown in the rectangle of Fig. 1e. Leukocytes clumped together around the *T. canis* (arrowheads) to form a granuloma. **g** The granuloma (arrow) was aggregated by inflammatory cells on day 28 PI. **h** The areas around the granuloma (arrow) showed chronic inflammation on day 56 PI

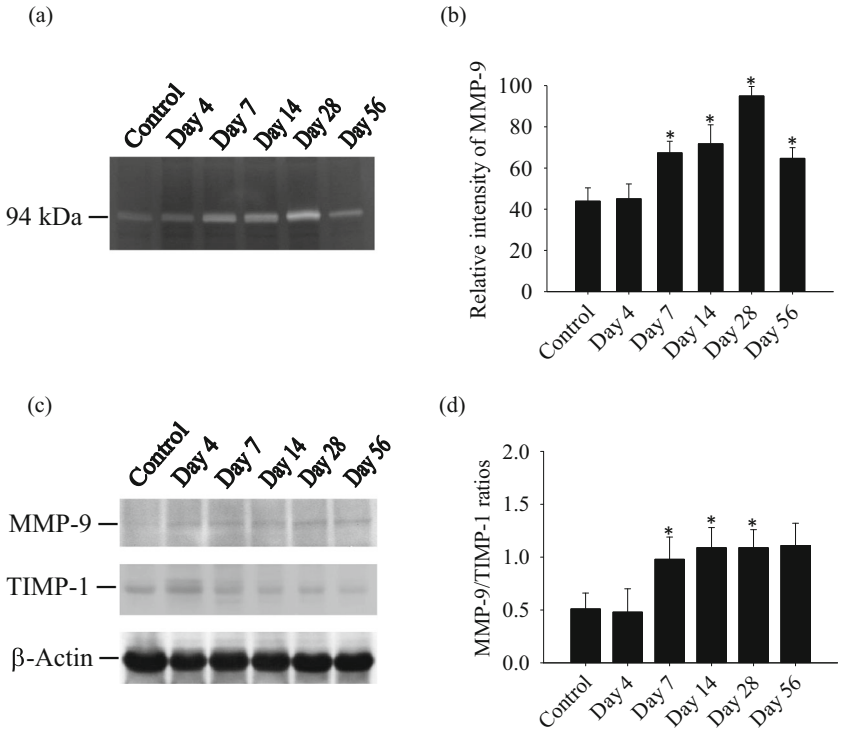

Fig. 2 Matrix metalloproteinase-9 (MMP-9) in a *T. canis*-infected eye. **a** The MMP-9 activities were analyzed by using gelatin zymography and time-course studies in the eyes of mice. **b** Quantitative analysis of MMP-9 was performed with a computer-assisted imaging densitometer system. **c** The bands of MMP-9 and TIMP-1 were analyzed by using Western blot and time-course studies in the eyes of mice. β-actin was used as a loading control. **d** Quantitative analysis of MMP-9/TIMP-1 ratios was performed with a computer-assisted imaging densitometer system. Asterisk indicates statistically significant difference in comparison with controls. Bars represent mean ± SD from three independent experiments performed twice

was stained with 0.25% Coomassie brilliant blue R-250 (Sigma, USA) for 1 h and destained in 15% methanol/7.5% acetic acid. Gelatinase activity was detected as unstained bands on a blue background. Quantitative analysis of the gelatinolytic enzyme was performed with a computer-assisted imaging densitometer system, UN-SCAN-ITTM gel version 5.1 (Silk Scientific, USA).

Western blot analysis

The extracts of mouse eyes were centrifuged at 12,000×*g* for 10 min to remove debris. The protein concentration was analyzed by using a protein assay kit (Bio-Rad, Hercules, CA, USA), with BSA serving as the standard. In brief, 30 μg of proteins was separated by means of 10% SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. The membranes were incubated for 60 min in blocking buffer (PBST containing 5% nonfat milk powder) at room temperature. Afterward, the membrane was washed thrice with PBST. Membranes were probed with anti-mouse antibodies for mouse-anti-mouse MMP-2, MMP-9, TIMP-1, and TIMP-2 (R&D Systems, Minneapolis, MN) diluted 1:1000 in 1% BSA, and the loading control was probed with monoclonal antibody for β-Actin (Sigma, St. Louis, MO, USA) diluted 1:5000 in 1% BSA at 37 °C for 60 min. The membranes were washed thrice for 10 min each time in PBST. They were incubated with the horse radish peroxidase (HRP)-conjugated rabbit-anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:10000 in 1% BSA at 37 °C for 60 min. Then, they were detected by using the enhanced chemiluminescence method (Amersham Biosciences, Amersham, UK).

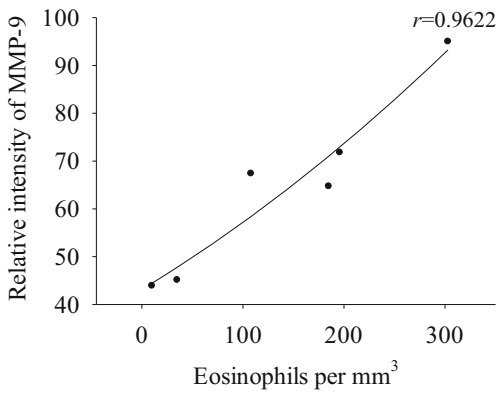

Fig. 3 Correlation of eosinophils with MMP-9 intensity. Eosinophils were correlated significantly ($r = 0.9662$; $P < 0.05$) with the MMP-9 intensity

Statistical analysis

The results for the different mouse groups were compared using the nonparametric Kruskal–Wallis test followed by post-testing using Dunn's multiple comparison of means. All results are presented as the mean ± standard deviation. The Spearman's ranking correlation test was used, and P values < 0.05 were considered statistically significant.

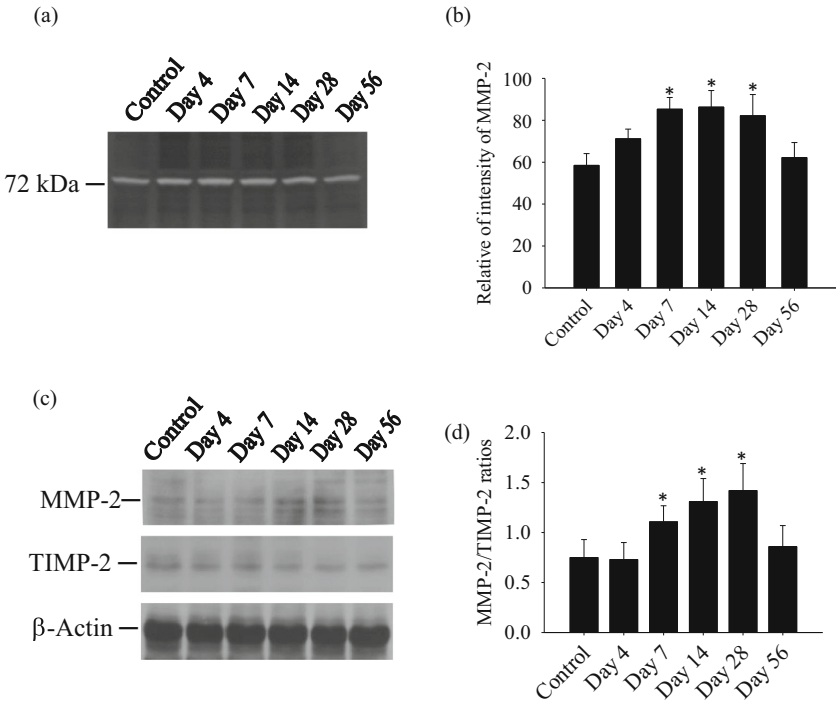

Fig. 4 Matrix metalloproteinase-2 (MMP-2) in a *T. canis*-infected eye. **a** The MMP-2 activities were analyzed by using gelatin zymography and time-course studies in the eyes of mice. **b** Quantitative analysis of MMP-2 was performed with a computer-assisted imaging densitometer system. **c** The bands of MMP-2 and TIMP-2 were analyzed by using Western blot and time-course studies in the eyes of mice. β-actin was used as a loading control. **d** Quantitative analysis of MMP-2/TIMP-2 ratios were performed with a computer-assisted imaging densitometer system. Asterisk indicates statistically significant difference in comparison with controls. Bars represent mean ± SD from three independent experiments performed twice

Results

Histological observations

Histological examination showed that *Toxocara* granulomas were unilateral in all infected mice. Intraocular granulomas in experimental groups (day 4, day 7, day 14, day 28, and day 56 PI) were 2/10 (20%), 2/10 (20%), 3/10 (30%), 3/10 (30%), and 3/10 (30%), respectively. However, larvae were only found in the granulomas of eyes on days 7 and 14 PI. The eye collected on day 4 PI had inflammatory cell infiltration. Furthermore, all eyes collected on days 7, 14, 28, and 56 PI had tight-structured granulomas surrounded by infiltrating leukocytes. In contrast, no inflammatory reactions were seen in the eye of control mice (Fig. 1).

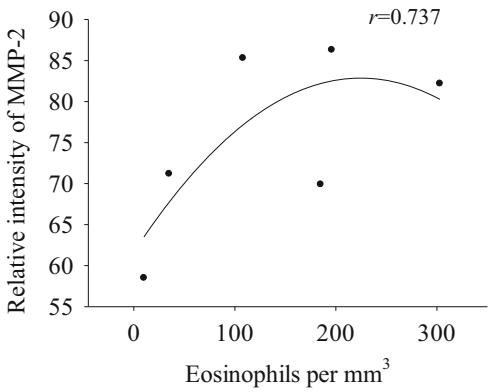

Fig. 5 Correlation of eosinophils with MMP-2 intensity. Eosinophils were correlated significantly ($r = 0.737$; $P < 0.05$) with the MMP-2 intensity

MMP-9 in the eye with *T. canis* infection

Substrate gel electrophoresis, with gelatin as substrate, was used to determine the levels of gelatinase activity at various time points in the eyes with *T. canis* infection. The collected samples of *T. canis*-infected eyes showed that the 94-kDa bands were MMP-9. MMP-9 activities remarkably increased on days 7, 14, 28, and 56 PI, as compared with control. Similarly, Western blot analysis of MMP-9/TIMP-1 ratios substantially increased on days 7, 14, 28, and 56 PI, as compared with control (Fig. 2).

Correlation of eosinophils with MMP-9 activity

The eosinophils in the blood of infected mouse were significantly increased from days 4 to 56 PI. Quantitative analysis showed eosinophils significantly correlated with the MMP-9 intensity (Fig. 3).

MMP-2 in the eye with *T. canis* infection

The collected samples of *T. canis*-infected eyes showed that the 72-kDa bands were MMP-2. MMP-2 activities remarkably increased on days 4, 7, 14, and 28 PI, as compared with control. To examine the expression patterns of MMP-2 and TIMP-2 in *T. canis*-infected eyes, we performed Western blot analysis. MMP-2/TIMP-2 ratios substantially increased on days 14 and 28 PI, as compared with control (Fig. 4).

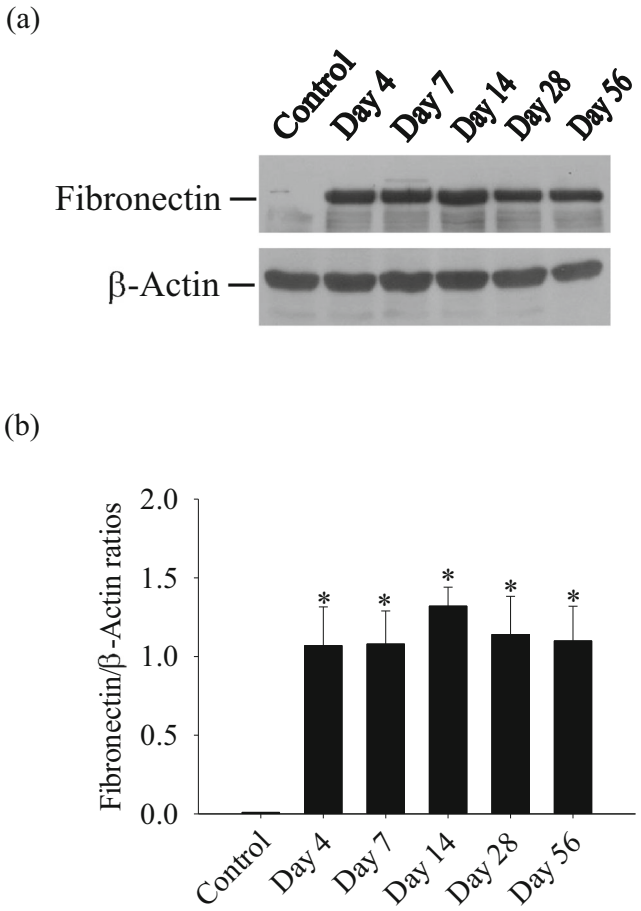

Fig. 6 Fibronectin protein levels in a *T. canis*-infected eye. **a** The 220-kDa fibronectin bands were detected on days 4, 7, 14, 28, and 56 post-inoculation (PI). **b** Quantitative analysis of fibronectin of the infected group was performed with a computer-assisted imaging densitometer system. Mean ± SD of three independent experiments performed twice. Asterisk indicates a statistically significant difference

Correlation of eosinophils with MMP-2 activity

The eosinophils assay results for the mouse blood after *T. canis* infection were increased significantly from days 4 PI to 56 PI. Quantitative analysis showed eosinophils significantly correlated with the MMP-2 intensity (Fig. 5).

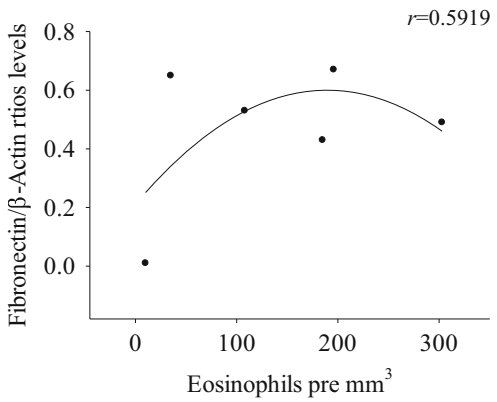

Fig. 7 Correlation between fibronectin and eosinophil counts. Fibronectin levels were significantly correlated ($r = 0.5919$; $P < 0.05$) with eosinophil counts

Fibronectin protein levels

Western blot results showed that the time-course studies of the 220 kDa of fibronectin remarkably increased ($P < 0.05$) from days 4 to 56 PI. Repeated experiments using different eye samples yielded consistent results (Fig. 6).

Correlation between eosinophil counts and fibronectin

Eosinophilia was observed in infected mice but not in uninfected mice. The eosinophil counts were significantly correlated with fibronectin processing (Fig. 7).

Discussion

The principal pathological entity in ocular toxocariasis is the eosinophilic abscess or granuloma. Eosinophil performs a major role in the eye's defense against parasites (Rockey et al. 1979). It is a prominent feature of several ocular immunopathologic reactions but is especially numerous in the intraocular and periocular granulomatous reactions caused by parasites (Rockey et al. 1981; Ashton 1960). In this study, few larvae were within the center of a granuloma on days 14 to 56 PI in histological sections. The possible explanations were discussed subsequently. (1) *Toxocara* third-stage larvae could migrate out of an early granuloma. Larvae were rapid tissue migrators, which had individual tracts in the tissues through which they migrate. (2) The larvae and their products or remnants may incite granuloma formation; their continued presence may not be necessary for the propagation of the immune response (Ghafoor et al. 1984; Shields 1984). Furthermore, we supposed that few larvae in the granuloma may be the larvae that were killed by inflammatory cells. However, the granuloma may be continuatively induced by the larva or its remnants in *T. canis*-infected eyes.

MMPs are involved in the corneal response to injury and infection (Wong et al. 2002; Ollivier et al. 2007). In ulcerating cornea, destruction of corneal ECM components has been attributed largely to the action of a family of MMPs that can collectively degrade virtually all ECM components. These assumptions are based primarily on the ability of different broad-spectrum MMP inhibitors to block corneal tissue loss or destruction (Wentworth et al. 1992; Barletta et al. 1996). MMP-9 increases immediately after superficial corneal wounding, and fungal infection perpetuates its upregulation. MMP-9 may also promote necrotizing inflammation and neovascularization with progressive corneal infection (Ma and Dohlman 2002; Yang et al. 2003). MMPs are being

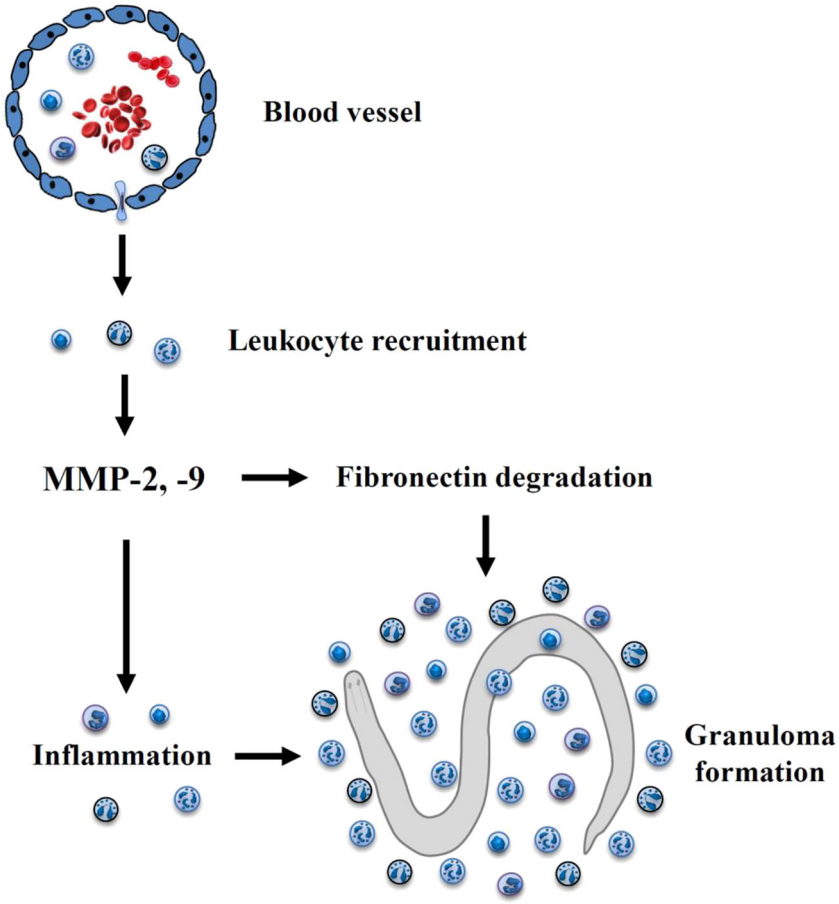

Fig. 8 The possible mechanisms for the ocular toxocariasis in corpus adiposum orbitae. *T. canis* larvae and leukocyte infiltrated from blood vessel both migrated into the corpus adiposum orbitae. Activated leukocytes secreted matrix metalloproteinase (MMP)-2 and MMP-9, leading to fibronectin degradation, and followed by a release of pro-inflammatory cytokines from ECM-binding sites, further activating the leukocytes. In addition, chemotactic, migration-inducing factors were released from ECM, causing an increase in infiltration of leukocytes evaded from the blood vessel through the endothelium and found a paved way through the degraded ECM

increasingly implicated in the pathogenesis of eye diseases (Sivak and Fini 2002). Elevated amounts of MMP-2 and MMP-9 are found in the aqueous humor and in infiltrating cells of both patients and animal models with uveal inflammation (Di Girolamo et al. 1996). In this study, the *Toxocara* induced a strong granuloma formation reaction, comparable to the reactions seen in infection. In addition, we discovered that MMP-2 and MMP-9 activities increased during early *Toxocara* infection but were returned to normal condition in late stage. These activated forms together with the enzymes released from the infiltrating inflammatory cells led to uncontrolled activation of MMPs, resulting in fibronectin degradation. We presumed that MMP-2 and MMP-9 may participate in granuloma formation in *Toxocara*-infected eye.

MMP family plays a significant role in many biological activities, including numerous immune responses, such as granuloma formation (Izzo et al. 2004; Parks et al. 2004). The expression of the two gelatinases (MMP-2 and MMP-9) at different stages of fibrosis suggests that MMP-9 could be rather linked to inflammation-induced tissue remodeling, whereas MMP-2 is associated with an impaired tissue remodeling, leading to pathological collagen deposition and interstitial fibrosis (Gueders et al. 2006). MMPs influence not only the immune response and clearance of pathogens in early disease course but also the progression to fibrosis within the final pathway of inflammation (Korpos et al. 2009). We further found that *Toxocara* infection remarkably increased MMP-2 and MMP-9 activities in the eye on days 7 to 28 PI. Leukocyte accumulation correlates with MMP-2 and MMP-9 activities in *T. canis*-induced granulomas. We supposed that MMP-2 and MMP-9 play substantial role in the early development of granulomas by promoting tissue remodeling and leukocyte recruitment into granulomas. However, the immune response was required to downregulate MMP activity and thus required for the generation of tight granulomas.

The clinical presentation and nature of infiltration are important factors to be considered when investigating corneal tissues for proteolytic activity following infection. In physiological circumstances, the balance between MMPs and their natural inhibitors is crucial in maintaining homeostasis of ECM proteins. In pathological tissue-destructive conditions, MMPs and their inhibitors are imbalanced (Pelletier et al. 1990). MMP-9 is one of these extracellular proteases and is released in tissues in response to pro-inflammatory stimuli. The sensitive detection of MMP-9 by gelatin zymography analysis makes it a suitable marker for objective scoring of eye inflammation. Given that MMP-9 is a prototypic activation product of various leukocyte types, gelatinolytic analysis can be used for evaluating acute or chronic inflammation.

Deregulation of TIMP or MMP activities leads to states of either exaggerated ECM turnover, often leading to remodeling via impaired repair and scar formation, or ECM accumulation, leading to fibrosis (Brew et al. 2000). An imbalance of the expression of MMPs and TIMPs and fibrogenic cytokine production seems to be associated with cumulative fibrosis (Hemmann et al. 2007). MMPs play key roles in leukocyte migration across the blood–retinal barrier in ocular infections. MMP-2 and MMP-9 are involved in the breakdown of physical barriers by degrading basement membranes and proteins associated with tight junctions or other membrane protein complexes (Cauwe et al. 2007). In our study, a higher ratio of MMP-2/TIMP-2 and MMP-9/TIMP-1 was associated with ocular granuloma formation. We presumed that *T. canis* larvae and leukocytes infiltrated from blood vessel both migrated into corpus adiposum orbitae. Activated leukocytes secreted MMP-2 and MMP-9, leading to fibronectin degradation, and followed by a release of pro-inflammatory cytokines from ECM-binding sites, which further activated the leukocytes. In addition, chemotactic, migration-inducing factors were released from ECM, thereby increasing the infiltration of leukocytes evaded from the blood vessel through the endothelium and found a paved way through the degraded ECM. The same factors attracted myofibroblasts, which synthesize ECM, in an attempt to limit the process (Fig. 8).

In this study, we suggested that imbalance of MMP-2/TIMP-2 and MMP-9/TIMP-1 may play a role in inflammatory cell infiltration and ECM degradation for the pathogenesis of ocular granuloma formation.

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Compliance with ethical standards The manuscript does not contain clinical studies or patient data.

Conflict of interest The authors declare that they have no conflict of interest.

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