Chlamydial Heat Shock Protein 60 Localizes in Human Atheroma and Regulates Macrophage Tumor Necrosis Factor-α and Matrix Metalloproteinase Expression

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Background—Recent evidence has implicated *Chlamydia pneumoniae* in the aggravation of atherosclerosis. However, the mechanisms by which this agent affects atherogenesis remain poorly understood. Chlamydiae produce large amounts of heat shock protein 60 (HSP 60) during chronic, persistent infections, and *C pneumoniae* localizes predominantly within plaque macrophages. Several studies have furnished evidence that endogenous (human) HSP 60 may play a role in atherogenesis. We tested here the hypothesis that atheroma contains chlamydial HSP 60 and that this bacterial product might stimulate macrophage functions considered relevant to atherosclerosis and its complications, such as production of proinflammatory cytokines as tissue necrosis factor-α (TNF-α) and matrix-degrading metalloproteinases (MMPs).

Methods and Results—Surgical specimens of human carotid atherosclerotic arteries (n=19) and normal arterial wall samples (n=7, 2 carotid arteries and 5 aortas) were tested immunohistochemically for the presence of chlamydial HSP 60 and human HSP 60. Macrophage localization of these antigens was assessed by double immunostaining. Murine peritoneal macrophages, maintained in serum-free conditions for 48 hours after harvesting, were incubated with *C pneumoniae*, chlamydial HSP 60, human HSP 60, or *Escherichia coli* lipopolysaccharide (LPS). Culture supernatants, collected at 24 hours for concentration-dependence experiments and at up to 72 hours for time-dependence experiments, were analyzed for TNF-α by ELISA and for MMP by gelatin zymography. Atherosclerotic lesions showed immunoreactive chlamydial HSP 60 in 47% (9 of 19) of the cases and human HSP 60 in 89% (17 of 19) of the cases. Chlamydial HSP 60 colocalized with human HSP 60 within plaque macrophages in 77% (7 of 9) of the cases. Nonatherosclerotic samples contained neither HSP. Both *C pneumoniae* and recombinant chlamydial HSP 60 induced TNF-α production by mouse macrophages in a concentration- and time-dependent fashion. *E coli* LPS and human HSP 60 produced similar effects. Similarly, *C pneumoniae* and HSPs induced MMPs in a concentration- and time-dependent manner. Heat treatment abolished the effect of *C pneumoniae* and HSPs on both TNF-α and MMP production, but it did not alter the ability of *E coli* LPS to induce these functions.

Conclusions—Chlamydial HSP 60 frequently colocalizes with human HSP 60 in plaque macrophages in human atherosclerotic lesions. Chlamydial and human HSP 60 induce TNF-α and MMP production by macrophages. Chlamydial HSP 60 might mediate the induction of these effects by *C pneumoniae*. Induction of such macrophage functions provides potential mechanisms by which chlamydial infections may promote atherogenesis and precipitate acute ischemic events. *(Circulation. 1998;98:300-307.)*

Key Words: proteins ▪ ischemia ▪ atherosclerosis

Substantial seroepidemiologic and some experimental evidence links *Chlamydia pneumoniae* with the pathogenesis and natural history of atherosclerosis. Airborne infection with this agent, often chronic and asymptomatic, is prevalent in the general population: An antibody titer against *C pneumoniae*, detectable in 40% to 50% of the adult population, correlates positively with the occurrence of coronary artery disease. *C pneumoniae* infection does not appear limited to the coronary arteries but can involve other segments of the vascular tree as well. Atheromatous lesions of the carotid arteries and of the lower extremities also contain *C pneumoniae*. Preliminary reports show that short courses of macrolide antibiotic therapy can reduce recurrent coronary events in patients with recent myocardial infarction or unstable angina and elevated anti-*C pneumoniae* antibody titers. Despite the various lines of evidence linking atheroma and *C pneumoniae*, the mechanisms by which this agent may affect vascular wall cells and atherogenesis remain poorly understood.

Atherosclerosis is largely viewed as a chronic inflammatory disease. In this respect, the life cycle of Chlamydiae, obligate intracellular pathogens, appears particularly interest-
Infection induces TNF-α, a product of activated T cells within atheroma, and interferon-γ, a product of activated T cells within atheroma, which indicates a chronic, persistent chlamydial infection, localizes within human atheroma; (2) chlamydial HSP 60 can activate macrophage TNF-α and MMP production, two functions relevant to atherogenesis and to lesional complications.

Methods

Reagents

Specific monoclonal antibodies against chlamydial HSP 60 and human HSP 60 were purchased from Affinity Bioreagents, Inc. Escherichia coli lipopolysaccharide (LPS) was purchased from Sigma. Formalin-inactivated C pneumoniae organisms were obtained from Washington Research Foundation. The inactivated C pneumoniae were used to provide a noninfectious source of chlamydial products. This preparation is antigenically intact and can elicit a specific immune response in mice (Kol, Libby, Lichtman, unpublished observations, 1997). Recombinant Chlamydia trachomatis HSP 60 was a generous gift of Dr Ying Yuan (Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Mont). Recombinant human HSP 60 was purchased from StressGen Biotechnologies Corporation, Victoria, British Columbia, Canada.

Immunohistochemistry

Surgical specimens of human carotid atherosclerotic arteries (n = 19) and normal arterial wall samples (n = 7, 2 carotid arteries and 5 aortas) were obtained by protocols approved by the Human Investigation Review Committee at the Brigham and Women’s Hospital. The studied samples were all from different subjects. Serial cryostat sections (5 μm) were cut, air dried onto microscope slides (Fisher Scientific), and fixed in acetone at −20°C for 5 minutes. Sections were preincubated with 0.3% hydrogen peroxidase for 20 minutes. One serial cross section from each lesion was used for each antibody: one for staining with anti-CD68 (Dako), one for staining with anti-chlamydial HSP 60 antibody, one for staining with anti-human HSP 60 antibody, and one for staining with control antibody (mouse IgG2, PharMingen; mouse IgG, myeloma protein MOMP-21, Sigma). Staining was performed with LSAB Kit Peroxidase (Dako), according to manufacturer’s instructions with light modifications; antibody binding was then visualized with 3-amino-9-ethylcarbazole (Dako). For single immunostaining, nuclear counterstaining was performed with hematoxylin (Sigma). For double immunostaining, sections stained with anti-chlamydial or anti-human HSP 60 were preincubated with avidin and biotin (Vector blocking kit) to block nonspecific binding of avidin/biotin complex. To identify macrophages within lesions, sections were then incubated overnight with primary antibody against CD68, followed by biotinylated secondary antibody (45 minutes; Vector laboratories) and avidin/biotin complex linked to alkaline-phosphatase (Vectastain ABC kit, Vector laboratories); antibody binding was visualized with fast blue (Sigma).

The number of positive samples are expressed in the “Results” section as percentages of the total number of samples examined per group, followed by the 95% confidence interval limits (CI). Fisher’s exact test was used for statistical comparison between unpaired data. A value of P ≤ 0.05 was considered significant.
Western Blotting

Because HSPs are well conserved among various species and share considerable homology, Western blotting was performed to ascertain specific recognition of chlamydial HSP 60 and human HSP 60 by their respective antibodies and to assess potential cross-reactivity. Recombinant human and chlamydia HSP 60 (1 μg/lane) were subjected to SDS/PAGE under reducing conditions and blotted to a polyvinylidene difluoride membrane (Millipore) in semidry conditions (Bio-Rad blotting apparatus). PBS containing 5% defatted dry milk and 0.1% Tween 20 was used to block membranes and to dilute primary antibodies and horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (Santa Cruz Biotechnology). Antibody binding was visualized by enhanced chemiluminescence (NEN-Dupont). The antibody against chlamydial HSP 60 recognized the chlamydial but not the human protein, whereas the anti-human HSP 60 antibody recognized the human but not the chlamydial protein (Figure 1).

Macrophage Isolation and Culture

C57BL/6 female mice (Sprague-Dawley) were maintained on normal chow diet in pathogen-free facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with regulations and standards of the United States Department of Agriculture, Department of Health and Human Services and National Institutes of Heath. Mice 8 to 12 weeks old were injected intraperitoneally with 1.5 mL of 4% thioglycollate broth (DIFCO). After 4 days, macrophages were collected by peritoneal lavage, washed with Hanks’ Balanced Salt Solution (Sigma), and resuspended in RPMI-1640 medium (BioWhittaker) supplemented with 10% fetal calf serum (HyClone) for plating at a density of 4×10⁵ cells/cm². After 2 hours, nonadherent cells were removed by washing with RPMI-1640, and the resultant monolayer was incubated in serum-free conditions for 48 hours before being used for experiments. The macrophage monolayer was >99% pure, as detected by specific immunostaining.

Experimental Conditions and Preparation of Conditioned Medium

After 48 hours in serum-free conditions, cells were incubated with C. pneumoniae, E. coli LPS, recombinant chlamydia HSP 60, human HSP 60, or with medium alone as a negative control. In the same experiments, before incubation, an aliquot of each reagent was heat-treated by boiling for 20 minutes. Concentrations used and incubation periods are indicated in individual experiments. Concentrations of C. pneumoniae are expressed as U/mL, which correspond to the number of microorganisms per milliliter of culture medium. Conditioned medium was collected at different time points and frozen for further analysis. For analysis of gelatinolytic activity, samples were first centrifuged (500g, 10 minutes, 4°C) and concentrated ×5 (Ultrafree centrifugal filter-4; Millipore).

Figure 2. Chlamydial heat shock protein (HSP) 60 colocalizes with human HSP 60 within atherosclerotic plaque macrophages. A, Human atherosclerotic plaque (magnification ×100) stained for macrophages (CD68, red). Rectangle indicates the macrophage-rich region (intimal plaque shoulder) sampled in high power views (×400, B, C, and D) of serial sections adjacent to the one depicted in A. B, Section stained with mouse IgGs as negative control yielded no staining. C, Double staining for chlamydial HSP 60 (red) and macrophages (CD68, blue). D, Double staining for human HSP 60 (red) and macrophages (CD68, blue). Arrowheads in C and D indicate macrophages (CD68+) that stain positively for either chlamydial or human HSP 60. Analysis of adjacent sections showed that both human and chlamydial HSP 60 colocalized within macrophage clusters. Lumen of the artery is at the top of each photomicrograph. Analysis of 7 of 9 samples (77%) showed similar results.
TNF-α Assay

TNF-α levels in culture supernatants were measured with a sandwich ELISA kit (R&D Systems). Samples were assayed in triplicate. Absorbance was measured in a Dynatech plate reader at 450 nm. Data are expressed as mean ± SD. Differences between experimental conditions were assessed by ANOVA with Bonferroni correction. A value of \( P \leq 0.05 \) was considered significant.

Gelatin Zymography

Gelatinolytic activity was assessed by SDS/PAGE of concentrated conditioned medium under nonreducing conditions in gels containing 8% polyacrylamide (Bio-Rad) and 2 mg/mL gelatin (Bio-Rad). To renature proteins after electrophoresis, SDS was removed from gels by washing at room temperature in 2.5% Triton X-100 (VWR Scientific). Gels were then incubated overnight in a buffer containing 50 mmol/L Tris-HCl (pH 7.6), 15 mmol/L NaCl, 10 mmol/L CaCl₂, 0.02% NaN₃, and 0.1% Brij 35 (Sigma). To detect bands of gelatinolytic activity, gels were stained with 0.25% Coomassie brilliant blue R-250 (Sigma). For molecular weight standardization, prestained (Bio-Rad) or nonprestained (Gibco-BRL) molecular weight markers were used.

Results

Localization of Chlamydial and Human HSP 60 in Atherosclerotic Plaque

Immunohistochemical analysis of human carotid atherosclerotic lesions showed colocalization of chlamydial and human HSP 60 within plaque macrophages. Atherosclerotic lesions (n=19) showed immunoreactive human HSP 60 in 17 of the cases (89%; 95% CI 66.9% to 98.7%) and immunoreactive chlamydial HSP 60 in 9 of the cases (47%; 95% CI 24.5% to 71.1%). The two samples that were negative for human HSP 60 were negative also for chlamydial HSP 60. Incubation of tissue samples with control IgGs yielded no staining. Analysis of nonatherosclerotic tissue (n=7) showed no immunoreactivity with either antibody (0%; 95% CI 0% to 41%). Positivity ratios were significantly different between atherosclerotic and nonatherosclerotic tissues for both human (\( P \leq 0.001 \)) and chlamydial (\( P=0.022 \)) HSP 60. As both \textit{C. pneumoniae} and endogenous HSPs localize mainly within plaque macrophages, \textit{6,30} cellular association of both antigens was defined by double immunostaining with macrophage-specific antibody. Both chlamydial HSP 60 and human HSP 60 localized mainly within macrophage-rich areas. Little HSP 60 was found outside macrophage-rich areas. Analysis of serial double-stained sections showed that chlamydial HSP 60 colocalized with human HSP 60 in 7 out 9 specimens examined (77%; 95% CI 40.0% to 97.2%) (Figure 2).

Induction of TNF-α Expression in Macrophages by Chlamydial Products

We explored the functional consequences of HSP 60 localization within macrophages by monitoring the production of TNF-α, a
cytokine known to be produced by these cells in atheroma. Both *C. pneumoniae* (Figure 3) and purified recombinant chlamydial HSP 60 (Figure 4) induced a time- and concentration-dependent increase in TNF-α elaboration by macrophages; maximal induction by both these stimuli occurred after 6 hours and lasted up to 72 hours. *C. pneumoniae* induced TNF-α elaboration by macrophages to the same extent as maximally effective concentrations of *E. coli* LPS. Chlamydia HSP 60 had nearly the same stimulatory effect on TNF-α production as *E. coli* LPS (Figure 5A). Human HSP 60 also induced TNF-α to a similar extent (Figure 5A). Chlamydiae express their own genus-specific endotoxin, thus *C. pneumoniae* could induce TNF-α because of its own endotoxin or because of its HSP 60. To distinguish among these possibilities, we heat-treated the *C. pneumoniae*, the HSP 60s (both chlamydia and human), and the control *E. coli* endotoxin. Bacterial lipopolysaccharides exhibit thermostability, whereas most proteins are thermodabile. Heat treatment of *C. pneumoniae* or HSP 60 abrogated their ability to induce TNF-α but did not alter the effect of *E. coli* endotoxin (Figure 5B), thus indicating that the *C. pneumoniae* effect was mainly mediated by a heat-labile component rather than its lipopolysaccharide.

**Induction of MMP Expression in Macrophages by Chlamydial Products**

In addition to production of cytokines such as TNF-α, macrophages may contribute to plaque evolution and instability by elaborating matrix metalloproteinases capable of
degrading the plaque’s fibrous cap. We therefore tested whether *C pneumoniae* or chlamydial HSP 60 might regulate MMP expression by macrophages. *C pneumoniae* induced a time-dependent elaboration of gelatinases by macrophages (Figure 6A). A 105-kDa band, corresponding to MMP-9 appeared early (6 hours); a fainter 72-kDa band, corresponding to MMP-2 appeared later (48 hours), increasing up to 72 hours. Chlamydia HSP 60 also induced a time-dependent increase in MMP-9 (Figure 7A), reaching a peak at 12 to 24 hours and gradually decreasing after 48 and 72 hours; however, the HSP did not induce MMP-2 to same extent as *C pneumoniae*. The highest concentration of *C pneumoniae* or Chlamydia HSP 60 tested actually decreased MMP-9 activity (Figures 6 and 7B). This decrease in gelatinolysis might be due to generation of an inhibitor or to protein degradation by macrophages, although the mechanism is not clear.

As in the case of TNF-α induction, heat treatment reduced MMP-9 induction by *C pneumoniae* and chlamydial HSP 60, but not by *E coli* LPS, thus indicating that this property of the chlamydial components also does not depend on endotoxin. In addition, human HSP 60 also induced MMP-9 in a heat-sensitive manner (Figure 8).

**Discussion**

Increased chlamydial HSP 60 expression characterizes chronic, persistent chlamydial infections. The ongoing inflammatory response in atherosclerosis, including macrophages...
Chlamydial HSP 60 Induces Macrophage TNF-α and MMP Release

Figure 8. Induction of matrix-degrading metalloproteinase (MMP)-9 production in mouse macrophages by *Chlamydia pneumoniae*, chlamydial heat shock protein (HSP) 60, human HSP 60, and *Escherichia coli* lipopolysaccharide (LPS). Effect of heat-treatment. Mouse macrophages were maintained in serum-free conditions for 48 hours and then incubated with serum-free medium only (O, unstimulated control), nontreated (−), or heat-treated (+) *E. coli* LPS (1 μg/mL), *C. pneumoniae* (107 U/mL), chlamydial HSP 60 (1 μg/mL), and human HSP 60 (1 μg/mL). Conditioned medium was collected after 24 hours and analyzed for MMP by gelatin zymography. Heat treatment abolished the effect on MMP-9 (105-kDa band) production of *C. pneumoniae*, chlamydial HSP 60, and human HSP 60 but did not modify the effect of thermostable *E. coli* LPS. Three independent experiments yielded similar results.

phage activation, now widely recognized, can result from traditional risk factors such as the consequences of hyperlipidemia. However, the potential contribution of nontraditional risk factors, such as infectious agents, has recently garnered considerable interest. This study examined the localization of chlamydia HSP 60 within atheroma and tested the potential role of this specific molecular component of Chlamydiae in modulating macrophage functions linked to complications of atheroma, such as TNF-α and MMP expression.

This article reports 3 novel findings: (1) chlamydia HSP 60 colocalizes with human HSP 60 within atherosclerotic plaque macrophages; (2) HSP 60, either chlamydial or human, potently stimulates TNF-α and MMP-9 production by macrophages; and (3) when these effects are elicited by *C. pneumoniae*, they are mediated by a heat-labile component, possibly HSP 60, rather than a thermostable lipopolysaccharide.

Wick et al. have proposed that HSP 60/65 might promote atherosclerosis by stimulating autoimmunity. Our finding that chlamydia HSP 60 colocalizes with its homolog, human HSP 60, within plaque macrophages in the majority of cases (77%), suggests that bacterial HSP might play such a role. The homology between human and chlamydial HSP 60 suggests the possibility of antigenic mimicry. Our approach cannot distinguish whether chlamydial HSP 60 found in plaques was produced by those *C. pneumoniae* that were actively replicating within plaque macrophages or by those in a chronic, persistent infective state. However, chronic infection with *C. pneumoniae*, through the expression of HSP 60, might provoke an autoimmune reaction against human HSP 60. Indeed, patients with carotid atherosclerosis or coronary artery disease have high titers of antibodies against human HSP 60. *C. pneumoniae* HSP 60, like other heat shock proteins, has been previously considered to act intracellularly to maintain cellular protein stability during stressful conditions. We report here the surprising finding that HSP 60 itself can activate macrophage stimulation, an observation with potentially important pathologic implications in atheroma formation and evolution. Human HSP 60 shares with the chlamydial protein the ability to stimulate TNF-α and MMP-9 production by macrophages. This study used mouse rather than human macrophages to facilitate the development of an animal model. Preliminary experiments in our laboratory have shown similar results using human monocytes-derived macrophages (Kol, Lichtman, Libby, unpublished observations, 1998).

When endothelial cells or macrophages express HSP 60 on their surface and are exposed to antibodies against HSP 60, they are susceptible to complement-mediated or antibody-dependent cellular cytotoxicity. If this were the case in vivo, this mechanism of cell injury might contribute to the pathobiology of atherogenesis.

Although it might be tempting to consider *C. pneumoniae* infection as a possible primary cause of atherosclerotic lesion formation in some cases, the data currently available do not justify this conclusion. Infection of the vascular wall with *C. pneumoniae* is generally focal and does not affect all lesions examined, raising legitimate questions about the specificity and the biological significance of the detection of this agent within atheroma. However, a recent autopsy study showed greater frequency of chlamydial antigens in the cardiovascular tissue of patients who died of ischemic heart disease than in patients who died of noncardiac causes (64% versus 38%). Moreover, the effects of a focal infection might influence the pathobiology of the surrounding atherosclerotic environment.

This study sheds new light on the potential molecular triggers to macrophage activation during atherogenesis. Because HSP 60 is mainly expressed during chronic, persistent chlamydia infection, this study suggests that chronic stimulation of macrophages by bacterial products might promote inflammatory aspects of atherogenesis and hence the development of acute coronary syndromes. Certainly, local infections with agents such as *C. pneumoniae* will most likely potentiate the evolution of preexisting atheroma, to which macrophages have already been recruited by traditional risk factors such as hypercholesterolemia. However, one cannot exclude a priori that macrophage infiltration in response to a chronic arterial infection might in some cases instigate lesion formation. The use of antibiotics to treat chlamydial infection might remove this stimulus for lesion complication and thus diminish the likelihood of acute ischemic events. The positive results of
recent preliminary secondary prevention trials with macrolide antibiotics are intriguing in this regard.12,13 In conclusion, this study shows that chlamydial HSP 60 colocalizes with human HSP 60 within plaque macrophages and that HSP 60 from both species can induce macrophage production of TNF-α and matrix-degrading metalloproteinase-ases, two mediators of atherosclerosis complications. Chlamydial HSP 60 might produce such effects in macrophages harboring C. pneumoniae infection. These findings help to understand the molecular pathways by which C. pneumoniae might participate in atherogenesis and to explain the mechanisms of the epidemiologic and pharmacologic links between this infectious agent and the clinical manifestations of atherosclerosis.

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