Brief Rapid Communication

Chlamydia pneumoniae Infection of Vascular Smooth Muscle and Endothelial Cells Activates NF-κB and Induces Tissue Factor and PAI-1 Expression

A Potential Link to Accelerated Arteriosclerosis

Ralf Dechend, MD; M. Maass, MD; J. Gieffers, MD; R. Dietz, MD; C. Scheidereit, PhD; A. Leutz, PhD; D.C. Gulba MD, MSc

Background—Recent reports link C. pneumoniae infection of arteriosclerotic lesions to the precipitation of acute coronary syndromes, which also feature tissue factor and plasminogen activator inhibitor 1 (PAI-1) overexpression. We investigated whether or not C. pneumoniae can induce thrombogenicity by upregulation of procoagulant proteins.

Methods and Results—Human vascular endothelial and smooth muscle cells were infected with a strain of C. pneumoniae isolated from an arteriosclerotic coronary artery. Tissue factor, PAI-1, and interleukin-6 expression was increased in infected cells. Concomitantly, NF-κB was activated and IκBα degraded. p50/p65 heterodimers were identified as the components responsible for the NF-κB activity.

Conclusions—These data provide evidence that C. pneumoniae infection can induce procoagulant protein and proinflammatory cytokine expression. This cellular response is accompanied by activation of NF-κB. Our results demonstrate how C. pneumoniae infection may initiate acute coronary syndromes. (Circulation. 1999;100:1369-1373.)

Key Words: plasminogen activator inhibitor 1 ■ tissue factor ■ interleukins ■ arteriosclerosis ■ C. pneumoniae

Arteriosclerosis involves genetic and environmental influences. In addition to gradual arteriosclerotic progression, plaque erosion and rupture may occur with mural thrombosis, sudden luminal narrowing, or abrupt vessel closure. The clinical consequence is the acute coronary syndrome (ACS). Recent data from epidemiological, histopathological, and intervention studies suggest a role of C. pneumoniae infection in the pathogenesis of ACS. However, the molecular mechanisms are poorly understood. Macrophages may be recruited to the plaque causing weakening of the fibrous cap and subsequent plaque rupture. Thrombogenicity participates in the precipitation of ACS. We reasoned that C. pneumoniae infection might increase the thrombogenicity of infected plaques. We examined tissue factor (TF) and plasminogen activator inhibitor 1 (PAI-1) expression in human vascular smooth muscle (VSMC) and endothelial cells (EC). Increased TF, PAI-1, and interleukin-6 (IL-6) were found after C. pneumoniae infection. Enhanced thrombogenicity was accompanied by sustained activation of the nuclear transcription factor NF-κB p50/p65 heterodimer and the simultaneous degradation of the inhibitor protein IκBα. Our findings suggest that C. pneumoniae infection induces procoagulant protein and proinflammatory cytokine expression via NF-κB activation. We suggest that this process may precipitate ACS.

Materials and Methods

C. pneumoniae infection was established in primary cultures of human VSMCs (Clonetics, San Diego, Calif) and in the immortalized human venous endothelial cell line ECV-304 (ATCC CRL 1998). Stock suspensions were prepared from the vascular C. pneumoniae strain CV-4, isolated from an arteriosclerotic coronary artery as described earlier. C. pneumoniae growth was monitored 72 hours after infection using a fluorescent isothiocyanate-conjugated C. pneumoniae-specific monoclonal antibody (DAKO, Copenhagen, Denmark). Cells were also continuously checked for detachment from plates and cell lysis. Cells were harvested at different time points for electrophoretic mobility shift assay (EMSA) and lysed in whole cell lysis buffer. Labeling and binding reaction were performed as described. The DNA probe contained the κB site from the MHC-enhancer (H2K) or the H2B octamer binding site. EMSAs were repeated 3 times and representative gels are shown. Polyclonal antiserum to NF-κB proteins p50, p65, c-Rel, and RelB were purchased from Santa Cruz Biotechnology Inc, Calif. For immunoblotting, whole cell lysates were prepared, quantified by standard Bradford assay, and immunoblotting was performed as described. TF and PAI-1 were obtained from Loxo (GmbH, Dossenheim, Germany); IL-6 and tubulin from Genzyme (Framingham, Mass). Protein concentrations of the cellular supernatants were quantified for ELISA using the Bradford method. IL-6 (Quantikine Immunoassay, R&D Systems, Minneapolis Minn) and PAI-1 (American
Diagnostics, Greenwich, Conn) were determined by ELISA. TF activity was determined in cell extracts with a clotting based assay (ACTICLOT, Diagnostics International, Karlsdorf, Germany).

Results

Figure 1A shows infection of human ECs and VSMCs. Intracellular inclusion bodies are visible, they contain actively replicating C. pneumoniae of the CV-4 strain isolated from infected coronary plaques.10

Figure 1B shows TF, PAI-1, tubulin, and IL-6 expression in cellular EC lysates. TF expression was increased 4 hours after infection, reached maximum by 48 hours, and was sustained for the entire 72-hour study period. Increased PAI-1 expression plateaued at 4 hours and was sustained throughout the 72 hours (Figure 1B). Tubulin expression was not influenced by infection, whereas IL-6 expression was increased by 4 hours. Similar effects were found in VSMCs; however, C. pneumoniae-induced TF and PAI-1 expression was lower in VSMCs than in ECs, as demonstrated by quantification (Figure 1B). In the supernatant of infected ECs, PAI-1 and IL-6 levels were induced nearly 4- and 6-fold, respectively, and remained elevated throughout the 72 hours of study (Figure 1C, center and right). In addition, as shown by a functional assay, TF coagulant activity of the cellular extracts increased to a similar extent as TF protein expression (Figure 1C, left).

NF-κB is an important transcriptional regulator for TF and IL-6 expression.11,12 Moreover, additional data suggest that PAI-1 expression is also under the control of NF-κB.13,14 Furthermore, NF-κB is activated by microbial infection.15 EMSA results are shown in Figure 2A. NF-κB activity was increased at 4 hours after C. pneumoniae infection and persisted over 72 hours in ECs and VSMCs. However, the DNA-protein complex formation shows somewhat different time-steps in the two cell types. The induced DNA complexes observed at 4 hours postinfection in HASMCs remain almost constant during the entire observation period. In ECs, however, transient DNA-protein complex was detected 24 hours after infection, whereas IL-6 expression was increased by 4 hours. Similar effects were found in VSMCs; however, C. pneumoniae-induced TF and PAI-1 expression was lower in VSMCs than in ECs, as demonstrated by quantification (Figure 1B). In the supernatant of infected ECs, PAI-1 and IL-6 levels were induced nearly 4- and 6-fold, respectively, and remained elevated throughout the 72 hours of study (Figure 1C, center and right). In addition, as shown by a functional assay, TF coagulant activity of the cellular extracts increased to a similar extent as TF protein expression (Figure 1C, left).

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Discussion

The important findings in this study were that C. pneumoniae infection of human EC and VSMC resulted in TF, PAI-1, and IL-6 overexpression. Moreover, we demonstrated that C. pneumoniae infection resulted in NF-κB activation. NF-κB remained upregulated for at least 72 hours and was accompanied by 1κB degradation. Although C. pneumoniae belong to the eubacteria, they have no close relatives among the known bacterial genera and a direct activation of NF-κB by C. pneumoniae infection has not been shown thus far.

Plaque rupture and increased thrombogenicity are prime mechanisms of ACS.2,6 Because chronic C. pneumoniae infection is linked to the precipitation of ACS,3,4 we examined whether infections with a C. pneumoniae strain isolated from such a coronary plaque would increase the expression of prothrombotic proteins in vascular cells. We found induced and sustained expression of functionally active TF and PAI-1. Our results agree with reports from Rickettsia rickettsii-infected ECs overexpressing TF, PAI-1, IL-1, IL-6, and IL-8.16 Furthermore, infection of ECs with different Chlamydia strains also induce TF overexpression.17 Thus, the alteration of the epi/pericellular hemostatic protein expression observed here is not necessarily specific for the vascular C. pneumoniae strain that we used.

In addition to an increased procoagulation protein expression, functional cytokines are expressed in human arteriosclerotic lesions.1 IL-6 is an important mediator of inflammation in cardiovascular tissue.19 IL-6 is highly expressed in arteriosclerotic lesions,19 implicated in plaque instability,20 and in the pathogenesis of acute myocardial infarction.18 C. pneumoniae infection induced sustained cellular overexpression and secretion of IL-6. Recruitment of mesenchymal and immunocompetent cells, proliferation, and migration of VSMCs are the consequences of cytokine overexpression in the arteriosclerotic plaque.21 This state of affairs further perturbs the anticoagulant activities. The functional cooperation between products of the coagulation cascade and cytokine-mediated inflammatory response has been shown to transform a stable plaque into an unstable plaque.21 In this context, colocalization of human heat shock protein 60 (hsp) with chlamydial hsp 60 in macrophages was demonstrated. Both hsp forms induced proinflammatory cytokines such as TNF-α and metalloproteinases.5

TF and IL-6 are transcriptionally regulated by NF-κB.11,12 Moreover, data have been presented that PAI-1 protein expression is also under the control of NF-κB.13 However, these data are less compelling. In addition, inhibition of TNF-α- induced PAI-1, TF, and IL-6 activation was achieved when NF-κB activity was inhibited with the antioxidant pyrrolidine dithiocarbamate.14 Therefore, current knowledge suggests a common link between C. pneumoniae infection, TF, PAI-1, IL-6 expression, and NF-κB. We therefore investigated whether NF-κB activity is induced in response to C. pneumoniae infection. We showed that after infection, p50/p65 heterodimer activation was upregulated in association with degradation of 1κBα. Our findings therefore suggest that the C. pneumoniae-induced overexpression of TF, PAI-1, and IL-6 is transcriptionally regulated by NF-κB.

Recent data underscored the role of NF-κB in the pathogenesis of arteriosclerosis.15,22 ECs, VSMCs, and macrophages harvested and grown from arteriosclerotic lesions express activated NF-κB. Our findings are consistent with
Figure 1. A, C. pneumoniae infection of EC and HASMC. Immunofluorescence staining reveals large intracellular inclusion bodies containing actively replicating C. pneumoniae, 72 hours after infection. B, Effects of C. pneumoniae infection on cellular expression of TF, PAI-1, and IL-6. Immunoblot analysis of cellular extracts prepared from ECs (left) and HASMCs (right) at different time points after C pneumoniae or mock infection. Tubulin expression was determined as a control. Densitometry quantification values (NIH Image Pro-gram Version 1.61) are given in arbitrary units (AU) below each time point. C, Open and solid bars represent TF activity in EC extracts of mock-infected and infected cells, respectively (left). ■ and ○ represent supernatant PAI-1 and IL-6 levels in infected and mock-infected endothelial cells, respectively, determined by ELISA (center and right). Data and standard deviation represent mean of triplicates.
Figure 2. A, Top, kinetics of NF-κB activation in cellular extracts of ECs (left) and HASMCs (right) analyzed by EMSA. Arrows 1, 2, and 3 represent p50/p65, p50/p50, and unspecific complexes, respectively. Bottom, OCT-1 DNA binding activity on the H2B site, used as a control; oct-1 indicates octamer-binding transcription factor 1. B, Subunit composition of C. pneumoniae-induced NF-κB DNA binding activity. The antibody-supershifted complexes (AS) are indicated. Competition with unlabeled NF-κB and AP-1 oligonucleotide is demonstrated. C, IκBα protein level after infection with C. pneumoniae or mock infection analyzed by immunoblotting.
NF-κECs. Interestingly, Gram-negative cell wall composition, and the ability to infect C. pneumoniae, including obligate intracellular lifestyle, are closely related; however, they share characteristics with many other bacteria. Interestingly, R. rickettsii has been shown to mediate NF-κB-induced transcriptional activation, resulting in the inhibition of EC apoptosis. A similar mechanism in C. pneumoniae infection might provide an explanation for chlamydial persistence in the endothelium and a continuous inflammatory stimulus within the vascular wall. We are the first to show that NF-κB is activated in response to C. pneumoniae infection.

Our data suggest that chronic C. pneumoniae infection in the cellular components of plaques can result in increased cellular and epipericellular procoagulant protein expression and increased chemoattractant activity through activated NF-κB. Both effects could enhance the vulnerability of atherosclerotic plaques. Our results add to the understanding of the role of C. pneumoniae bacteria in the mechanism of accelerated atherosclerotic disease.

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References
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