Increased production of matrix metalloproteinases 1 and 3 by smooth muscle cells upon infection with *Chlamydia pneumoniae*

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Abstract

Atherosclerosis has been linked to *Chlamydia pneumoniae* infection. In atherosclerotic arteries chlamydiae infect macrophages, endothelial cells, and smooth muscle cells (SMC). It has been suggested that the proteolysis of the extracellular matrix by matrix metalloproteinases (MMPs) is involved in the destabilisation and rupture of atherosclerotic plaques. In this study we investigated the expression of several MMPs and tissue inhibitors of MMP (TIMPs) in *C. pneumoniae-*infected SMC using reverse transcription-polymerase chain reaction analysis. Chlamydial infection of SMC up-regulated the mRNA levels of MMP-1 (interstitial collagenase) and MMP-3 (stromelysin) but did not affect the expression of MMP-2 and -9 (gelatinases). Additionally, the levels of TIMP-1 and -2 mRNA remained unchanged upon infection. Cells infected with *C. pneumoniae* secreted increased quantities of MMP-1 and -3 proteins as demonstrated by enzyme-linked immunosorbent assays. The ability of *C. pneumoniae* to stimulate the production of MMP-1 and -3 by SMC may be important for its pathogenic role in the progression of atherosclerotic disease.

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Keywords: *Chlamydia pneumoniae*; Atherosclerosis; Smooth muscle cell; Matrix metalloproteinase

1. Introduction

Atherosclerosis is considered to be a chronic inflammatory disease of the arterial vessel wall [1]. Clinical studies have demonstrated an association of *Chlamydia pneumoniae* with coronary and carotid artery disease and abdominal aortic aneurysms ([2]; reviewed in [3]). The plausibility of the hypothesis that *Chlamydia* plays a causal or contributory role in the pathogenesis of atherosclerosis is based on the specific nature of this intracellular pathogen. Chlamydiae frequently cause persistent infections. It is known that urogenital diseases caused by *Chlamydia trachomatis* are associated with persistent infection leading to chronic inflammation and fibrosis [4].

*Ch. pneumoniae* was found in atherosclerotic plaques of different stages of development: early lesions (fatty streaks), fibrous, and complicated (vulnerable) plaques [5]. Besides endothelial cells and macrophages chlamydiae infect smooth muscle cells (SMC) in atherosclerotic arteries [6,7]. Studies on animal and cell culture models have offered mechanisms by which *Chlamydia pneumoniae* may initiate or modulate the formation of fatty streaks as well as fibrous atherosclerotic lesions (reviewed in [3]).

Unstable angina, acute coronary syndrome and myocardial infarction are mostly due to plaque destabilisation that results in plaque rupture and subsequent thrombosis [8]. The stability of an atherosclerotic plaque is determined by the integrity and collagen content of its fibrous cap overlying the central lipid core. Vulnerable lesions are characterised by a thinned and collagen-poor fibrous cap, compared to stable fibrous plaques [9,10]. Several studies have suggested that the overexpression of matrix metalloproteinases (MMPs) in atherosclerotic lesions not only modulates the arterial remodelling but also contributes to plaque vulnerability and rupture by degrading extracellular matrix proteins of the fibrous cap [11–13].

MMPs are zinc-dependent endopeptidases that are di-
vided into collagenases, stromelysins, gelatinases and other enzymes. They are secreted as latent proenzymes and require cleavage to attain proteolytic activity. Enzymatic activities of MMPs are counter-regulated by tissue inhibitors of MMP (TIMPs) binding to active or latent MMP forms [14]. As shown by immunocytochemistry, endothelial cells, macrophages and SMC are important producers of MMPs in atherosclerotic vessels [9,15,16]. A recent study has reported that the infection of macrophages with C. pneumoniae results in an up-regulation of MMP-2 (72-kDa gelatinase) and MMP-9 (92-kDa gelatinase) whereas the synthesis of MMP-1 (interstitial collagenase) and MMP-3 (stromelysin-1) is not influenced [17]. In the atherosclerotic plaque the interaction of C. pneumoniae with SMC may also contribute to an increased MMP expression. Therefore, in this study we investigated the effect of C. pneumoniae infection on the production of MMP-1, -2, -3 and -9 by SMC in vitro.

2. Materials and methods

2.1. Chlamydia propagation, cell culture, and infection of SMC

C. pneumoniae strain TW-183 (obtained from the Institute of Ophthalmology, London, UK) was propagated in buffalo green monkey (BGM) cells [18]. Infectivity titres of chlamydial stocks were quantified by titrating the number of inclusion-forming units (IFU) ml⁻¹ in BGM cells. These titres were used to determine the infectious doses for SMC. Chlamydial stocks were checked for Mycoplasma contamination by culture and polymerase chain reaction (PCR) by the Bundesinstitut für Gesundheitliche Verbraucherschutz und Veterinärmedizin, Jena, Germany. Only Mycoplasma-free stocks were used for further experiments.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Primer pair*</th>
<th>Product size (bp)</th>
<th>Reference</th>
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<tr>
<td>MMP-1</td>
<td>5'-ATGCCACAAATCTCCCTTTACTCC-3', 5'-TTTCCCTAGAAAAGCCACCATCG-3'</td>
<td>247</td>
<td>[19]</td>
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<tr>
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<td>5'-CAAGGACCGTGTTTACGAGCC-3', 5'-ATGCCATCCGGCATCCTGCG-3'</td>
<td>375</td>
<td>[19]</td>
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<tr>
<td>MMP-3</td>
<td>5'-GAACATGCAAAAGGCTACACAC-3', 5'-AAATGAAACGCTTCCTGTAG-3'</td>
<td>463</td>
<td>[20]</td>
</tr>
<tr>
<td>MMP-9</td>
<td>5'-CATTTGCTCCAAGATGGAATATAC-3', 5'-CGAGATTCGATGCTGACATG-3'</td>
<td>515</td>
<td>[20]</td>
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<tr>
<td>TIMP-1</td>
<td>5'-CTCTGGAAAAAGCAATTATATTGGG-3', 5'-GTTGAGGCTTACCTATTATGGG-3'</td>
<td>507</td>
<td>[20]</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>5'-CTGTTGAGGAGCTTCAGTGA-3', 5'-GTTCCAGAGGCTCGTGACCTAA-3'</td>
<td>105</td>
<td>[21]</td>
</tr>
</tbody>
</table>

*Top row, sense strand; bottom row, antisense strand.

Human vascular SMC (1210611, Cell Lining, Berlin, Germany) were subcultured in SMC growth medium 2 (PromoCell, Heidelberg, Germany). The cells stained positively with antibodies to smooth muscle α-actin (monoclonal mouse IgG, clone 1A4; Dako, Hamburg, Germany) and were characterised by the appearance of numerous bundles of α-actin filaments.

SMC were seeded into 35-mm-diameter culture wells or into 11-mm-diameter culture tubes containing a glass coverslip. Before infection, confluent SMC were maintained in basal medium containing 2% foetal calf serum (FCS) but no antibiotics for 72 h. Cell monolayers were infected with chlamydiae at multiplicities of infection (MOIs; IFU per cell) of 1, 5 and 10. After centrifugation at 4000 × g at 37°C for 45 min, the inoculum was decanted, and the cells were further incubated with basal medium containing 2% FCS but no antibiotics. For mock-infected cultures, the cells were centrifuged with a harvest of uninfected BGM cells. For heat inactivation, chlamydial suspensions were held at 75°C for 10 min prior to inoculation onto cell monolayers. For UV inactivation, chlamydial suspensions were placed under a UV lamp (15 W at 30 cm) for 15 min.

For determination of chlamydial inclusions, coverslips were removed from the culture tubes at 72 h after infection, fixed with methanol, and stained with fluorescein isothiocyanate (FITC)-conjugated antibody to chlamydial lipopolysaccharide (LPS) (Imag; Dako). The percentage of inclusion-containing cells was calculated from determination of inclusions in 20 randomly selected ×400 microscopic fields.

2.2. Reverse transcription (RT)-PCR analysis

Total RNA was prepared from cell monolayers using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. First-strand cDNA was reverse transcribed from 1 μg of RNA in a total reaction volume of 20 μl with 15 U of avian myeloblastosis
virus reverse transcriptase and 0.5 μg of oligo(dT)$_{15}$ primer, using the Promega reverse transcription system (Promega, Mannheim, Germany) as instructed by the manufacturer.

Each 25 μl of PCR mixture contained 0.5 μl of cDNA (corresponding to 25 ng of RNA), 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.2 mM each dNTP, 1.5 mM MgCl$_2$, 0.4 μM sense and antisense primers (Table 1), and 1.25 U of Taq DNA polymerase (Promega). cDNA was amplified for pyruvate dehydrogenase (PDH) as reference. Twenty-five cycles of amplification were carried out in a TRIO-Thermoblock (Biometa, Göttingen, Germany). Reactions consisted of an initial incubation at 95°C for 7 min and then cycling at 95°C for 30 s, 62°C (MMP-1, -2, and -9 primers; TIMP-1 and -2 primers) or 60°C (MMP-3 and PDH primers) for 30 s, and 72°C for 1 min, with a final incubation at 72°C for 10 min. Negative controls were performed by omitting RNA from cDNA synthesis and PCR amplification (data not shown). Products were electrophoresed on 1% agarose gels containing SYBR Green I (Molecular Probes; purchased from MoBiTec, Göttingen, Germany) and visualised on a UV transilluminator. For comparison of the bands, photographs of the products were scanned and the volumes (OD$_{U \text{mm}^2}$) of the band images were quantitated with MultiAnalyst software (Bio-Rad, Munich, Germany). All MMP and TIMP signals were normalised against the PDH signal from the same sample. All results shown are representative of two separate experiments.

The specificity of the PCR products was confirmed by the presence of a DNA band of the expected size (Table 1) and by sequencing in an automated ABI Prism 310 genetic analyser (Perkin Elmer, Applied Biosystems, Weiterstadt, Germany) as previously described [18].

### 2.3. MMP assays

Supernatants of infected and mock-infected cultures were collected at 24, 48 and 72 h after infection and stored at −30°C until assayed. Levels of MMP-1 proenzyme were measured by quantitative enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Wiesbaden, Germany). Active MMP-1 was analysed using the Biotrak MMP-1 activity assay system (Amersham Pharmacia Biotech Europe, Freiburg, Germany) that measures the cleavage of a chromogenic peptide substrate. Total MMP-3 and -9 levels were examined by ELISA (R&D Systems). The sensitivities were 0.021 ng ml$^{-1}$ for pro-MMP-1, 0.1 ng ml$^{-1}$ for active MMP-1, 0.009 ng ml$^{-1}$ for MMP-3, and 0.156

<table>
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<tr>
<th>MOI*</th>
<th>Inclusion-containing SMC (%)b</th>
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<tr>
<td>1</td>
<td>7 (1)</td>
</tr>
<tr>
<td>5</td>
<td>16 (1)</td>
</tr>
<tr>
<td>10</td>
<td>21 (2)</td>
</tr>
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*Cells grown on 11-mm-diameter coverslips were infected with indicated doses of *C. pneumoniae* TW-183.

b Intracellular inclusions were counted after staining with FITC-conjugated antibody to chlamydial LPS (Imagen; Dako). Infection is expressed as percentage of inclusion-containing cells (with standard deviations). Values are means of three experiments.

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Fig. 1. Expression of MMP and TIMP genes in SMC infected with *C. pneumoniae* TW-183. Cells grown in 35-mm-diameter culture wells were infected at various MOIs. Total RNA was extracted at 24 h after infection. mRNA levels were determined by RT-PCR.

Table 2

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>1</td>
<td>0.2</td>
<td>1</td>
<td>&lt; 0.1</td>
<td>&lt; 0.1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>0.4</td>
<td>1.2</td>
<td>0.3</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>10</td>
<td>0.8</td>
<td>1.1</td>
<td>0.1</td>
<td>&lt; 0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*Figures show the ratio of gene expression to PDH.*
ng ml⁻¹ for MMP-9. All assays were performed according to the manufacturer’s protocols.

2.4. Statistical analysis

The data of MMP assays were expressed as means with standard deviation. Student’s t test was used to assess the significance of observed differences. A P value ≤ 0.05 was considered statistically significant.

3. Results

3.1. MMP and TIMP mRNA levels in C. pneumoniae-infected SMC

SMC were infected with C. pneumoniae TW-183 at various MOIs. The resultant percentages of Chlamydia-positive cells in infected cultures are shown in Table 2. In first experiments we examined the expression of MMP and TIMP mRNAs in SMC using RT-PCR analysis. The results are summarised in Fig. 1. Mock-infected cells produced MMP-2, TIMP-1 and TIMP-2 mRNA, but showed only little expression of MMP-1, -3 and -9 genes. The infection of SMC with C. pneumoniae markedly increased the amounts of MMP-1 and -3 mRNA in a dose-dependent manner. In contrast, the expression of the MMP-9 gene was not induced, and the level of MMP-2 mRNA remained unchanged. C. pneumoniae infection did not affect the expression levels of the TIMP-1 and -2 genes in SMC. The selective up-regulation of MMP-1 and -3 mRNA was observed at 24, 48 and 72 h after infection (data not shown). As demonstrated in Fig. 2, the enhancement of MMP-1 and -3 mRNA levels in Chlamydia-infected SMC required the viability of the bacterial organisms because heat- and UV-inactivated chlamydiae did not increase the expression of both MMPs above the baseline levels for mock-infected cells.

3.2. C. pneumoniae stimulates the production of MMP-1 and -3 by SMC

Further experiments evaluated whether enhanced mRNA levels of MMP-1 and -3 in Chlamydia-infected SMC were associated with an increased secretion of MMP proteins from the cells. To quantitate MMP-1 proenzyme in culture supernatants we used an immunoassay. The active form of MMP-1 was measured by a colorimetric activity assay. At an MOI of 5, C. pneumoniae significantly enhanced the production of the MMP-1 proenzyme by fourfold over the control level at 72 h after infection (Fig. 3A). The level of active MMP-1 was 10-fold higher in Chlamydia-infected cultures than in mock-infected controls at 72 h after infection (Fig. 3B).

MMP-3 was analysed using an immunoassay that detects both inactive and active enzyme. In conditioned me-

dium collected from C. pneumoniae-infected SMC a significant increase in the amounts of MMP-3 was found, compared to mock-infected cells (Fig. 4).

It has been reported that macrophages produce MMP-9 upon chlamydial infection [17]. Infected SMC showed only low levels of MMP-9 mRNA (Fig. 1). Neither Chlamydia-infected nor mock-infected SMC secreted detectable amounts of MMP-9 protein.

4. Discussion

The activation of synthetic functions of SMC essentially contributes to the pathogenesis of atherosclerosis [1]. This study shows that C. pneumoniae infection up-regulates the production of MMP-1 and -3 by SMC.

Mock-infected SMC expressed MMP-2 and TIMP mRNAs. These factors are also present in non-diseased arteries [9,11]. In contrast, atherosclerotic arteries display an increased expression of MMP-1, -3 and -9 [9,11]. We have found a selective up-regulation of MMP-1 and -3 in SMC upon chlamydial infection whereas the production of MMP-9 was not induced. However, recent studies have demonstrated that MMP-9 is secreted by macrophages in response to infection with C. pneumoniae or stimulation with chlamydial heat shock protein 60 [17,22]. Therefore,
It can be concluded that the chlamydial infection of SMC and macrophages results in an increased production of three important MMPs that are known to be expressed in atherosclerotic plaques.

The consequences of MMP production in atherosclerosis are not completely understood [23]. MMP-1 (interstitial collagenase) mediates the initial cleavage of collagen fibrils of type I to III; the collagen fragments are further degraded by other proteases such as MMP-2 and -9 (gelatinases) or MMP-3 (stromelysin) [14]. Experimental data from murine atherosclerotic models suggest that at an early stage MMP-1 and -3 could have beneficial effects on the arterial remodelling by preventing an excessive deposition of matrix proteins [23,24]. In contrast, MMPs may also play an important role in later steps of plaque destabilisation [9,10]. Collagen type I is a major matrix component of the fibrous cap and the integrity of the collagenous skeleton determines the stability of the plaque [9]. Therefore, an overexpression of MMP-1 and other MMPs may promote the vulnerability of atherosclerotic lesions and contribute to the risk of plaque rupture [9,11,23]. This suggestion is supported by in situ analyses which demonstrated a co-localisation of increased levels of MMP-1 with collagenolysis in vulnerable versus fibrous plaques [13]. Alternatively, the overexpression of MMP-1 and -3 in the atherosclerotic vessel wall may lead to proteolytic injury to the media, thus promoting aneurysmal dilatation [25,26].

TIMPs function as inhibitors of MMPs [14]. TIMP-1 primarily binds to MMP-1, -3 and -9 whereas TIMP-2 is associated with MMP-2. *C. pneumoniae* did not affect the expression of the TIMP-1 gene. This observation indicates that the ratios of MMP-1 and -3 to their inhibitor were increased in infected SMC cultures.

Several growth factors and cytokines have been described as inducers of MMP expression [27,28]. Further studies are needed to clarify whether *Chlamydia*-mediated MMP synthesis is caused by a direct effect of chlamydial infection on SMC or soluble factors released from infected cells. Moreover, it is of interest to examine the production of factors that can activate MMP proenzymes in *Chlamydia*-infected cell cultures.

Although the precise role of MMPs in the pathogenesis of atherosclerosis has yet to be defined, the finding that *C. pneumoniae* stimulates the production of MMP-1 and -3 by SMC provides a hypothesis how this pathogen may promote destabilisation of atherosclerotic plaques or contribute to the formation of aneurysms.

**Fig. 3.** Release of MMP-1 from *C. pneumoniae*-infected SMC in comparison to mock-infected cells. A: Pro-MMP-1 was measured by ELISA. B: Active MMP-1 was determined using the Biotrak MMP-1 activity assay system. ◆, mock-infected; ▼, MOI 1; ▲, MOI 5. Cells were cultured in 11-mm-diameter tubes. Values are means (with standard deviations) of four experiments. *P ≤ 0.01; **P ≤ 0.05; ***P ≤ 0.001 compared to mock-infected cells (Student’s t-test).

**Fig. 4.** Detection of MMP-3 in culture supernatants of *C. pneumoniae*-infected SMC by ELISA. ◆, mock-infected; ▼, MOI 1; ▲, MOI 5. Cells were cultured in 11-mm-diameter vials. Values are means (with standard deviations) of four experiments. *P ≤ 0.01 compared to mock-infected cells (Student’s t-test).
Acknowledgements

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