Chlamydia pneumoniae Stimulates Proliferation of Vascular Smooth Muscle Cells Through Induction of Endogenous Heat Shock Protein 60

Satoru Hirono, Elena Dibrov, Cecilia Hurtado, Annette Kostenuk, Robin Ducas, Grant N. Pierce

Abstract—Chlamydia pneumoniae infection has been linked with atherosclerosis. However, the mechanism responsible for the atherogenic effects of C pneumoniae remains unclear. Heat shock proteins (HSPs) have been found in atherosclerotic lesions. HSPs of HSP70 and HSP90 families are involved in the regulation of cell cycle progression and cell proliferation. We assessed the hypothesis that HSP60 is induced in vascular cells infected with C pneumoniae and stimulates cell proliferation. Rabbit vascular smooth muscle cells (VSMCs) and human umbilical vein endothelial cells (HUVECs) were infected with C pneumoniae. Western blot analysis demonstrated the induction of endogenous HSP60 expression in C pneumoniae–infected VSMCs. C pneumoniae infection significantly increased the number of VSMCs, and the mitogenic effect correlated with the expression level of endogenous HSP60. In contrast to VSMCs, C pneumoniae infection had no effect on the expression level of HSP60 and did not stimulate cell proliferation in HUVECs. Exogenous addition of recombinant chlamydial HSP60 had no mitogenic effect on VSMCs and HUVECs. However, overexpression of HSP60 within VSMCs by infection with adenovirus encoding human HSP60 resulted in a significant increase in cell numbers compared with uninfected VSMCs. These results suggest that overexpression of endogenous HSP60 may be a central intracellular event responsible for the mitogenic effects induced by C pneumoniae infection. In addition to C pneumoniae, other infectious agents and atherogenic risk factors may also stimulate VSMC proliferation and contribute to the lesion formation through the induction of HSP60. (Circ Res. 2003;93:710-716.)

Key Words: atherosclerosis ■ infection ■ inflammation ■ coronary disease

Atherosclerosis is now recognized as a chronic inflammatory process, and it is becoming increasingly clear that an infection may be an important initiating component within the atherogenic process.1–3 Chlamydia pneumoniae is one infectious agent that has received particular attention as a potent atherogenic stimulus. Correlative studies support a possible relationship between atherosclerosis and C pneumoniae infection.4,5 Pathological studies have revealed the localization of C pneumoniae in atherosclerotic lesions.6,7 Several animal models have confirmed a causal role of C pneumoniae in atherogenesis8–12; however, the precise mechanism(s) by which C pneumoniae promote atherosclerosis still remains unclear.

Dysregulated proliferation of vascular cells in response to environmental stimuli plays a key role in the development of atherosclerosis. Although C pneumoniae–mediated activation of macrophages and vascular cells has been extensively studied,5,13–15 only limited studies have addressed the possible role for C pneumoniae infection in cell proliferation.16 The mechanism responsible for the proliferative action of C pneumoniae is unclear.

Growing evidence indicates that heat shock protein (HSP) 60 may serve as a possible link between C pneumoniae infection and atherosclerosis.17,18 HSPs of the HSP60 family are phylogenetically highly conserved, facilitating immunological cross-reactions between chlamydial and human HSP60. Wick and colleagues have hypothesized that autoimmune reactions against HSP60 may play an important role in atherogenesis.19 They first reported that serum antibodies to mycobacterial HSP65 were increased in subjects with atherosclerosis.20 Recently, they have shown that titers of anti-HSP65 antibody correlate with seropositivity to C pneumoniae,21 indicating a role for C pneumoniae infection in the production of these antibodies. Because serum antibodies against chlamydial HSP60 from subjects with atherosclerosis cross-react with human HSP60 and mediate endothelial cytotoxicity,22 it is suggested that humoral immune reactions to HSP60 may play an important role in vascular endothelial injury, a key process in the early stages of atherosclerosis. Interestingly, two independent groups have shown that an elevated anti-human HSP60 antibody level is a risk factor for coronary atherosclerosis, especially when it is present with C pneumoniae infection.23,24
In addition to its antigenic properties, both chlamydial and human HSP60 might mimic the ability of C. pneumoniae to stimulate the activation of macrophages/monocytes and vascular cells. Recent studies have also implicated HSPs in cell growth and proliferation. In this regard, the finding that chlamydial HSP60 colocalizes with human HSP60 within atherosclerotic plaque macrophages is of great interest. It suggests that C. pneumoniae may induce the expression of endogenous HSP60 together with its own HSP60 in vascular cells, accelerating the development of atherosclerotic lesions. However, it is not known whether endogenous HSP60 is induced in vascular cells by C. pneumoniae infection. It is also unknown if endogenous HSP60 can directly stimulate proliferation of vascular cells.

In the present study, we tested whether C. pneumoniae infection stimulates proliferation of vascular smooth muscle cells (VSMCs) and endothelial cells and whether C. pneumoniae infection induces endogenous HSP60 in these cells. We also tested the hypothesis that induction of endogenous HSP60 may stimulate cell proliferation.

**Materials and Methods**

**Cell Culture**

VSMCs were harvested from the aorta of New Zealand White rabbits using the explant technique as previously described. To induce differentiation, VSMCs were placed in a serum-free Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen Corp.) supplemented with transferrin (5 μg/mL), selenium (1 nmol/L), ascorbate (200 μmol/L), and insulin (10 nmol/L) for 5 to 6 days before the experiments. Human umbilical vein endothelial cells (HUVECs) were obtained from Cell Applications, Inc (San Diego, Calif) and maintained in endothelial cell growth medium (ECGM; Cell Applications, Inc).

**Chlamydia pneumoniae**

C. pneumoniae AR39 strain was obtained from the University of Washington, Seattle, Wash. The organism was propagated in HL cells and purified by Hypaque gradient centrifugation. The purified organism was resuspended in chlamydial transport medium, sucrose phosphate glutamic acid, and stored at −70°C until use. The titer of C. pneumoniae was determined in cycloheximide-treated HL cells, and concentrations used were expressed as inclusion-forming units (IFU) per mL.

**Chlamydial HSP60**

Recombinant C. pneumoniae HSP60 was expressed in Escherichia coli and purified by using Qiagen expression vector QIAexpress System (Qiagen Inc). The pQ60 expression vector that contains C. pneumoniae HSP60 gene (kind gift of Dr Jane C. Goodall, University of Cambridge, Cambridge, UK) was transformed into E. coli M15. This enabled the recombinant protein to be expressed as a fusion protein tagged with C-terminal 6-histidine residues. The recombinant protein was purified by affinity chromatography with Ni-nitrilotriacetic acid resin, aliquoted, and stored at −20°C. No endotoxin was detectable in this preparation by Limulus amebocyte lysate assay (E-TOXATE kit, Sigma Chemical Company). Protein concentration was determined by the Lowry method. The homogeneity of the recombinant protein was confirmed by SDS-PAGE and Coomassie blue staining. Western blot analysis (Figure 1) showed that the protein was specifically recognized by anti-chlamydial HSP60 antibody (Affinity Bioreagents Inc).

**Immunocytochemistry**

VSMCs and HUVECs were seeded on glass coverslips in 24-well plates at 3 × 10^4 cells/well and inoculated with C. pneumoniae. After 48 hours, the infected cells were fixed with 100% methanol and then incubated with anti-Chlamydia monoclonal antibody (Chemicon International Inc). Inclusion bodies were visualized by staining with FITC-conjugated anti-mouse IgG (Sigma Chemical Company). The samples were examined by confocal fluorescence microscopy.

**Cell Proliferation Assay**

VSMCs and HUVECs were seeded at 5 × 10^4 cells/well in 96-well plates and incubated with C. pneumoniae or chlamydial HSP60 in DMEM containing 1% fetal bovine serum (FBS; Invitrogen Corp.) and ECGM, respectively. After 48 hours, the number of living cells was determined by a colorimetric enzyme assay (CellTiter 96 Cell Proliferation Assay; Promega Corporation) based on a cytoplasmic enzyme activity present in viable cells. The absorbance of a formazon product in tissue culture media was measured at 500 nm using a microplate reader.

**Direct Cell Counting**

VSMCs were seeded at 2 × 10^4 cells/well in 24-well plates and incubated with C. pneumoniae in DMEM/1% FBS for 48 hours. The attached cells were harvested by trypsinization and cell number was determined using a hemocytometer.

**Western Blot Analysis**

VSMCs and HUVECs were seeded at 1.5 × 10^4 cells/well in 6-well plates and incubated with C. pneumoniae in DMEM/1% FBS and ECGM, respectively. VSMCs were also exposed to high temperature to determine whether endogenous HSP60 is induced in response to heat stress (for detail, see the expanded Materials and Methods section in the online data supplement available at http://www.circresaha.org). After 48 hours, cells were harvested by trypsinization and counted using a hemocytometer. Cells (5 × 10^4 cells/lane) were washed with PBS and lysed in sample buffer. Proteins were separated on a 9% SDS polyacrylamide gel and transferred electrophoretically onto a nitrocellulose membrane. The membrane was incubated with anti-mammalian HSP60 monoclonal antibody (StressGen Biotechnologies Corp), which shows no cross-reactivity with bacterial HSP60 (Figure 1) or anti-proliferating cell nuclear antigen (PCNA) monoclonal antibody (Sigma Chemical Company). HRP-conjugated anti-mouse IgG (Chemicon International Inc) was used as a secondary antibody, and the immunologically cross-reacting band was visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce). Expression levels of HSP60 and PCNA were quantified by densitometry.

**Adenoviral Vectors**

Recombinant adenovirus-expressing human HSP60 (Ad-HSP60) was constructed using AdEasy Vector System (Qbiogen). Human HSP60 cDNA (kind gift of Dr Radhey S. Gupta, McMaster Univer-
In agreement with previous studies,35 VSMCs and HUVECs were capable of supporting the growth of C. pneumoniae in vitro. Multiple inclusion bodies were observed in both VSMCs and HUVECs after 48 hours of C. pneumoniae infection (Figure 2).

C. pneumoniae Stimulates VSMC Proliferation

VSMC proliferation was stimulated at 48 hours after inoculation with C. pneumoniae in a concentration-dependent manner. The cytoplasmic enzyme activity assay showed that C. pneumoniae (5×10⁴ and 1×10⁵ IFU/mL) significantly increased the number of VSMCs compared with uninfected controls (Figure 3). The mitogenic effect was confirmed by direct cell counting. The increase in cell number (seeded at concentration (10 μg/mL), chlamydial HSP60 decreased the number of VSMCs by 8% and HUVECs by 11% compared with controls incubated with the medium alone. The cytotoxic
effect of HSP60 has been shown previously. The concentrations of recombinant chlamydial HSP60 used in this experiment were chosen based on quantitative analyses by Western blot using the anti-chlamydial HSP60 antibody. We calculated that the amount of chlamydial HSP60 in 10^5 IFU of C. pneumoniae was equivalent to 0.3 to 1.0 μg of the recombinant protein. Thus, the mitogenic effect of C. pneumoniae is not mediated by the extracellular HSP60.

Overexpression of Endogenous HSP60 Stimulates VSMC Proliferation

These observations led us to hypothesize that the mitogenic effect of C. pneumoniae is achieved through the induction of intracellular HSP60. However, other components of C. pneumoniae, such as chlamydial lipopolysaccharide (LPS), may also play a role in the mitogenic effect. To eliminate the possible contribution of other chlamydial components and to determine whether endogenous HSP60 by itself could stimulate cell pro-
Recent studies have revealed a strong association between infection and atherosclerosis. \(^\text{2–12}\) HSP60 has attracted attention as a possible link between \(C\) pneumoniae infection and atherosclerosis. \(^\text{17,18}\) HSPs are a family of highly conserved ubiquitous proteins that are induced by various stress stimuli and act as molecular chaperones to restore cellular homeostasis. HSPs fulfill a wide range of functions in cytoprotection and the intracellular assembly, folding, and translocation of proteins. Evidence has accumulated that HSPs of the HSP70 and HSP90 families are associated with key molecules of the cell cycle regulatory system and play important roles in the process of cell growth and differentiation. \(^\text{26}\) However, it is not known whether HSP60 is involved in the process.

Our data demonstrates that \(C\) pneumoniae infection stimulates VSMC proliferation. It is possible that a number of factors found in \(C\) pneumoniae, including chlamydial LPS, may be responsible for the mitogenic effect. Our study, however, has focused on one specific factor, HSP60. We have conclusively proven that VSMC proliferation was achieved through the induction of endogenous HSP60 expression. This conclusion is supported by several observations. First, \(C\) pneumoniae infection induced the expression of endogenous HSP60 and stimulated VSMC proliferation. The mitogenic effect correlated with the expression level of endogenous HSP60. As expected, this effect was accompanied by an increase in the expression level of PCNA, a protein involved in cell cycle progression. Second, exogenous addition of chlamydial HSP60 had no mitogenic effect on VSMCs. Third, the inability of \(C\) pneumoniae to stimulate HUVEC proliferation was associated with an inability to induce HSP60 expression. Fourth, and most importantly, overexpression of human HSP60 in VSMCs by adenoviral infection stimulated cell proliferation. This is the first report that shows HSP60 induction directly stimulates cell proliferation.

\(C\) pneumoniae are extremely rich in HSP60 that is highly antigenic. \(^\text{37}\) Chlamydial and human HSP60s possess a considerable homology and are thought to function in at least two ways to promote atherosclerosis: first by cross-reactive immune responses involving host (human) HSP60 homologues\(^\text{19}\) and second as signal transducers that activate macrophages and vascular cells such as endothelial cells and VSMCs. \(^\text{13,25}\) It has been suggested that \(C\) pneumoniae infection of the vascular wall may induce expression of endogenous HSP60 together with its own HSP60 within vascular cells. Although the colocalization of chlamydial and human HSP60 in plaque macrophages has been demonstrated, \(^\text{13}\) it is not clear whether \(C\) pneumoniae infection itself is sufficient to induce endogenous HSP60 expression in vascular cells. In the present study, we have convincingly demonstrated that \(C\) pneumoniae induces endogenous HSP60 in VSMCs.

In contrast to VSMCs, \(C\) pneumoniae infection had no mitogenic effect and indeed caused significant cell death in HUVECs. This is not unexpected. Endothelial cells have been shown to be less susceptible to \(C\) pneumoniae infection. \(^\text{38}\) Our results may provide an answer for this cell-specific difference. The discrepancy in cell response to infection may be due to the difference in expression level of HSP60. HSP60 is induced by infection in VSMCs but not in HUVECs. The basal level of HSP60 is already higher in VSMCs than HUVECs, as HSP60 is mainly located in mitochondria. Because of the cell-stabilizing and anti-apoptotic role for HSP60, \(^\text{38}\) VSMC may survive the extreme form of stress caused by infection and escape apoptotic cell death. From a different point of view, the inability to increase endogenous HSP60 in HUVECs may be a protective mechanism that prevents further dissemination of the microbes. This may also explain why \(C\) pneumoniae infection was most prominently observed in VSMCs in the atherosclerotic lesions. \(^\text{6,7}\)

In the present study, exogenous addition of recombinant chlamydial HSP60 had no mitogenic effect on VSMCs. This result is inconsistent with the previous observation by Sasu et al. \(^\text{16}\) They reported that the mitogenic effect of \(C\) pneumoniae on VSMCs could be mimicked by exogenous chlamydial HSP60 via a Toll-like receptor (TLR) 4-mediated signaling pathway. However, they also observed that inactivation of \(C\) pneumoniae by UV-light or heat (56°C) treatment significantly attenuated its mitogenic effect, \(^\text{16}\) suggesting that active infection of the live organism may also play an important role in cell proliferation. Our results agree with those of Sasu et al. \(^\text{16}\) in that \(C\) pneumoniae infection is critical to the proliferative action of HSP60. However, our data convincingly demonstrates that it is the induction of endogenous, intracellular HSP60 that induces cell proliferation, not the addition of extracellular HSP60. This does not rule out the possibility that extracellular HSP60 may have a proliferative effect under some conditions. For example, several groups have reported that the expression of TLR4 is upregulated by bacterial LPS and certain cytokines in human monocyes and endothelial cells. \(^\text{39,40}\) If \(C\) pneumoniae infection induces TLR4 expression on VSMCs together with endogenous HSP60, which could be subsequently released into the extra-
extracellular HSP60 may still act as a signal transducer via the upregulated TLR4 to stimulate cell proliferation. Our data definitively demonstrated, however, that rabbit VSMCs do not respond to extracellular HSP60 in the basal state. Our data also identify the important proliferative role of HSP60 as an intracellular protein.

The mechanism by which induction of intracellular HSP60 stimulates cell proliferation is unclear. However, it is reasonable to hypothesize that HSP60 interacts with one or more of the key intracellular proteins involved in cell cycle regulation. Our results and previous findings would support such a hypothesis. It has been shown that HS70 and HS90 interact with key regulatory molecules of cell cycle progression and stimulate the entry of cells into the S-phase. If other families of HSPs participate in cell proliferation through interactions with cell cycle regulatory proteins, the same may be true for HSP60. Our own preliminary results support this contention. Overexpression of HSP60 did stimulate the expression of the cell cycle protein, PCNA.

Because overexpression of HSP60 in VSMCs resulted in a significant increase in cell numbers, it is suggested that any other stress stimuli that induce HSP60 in cells could be potentially mitogenic. In vascular cells, induction of HSPs has been observed in response to various stress stimuli, including oxidized low-density lipoprotein, cytokines, shear stress, and hypertension, all of which are known to contribute to atherogenesis. Therefore, induction of HSP60 by a variety of atherogenic risk factors may contribute to the formation of atherosclerotic lesions through VSMC proliferation. The implications of the present study may extend beyond these risk factors as well. It has been suggested that other bacteria, such as Helicobacter pylori and oral bacteria, and viruses, such as cytomegalovirus and herpesviruses, can also initiate the inflammatory process that leads to atherogenesis. This indicates that there may be a common intracellular signaling pathway for all of these infectious agents. HSPs are induced by a variety of infectious stimuli. On the basis of our results, therefore, it is plausible to suggest that HSP60 may represent an important component of that common signaling pathway.

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References


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Materials and Methods

Western Blot Analysis of HSP60 in heat-stressed VSMCs

VSMCs were seeded at 1.5x10^5 cells/well in 6-well plates, heated at 42°C for 1 hour and then incubated at 37°C in DMEM/0.5%FBS for up to 24 hours. Cells were harvested by trypsinization at 2, 6 and 24 hours after the heat treatment. Cells (5x10^4 cells/lane) were washed with PBS and lysed in sample buffer. Proteins were separated on a 9% SDS polyacrylamide gel and transferred electrophoretically onto a nitrocellulose membrane. The membrane was incubated with anti-mammalian HSP60 monoclonal antibody (StressGen Biotechnologies Corp., Victoria, BC). HRP-conjugated anti-mouse IgG (Chemicon International Inc.) was used as a secondary antibody, and the immunologically cross-reacting band was visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Expression levels of HSP60 were quantified by densitometry.
Results

Heat Stress Induces Endogenous HSP60 Expression in VSMCs

Western blot analysis demonstrated that endogenous HSP60 expression was induced in VSMCs after exposure to high temperature. The expression level of HSP60 was increased 1.8-fold at 6 hours and 2.9-fold at 24 hours after heat stress (at 42°C for 1 hour) relative to untreated controls (Figure 1).
**Figure Legends**

**Figure 1.** Western blot analysis of HSP60 in VSMCs after exposure to high temperature. VSMCs were heated at 42°C for 1 hour and then incubated at 37°C in DMEM/0.5%FBS up to 24 hours. Cell (5×10^4 cells/lane) were subjected to 9% SDS-PAGE and blotted with anti-mammalian HSP60 antibody. Densitometric analysis revealed 1.8- and 2.9-fold increases of HSP60 expression in VSMCs at 6 and 24 hours after heat treatment, respectively.
Online Figure 1

60 kDa

Heat stress
-  +
-  +
6 hrs  24 hrs