Identification and Characterization of Chlamydia pneumoniae-Specific Proteins That Activate Tumor Necrosis Factor Alpha Production in RAW 264.7 Murine Macrophages

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Chlamydia pneumoniae is a common respiratory pathogen, which activates macrophages to induce inflammatory cytokines that may promote atherosclerosis. However, the antigens that induce macrophage activation have not been well defined. In the current study, three chlamydial proteins which are recognized during human infection, outer membrane protein 2 (OMP2) and two 53-kDa proteins (Cpn 0980 and Cpn 0809), were investigated to determine whether they activate macrophages and, if they do, what mechanism they use for this activation. It was shown that these three proteins could (i) induce expression of tumor necrosis factor alpha (TNF-α) and tissue factor and (ii) induce phosphorylation of p44/42 mitogen-activated protein kinases (MAPK) and activation of early growth response factor 1 (Egr-1). Control proteins, the N-terminal fragment of polymorphic membrane protein 8 and the thioredoxin portion of the fusion protein, had no effect on macrophages. Treatment of cells with a MEK1/2 inhibitor, U0126, dramatically reduced the phosphorylation of ERK, activation of Egr-1, and expression of TNF-α in macrophages treated with recombinant proteins. Toll-like receptors (TLRs) act as sensors for microbial antigens and can signal via the MAPK pathway. Chlamydial protein-induced expression of TNF-α was significantly reduced in macrophages lacking TLR2 or TLR4. These findings suggest that C. pneumoniae may activate macrophages through OMP2, Cpn 0980, and Cpn 0809 in addition to chHSP60 and that activation occurs via TLR2 or TLR4, Egr-1, and MAPK pathways.

Atherosclerosis is thought to result from a protective, inflammatory fibroproliferative response against chronic injury over a long period of time (43). The earliest stages of atherosclerosis are characterized by invasion of the intima by mononuclear phagocytes (44). Several lines of evidence indicate that Chlamydia pneumoniae, an obligate intracellular bacterium associated with respiratory tract infection, may play a role in progression of atherosclerosis (9, 47). An association between anti-C. pneumoniae antibodies and cardiovascular disease was found in several seroepidemiological studies (13, 46, 50). Recent clinical studies have suggested that infection with C. pneumoniae is associated with increased plaque instability and thrombogenesis during acute coronary events (14, 52). Although C. pneumoniae has been detected in smooth muscle cells and endothelial cells in atheroma (28a, 28b), it is localized mainly in macrophage-derived foam cells (28b). In vitro studies have shown that C. pneumoniae can induce macrophage foam cell formation (22, 23), and in vivo studies have shown that C. pneumoniae infection accelerates lesion progression in animal models (5, 8). It has been hypothesized that C. pneumoniae infection of macrophages is a key factor for dissemination to the vasculature, establishment of persistent infection in the atheroma, and exacerbation of lesion progression.

It has been reported that C. pneumoniae infection of monocytes/macrophages triggers the secretion of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) (19, 24), and production of tissue factor (TF) (4). TNF-α is found in atherosclerotic plaques and can induce a number of vascular cell functions relevant to atherogenesis (36). TNF-α is thought to play an important role in the inflammatory processes involved in atherogenesis (42). TF, a potent prothrombotic molecule, is thought to play an important role in stimulating thrombus formation after plaque rupture (4). The mechanisms by which C. pneumoniae activates monocytes to produce cytokines and TF have been investigated. C. pneumoniae has been shown to activate dendritic cells via NF-κB and Toll-like receptor 2 (TLR2)/TLR4-dependent pathways to express cytokines (38). However, Netea et al. reported that only the TLR2-dependent pathway stimulates cytokine production by sonicated C. pneumoniae (32). In vivo studies also showed that TLR2 and TLR4 are involved in C. pneumoniae-induced cytokine release (12, 40). C. pneumoniae induction of TF expression in mouse macrophages is mediated in part by early growth response factor 1 (Egr-1), signaling through TLR4, and activation of the MEK-ERK1/2 pathway. Egr-1 is a nuclear transcription factor and plays a role in the regulation of the expression of growth factors such as platelet-derived growth factor, cytokines such as TNF-α, and adhesion molecules such as ICAM. Thus, C. pneumoniae signaling through a TLR activates expression of chemokines and cytokines by macrophages that are key components of atherosclerosis.

However, the specific antigens of C. pneumoniae that mediate macrophage activation have not been well defined. Recent studies have shown only that chHSP60 can activate macrophages through the TLR4 and NF-κB pathway (7). To determine whether other relevant antigens also activate macrophages, three chlamydial proteins, outer membrane protein 2 (OMP2) and two 53-kDa proteins (Cpn 0980 and Cpn 0809),
that are recognized frequently during human infection (10, 35, 55) were investigated. The 53-kDa proteins appear to be putative outer membrane proteins (16). OMP2 is a cysteine-rich protein and a major component of the chlamydial cell wall. Although OMP2 is poorly surface accessible to antibody binding in intact cells (58), pronounced antibody responses to OMP2 occur following C. pneumoniae infection (34, 57). Therefore, in the current study, whether these antigens stimulate macrophage functions relevant to atherosclerosis and, if they do, what mechanism they use for stimulation were investigated.

### MATERIALS AND METHODS

**Cell culture.** RAW 264.7 cells, a murine macrophage cell line (ATCC, Manassas, VA), were grown in Dulbecco modified Eagle medium (Gibco BRL, Langley, OK) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

**Isolation of murine peritoneal macrophages.** To harvest peritoneal macrophages, mice were euthanized by cervical dislocation, and the peritoneal cavities were washed three times with 10 ml of macrophage culture medium. Peritoneal macrophages, mice were euthanized by cervical dislocation, and the peritoneal cavities

**Production and purification of the recombinant proteins.** Transformed *Escherichia coli* TOP10 cells (200-ml cultures) were induced with 1 mM arabinose, grown to an optical density at 600 nm of 1.5, and centrifuged at 3,000 g for 10 min. The cells were resuspended in 100 mM NaH2PO4, 10 mM Tris-Cl, 8 M urea (pH 8), lysed on ice for 30 min, sonicated, and centrifuged at 3,000 x g for 15 min. The supernatant was washed three times with 10 M of macrophage culture medium. Peritoneal macrophages were plated into six-well culture plates containing coverslips at a concentration of 1 x 10^5 macrophages/well, which yielded 90% to 100% confluence. After overnight incubation, nonadherent cells were removed by washing with Hanks balanced salt solution. Adherent cells were cultured for 3 days with fresh macrophage culture medium.

**Cloning and expression of the chlamydial protein genes and gene fragments.** *C. pneumoniae* DNA was purified by using a standard protocol (49). The complete OMP2 and Cpn 0980 genes were amplified, while N-terminal fragments of the Cpn 0809 and polymorphic membrane protein 8 (PMP 8N) genes were amplified. The forward and reverse primers used are shown in Table 1. Cpn 0980 and Cpn 0809 recombinant protein genes were cloned and expressed as described previously (10). The remaining amplified products (OMP2 and PMP 8N genes) were ligated directly into the pBdATop/Thio vector (Invitrogen, Carlsbad, CA) by TA cloning and expressed by using the Topo/Thio fusion expression system according to the directions of the manufacturer.

**RESULTS**

Chlamydial recombinant proteins induce expression of TNF-α and TF. RAW 264.7 cells are continuously growing cells that are activated by LPS to induce secretion of TNF-α (18, 26). It has been reported that *C. pneumoniae* can stimulate macrophages to secrete TNF-α (19, 24) and to express TF (4). To investigate whether these proteins elicit RAW 264.7 cells to secrete TNF-α, the individual recombinant proteins were incubated with RAW 264.7 cells for 24 h, and the supernatants were assayed for TNF-α. Stimulation of RAW 264.7 cells with LPS, the positive control, induced the secretion of TNF-α (Fig. 1A). All three recombinant proteins induced the expression of TNF-α in a dose-dependent manner, like LPS (Fig. 1A). In contrast, the negative controls, the thioredoxin portion of the fusion protein and PMP 8N, did not activate the expression of TNF-α. Similar to the induction of TNF-α expression, the expression of TF was also induced by Cpn 0809, Cpn 0980, OMP2, and LPS but not by PMP 8N, a recombinant protein which is known not to activate endothelial cells (33) (Fig. 1B).

### Table 1. Primers used for amplification of recombinant proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Forward primer (5'→3')</th>
<th>Reverse primer (5'→3')</th>
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<tr>
<td>OMP2</td>
<td>CATCTTCAGCTGACACG</td>
<td>GAGGCTTGGGAGACAG</td>
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<tr>
<td>Cpn 0980</td>
<td>ACGCTGTTTGGTTGGT</td>
<td>TGGAGTTGAGTGGTTAGG</td>
</tr>
<tr>
<td>Cpn 0809</td>
<td>GGGAACTTTCTGCTATTTTT</td>
<td>GAGGAGCTGTTGGTGGTTAGG</td>
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<tr>
<td>PMP 8N</td>
<td>AGACTGCGAAGAATCAGC</td>
<td>GAGGAGCTGTTGGTGGTTAGG</td>
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The induction of TNF-α expression by recombinant proteins (OMP2, Cpn 0809, and Cpn 0980) mediated through the MEK-ERK1/2 pathway. Since it was previously shown that expression of TF by C. pneumoniae occurs through activation of Egr-1 and the MEK-ERK1/2 pathway (4), whether OMP2, Cpn 0809, and Cpn 0980 activated Egr-1 induction and TF expression was investigated. As shown in Fig. 3A, all three recombinant proteins induced Egr-1 protein expression in the nuclear fraction in a dose-dependent manner. The maximum induction was observed at 1 h (Fig. 3B). Proliferating cell nuclear antigen was used as an internal control for standardization (Fig. 3B). As expected, the total cellular Egr-1 was not affected (Fig. 3C). In contrast, bovine serum albumin, thioredoxin, and PMP 8N had no effect on Egr-1 induction (Fig. 3A and B). To determine if the MEK-ERK1/2 pathway was required for induction of Egr-1, the effect of U0126 on Egr-1 expression in RAW 264.7 cells was examined. As shown in Fig. 3B, U0126 inhibited induction of Egr-1. These data confirmed that the induction of the MEK-ERK1/2 pathway by C. pneumoniae antigens occurred through Egr-1.

TNF-α induction by chlamydial antigens is diminished in TLR2- and TLR4-deficient murine macrophages. Macrophages play a key role in early innate immune responses and production of proinflammatory cytokines upon activation. Therefore, to investigate the potential involvement of TLR2 and TLR4 signaling in the induction of TNF-α by OMP2, Cpn 0809, and Cpn 0980, macrophages from TLR2 or TLR4 knock-out mice were tested and compared with wild-type macrophages. TLR2- and TLR4-deficient murine macrophages.

Recombinant antigens stimulate phosphorylation of p42/44 mitogen-activated protein kinases (MAPK) in mouse macrophages. The finding that TNF-α induction by recombinant proteins was inhibited by U0126 suggested that the recombinant antigens activate phosphorylation of ERK1/2. Immuno blot analysis showed that the expression of phosphor-ERK1/2 was activated by recombinant OMP2, Cpn 0809, and Cpn 0980 at concentrations ranging from 0.5 to 5 μg/ml (Fig. 2A). To standardize sample loading, total ERK was used as the internal control. A time-dependent increase in phosphor-ERK1/2 activity was observed from 15 min to several hours (Fig. 2B). As expected, PMP 8N did not have a stimulatory effect on ERK1/2 phosphorylation (Fig. 2B). To confirm the specificity of the recombinant antigen-induced phosphorylation of ERK1/2, macrophage cultures were preincubated with the MEK inhibitor U0126 for 30 min before treatment with recombinant proteins. U0126 inhibited induction of ERK1/2 phosphorylation (Fig. 2A). In contrast to the phosphor-ERK1/2 results, no phosphorylation of p38 and JNK was observed (Fig. 2C).

Induction of Egr-1 expression by recombinant antigens is mediated through the MEK-ERK1/2 pathway. Since it was previously shown that expression of TF by C. pneumoniae occurs through activation of Egr-1 and the MEK-ERK1/2 pathway (4), whether OMP2, Cpn 0809, and Cpn 0980 activated Egr-1 induction and TF expression was investigated. As shown in Fig. 3A, all three recombinant proteins induced Egr-1 protein expression in the nuclear fraction in a dose-dependent manner. The maximum induction was observed at 1 h (Fig. 3B). Proliferating cell nuclear antigen was used as an internal control for standardization (Fig. 3B). As expected, the total cellular Egr-1 was not affected (Fig. 3C). In contrast, bovine serum albumin, thioredoxin, and PMP 8N had no effect on Egr-1 induction (Fig. 3A and B). To determine if the MEK-ERK1/2 pathway was required for induction of Egr-1, the effect of U0126 on Egr-1 expression in RAW 264.7 cells was examined. As shown in Fig. 3B, U0126 inhibited induction of Egr-1. These data confirmed that the induction of the MEK-ERK1/2 pathway by C. pneumoniae antigens occurred through Egr-1.

Blockage of ERK1/2 phosphorylation inhibits the production of TNF-α. The induction of TNF-α expression by LPS is mediated by activation of the MEK-ERK1/2 pathway in macrophages (17). Our previous study showed that stimulation of TF production by C. pneumoniae occurred through the MEK-ERK1/2 and Egr-1 signaling pathway in RAW 264.7 cells (4). To determine if the MEK-ERK1/2 pathway is required for the induction of TNF-α expression by recombinant OMP2, Cpn 0809, and Cpn 0980 in macrophages, the induction of TNF-α was analyzed in the presence of U0126, an inhibitor of the upstream protein kinase, MEK1/2. Pretreatment with U0126 inhibited the recombinant antigen-induced production of TNF-α significantly (Fig. 1A). These results indicate that induction of TNF-α occurs through the MEK-ERK1/2 pathway. However, when cells were treated with a higher concentration of recombinant antigens (5.0 μg/ml), although TNF-α production was significantly decreased by U1026, it was not ablated. This suggests that TNF-α induction by OMP2, Cpn 0980, and especially Cpn 0809 can also occur through MEK-ERK1/2-independent pathways.

FIG. 1. Effects of recombinant proteins on TNF-α and TF expression in RAW 264.7 cells. (A) Effects of U0126 on the induction of TNF-α production by recombinant antigens. RAW 264.7 cells were preincubated with 10 μM U0126 for 30 min. After this, macrophages were washed and treated with recombinant proteins for 24 h in fresh medium. The culture supernatants were collected and assayed for TNF-α production. The data are the means ± standard deviations from two wells. One asterisk, *P < 0.05 for a comparison of control and treated cultures; two asterisks, **P < 0.05 for a comparison of recombinant protein- and U0126-treated cultures. (B) Effects of recombinant proteins on TF expression. The data are the means ± standard deviations from two wells. One asterisk, *P < 0.05 for a comparison of control and treated cultures. C. pn infect, C. pneumoniae infection.
contrast, LPS, which is known to signal through TLR4, stimulated TNF-α expression equally in TLR2 knockout and wild-type macrophages (41). Interestingly, TNF-α expression was also decreased in TLR4 knockout macrophages. These findings suggest that OMP2, Cpn 0809, and Cpn 0908 could stimulate TNF-α expression through both TLR2- and TLR4-mediated signaling pathways.

**DISCUSSION**

Strong evidence indicating that there is an association between *C. pneumoniae* and atherosclerosis has been presented previously. Since inflammatory processes are essential components of atherogenesis (36), induction of chronic inflammatory reactions by chlamydial infection may promote the progression of atherosclerosis. *C. pneumoniae* infection can trigger monocytes/macrophages to secrete proinflammatory cytokines such as TNF-α (19, 24) and TF (4). All three recombinant antigens stimulated the secretion of TNF-α (Fig. 1A) and TF (Fig. 1B) in macrophages. It has been reported that induction of TNF-α in macrophages with LPS is mediated by activation of the MEK-ERK1/2 pathway and NF-κB translocation (17). Activation of the MEK-ERK1/2 kinase by *C. pneumoniae* infection has been demonstrated in endothelial cells and smooth muscle cells (27, 51). Our previous study also showed that stimulation of TF production by *C. pneumoniae* was mediated through the MEK-ERK1/2 and Egr-1 signaling pathway in RAW 264.7 cells (4). However, the specific *C. pneumoniae* antigens that activate MEK-ERK1/2 were not determined.

Three such antigens, Cpn 0980, Cpn 0809, and OMP2, were identified in the current study (Fig. 2A). The specificity of the ERK1/2 activation was confirmed by treatment with the MEK inhibitor U0126 (Fig. 2A). Pretreatment of macrophages with U0126 also significantly decreased the expression of TNF-α production by chlamydial antigens (Fig. 1A). These findings indicate that chlamydial antigens could activate TNF-α secretion by macrophages via phosphorylation of the MEK-ERK1/2 pathway. This suggests that activation of MAPK pathways may be a common mechanism for *C. pneumoniae* infection to trigger cellular responses. In contrast, the effect of LPS on stimulation of TNF-α expression in RAW 264.7 cells was not affected by U0126. These findings are consistent with reports demonstrating that in addition to ERK 1/2, LPS-induced TNF-α expression occurs via p38 (2, 45) and JNK (3, 29) signaling. In contrast, *C. pneumoniae* antigens did not activate JNK and p38 kinases in this study (Fig. 2C). Our data differed from those reported by Krüll et al. (28), who showed that *C. pneumoniae* activated p38 in epithelial cells, suggesting that other *C. pneumoniae* antigens may stimulate different pathways. It also has been reported that *C. pneumoniae* (2, 38, 51), chHSP60 (2, 7), or chlamydial PMP 20 and 21 (33) activate...
NF-κB translocation in several cell types, which was not addressed in this study.

Activation of ERK1/2 induces phosphorylation and activation of the transcription factors c-jun, c-fos, and Egr-1 (17, 54, 59). The activation of ERK1/2 by OMP2, Cpn 0809, and Cpn 0980 was correlated with an increase in the expression and phosphorylation of Egr-1 (Fig. 3A) in the nucleus. Furthermore, inhibition of MEK-ERK1/2 reduced expression of TNF-α and Egr-1 (Fig. 3B), further supporting the hypothesis that the ERK1/2 signal transduction cascade has a role in the activation of macrophages by these chlamydial antigens (Fig. 3B). This finding is consistent with the findings of a previous study performed by Guha et al. (17) showing that in human monocytes treated with E. coli-derived LPS there is activation of the MEK-ERK1/2 kinase pathway and increased expression of Egr-1 and TNF-α. Egr-1 is abundantly expressed in human atherosclerotic lesions, and its expression is induced in the aortas of low-density lipoprotein receptor-deficient mice fed a high-cholesterol diet (30). Egr-1 is known to regulate the expression of a plethora of proatherogenic factors (4). Thus, activation of Egr-1 in macrophages by C. pneumoniae is likely to have a broad effect on the atherogenic process.

Binding of bacterial products to TLRs can activate the MAPK pathway (20). It has previously been shown that TLRs mediate translation of microbial recognition into expression of specific subsets of chemokines and cytokines by macrophages and dendritic cells (6, 21, 53, 54). Furthermore, TLR2 and TLR4 have been shown to induce different sets of chemokines and cytokines, implying that the innate immune response can be customized for different pathogens (11, 15, 39). Recent evidence has shown that C. pneumoniae activates the innate immune system via TLRs. However, different studies have reported differences in TLR utilization depending on whether live organisms, sonicated organisms, or individual antigens were tested. For example, recognition of live and sonicated organisms occurred mainly through TLR2, and TLR4 played a minor role in recognition of live organisms (32, 38). Bulut et al. (7) reported that chHSP60 activates macrophages and endothelial cells through TLR4, while Vabulas et al. (56) reported that chlamydial chHSP60 was recognized by both TLR2 and TLR4. In this study, OMP2 and two 53-kDa chlamydial proteins were shown to induce TNF-α signaling through both TLR2 and TLR4 (Fig. 4). Activation of macrophages by the recombinant antigens was not due to endotoxin contamination, since heat treatment abolished the increase in chlamydial antigen-induced TNF-α expression (Fig. 4). These findings suggest that chlamydial proteins activate macrophages through both TLR2 and TLR4. Because TLRs play a critical role in inflammatory signaling, the finding that several chlamydial antigens signal through TLR2 and TLR4 suggests one mechanism by which chronic infection promotes the inflammatory process of atherosclerosis.

In conclusion, the present study demonstrated that in addition to chHSP60, other chlamydial proteins recognized during human infection induce the expression of TNF-α in macrophages and that this induction involves Egr-1 and the MEK-ERK1/2 kinase pathway. TNF-α expression is predominantly dependent on binding of chlamydial proteins to the TLR2 but can occur in part through TLR4. These observations should
facilitate elucidation of the cellular and molecular mechanisms by which C. pneumoniae infection promotes atherosclerosis.

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