Elevated concentrations of matrix metalloproteinase-12 and elastin degradation products in the sera of pregnant women infected with *Toxoplasma gondii*

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Although the expression of matrix metalloproteinase-12 (MMP-12) appears to be increased in most inflammatory diseases, the role of this enzyme in the pathogenesis of toxoplasmosis in pregnant women, if any, is unknown. In a recent study in Taiwan, the serum concentrations of MMP-12 and its substrate elastin were evaluated in pregnant women with *Toxoplasma gondii* infection. Compared with the healthy pregnant and non-pregnant women used as controls, the pregnant women with toxoplasmosis had significantly higher serum concentrations of MMP-12 and significantly higher levels of elastin synthesis and degradation. Interaction between MMP-12 and elastin in the serum samples was confirmed by co-immunoprecipitation. It seems possible that MMP-12 may contribute to elastin degradation occurring during the pathogenesis of toxoplasmosis in pregnant women.

Toxoplasma gondii is an intracellular coccidian that has evolved to infect a wide variety of warm-blooded organisms, including humans, and is the cause of toxoplasmosis, one of the more common parasitic zoonoses world-wide (Sabin and Olitsky, 1937; Tenter *et al.*, 2000). *Toxoplasma* establishes a systemic infection following its ingestion. To do so, it must cross the intestinal epithelium, basement membrane, and lamina propria. Active traversal of cellular barriers represents a novel means of tissue dissemination and is used by the parasite to gain access to biologically restricted organs, including the brain and the placenta, in its host (Barragan and Sibley, 2002). To reach

such ‘immunoprivileged’ sites, *T. gondii* must degrade the extracellular matrix (ECM; Da Gama *et al.*, 2004; Buache *et al.*, 2007). The upregulation of the migratory capacity of *T. gondii* is an important component in the pathogenesis of toxoplasmosis (Barragan and Sibley, 2002).

Matrix metalloproteinases (MMP) are structurally related enzymes — members of a family of neutral proteinases involved in normal and pathological processes in the central nervous system (CNS) of mammals (Lukes *et al.*, 1999) — that are collectively capable of degrading essentially all of the components of ECM (Shapiro, 1998). Normally released into the extracellular space, they break down the ECM to allow cell growth and to facilitate remodelling (Lukes *et al.*, 1999). One MMP, MMP-12, is an elastin-degrading proteinase, with a 54-kDa proenzyme that is processed into a

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45-kDa active form (Matsumoto *et al.*, 1998). Elastin, which accounts for up to 4% of the ECM of brain microvessels (Faris *et al.*, 1982), contains two cysteine residues that may be important for the protein's interaction with other ECM components (Rosenbloom, 1984). The elastic properties of many tissues, such as the lung, dermis and large blood vessels, are attributable to the presence of elastin fibres in the extracellular space (Rosenbloom, 1984). Elastin is an important factor in the development of vascular wall alterations (Nicoloff *et al.*, 2000) and is largely responsible for maintaining the elasticity of the vascular wall (Robert *et al.*, 1984). During pregnancy, the uterine spiral arteries of placental mammals, including humans, are remodelled to allow the placenta to meet the increasing requirement for nutrients and oxygen. It is MMP-12, derived from trophoblasts and vascular smooth-muscle cells, that mediates elastin degradation during this vascular remodeling (Harris *et al.*, 2010).

To date, MMP-12 and its substrate elastin have not been the object of investigation in healthy pregnant women or pregnant women with toxoplasmosis. The main aim of the present study was to evaluate serum concentrations of MMP-12 and elastin in pregnant women with and without *T. gondii* infection, to see if MMP-12 might be involved in the pathogenesis of toxoplasmosis during pregnancy.

SUBJECTS AND METHODS

Subjects and Serum Sampling

The sera investigated were separated from blood samples collected, in Taiwan, from 220 pregnant women during the third trimester (i.e. weeks 38–40 of the gestation, while the women were in confinement). Although 194 of the women appeared healthy when sampled, the other 26 had toxoplasmosis, as confirmed by positivity in commercial, enzyme-linked immuno-assays (Euroimmun, Cardiff, U.K.) for anti-*T.*

gondii IgM (six cases) or anti-*T. gondii* IgG (20 cases). The women found seropositive for anti-*T. gondii* IgM were all found to show low IgG avidity in another commercial enzyme-linked immuno-assay (Ani Labsystems, Vantaa, Finland), indicating that they had recent infections. As controls, sera were also collected from healthy non-pregnant women, who were seronegative for toxoplasmosis and gave blood voluntarily, with their informed consent.

Ethical Approval

The study protocol was approved by the Research and Ethical Review Committees and Internal Review Board of Chung Shan University Hospital in Taichung, Taiwan.

Antibodies

The two primary antibodies used in the western blots (see below) were rabbit anti-human-MMP-12-pro-enzyme monoclonal antibody (Abcam, Cambridge, U.K.) and goat anti-human-elastin polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The corresponding secondary antibodies were horseradish-peroxidase (HRP) conjugates of anti-rabbit-IgG and anti-goat-IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), respectively.

Western-blot Analysis

The protein contents of the serum samples were determined using commercial protein assays (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as the standard. A subsample of each serum was mixed with an equal volume of loading buffer [62.5 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulphate, 5% (v/v) 2-mercaptoethanol and 0.05% (v/v) Bromophenol Blue], and then the mixture was boiled for 5 min before being subjected to electrophoresis on a 10% (w/v) polyacrylamide gel. The separated proteins were then electrotransferred to a nitrocellulose membrane, at a constant

current of 30 mA overnight. After three 30-min washes in 0.15 M phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 20 (PBS-T), at room temperature, the membrane was soaked in a 1:1000 dilution of a primary antibody at 37°C for 1 h. After another three washes in PBS-T, the membrane was incubated, again at 37°C for 1 h, with a 1:5000 dilution of the relevant HRP-conjugated secondary antibody. The reactive protein was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) and quantified using a computer-assisted imaging densitometer (UN-SCAN-IT; Silk Scientific, Orem, UT).

Co-immunoprecipitation

To prevent nonspecific adsorption, protein A/G agarose beads (Santa Cruz Biotechnology) were washed five times in 0.15 M PBS and then incubated with 5% (w/v) BSA at 4°C for 30 min. The suspension was centrifuged twice (at $10,000 \times g$ for 2 min each time) and then the supernatant solution was discarded.

Anti-elastin antibody (as used in the previous western blots) was incubated with the serum samples at 4°C overnight and collected by binding to the protein A/G agarose beads. The beads were washed twice in dissociation buffer [0.5 M Tris-HCl (pH 8.0), 120 mM NaCl, and 0.5% (w/v) Triton X-100] before the bound proteins were resolved by SDS-PAGE and MMP-12 association was determined by western-blot analysis.

Statistical Analysis

Results for the three groups of subjects (i.e. the pregnant women with and without toxoplasmosis and the healthy non-pregnant women) were compared using the non-parametric Kruskal-Wallis test followed by post-hoc analysis using Dunn's multiple comparison of means. A *P*-value of <0.05 was considered indicative of a statistically significant difference.

RESULTS

Serum MMP-12

The results of the western blotting and subsequent quantitative analysis indicated not only that the sera of pregnant women (with or without toxoplasmosis) contained significantly higher concentrations of MMP-12 than the sera of healthy non-pregnant women but also that the sera of pregnant women with toxoplasmosis contained significantly higher concentrations of MMP-12 than the sera of healthy pregnant women or healthy non-pregnant women (Fig. 1).

Serum Elastin

The results of the corresponding western blotting for elastin (and subsequent quantitative analysis) indicated that the serum concentrations of both the 68-kDa and 55-kDa proteins were significantly higher in pregnant women (with or without toxoplasmosis) than in healthy non-pregnant women. In addition, the serum concentrations of 25-kDa degraded elastin were significantly higher in the pregnant women with toxoplasmosis than in the healthy pregnant women or the healthy non-pregnant women (Fig. 2).

Interaction of MMP-12 and Elastin in Serum

The results of the co-immunoprecipitation indicated that MMP-12 in the sera of pregnant women with toxoplasmosis did interact with elastin (Fig. 3).

DISCUSSION

Human parturition is multi-step process that includes myometrial contraction, cervical ripening, foetal membrane rupture, and detachment of the placenta and foetal membranes from the maternal uterus (Li *et al.*, 2004). Some of these processes require the degradation or remodelling of macromolecules of the ECM, by proteolytic

FIG. 1. Mean concentrations of matrix metalloproteinase-12 (MMP-12) in the sera of healthy non-pregnant women (HC), healthy pregnant women (PC) and pregnant women infected with *Toxoplasma gondii* (Infection), as determined by western blotting. The infected women investigated had been found seropositive for anti-*T. gondii* IgM [(a) and (b)] or anti-*T. gondii* IgG [(c) and (d)]. The densitometer readings that were significantly greater than the corresponding value for the healthy non-pregnant women (*) or the values for both the healthy non-pregnant women and healthy pregnant women (†) are indicated ($P < 0.05$ for each comparison). The numbers in parentheses indicate the numbers of subjects in each group whereas the error bars indicate S.D.

enzymes (Woessner, 1991). One such enzyme, MMP-12, is known to play an important role in the remodelling of uterine arteries during pregnancy, at least in rats (Kelly *et al.*, 2003), and might represent a means by which vasodilatation is maintained in later pregnancy. Later still, elevated levels of MMP-12 post-partum may contribute to vessel regression and the return to a non-pregnant physiological state (Kelly *et al.*,

2003). In the present study, not only were the MMP-12 concentrations in the sera of healthy pregnant women (in a state of confinement) found to be significantly higher than those in the sera of healthy non-pregnant women, but also the serum concentrations of this enzyme in pregnant women with *T. gondii* infections were found to be significantly higher than those seen in healthy pregnant women. These results

FIG. 2. Mean concentrations of elastin or elastin degradation products in the sera of healthy non-pregnant women (HC), healthy pregnant women (PC) and pregnant women infected with *Toxoplasma gondii* (Infection), as determined by western blotting. The infected women investigated had been found seropositive for anti-*T. gondii* IgM [(a) and (b)] or anti-*T. gondii* IgG [(c) and (d)]. The densitometer readings that were significantly greater than the corresponding value for the healthy non-pregnant women (*) or the values for both the healthy non-pregnant women and healthy pregnant women (†) are indicated ($P < 0.05$ for each comparison). The numbers in parentheses indicate the numbers of subjects in each group whereas the error bars indicate S.D.

indicate that, although MMP-12 may participate in a physiological process required for labour, overexpression of this enzyme may result in the pathological degradation of the ECM in pregnant women with toxoplasmosis.

Although, in mammals, elastin is encoded by a single gene and is secreted as a 60- to 70-kDa monomer called tropoelastin (Mecham, 2008), it is common to detect multiple bands by SDS-PAGE after tropoelastin

purification. Major bands of approximately 55, 45, 25 and 15 kDa are the degradation products of tropoelastin, resulting from cleavage by tryptic-like proteases (Mecham *et al.*, 1997; Mecham, 2008). Tropoelastin is readily degraded by MMP-12, having at least 89 cleavage sites for this elastinolytic enzyme (Heinz *et al.*, 2010). The cleavage-site specificity of MMP-12 for tropoelastin follows the order Ala>Leu>Lys>Val/Tyr>Gly (Heinz *et al.*, 2010). The 68-kDa

FIG. 3. Interaction between matrix metalloproteinase-12 (MMP-12) and elastin from the sera of healthy non-pregnant women (HC), healthy pregnant women (PC) and pregnant women infected with *Toxoplasma gondii* (Infection), as demonstrated by co-immunoprecipitation. The sera were subjected to immunoprecipitation with anti-elastin antibody followed by anti-MMP-12 immunoblotting. The infected women investigated had been found seropositive for anti-*T. gondii* IgM (a) or anti-*T. gondii* IgG (b).

protein detected in the present study was assumed to be tropoelastin, while the 55-, 45- and 25-kDa proteins observed were assumed to be degradation products resulting from tropoelastin cleavage by proteinases. Growth and remodelling of elastin involve highly coordinated interactions between cells, cytokines, proteinases and the ECM. In the serum, the products of elastin-fibre degradation (i.e. elastin-derived peptides) have distinct cellular effects as they interact with receptor complexes on cell membranes (Hinek *et al.*, 1993; Privitera *et al.*, 1998). These interactions activate intracellular signalling pathways that lead to diverse cellular events, such as increased elastase production, release of free radicals, cell proliferation, and chemotactic

activity of monocytes, fibroblasts and tumour cells (Fülöp *et al.*, 1998; Labat-Robert, 2004). The disruption of elastin is not just an end product of elastin turnover but may also be an important contributor to the pathogenesis of tissue injury (Konova *et al.*, 2009). In the present study, the levels of both the synthesis and degradation of elastin appeared to be significantly higher in pregnant women with toxoplasmosis than in healthy pregnant women or in healthy non-pregnant women. Increased elastin degradation may therefore have an important role in the pathogenesis of *T. gondii* infection during pregnancy.

Various MMP may be essential for the breakdown of vessel walls and the degradation of several macromolecular components of the ECM. One member of the MMP family, MMP-12, is able to degrade elastin and various basement-membrane components (Vaalamo *et al.*, 1999), elastin representing a critical component of the aortic-wall structure and a matrix substrate for MMP-12 (Curci *et al.*, 1998). In the present study, co-immunoprecipitation was used to demonstrate the interaction between MMP-12 and elastin from the sera of pregnant women with toxoplasmosis, supporting the possibility that MMP-12-related damage to elastin forms part of the pathology of *T. gondii* infection in humans (and, presumably, other mammals). Upregulation of MMP-12 during toxoplasmosis may lead to the disruption of the basement membrane, leucocyte recruitment, and tissue damage. The enzyme may serve as a useful marker in pregnant women with *T. gondii* infection.

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