Chlamydia pneumoniae inhibits corticosteroid-induced suppression of metalloproteinase-9 and tissue inhibitor metalloproteinase-1 secretion by human peripheral blood mononuclear cells

Chan-Sun Park,1 Yoon Su Lee,2 Hyouk-Soo Kwon,2 Taehoon Lee,2 Tae-Bum Kim,2 Keun-Ai Moon,3 Bin Yoo,4 Hee-Bom Moon2 and You Sook Cho2

Correspondence
You Sook Cho
yscho@amc.seoul.kr

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1Department of Internal Medicine, Haeundae Paik Hospital, Inje University, Busan, Republic of Korea
2Department of Allergy and Clinical Immunology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea
3Asan Institute for Life Science, Seoul, Republic of Korea
4Department of Rheumatology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea

Chlamydia pneumoniae infection has been suggested to be associated with severe asthma characterized by persistent airway limitation, which may be related to airway remodelling. We investigated whether C. pneumoniae infection affected the secretion of metalloproteinase-9 (MMP9) and tissue inhibitor metalloproteinase-1 (TIMP1), and altered the responsiveness of inflammatory cells to corticosteroids. Human peripheral blood mononuclear cells (PBMCs) were cultured in vitro in the presence or absence of C. pneumoniae. Secretion of both MMP9 and TIMP1 was strongly suppressed by dexamethasone treatment in uninfected cells. MMP9 secretion was also significantly inhibited by dexamethasone in C. pneumoniae-infected cells, but TIMP1 secretion was not; hence the MMP9 to TIMP1 ratio decreased. Interestingly, expression of human glucocorticoid receptor β, which is believed to confer resistance to corticosteroids, was enhanced by dexamethasone treatment in C. pneumoniae-infected PBMCs. We conclude that C. pneumoniae infection may promote airway remodelling by decreasing the ratio of MMP9 to TIMP1 secreted by inflammatory cells, and by altering cellular responsiveness to corticosteroids.

INTRODUCTION

Infection with Chlamydia pneumoniae, an obligately intracellular pathogen, has been suggested to be associated with severe non-atopic asthma (Cook et al., 1998), corticosteroid-dependent or -resistant asthma (Black et al., 2000; Hahn et al., 1998; von Herten et al., 2002), and asthma with persistent airflow limitation (Pasternack et al., 2005; ten Brinke, 2008). C. pneumoniae has an innate propensity to persist, and can cause chronic or latent infections in various structural and immune cells. The infected cells release large amounts of various pro-inflammatory mediators, such as tumour necrosis factor (TNF)-α, interleukin (IL)-8, IL-6, interferon-γ and basic fibroblast growth factor, and upregulate adhesion molecules (Kaukoranta-Tolvanen et al., 1996; Kol et al., 1999; Rödel et al., 2003). Recently, we demonstrated that C. pneumoniae infection stimulated expression of vascular endothelial growth factor, CCL20, tissue inhibitor of metalloproteinase (TIMP)-1 and TGFβ in human bronchial epithelial cells and increased intracellular reactive oxygen species, and these events were associated with MAPK activation (Kim et al., 2009; Park et al., 2010). This suggests that C. pneumoniae infection aggravates inflammation and promotes remodelling in asthmatic airways.

Airway remodelling involves structural changes that are probably associated with persistent airway inflammation and irreversible airway obstruction. It has been suggested that the levels of matrix metalloproteinase (MMP)-9 and its physiological inhibitor, TIMP1, in lung tissues are related to certain asthma phenotypes and that these two entities play important roles in airway remodelling (Gueders et al., 2006;
Kelly & Jarjour, 2003). Furthermore, it has been reported that infected human bronchial epithelial cells show enhanced TIMP1 secretion, which suggests that C. pneumoniae may participate in airway remodelling by altering the MMP9 to TIMP1 ratio (Park et al., 2010).

It has also been reported that certain infections reduce sensitivity to glucocorticoids (GCs) (Fakhri et al., 2004; Li et al., 1999), and that C. pneumoniae enhances the proliferation of human peripheral blood mononuclear cells (PBMCs) and reduces their corticosteroid responsiveness via a TNF-α-dependent pathway (Cho et al., 2005). Insensitivity to GCs is a key aspect of the development of severe asthma with airway remodelling. Although the precise cause of corticosteroid resistance is unknown, there is evidence that the development of corticosteroid-resistant or -insensitive asthma is associated with the modulation of glucocorticoid receptor (GR) function (Adcock & Barnes, 2008; Hamid et al., 1999). In addition, recent studies of asthma patients have demonstrated increased expression of GRβ, an endogenous inhibitor of GC action, in PBMCs and airway T cells, and a reduced ratio of GRα positive to GRβ positive cells (Hamid et al., 1999; Leung et al., 1997; Sousa et al., 2000). These findings suggest that GC insensitivity is linked to increased expression of GRβ.

It appears, therefore, that C. pneumoniae infection affects the secretion of MMP9 and TIMP1 from inflammatory cells, and causes corticosteroid insensitivity. In the present study, we evaluated the effect of C. pneumoniae infection of inflammatory cells on the secretion of MMP9 and TIMP1, and identified a possible mechanism responsible for altered responsiveness to corticosteroids.

METHODS

Culture and preparation of C. pneumoniae. C. pneumoniae was cultured and prepared as previously described (Cho et al., 2005; Park et al., 2010). In brief, strain TW183 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and propagated in HEP-2 cells (ATCC) grown in Eagle’s minimum essential medium (Sigma) supplemented with 10% fetal calf serum, L-glutamine (2 mM), non-essential amino acids, gentamicin (10 mg l⁻¹), vancomycin (50 mg l⁻¹), amphotericin B (2 mg l⁻¹) and HEPES buffer solution (10 mM; Gibco-BRL Life Technologies). Confluent monolayers were infected with C. pneumoniae by centrifuging (2000 g, 35 °C, 45 min) infectious inocula onto host cells. The supernatants were then replaced by serum-free growth medium containing cycloheximide (1 mg l⁻¹; Sigma).

Infected cells were cultured in a 35 °C, 5% CO₂ saturated humidified incubator, harvested on day 3 or 4, and disrupted by two cycles of freezing/thawing and ultrasonication; similar harvests were pooled. After centrifugation at 1000 g for 5 min to remove cell debris, bacteria were concentrated by high-speed centrifugation at 25 000 g for 25 min. Pellets were resuspended in PBS (pH 7.4), mixed with an equal volume of sucrose–phosphate–glutamic acid buffer, aliquoted and frozen at −70 °C.

C. pneumoniae titres were determined using an immunofluorescence assay. Briefly, HEP-2 cells were infected with serial dilutions of bacterial stock, cultured for 65 h, fixed with methanol–acetone (1:1) and incubated with antibody to the major outer-membrane protein (mouse IgG3 anti-C. pneumoniae mAb; Dako), followed by FITC-conjugated secondary antibody (goat F(ab′)₂, anti-mouse IgG; Sigma). After counting C. pneumoniae inclusions under a fluorescence microscope and correcting for dilution factors, bacterial titres were expressed as inclusion forming units (IFU) per cell.

Preparation of human PBMCs and infection with C. pneumoniae. Heparinized venous blood from healthy volunteers was layered on a Ficoll-Paque density gradient. The interfacial mononuclear cell fraction was washed with Dulbecco’s PBS by centrifugation and resuspension in RPMI 1640 medium (BioWhittaker). Cells so obtained were cultured in RPMI 1640 medium, supplemented with 10% heat-inactivated (30 min at 56 °C) fetal calf serum, 40 mmol l⁻¹ glutamine, 100 U penicillin G sodium ml⁻¹, 100 U streptomycin ml⁻¹ and 20 mmol HEPES buffer solution 1 M (Gibco-BRL Life Technologies).

Immediately after inoculating C. pneumoniae at 0.4 IFU per cell, samples were centrifuged at 35 °C for 1 h at 550 g to facilitate contact between cells and bacteria. Infection of the PBMCs was confirmed using the immunofluorescence assay described above. The study protocol was approved by the independent ethical review board of our institute, and written informed consent was obtained.

Measurements of MMP9 and TIMP1, and responsiveness to corticosteroids. Initially, we evaluated whether C. pneumoniae had altered the secretions of MMP9 and TIMP1. Culture supernatants of PBMCs were sampled 65 h after C. pneumoniae infection, and the concentrations of TIMP1 and MMP9 were determined by using commercial ELISA kits (R&D Systems). Next, to evaluate responsiveness to corticosteroids, infected and uninfected PBMCs were cultured in the presence or absence of dexamethasone (10⁻⁶ M). Levels of MMP9 and TIMP1 in supernatants were measured in the same manner. To clarify the effects of C. pneumoniae infection on the production of MMP9 and TIMP1 by dexamethasone treatment, we calculated the percentage ratio of MMP9 or TIMP1 levels in supernatant after dexamethasone treatment over those without dexamethasone treatment. We also calculated the molar ratio of MMP9 over TIMP1 under each experimental condition.

Expression of GRβ on PBMCs. Expression of GRβ in infected and uninfected PBMCs was evaluated immunohistochemically. Cells were prepared by the cyto spin technique 65 h after infection and incubated for 15 min at room temperature with a permeabilizing solution [PBS containing 0.5% (w/v) BSA, 0.1% (v/v) Tween 20 and 0.1% (w/v) saponin; Sigma-Aldrich]. The permeabilizing solution was then decanted and a commercial blocking solution (Superblock; Scytek) was added for 15 min at room temperature. The blocking solution was then aspirated off and discarded. Next, the cytopsins were incubated with affinity-purified polyclonal antibodies to human anti-GRβ (Southern Biotechnology Associates) diluted in permeabilizing solution. Purified non-immune rabbit IgG (Southern Biotechnology Associates) was used as a control. After incubation overnight at 48 °C, the cells were washed in PBS containing 0.1% Tween 20 for 15 min at room temperature with gentle agitation, incubated with a goat anti-rabbit F(ab′)₂ FITC conjugate (Dako) for 30 min at room temperature, and again washed in PBS containing 0.1% Tween 20. The preparations were mounted, and examined by fluorescence microscopy.

Statistical analysis. All data are presented as means ± SEMs. Statistical comparisons were made with the Mann–Whitney U test using spss software (version 12.0 for Windows). P-values below 0.05 were considered statistically significant.
RESULTS

Effects of C. pneumoniae infection on the production of MMP9 and TIMP1 by PBMCs and on their responses to corticosteroids

No difference was found between infected and uninfected PBMCs in terms of secreted MMP9 levels. However, secretion of MMP9 by both infected and uninfected PBMCs was significantly decreased by dexamethasone (Fig. 1a). *C. pneumoniae* infection slightly, but significantly, reduced the inhibitory effect of dexamethasone treatment on MMP9 secretion (15% vs 36%, *P*<0.01) (Fig. 1b).

Secretion of TIMP1 was increased by *C. pneumoniae* infection. In uninfected PBMCs, dexamethasone suppressed TIMP1 secretion, as did MMP9 secretion; however, it had no detectable effect on TIMP1 secretion in infected PBMCs (Fig. 2a). Accordingly, the inhibitory effect of corticosteroid on TIMP1 secretion was much weaker in infected PBMCs than in uninfected PBMCs (26% vs 88%, *P*<0.01) (Fig. 2b).

We also assessed the effect of infection on the MMP9/TIMP1 molar ratio: the ratio was significantly lower in infected than in uninfected PBMCs. Interestingly, dexamethasone treatment decreased the MMP9/TIMP1 ratio more in the infected PBMCs than in the uninfected PBMCs although dexamethasone reduced the ratio in both infected and uninfected PBMCs (Fig. 3).

Expression of GRβ in *C. pneumoniae*-infected PBMCs

To investigate the mechanism underlying the altered response to corticosteroid treatment in infected PBMCs, we examined the expression of intracellular GRβ immunohistochemically using a confocal microscope. Importantly, GRβ staining was much stronger in the infected cells than in the uninfected cells. Staining was also significantly stronger in the infected cells after dexamethasone treatment (Fig. 4). The changes in expression of MMP9, TIMP1 and GRβ in the various experiments reported above are summarized in Table 1.

DISCUSSION

The present study shows that *C. pneumoniae* infection alters the secretion of MMP9 and TIMP1 by human PBMCs, especially in the presence of corticosteroids. The altered MMP9 and TIMP1 levels and molar ratios suggest that this atypical respiratory bacterium is related to airway remodelling. We also found that corticosteroid treatment of the infected PBMCs significantly increased expression of GRβ, indicating that reduced responsiveness to corticosteroids may contribute to airway remodelling.

Interestingly, *C. pneumoniae* can exist in a latent form (Beatty *et al.*, 1994), and immune responses induced by it may increase the severity of various chronic diseases. In fact, several authors have suggested an association between *Chlamydia pneumoniae* infection and tissue damage and remodelling in chronic diseases such as atherosclerosis (Watson & Alp, 2008), Alzheimer’s disease (Balin *et al.*, 2008), conjunctival scarring (Wright *et al.*, 2008) and tubal obstruction in chronic salpingitis (Patton *et al.*, 1990). Thus, persistent or recurrent *C. pneumoniae* infection of asthmatic airways could accelerate disease progression and perpetuate inflammation and fibrosis, so leading to tissue remodelling. Indeed, it has been reported that chronic and repeated *C.
pneumoniae infection in mice increases IL-4 expression and the thickness of airway subepithelial membranes (Chen et al., 2009). However, while it has been speculated that C. pneumoniae infection plays an important role in the pathogenesis of asthma from epidemiological data, the precise mechanism of C. pneumoniae infection in the pathogenesis of airway remodelling remains unclear.

Accumulating evidence indicates that both proteolytic and antiproteolytic activities and their balance play important roles in tissue remodelling, especially in relation to extracellular matrix (ECM) deposition and fibrosis (Gueders et al., 2006; Kelly & Jarjour, 2003). Matrixins, a family of proteases, of which MMP9 is the predominant form in pulmonary tissue, can digest components of the ECM, while TIMP1, a major specific inhibitor of MMP9, has fibrogenic properties and promotes the multiplication of fibroblasts and myofibroblasts (Gueders et al., 2006). Increased levels of MMP9 have been detected in the bronchoalveolar lavage fluid, sputum and sera (Bossé et al., 1999; Mautino et al., 1997; von Hertzen, 2002) of asthmatic patients and there is a significant correlation between MMP9 level, decreased forced expiratory volume in 1 s (FEV1) and irreversible airway obstruction (Boulay et al., 2004; Mattos et al., 2002).

Since MMP9 is stoichiometrically inhibited by TIMP1 (Gueders et al., 2006), the ratio of MMP9 to TIMP1 evaluated in this study is critical for understanding the precise role of these enzymes in the pathogenesis of airway remodelling in asthma. In fact, the decreased MMP9 to TIMP1 ratio in the bronchial secretion of asthmatic patients has been clearly shown to be associated with chronic airflow obstruction (Bossé et al., 1999; Matsumoto et al., 2005).

In connection with the suggested role of C. pneumoniae in the pathogenesis of asthmatic airway remodelling, we have attempted to evaluate the hypothesis that intracellular infection with this organism is associated with alterations not only in the secretion of MMP9 and TIMP1 individually but also in their ratio. We have shown that C. pneumoniae infection alters the ratio in favour of TIMP1, especially in response to corticosteroid treatment. Although corticosteroids are considered the most effective anti-inflammatory drugs for the treatment of asthma, their effects on the production of tissue enzymes involved in airway remodelling have not been thoroughly investigated. It has been
reported that corticosteroids lower the levels of MMP9 and TIMP1 in induced sputum samples from asthmatics (Pujols et al., 2004), whereas others have found that corticosteroid treatment affects the MMP9/TIMP1 ratio rather than just absolute MMP9 and TIMP1 levels (Hamid et al., 1999; Mattos et al., 2002). The present study shows that the production of MMP9 and TIMP1 is modulated and the MMP9/TIMP1 ratio is reduced by the infected PBMCs, especially by corticosteroid treatment. On the basis of our data showing a decreased MMP9/TIMP1 ratio in C. pneumoniae-infected cells, it is likely that C. pneumoniae infection aggravates airway limitation and leads to airway remodelling, especially in asthma patients treated with corticosteroids.

In the current study, we used PBMCs instead of alveolar macrophages and bronchial epithelial cells. Since the functions of PBMCs are clearly not identical to those of alveolar macrophages, our results can only suggest the role of inflammatory cells in the consequences of infection with C. pneumoniae. Moreover, the in vitro experiments in this study do not simulate the chronic infectious state. Although we have previously demonstrated that C. pneumoniae infection enhances the secretion of TIMP1 in human bronchial epithelial cells (Park et al., 2010), and PBMCs may have similar functions to inflammatory cells of the lung, the results of the present study provide no direct evidence for an association between chronic airway infection by C. pneumoniae and airway remodelling in asthmatic airways. Further studies are needed to obtain such evidence.

Epidemiological studies have suggested that chronic infection with C. pneumoniae is associated with severe corticosteroid-resistant or -dependent asthma. We have also shown that C. pneumoniae infection induces corticosteroid resistance, since the survival of C. pneumoniae-infected immune cells in response to dexamethasone treatment is enhanced (Cho et al., 2005). Recently, it has been suggested that GRβ is a dominant-negative inhibitor of GRα-mediated transactivation of target genes, and that it promotes GC resistance by altering transcription at GC-inducible, AP-1 inducible or NF-κB-inducible promoters (Pujols et al., 2004, 2007). In fact, increased expression of GRβ on PBMCs and airway T cells and a reduced ratio of GRα-positive to GRβ-positive cells have been reported in GC-resistant asthma patients (Hamid et al., 1999; Leung et al., 1997; Sousa et al., 2000).

A limitation of this study is the lack of direct data on GRα levels, although several recent studies have demonstrated that nuclear translocation of GRα and transactivation by it are reduced by transduction of the GRβ gene, and that GRβ has a dominant-negative effect on GRα-mediated trans-repression (Goleva et al., 2006; Taniguchi et al., 2010).

Table 1. Summary of the effects of C. pneumoniae infection on the expression of MMP9, TIMP1 and GRβ, with or without dexamethasone treatment

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Fig. 4. Expression of GRβ on C. pneumoniae-infected PBMCs. After 65 h of infection with and without dexamethasone, cells were treated with human anti-GRβ and GR goat anti-rabbit F(ab')2 FITC conjugate and examined by confocal microscopy (×400). Results of three independent experiments.
However, our findings can at least partly explain how the enhanced expression of GRβ contributes to the altered responsiveness to corticosteroids in *C. pneumoniae*-infected PBMCs. Further work is required to elucidate the precise mechanism of corticosteroid resistance.

**Conclusions**

We believe that this is the first study to suggest that *C. pneumoniae*-infected inflammatory cells may promote airway remodelling as a consequence of a reduced MMP9/TIMP1 ratio caused by their decreased responsiveness to corticosteroids. Further studies are needed to document the effect of *C. pneumoniae* on various cell types, including alveolar macrophages and bronchial epithelial cells.

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