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Induction of tumour necrosis factor, interleukin-1β and matrix metalloproteinases in pulmonary fibrosis of rats infected with *Angiostrongylus cantonensis*

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Abstract

In angiostrongyliasis, chronic parasite-induced granuloma formation can lead to tissue destruction and fibrosis. Here, the histomorphology of granulomatous fibrosis and proteinase production in the lungs of *Angiostrongylus cantonensis*-infected Sprague-Dawley rats were investigated. The relationship between metalloproteinases and granulomatous fibrosis was investigated following infection of each rat with 60 infective larvae. Granulomata and fibrosis were marked in the lungs of rats on day 180 post-inoculation. Reverse transcriptase polymerase chain reaction of lung mRNA showed an up-expression of proinflammatory cytokine including tumour necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β). According to Western blot analysis, matrix metalloproteinase-2 (MMP-2) proenzyme was presented in the lungs of uninfected and infected rats, and partial conversion of 72 kDa proenzyme to the 64 kDa active form occurred in infected rats. In addition, increased protein levels of MMP-9 and MMP-13 were detected in infected lungs, but were undetectable in controls. The results suggest that TNF-α, IL-1β, MMP-2, -9, and -13 may be associated with the granulomatous fibrosis.

Introduction

Granulomatous fibrosis results from an imbalance in the equilibrium of the normal processes of synthesis and degradation of extracellular matrix components. The fibrotic response is generally considered an irreversible process and is characterized by a progressive accumulation of connective tissue proteins (Crouch, 1990). Recently, matrix metalloproteinases (MMPs) have been shown to be key enzymes in the process of lung fibrosis (Winkler & Fowlkes, 2002). MMPs are a family of zinc metallo-endopeptidases that regulate cell-matrix composition (Matrisian, 1992). Amongst more than 20 kinds of MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are considered to be especially important in the degradation of the extracellular matrix and to play a prominent role in clinical pulmonary disease (Fukuda *et al.*, 1998; Ohnishi *et al.*, 1998; Cataldo *et al.*, 2000). The expression of MMP by immune cells is greatly modulated by inflammatory mediators such as tumour necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-4 (IL-4) (Mauviel, 1993; Birkedal-Hansen, 1995).

Angiostrongylus cantonensis is a parasitic nematode species living in the rat pulmonary artery and with a typically complex life-cycle (Alicata, 1965). Third-stage larvae (L3) orally infect the final host, and are carried in the blood to the central nervous system, where they moult twice to become immature adults. Twenty-six to twenty-nine days after host exposure, worms migrate to the heart

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and lungs through the cerebral venous circulation. Once reaching the branches of the pulmonary artery, they grow rapidly, attain sexual maturity, and release eggs. The eggs, embryonate for 1 week and then hatch in the lung parenchyma (Alicata, 1965).

In order to investigate the expression of cytokine (TNF-α and IL-1β) and metalloproteinases (MMP-2, -9, -13) in parasite-induced granulomatous fibrosis, a rat experimental model was developed to examine the production of pro-inflammatory cytokines and metalloproteinases in the lung fibrosis of *A. cantonensis*-infected rats.

Materials and methods

Experimental animals

Five-week-old male Sprague-Dawley rats were purchased from the National Laboratory Animal Center, Taipei, Taiwan. They were maintained with a 12-h light :12-h dark photoperiod, provided with commercial rodent food (Purina Laboratory Chow; Ralston-Purina, St Louis, Missouri, USA) and water *ad libitum*, and kept in the laboratory for more than one week before experimental infection.

Larval preparation

Infective larvae (L3) of *A. cantonensis* were obtained from naturally infected giant African snails *Achatina fulica* collected from fields in Pingtung County, southern Taiwan. Larvae within tissues were recovered using the method of Parsons & Grieve (1990) with slight modifications. Briefly, the shell of each snail was crushed, the tissues homogenized and digested in a pepsin-HCl solution (pH 1–2, 500 I.U. pepsin / g^{-1} tissue), and incubated with agitation at 37°C in a waterbath for 2 h. Host cellular debris was removed by centrifugation at 1400 *g* for 10 min and larvae in the sediment were observed under a microscope. Morphological criteria for identification of the L3 of *A. cantonensis* were a length of 425 to 524 μm and a width of 23 to 34 μm and the tail always terminates in a fine point (Ash, 1970). To confirm identification, 60 L3 were fed to each of five rats, the brains of two of which were examined 2 to 3 weeks later for evidence of infection. The remaining three rats were sacrificed 5 to 6 weeks post-infection to reveal adult *A. cantonensis* in the pulmonary arteries. The morphology of the adult worms was consistent with that described for *A. cantonensis*. The males measured 14–15 mm in length, having a tail with copulatory bursa and long spicules; females measured 24–26 mm in length, with a characteristic barber-pole appearance (Lindo *et al.*, 2002).

Infection of rats

A total of 30 male rats were randomly allocated to uninfected control and infected groups of 15 rats each. Rats were prohibited food and water for 12 h before infection. The infected group received 60 *A. cantonensis* larvae by oral inoculation and were sacrified on day 180 post-inoculation (PI). The control rats received water and were sacrified on day 180 PI.

Histology

Rat lungs were fixed separately in 10% neutral buffered formalin for 24 h, 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 cut at a 5 μm thickness for each organ from each mouse. Sections were deparaffinized, stained with haematoxylin and eosin using standard techniques and examined under a light microscope.

Reverse transcriptase polymerase chain reaction (RT–PCR) analysis

Total RNA from lung tissues was isolated using Trizol reagent (Invitrogen, Carslbad, California, USA), according to the manufacturer's instructions. One microgram of total RNA was used for first strand cDNA synthesis in 20 μl of reaction volume using 50 units of Superscript^{TM} II reverse transcriptase (Invitrogen, Carslbad, California, USA). Polymerase chain reaction (PCR) was performed under standard conditions using *Taq* DNA polymerase (Invitrogen, Carslbad, California, USA) and primers. Forward (5′-3′) and reverse (5′-3′) primers, respectively, were 5′-GGT CGG TGT GAA CGG ATT TG-3′ and 5′-GCC TTC TCC ATG GTG GTG AA-3′ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Teng *et al.*, 2004), 5′-TGA GCA CAG AAA GCA TGA TC-3′ and 5′-CAT CTG CTG GTA CCA CCA GTT-3′ for TNF-α (Teng *et al.*, 2004), 5′-CAC CTC TCA AGC AGA GCA CAG -3′ and 5′-GGG TTC CAT GGA GAA GTC AAC-3′ for IL-1β (Teng *et al.*, 2004). PCR cycling conditions for GAPDH, TNF-α and IL-1β were denaturation at 94°C for 45 s, annealing at 55°C for 1 min, primer extension at 72°C for 2 min, and then holding at 4°C; this was repeated for 30 cycles for TNF-α and IL-1β, and 25 cycles for GAPDH. Ten microlitres of the amplified product were then subjected to electrophoresis in 3% agarose gels containing 20 $\mu g\,ml^{-1}$ ethidium bromide in Tris borate-EDTA buffer. Gels were visualized on a UV transilluminator (Taipei, Taiwan), and digital images were taken using a DGIS-5 Digital Gel Image System (Taipei, Taiwan).

Western blot analysis

Rat lungs were homogenized in buffer containing 0.1% Triton X-100, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM K_2HPO_4. The homogenates were then centrifuged at 12,000 *g* at 4°C for 10 min, and the protein contents of the supernatants were determined with protein assay kits (Bio-Rad, Hercules, California, USA) using BSA as the standard. An equal volume of loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulphate (SDS), 5% 2-mercaptoethanol, 0.05% bromophenol blue) was added to the samples, which contained 30 μg of lung tissue protein. The mixture was boiled for 5 min prior to electrophoresis on SDS-polyacrylamide gel under non-reducing

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conditions and electrotransferred to nitrocellulose membrane at a constant current of 190 mA for 90 min. The membrane was subsequently saturated with PBS containing 0.1% Tween 20 for 30 min at room temperature and allowed to react with primary antibodies (mouse anti-rat MMP-2, -9, and -13 polyclonal antibody; NeoMarkers, Fremont, California, USA) diluted 1:100 at 37°C for 1 h. The membrane was then washed three times with PBS containing 0.1% Tween 20 (PBS-T), followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) diluted 1:5000 at 37°C for 1 h to detect the bound primary antibody. The reactive protein was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). To confirm equivalent protein loading, membranes were stripped by incubation in 62.5 mM of Tris-HCl (pH 6.8), 2% SDS, and 100 mM 2-mercaptoethanol at 55°C, subsequently washed with PBS-T, and reprobed with anti-β-actin antibody (dilution 1:500).

Statistical analysis

All results are presented as mean $\pm$ standard deviation (S.D.). The nonparametric Mann-Whitney test was used for comparisons between each of the two groups. P values of <0.05 were considered statistically significant.

Results

Histology

Non-inoculated lung tissue showed normal alveolar structure with thin alveolar septa and a typical aspect of a small blood vessel (fig. 1a,b). In infected lung tissue, the alveolar space had filled with fibrotic granulomata, and a dense circumferential accumulation of collagen surrounded each granuloma with first stage larvae occupying the centre (fig. 1c,d).

RT–PCR analysis of TNF-α and IL-1β mRNA in the lungs

In order to explore the role of TNF-α and IL-1β in granulomatous fibrosis, the expression of TNF-α and IL-1β in the granulomatous fibrosis was examined. Total RNAs isolated from rat lungs were assayed by RT–PCR analysis using TNF-α, IL-1β, and GAPDH-specific primers. The mRNA expression of TNF-α and IL-1β was expressed significantly in *A. cantonensis*-infected rats compared with uninfected controls (fig. 2).

Detection of protein levels of MMP-2, -9, and -13

Western blotting revealed that the proenzyme of 72 kDa bands presented in *A. cantonensis*-infected rats and uninfected controls. Significantly, the active form of 64 kDa bands presented only in infected rats. In addition, increased protein levels of 94 kDa MMP-9 and 60 kDa MMP-13 were detected in infected lungs, but not in controls (fig. 3).

Discussion

Eggs and the first-stage larvae of *A. cantonensis* were observed in the lungs of rats and induced granulomatous fibrosis in alveoli on day 180 PI. The active form of MMP-2 increased in rats with pulmonary granulomatous fibrosis on day 180 PI. Protein levels of MMP-9 and MMP-13 were also increased along with the pathological changes. These data indicate that increased MMP-2, -9, and -13 coincided with pathological changes in the lungs and are consistent with the concept that matrix degradation occurs in the inflammatory and fibrotic reactions (Dunsmore & Rannels, 1996).

MMP-9 is a useful marker for angiostrongyliasis meningitis (Lai *et al.*, 2004; Lee *et al.*, 2004) and may be associated with the degeneration of Purkinje cells in the mouse cerebellum infected with *A. cantonensis* (Chen *et al.*, 2004). Pathological findings in reported cases of angiostrongyliasis are mainly in the central nervous system, but pulmonary injury has also been noted including pneumonia, granulomatous reactions of pulmonary vessels, and pulmonary haemorrhage (Yii *et al.*, 1968). Hsu *et al.* (2005) indicated that granulomatous fibrosis was strongly associated with MMP-2 and MMP-9, and these enzymes may be useful markers in *A. cantonensis*-infected rat lungs. In addition to MMP-2 and MMP-9, the present study has also demonstrated that MMP-13 is associated with lung fibrosis.

MMPs are involved in the degradation of extracellular matrix in most chronic inflammatory diseases (Dunsmore & Rannels, 1996). Gelatinases show a catalytic activity towards denatured fibrillar collagens (gelatin), and degrade native collagens (type-IV collagen as well as type-I, -III, -V and -XI collagens) and other structural proteins (fibronectin, laminin, elastin and proteoglycan core protein) (O'Connor & FitzGerald, 1994; Pardo & Selman, 1996). Excessive MMP-2 and MMP-9 production might play a role in basement membrane disruption, thus enhancing fibroblast invasion to the alveolar spaces (Selman *et al.*, 2000). It is well appreciated that dysregulation of gelatinases in the form of excessive matrix formation can result in fibrotic disease. MMP-9 may be linked to inflammatory process-induced tissue remodelling, whereas MMP-2 may be associated with an impaired tissue remodelling leading to collagen deposition and subepithelial fibrosis (Corbel *et al.*, 2003). In this context, the extracellular matrix component degradation of lung fibrosis might be associated with the secretion of proteinases of MMP-2, -9, and -13.

The importance of cytokines produced during fibrosis is well documented, demonstrating that these mediators have the potential of orchestrating and amplifying inflammation and fibrotic responses (Gharaee-Kermani & Phan, 2001). Several studies have demonstrated the key role of proinflammatory cytokines such as TNF-α and IL-1β (Sime *et al.*, 1998; Kolb *et al.*, 2001). Human and experimental studies have highlighted the potential profibrotic roles of additional cytokines, including IL-4, IL-5, IL-10 and IL-13, all classified as belonging to the class of Th2 type

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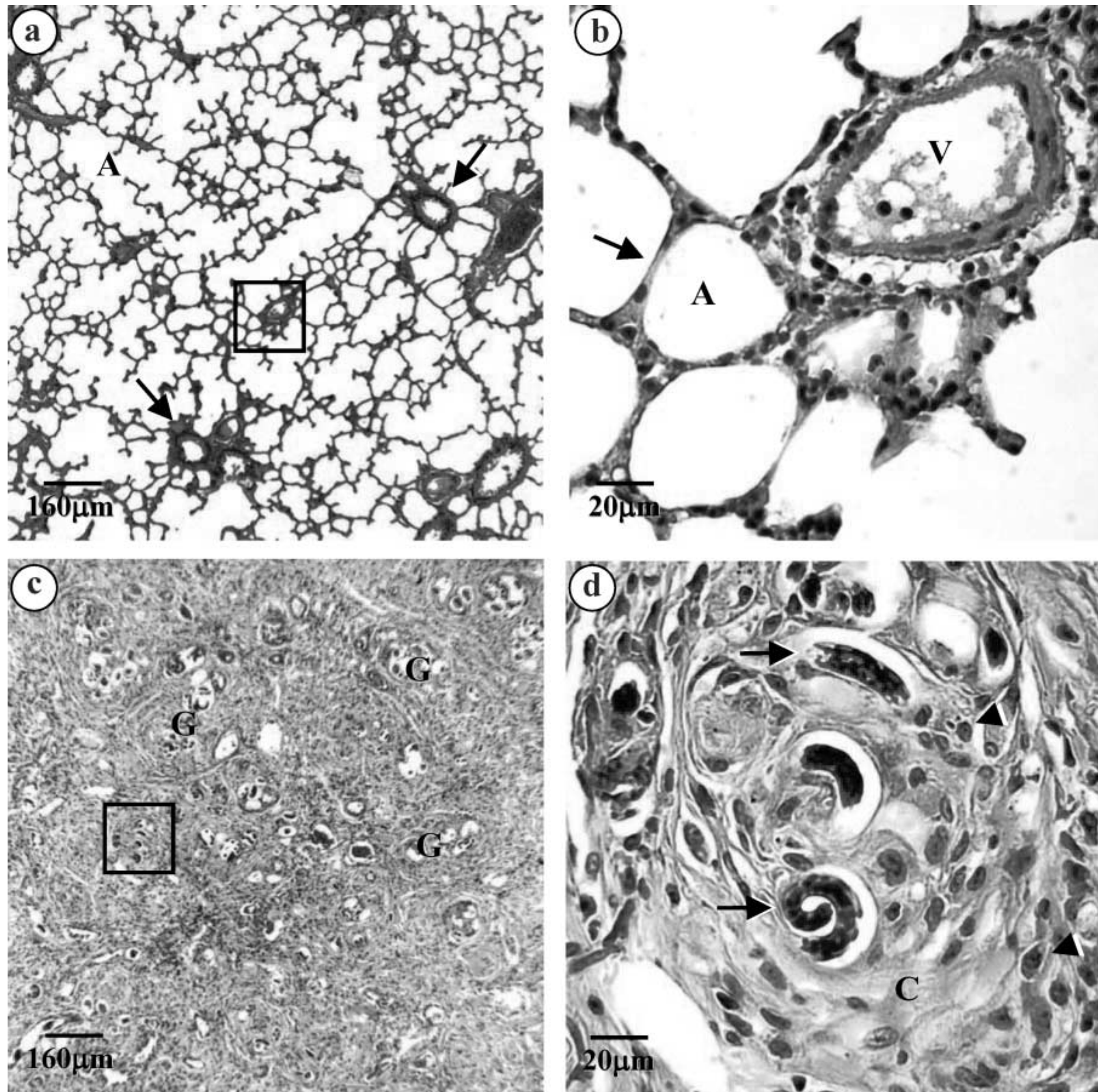

Fig. 1. Histology of non-inoculated and infected lung tissue of rats with *Angiostrongylus cantonensis* on day 180 post-inoculation (PI). (a) Non-inoculated lung tissue showing normal alveolar structure (A) and small blood vessel (arrows). (b) Enlargement of the portion shown in the rectangle of fig. 1a. Alveolar structure (A) with thin alveolar septa (arrow) and a typical aspect of a small blood vessel (V). (c) Alveolar space filled with fibrotic granulomata (G) on day 180 PI. (d) Enlargement of the portion shown in the rectangle of fig. 1c. showing a fibrotic granuloma with infiltrating leukocytes (arrowheads), a dense circumferential accumulation of collagen (C) surrounding the granulomata, and first stage larvae of *A. cantonensis* (arrows) occupying the centre.

cytokines (Wallace *et al.*, 1995; Gharaee-Kermani & Phan, 1997; Lee *et al.*, 2002; Huaux *et al.*, 2003).

MMP expression by immune cells is greatly modulated by inflammatory mediators, such as TNF-α and IL-1β (Mauviel, 1993; Birkedal-Hansen, 1995). TNF-α and IL-1β administered individually and in combination with interferon-gamma (IFN-γ) have been shown to augment the production of several matrix-degrading enzymes (Zhu *et al.*, 2001). Significantly, this study shows there is an increased expression of TNF-α and IL-1β corresponding to MMP-13 production and granulomatous fibrosis, indicating that TNF-α and IL-1β may be associated with MMP-13 production or granulomatous fibrosis. These results are similar to those of Siwik *et al.* (2000), which showed that TNF-α and IL-1β decrease collagen

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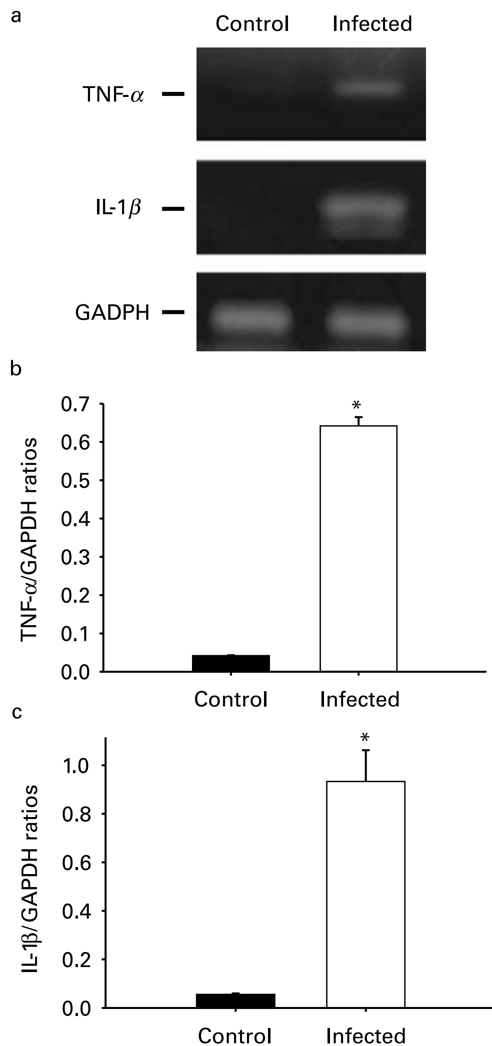

Fig. 2. Reverse transcriptase polymerase chain reaction analysis of TNF-α and IL-1β mRNA in the lungs of rats infected with *Angiostrongylus cantonensis* on day 180 post-inoculation compared with controls. (a) The mRNA expression of TNF-α and IL-1β were significant in infected rats compared with controls. GAPDH mRNA was used as a loading control. (b–c) Densitometric scanning quantification was expressed as the ratio of the signal intensity of TNF-α, IL1β mRNA to that of GAPDH at each group, respectively.

synthesis and increase MMP-13 activity in cardiac fibroblasts *in vitro*. Thus, these cytokines may, at least in part, be responsible for mediating the process of granulomatous fibrosis.

In conclusion, this study clarifies that *A. cantonensis* induced the production of pro-inflammatory cytokines (TNF-α and IL-1β) and metalloproteinases (MMP-2, -9 and -13). Furthermore, it raised the possibility that these cytokines could regulate the production and secretion of these metalloproteinases. To investigate this possibility, further experimental studies will be needed.

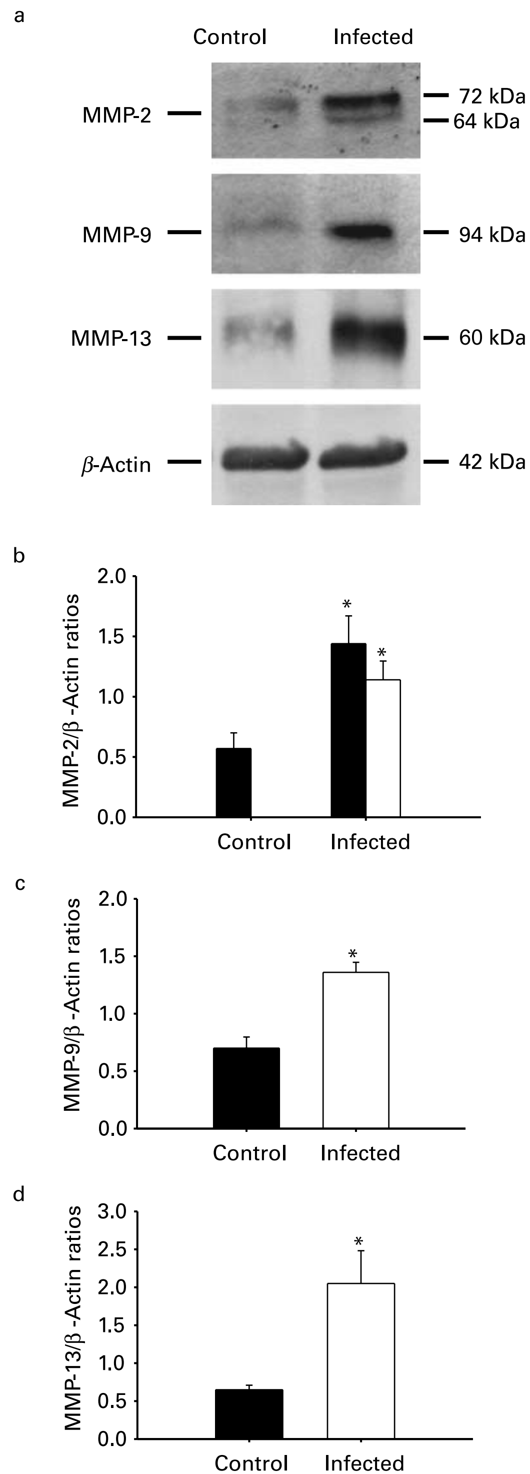

Fig. 3. Detection of protein levels of MMP-2, -9, and -13. (a) The protein level in rats infected with *Angiostrongylus cantonensis* on day 180 post-inoculation showed a significant increase ($P < 0.05$) compared with uninfected controls. β-actin was used as a loading control. (b–d) Densitometric scanning quantification was expressed as the ratio of the protein levels of MMP-2, MMP-9, MMP-13 to that of β-actin at each group, respectively. ■, 72 kDa; □, 64 kDa.

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