Chlamydia pneumoniae and Chlamydial Heat Shock Protein 60 Stimulate Proliferation of Human Vascular Smooth Muscle Cells via Toll-Like Receptor 4 and p44/p42 Mitogen-Activated Protein Kinase Activation

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Abstract—An early component of atherogenesis is abnormal vascular smooth muscle cell (VSMC) proliferation. The presence of Chlamydia pneumoniae in many atherosclerotic lesions raises the possibility that this organism plays a causal role in atherogenesis. In this study, C pneumoniae elementary bodies (EBs) rapidly activated p44/p42 mitogen-activated protein kinases (MAPKs) and stimulated proliferation of VSMCs in vitro. Exposure of VSMCs derived from human saphenous vein to C pneumoniae EBs (3×10^7 inclusion forming units/mL) enhanced bromodeoxyuridine (BrdU) incorporation 12±3-fold. UV- and heat-inactivated C pneumoniae EBs also stimulated VSMC proliferation, indicating a role of direct stimulation by chlamydial antigens. However, the mitogenic activity of C pneumoniae was heat-labile, thus excluding a role of lipopolysaccharide. Chlamydial hsp60 (25 μg/mL) replicated the effect of C pneumoniae, stimulating BrdU incorporation 7±3-fold. Exposure to C pneumoniae or chlamydial hsp60 rapidly activated p44/p42 MAPK, within 5 to 10 minutes of exposure. In addition, PD98059 and U0126, which are two distinct inhibitors of upstream MAPK kinase 1/2 (MEK1/2), abolished the mitogenic effect of C pneumoniae and chlamydial hsp60. Toll-like receptors (TLRs) act as sensors for microbial antigens and can signal via the p44/p42 MAPK pathway. Human VSMCs were shown to express TLR4 mRNA and protein, and a TLR4 antagonist abolished chlamydial hsp60–induced VSMC proliferation and attenuated C pneumoniae–induced MAPK activation and VSMC proliferation. Together these results indicate that C pneumoniae and chlamydial hsp60 are potent inducers of human VSMC proliferation and that these effects are mediated, at least in part, by rapid TLR4-mediated activation of p44/p42 MAPK. (Circ Res. 2001;89:244-250.)

Key Words: Chlamydia pneumoniae n heat shock proteins n vascular smooth muscle n cell division n mitogen-activated protein kinases

Atherosclerosis is an inflammatory disease, with the earliest stages characterized by the invasion of the intima by mononuclear phagocytes and by intimal hyperplasia.1 Accumulating evidence indicates that chronic infection with the ubiquitous respiratory pathogen Chlamydia pneumoniae, a Gram-negative obligate intracellular bacterium, may be an additional risk factor for atherosclerosis. Macrophages are thought to become infected with C pneumoniae in the respiratory tract and then enter the circulation and cross the endothelium at sites of preexisting vascular inflammation. The first report linking C pneumoniae to atherosclerosis identified the organism by electron microscopy in coronary atherosclerotic plaques and localized it to intimal smooth muscle cells (SMCs).2 C pneumoniae has been found frequently in lesions of the aorta, iliac, carotid, and coronary arteries,3,5 but is rarely found in normal arterial tissue.6 In vitro evidence supports the notion that C pneumoniae can infect human arterial SMCs.7–9 However, it is not clear whether C pneumoniae organisms that have been identified within SMCs of human atheromas are actively replicating or viable. Irrespective of whether C pneumoniae replicates within SMCs in vivo, its presence in atherosclerotic lesions raises the issue of whether the organism plays a causal role or is an innocent bystander.3,10 Evidence supporting a causal role of chlamydia in atherogenesis comes from recent reports that C pneumoniae and chlamydial antigens can activate mononuclear phagocytes and vascular cells, including SMCs. A heat-stable component of C pneumoniae induces macrophage foam cell formation,11 and this effect is replicated by chlamydial lipopolysaccharide (LPS). On the other hand, a heat-labile component of C pneumoniae stimulates oxidation of LDL12 and synthesis of...
proinflammatory cytokines, including interleukin (IL)–1, IL-6, and tumor necrosis factor (TNF)–α, by human blood mononuclear cells. Heat shock protein 60 (hsp60) is a highly expressed chlamydial protein that can activate macrophages and vascular cells by mechanisms that are not well characterized. Chlamydial hsp60 mimics the ability of C pneumoniae to stimulate LDL oxidation by human blood mononuclear cells. Chlamydial hsp60 also induces synthesis of TNF-α and matrix-degrading metalloproteinases by mouse macrophages and expression of adhesion molecules by human endothelial cells. We undertook the present study to determine whether C pneumoniae or chlamydial hsp60 stimulates proliferation of human vascular SMCs (VSMCs), a process responsible for intimal hyperplasia in early atherosclerotic lesions.

A crucial component of signaling via classical growth factors involves sequential activation of Ras, Raf, and p44/42 mitogen-activated protein kinase (MAPK). Exposure to C pneumoniae induces rapid activation of p44/42 MAPK in human umbilical vein endothelial cells, although the signaling pathway that links chlamydia to MAPK activation is not known. Recent studies have documented the role of transmembrane Toll-like receptors (TLRs) in cellular activation by microbial pathogens. Microbial antigens may interact with the extracellular domain of TLRs and subsequently activate multiple intracellular signaling pathways. Bacterial LPS–induced activation of nuclear factor (NF)–κB and p44/42 MAPK pathways has been extensively studied, and is now known to involve TLR4. An inhibitor of TLR4-mediated LPS signaling also abolished induction of inflammatory cytokine synthesis after exposure to Chlamydia trachomatis, a related species of chlamydia. The present study tested whether C pneumoniae stimulates human VSMC proliferation via activation of TLR4 and/or p44/42 MAPK.

Materials and Methods

Human VSMC Culture

VSMCs were obtained by explant technique from saphenous veins harvested for coronary artery bypass surgery at New England Medical Center and from segments of pulmonary artery harvested from organ donors (National Disease Research Interchange, Philadelphia, PA). VSMCs were cultured in DMEM supplemented with 10% FCS, glutamine, penicillin, streptomycin, and fungizone, and used at passages 2 to 6.

Propagation and Purification of C pneumoniae

The AR39 strain of C pneumoniae was inoculated into HEp-2 cells at a multiplicity of infection of 10. After 48 to 72 hours, HEP-2 cells were harvested on ice and sonicated. Elementary bodies (EBs) were harvested on ice and sonicated. Elementary bodies (EBs) were inactivated by exposure to UV light at 280°C. In some cases, EBs were inactivated by exposure to UV light at 280°C. In some cases, EBs were inactivated by exposure to UV light or by using 0.1% Triton X-100. In others, the TLR4 antagonist Rhodobacter sphaeroides diphasphoryl lipid A (RSLA; 1 μg/mL) was added just before addition of C pneumoniae or hsp60. After 48 hours, BrdU was added and cells were incubated an additional 24 hours. To immunostain for BrdU, cells were washed in PBS, fixed in 3.7% formaldehyde (15 minutes), and permeabilized in ice-cold methanol (3 minutes). Nonspecific binding sites were blocked with 10% normal horse serum, and the cells were incubated for 90 minutes at 37°C with BrdU monoclonal antibody (mAb; Amersham) and DNease I (10 U/mL), and then 45 minutes at room temperature with Texas Red–coupled donkey anti-mouse IgG. Cell nuclei were stained with Hoechst 33342 and evaluated by epifluorescence microscopy. For each treatment group, 200 cells were analyzed by an observer blinded to the specimen treatment.

Cell Proliferation

VSMCs were plated in 12-well plates (5000 cells/cm²), incubated overnight, and the numbers of attached cells determined by hemocytometer (day 0 cell counts). VSMCs were then exposed to C pneumoniae or DMEM/1% FCS alone, and final cell counts were determined after 96 hours.

p44/p42 MAPK Phosphorylation

Cells were rapidly frozen in liquid nitrogen and then harvested in buffer containing (in mmol/L) NaCl 150, HEPES (pH 7.4) 50, NaVO4 1, and NaF 1; 1% Triton X-100; 10% glycerol; and protease inhibitor cocktail (Boehringer Mannheim). Cell lysates were gently rotated (15 minutes, 4°C), centrifuged (16 000g, 20 minutes, 4°C), and the supernatants stored at −80°C. Cell proteins (40 μg) were separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were incubated sequentially with 5% nonfat dry milk, rabbit antibodies specific for threonine- and tyrosine-phosphorylated p44/p42 MAPK (New England Biolabs), biotinylated goat anti-rabbit IgG, and proteinase inhibitor cocktail (Boehringer Mannheim). Cell lysates were gently rotated (15 minutes, 4°C), centrifuged (16 000g, 20 minutes, 4°C), and the supernatants stored at −80°C. Cell proteins (40 μg) were separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were incubated sequentially with 5% nonfat dry milk, rabbit antibodies specific for threonine- and tyrosine-phosphorylated p44/p42 MAPK (New England Biolabs), biotinylated goat anti-rabbit IgG, and streptavidin-biotinylated alkaline phosphatase complex. Blots were developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. To verify equivalency of cell extracts, replicate blots were incubated with rabbit antibodies specific for total p44/p42 MAPK. NIH Image 1.61 was used to quantify bands.

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was isolated from VSMCs using an RNeasy kit (Qiagen). cDNA (2 μg) was reverse-transcribed for 1 hour at 37°C with 200 units of Moloney murine leukemia virus reverse transcriptase and oligo(dT) primer, in a volume of 20 μL. The reaction was terminated at 95°C, and 2 μL of first-strand cDNA added to each PCR reaction. The primer sets used to amplify TLR4 were 5′-TGGCGGTTCATACATAAA-3′ and 5′-CCATCCGAATTATAAGAAAGTC-3′, yielding a 413-bp fragment, and 5′-TTGGTGCACGCATCTACCA-3′ and 5′-TTCCTGCAATGATCGATCTGTA-3′, yielding a 299-bp fragment. The primer sets used to amplify TLR2 were 5′-ACTGGGATTGATGATGAGT-3′ and 5′-GAATATGCAGCC-3′, yielding a 949-bp fragment and 5′-AGGCGCATCCTGCCAGACACT-3′ and 5′-AGCCAGGCCACATATTTC-3′, yielding a 519-bp fragment. Primers were annealed at 55°C, and samples were amplified for 25 cycles.
C pneumoniae after exposure of saphenous vein SMC cultures to viable BrdU incorporation was markedly increased 48 to 72 hours.

VSMCs were gently scraped from culture plates with a soft rubber scraper in PBS containing 1% BSA and 1 mmol/L EDTA. Cells were harvested by exposure to UV light or heat (56°C) only partially attenuated the mitogenic effect. B and D, Heating to 100°C abolished the mitogenic activity of C pneumoniae (3 × 10⁷ IFU/mL; n = 5 experiments) and chlamydial hsp60 (25 μg/mL; n = 3 experiments). *P < 0.02, significantly different from VSMCs exposed to medium alone; †P < 0.02, significantly different from VSMCs exposed to C pneumoniae or chlamydial hsp60 without prior heat inactivation.

Immunostaining and Flow Cytometric Analysis

VSMCs were gently scraped from culture plates with a soft rubber scraper in PBS containing 1% BSA and 1 mmol/L EDTA. Cells were incubated 1 hour with HAT125 (aTLR4; a gift from K. Miyake, Saga Medical School, Saga, Japan), TL2.1 (aTLR2/26), or control mouse IgG (each 10 μg/mL), followed by FITC-labeled donkey anti-mouse IgG. Cells were then analyzed for intensity of FITC fluorescence in a FACSCalibur flow cytometer (Becton Dickinson). Immunoreactive TLR4 was localized in individual SMCs by epifluorescence microscopy after indirect immunofluorescent staining. VSMCs were fixed with formaldehyde and then incubated with goat polyclonal antibody specific for human TLR4 (Santa Cruz Biotechnology), followed by Texas Red–coupled donkey anti-goat IgG.

Results

A Heat-Labile Component of C pneumoniae Induces Proliferation of VSMCs

BrdU incorporation was markedly increased 48 to 72 hours after exposure of saphenous vein SMC cultures to viable C pneumoniae EBs. Exposure to C pneumoniae (10⁷ inclusion forming units [IFU/mL]) stimulated DNA synthesis 2.2-fold relative to control VSMCs (Figure 1A; n = 5 experiments). Exposure to C pneumoniae (3 × 10⁷ and 5 × 10⁷ IFU/mL) produced 5.6-fold and 5.1-fold increases in BrdU incorporation, respectively. C pneumoniae (3 × 10⁷ IFU/mL) produced maximal stimulation of VSMC proliferation and was chosen for further study. In 10 experiments with saphenous vein SMCs from different patients, C pneumoniae enhanced BrdU incorporation 12 ± 3-fold (range 2.4- to 31-fold), with the largest increases elicited in cells with lower baseline proliferation rates. Exposure to C pneumoniae (3 × 10⁷ IFU/mL) stimulated BrdU incorporation 3.4 ± 0.1-fold in three experiments with SMCs derived from human pulmonary artery (BrdU-positive cells increased from 9.2 ± 0.9% to 30.8 ± 3.6%; P < 0.001). Direct cell counting confirmed that C pneumoniae–induced DNA synthesis was followed by cell replication. The increment in cell number (from 4941 cells/cm² on day 0) was ≈3-fold greater in saphenous vein SMCs incubated 96 hours with C pneumoniae (10 111 ± 1526 cells/cm²), compared with VSMCs incubated in medium alone (DMEM/1% FCS; 3569 ± 158 cells/cm²).

Mitogenic Effect of C pneumoniae Does Not Require Active Infection

The proliferative response elicited by viable C pneumoniae EBs could be triggered by a specific chlamydial antigen or may be secondary to an active infection within VSMCs. To determine whether infection of SMCs contributed to this mitogenic effect, the effect of viable EBs was compared with that of EBs that had been inactivated by exposure to UV light or heat (56°C). Exposure to C pneumoniae (3 × 10⁷ IFU/mL) stimulated BrdU incorporation 5.6-fold, whereas exposure to UV-inactivated or 56°C-inactivated C pneumoniae stimulated BrdU incorporation 3.3-fold and 2.7-fold, respectively (Figure 1B; n = 5 experiments). These results indicate that the effect of C pneumoniae is mediated, at least in part, via recognition of a chlamydial antigen, although infection may enhance this effect.

Molecular components of C pneumoniae that can activate cells include LPS, major outer membrane protein, and hsp60. The activity of LPS is not affected by heating to 100°C; whereas the activity of most proteins is destroyed. The mitogenic effect of C pneumoniae was abolished by heating to 100°C in five independent experiments (Figure 1B), consistent with a role of a chlamydial protein, and ruling out a primary role of LPS.

Chlamydial hsp60 Is a Heat-Labile Inducer of VSMC Proliferation

BrdU incorporation was markedly increased in human VSMCs exposed to chlamydial hsp60 for 72 hours. The percentage of cells that incorporated BrdU increased 5.3-fold, 6.7-fold, 20-fold, and 22.4-fold in VSMCs exposed to 5, 10, 25, and 50 μg/mL chlamydial hsp60, relative to control VSMCs (Figure 1C). Chlamydial hsp60 (25 μg/mL) produced near-maximal stimulation of VSMC proliferation and was chosen for further study. Notably, exposure to chlamydial hsp60 (25 μg/mL) produced a mitogenic effect similar to that of C pneumoniae (3 × 10⁷ IFU/mL). In five experiments with VSMCs from different patients, chlamydial hsp60 enhanced BrdU incorporation 7 ± 3-fold (range 2.2- to 20-fold), with the largest increases elicited in cells with low baseline proliferation rates. The effect of chlamydial hsp60 (25 μg/mL) was also abolished by heating to 100°C (Figure 1D).

C pneumoniae and Chlamydial hsp60 Activate p44/p42 MAPK in Human VSMCs

To determine whether exposure to C pneumoniae activates p44/p42 MAPK in VSMCs, cells were left untreated or were
stimulated with *C. pneumoniae* \((3 \times 10^7 \text{ IFU/mL})\) or chlamydial hsp60 \((25 \mu g/mL)\) for 5 to 30 minutes. Cell lysates were analyzed by Western blot using a polyclonal antibody specific for the active, dually phosphorylated forms of p44/p42 MAPK. Levels of phosphorylated p44 and p42 MAPK were increased in VSMCs after only a 5-minute exposure to *C. pneumoniae*, were maximally increased at 10 minutes (11-fold and 3-fold, respectively), and remained elevated after 30 minutes (Figure 2, left). The levels of phosphorylated p44 and p42 MAPK were likewise increased (3-fold and 2-fold, respectively) in VSMCs incubated 10 minutes with chlamydial hsp60 \((25 \mu g/mL)\) (Figure 2, right). In contrast, total p44/p42 MAPK levels were similar in all groups. Phosphorylated p44 and p42 MAPKs were similarly increased in VSMCs incubated for 10 minutes with 10% FCS \((6\text{-fold and } 2\text{-fold, respectively; data not shown). These results indicate that MAPK is rapidly activated after exposure to *C. pneumoniae* or chlamydial hsp60. 

**MEK Inhibition Abolishes *C. pneumoniae*–Induced VSMC Proliferation**

Two specific inhibitors of MEK, PD98059 and U0126, were used to assess the role of MEK-induced p44/p42 MAPK activation in the mitogenic effect of *C. pneumoniae*. U0126 \((10 \mu \text{mol/L})\) largely attenuates p44/p42 MAPK activation and is equally effective at inhibiting MEK1 and MEK2. Concentrations of PD98059 5-fold to 10-fold higher are required to produce similar inhibition of MEK activity, and MEK1 is preferentially inhibited. VSMCs were pretreated for 30 minutes with vehicle, PD98059 \((50 \mu \text{mol/L})\), or U0126 \((10 \mu \text{mol/L})\) before exposure to *C. pneumoniae* or medium alone. BrdU incorporation increased 8.6-fold in VSMCs exposed to *C. pneumoniae* \((3 \times 10^7 /\text{ mL})\) in the absence of a MEK inhibitor (Figure 3). The mitogenic effect of *C. pneumoniae* was inhibited by 90% in VSMCs pretreated with PD98059 and was abolished in VSMCs pretreated with U0126. U0126 likewise inhibited the mitogenic effect of chlamydial hsp60 \((25 \mu g/mL)\), by 73% and 100%, in two independent experiments (data not shown). 

**Human VSMCs Express TLR4 mRNA and Protein**

RT-PCR analysis indicated that saphenous vein SMCs express TLR4 but not TLR2 mRNA. cDNA was prepared from VSMCs derived from three different patients and amplified by primers specific for TLR2 or TLR4. Each VSMC cDNA sample yielded TLR4 PCR product of similar size to that obtained from a human bone marrow cDNA library, when amplified with either TLR4 primer pair (Figure 4A). In

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**Figure 2.** *C. pneumoniae* and chlamydial hsp60 activate p44/p42 MAPK in saphenous vein SMCs. VSMCs were serum-deprived for 24 hours (DMEM/1% FCS) and left untreated or stimulated with *C. pneumoniae* \((3 \times 10^7 \text{ IFU/mL}; \text{ left panel})\) or chlamydial hsp60 \((25 \mu g/mL; \text{ right panel})\). Cell lysates (40 \mu g protein) were analyzed by Western blot analysis using an antibody specific for active phospho-p44/p42 MAPK (top) or total MAPK (bottom).

**Figure 3.** *C. pneumoniae*–induced proliferation is abolished by MEK inhibitors. VSMCs derived from human saphenous vein were preincubated 30 minutes with or without PD98059 \((50 \mu \text{mol/L})\) or U0126 \((10 \mu \text{mol/L})\) and then exposed to *C. pneumoniae* \((3 \times 10^7 \text{ IFU/mL})\) for 72 hours, with BrdU incorporation determined during the last 24 hours (\(n = 4\) experiments). \(P^* < 0.03\), significantly different from VSMCs exposed to medium alone; \(P^* < 0.02\), significantly different from VSMCs exposed to *C. pneumoniae* without MEK inhibitors.

**Figure 4.** VSMCs express TLR4 mRNA and protein but do not express TLR2. A, RT-PCR analysis of saphenous vein SMC cDNA; 25 cycles of amplification with two distinct TLR4-specific primer sets produced the expected 413-bp (left) and 299-bp (right) products. TLR4 product was absent when cDNA was excluded from the reaction (\(2\)). cDNA prepared from human bone marrow (HBM) yielded product of similar size. B, VSMCs were incubated with mAb HTA125 (\(\text{a} \text{TLR4)}\), TL2.1 (\(\text{a} \text{TLR2)}\), or control mouse IgG \((10 \mu g/mL)\), followed by anti-mouse IgG-FITC, and analyzed by flow cytometry. Relative fluorescent intensity and relative cell number are shown on the \(x\)- and \(y\)-axes, respectively. The mean relative fluorescent intensities of each group are shown in parentheses. C, VSMCs were stained with goat anti-human TLR4 Ab, followed by anti-goat IgG Texas Red, and Hoechst 33342. Membrane-associated TLR4 was visualized by epifluorescence microscopy.
contrast, two sets of primers specific for TLR2 produced no PCR product with cDNAs from VSMCs, but yielded the expected TLR2 PCR product when human bone marrow cDNA was amplified (data not shown).

Human VSMCs also expressed immunoreactive TLR4, but not TLR2, as determined by immunostaining followed by flow cytometric analysis or fluorescence microscopy. The mean relative fluorescent intensities of VSMCs were 81, 97, and 253, when incubated in the presence of control IgG, TLR2 mAb, or TLR4 mAb, respectively (Figure 4B). Flow cytometric analysis of VSMCs derived from a different patient yielded similar results (not shown). Microscopic analysis of VSMCs that were immunostained with polyclonal TLR4 antibodies revealed specific staining that appeared localized to the cell membrane (Figure 4C). In contrast, TLR2 antigen was not detected in VSMCs by immunostaining with TLR2 mAb (data not shown).

**RSLA Inhibits C pneumoniae–and Chlamydial hsp60–Induced VSMC Proliferation**

Diphosphoryl lipid A prepared from *R. sphaeroides* (RSLA) is a competitive antagonist of LPS signaling and may act as a competitive inhibitor of TLR4. Therefore, RSLA was used to investigate the role of TLR4 in recognition of *C pneumoniae* and hsp60 by VSMCs. Addition of RSLA (1 μg/mL) to VSMC cultures immediately before addition of chlamydial hsp60 abolished the subsequent proliferative response to the chlamydial protein (Figure 5; *P*<0.01; n=5 experiments). The mitogenic effect of FCS was not altered by addition of RSLA, indicating that the lipid A analogue does not have nonspecific effects on VSMC proliferation. RSLA attenuated proliferation induced by *C pneumoniae* by 46±16% (Figure 5; *P*<0.05, n=7 experiments). These results support the hypothesis that *C pneumoniae* and chlamydial hsp60 stimulate human VSMC proliferation via activation of TLR4.

**Discussion**

Identification of subendothelial macrophages in early atherosclerotic lesions led to the hypothesis that atherosclerosis is an inflammatory disease. More recently, correlative studies have supported a possible association between atherosclerosis and chronic infection with the Gram-negative obligate intracellular bacterium *C pneumoniae*. *C pneumoniae* is a common cause of respiratory tract infections, with most adults experiencing several infections over the course of a lifetime. *C pneumoniae* bacteria occur frequently in atherosclerotic lesions, but only rarely in normal arterial tissue. Evidence that *C pneumoniae* can induce proatherogenic effects on mononuclear phagocytes and vascular cells supports a role for the organism in the pathogenesis of vascular disease. The present studies have four principal findings. First, *C pneumoniae* and chlamydial hsp60 are potent stimuli to human VSMC proliferation. Second, the mitogenic effect of *C pneumoniae* EBs is independent of chlamydial LPS and is likely mediated by a heat-labile chlamydial protein. Third, p44/p42 MAPK activation is crucial to *C pneumoniae* and chlamydial hsp60–induced VSMC proliferation. Finally, the mitogenic effects of both *C pneumoniae* and hsp60 involve TLR4.

Several observations support the hypothesis that the mitogenic effect of *C pneumoniae* is mediated, at least in part, by rapid recognition of a heat-labile component of the organism. First, p44/p42 MAPK is activated 5 minutes after exposure to the organism, and its activation is crucial to the subsequent increase in proliferation. Second, the mitogenic effect is mimicked by chlamydial hsp60. Third, the mitogenic effect can occur in the absence of active infection. The developmental cycle of *C pneumoniae* is biphasic. EBs released into the extracellular space are metabolically inactive, yet infectious, and can be taken up by cells. Once inside the cell, chlamydia reside within inclusions in the cytoplasm, utilizing the host cell’s metabolic machinery to develop into mature reticulate bodies, divide, and create new infectious EBs, which are released on host cell lysis. In the present study, *C pneumoniae* EBs that were rendered noninfectious by exposure to UV light or heat (56°C) also stimulated proliferation of VSMCs, although the response was partially attenuated. These results suggest that the mitogenic effect can occur in the absence of secondary events linked to active infection. However, they do not rule out the possibility that active infection may contribute to SMC activation in other settings, either in SMCs cultured in vitro or in atherosclerotic lesions in vivo.

The mitogenic activity of *C pneumoniae* was heat-labile, ruling out a primary role of chlamydial LPS. Evidence for a possible role of chlamydial hsp60 was provided by the fact that chlamydial hsp60 replicated the mitogenic effect of *C pneumoniae*. Recent studies suggest that chlamydial hsp60 can activate cells in a manner similar to IL-1 or LPS. Chlamydial hsp60 induces synthesis of TNF-α and matrix-degrading metalloproteinases by mouse macrophages, expression of adhesion molecules by human endothelial cells, and oxidation of LDL by human blood mononuclear cells. Chlamydial hsp60 is found in macrophage-rich areas of human atherosclerotic plaque. Whether the presence of chlamydial hsp60 is due to an active or persistent *C pneumoniae* infection or to the persistence of the hsp60 antigen is not known. It has been proposed that *C pneumoniae* can establish persistent infections that could serve as a source of...
antigen to chronic inflammatory responses. In this regard, it is interesting that the related organism, *C trachomatis*, continues to synthesize hsp60 during a persistent infection, whereas synthesis of major outer membrane protein and LPS is greatly reduced.34 The fact that chlamydial hsp60 is present in atherosclerotic lesions and is also a potent human VSMC mitogen supports a possible role of chlamydial hsp60 in intimal hyperplasia.

Recent evidence supports the hypothesis that hsp60 activates cells via TLRs, the sensors of the innate immune system. TLRs are transmembrane proteins with an extracellular domain consisting of leucine-rich repeats involved in recognition of microbial components. To date, nine TLRs have been identified in humans, but only a few of their ligands have been established.17 In one study, human hsp60 stimulated TNF-α and nitric oxide production in mouse macrophages, whereas macrophages derived from C3H/HeJ mice that express a nonfunctional form of TLR4 were nonresponsive to hsp60, supporting a role of TLR4 in human hsp60–induced cell activation.35 In another study,36 expression of CD14 conferred responsiveness to human hsp60 in U937 cells, an astrocytoma cell line that expresses TLR4 but not TLR2,37 thereby implicating a CD14-dependent TLR in hsp60 signaling. Human VSMCs used in the present study expressed TLR4 mRNA and protein but did not express TLR2 mRNA or protein, likewise suggesting a role of TLR4. Studies with RSLA, a lipid A analogue that antagonizes LPS-induced signaling,38 further supported a role of TLR4 rather than TLR2 in the mitogenic effect of both *C pneumoniae* and chlamydial hsp60. RSLA appears to be a specific inhibitor of TLR4, given that it markedly attenuates cellular activation by LPS,31 and by lipoteichoic acid,39 an active component of Gram-positive bacteria that can activate TLR4.40 In contrast, RSLA does not inhibit macrophage activation by either heat-killed *Staphylococcus aureus* or extracts of *Mycobacterium tuberculosis*.31,41 Organisms that stimulate TLR2.40,41 However, it is possible that a distinct TLR is also expressed in VSMCs and is inhibited by RSLA. Further studies are required to establish whether RSLA attenuates signaling by other TLRs.

The cytosolic domains of TLRs have high homology with the intracellular domain of the type I IL-1 receptor. As predicted by this homology, TLRs and the IL-1 receptor recruit and activate common intermediate proteins, leading to activation of the p44/p42 MAPK pathway and NF-κB. After binding of their respective ligands, TLR4 and the IL-1 receptor associate with an adapter protein, MyD88,42 leading to recruitment of IL-1 receptor–associated kinases (IRAKs) to the receptor complex. IRAKs then interact with TRAF6, a member of the TNF receptor–associated factor family. TRAF6 activation, in turn, can lead to phosphorylation of p44/p42 MAPK by undefined mechanisms.43 The p44/p42 group of MAPKs is a central component of signaling via growth factors. Sequential activation of Ras and Raf activates MEK. MEK then activates MAPK by dual phosphorylation of key threonine and tyrosine residues, and MAPK in turn phosphorylates serine and threonine residues on several transcription factors, including c-Myc, activator protein-1, NF–IL-6, activating transcription factor-2, and Elk-1, leading ultimately to cell growth and differentiation.44 *C pneumoniae* induces rapid p44/p42 MAPK activation in human VSMCs, as previously reported in human umbilical vein endothelial cells.45 The mechanism of chlamydia-induced MAPK activation in human VSMCs and endothelial cells is not known, but given the rapidity of the effect, it is likely to be mediated by direct recognition of a chlamydial component, and in SMCs may involve chlamydial hsp60–induced activation of TLR4.

The present studies have shown that *C pneumoniae* is a potent stimulus for human VSMC proliferation. Furthermore, the mitogenic effect is mediated by rapid TLR4-mediated recognition of *C pneumoniae*, which may be due in part to recognition of chlamydial hsp60 and subsequent activation of p44/p42 MAPK. The mitogenic effect of *C pneumoniae* may contribute to intimal hyperplasia during the early stages of atherogenesis.

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