Involvement of the Antimicrobial Peptide LL-37 in Human Atherosclerosis

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Objective—Antimicrobial peptides are effector molecules of the innate immune system. To understand the function of vascular innate immunity in atherosclerosis, we investigated the role of LL-37, a cathelicidin antimicrobial peptide, in the disease process.

Methods and Results—Using real-time polymerase chain reaction, we found a 6-fold increase in human cationic antimicrobial protein 18/LL-37 transcript in human atherosclerotic lesions compared with normal arteries. Immunohistochemical analysis of atherosclerotic plaques showed that LL-37 was expressed mainly by macrophages and some endothelial cells. Western blot demonstrated existence of active LL-37 peptide and abundant proprotein in atheroma specimens. To understand the functional implication of LL-37 production in atherosclerosis, the transcription profile was assessed in endothelial cells treated with LL-37. Our data show that LL-37 induces expression of the adhesion molecule intercellular adhesion molecule-1 and the chemokine monocyte chemoattractant protein 1 in endothelial cells. Intriguingly, Chlamydia pneumoniae withstood the antimicrobial activity of LL-37 in vitro, although inflammatory response was induced on infection.

Conclusion—LL-37 is produced in atherosclerotic lesions, where it may function as an immune modulator by activating adhesion molecule and chemokine expression, thus enhancing innate immunity in atherosclerosis. (Arterioscler Thromb Vase Biol. 2006;26:1551-1557.)

Key Words: atherosclerosis • antimicrobial peptide • immune system • inflammation • infection

Antimicrobial peptides, identified by their ability to kill bacteria, fungi, and certain viruses, are conserved in species as diverse as plants, insects, and mammals and are main components of the innate immune system. Cathelicidins comprise a family of antimicrobial peptides characterized by a conserved cathelin-like domain and a variable C-terminal region with antimicrobial properties. Human cathelicidin or human cationic antimicrobial protein 18 (hCAP18)/LL-37 is the only cathelicidin member identified in humans so far. From hCAP18/LL-37, a 37-aa peptide, LL-37, is generated after proteolytic cleavage. hCAP18/LL-37 was initially isolated from neutrophils and subsequently shown to be expressed by epithelial cells lining the most common entry sites of bacteria, namely the intestine, skin, and the airways. Recently, LL-37 has been identified in inflammatory cells such as monocytes, macrophages, T cells, and mast cells, and its expression is induced in some inflamed tissues. Intriguingly, some microorganisms induce the expression of LL-37, whereas others suppress it, probably as an immune escape mechanism. LL-37 has been shown to be bactericidal against a wide variety of microorganisms, including both Gram-positive and Gram-negative bacteria.

In addition to antimicrobial effects, LL-37 has many other biological functions. For instance, LL-37 is chemotactic for mast cells, neutrophils, monocytes, and T cells. It can induce mast cell degranulation and subsequent histamine release. LL-37 plays a role in wound healing by promoting neovascularization and re-epithelialization of healing skin. LL-37 also regulates differentiation and maturation of dendritic cells. Thus, there is mounting evidence that LL-37 constitutes an important regulator and effector molecule of innate immunity.

Because LL-37 plays a role in host defense and in inflammation elicited by inflammatory diseases of the skin and airways such as psoriasis and sarcoidosis, and is upregulated in cutaneous injury and pulmonary infections, we hypothesized that this peptide might contribute to the innate immunity involved in atherosclerosis.

Infection with the obligate intracellular Gram-negative bacterium Chlamydia pneumoniae is recognized as a major...
cause of sinusitis, pharingitis, bronchitis, and pneumonia. Seroepidemiological studies indicate that C. pneumoniae represents by far the most prevalent chlamydial infection, affecting ≥50% of the population worldwide. Coronary heart disease and myocardial infarction have been associated to C. pneumoniae infection by seroprevalence studies24,25 and by the direct detection of the organism within atherosclerotic plaques26-27; nevertheless, precise effects of vascular innate immune system against C. pneumoniae infection are largely unknown.

In this study, we show that LL-37 is expressed in human atherosclerotic lesions and that it induces inflammatory responses in human endothelial cells. C. pneumoniae is not sensitive to LL-37 and can withstand high levels of the antimicrobial peptide.

Methods
Detailed Materials and Methods are provided in the expanded methods, available online at http://atvb.ahajournals.org.

Specimen Collection
Atherosclerotic plaque samples were obtained from patients undergoing carotid endarterectomy. Healthy vessels were represented by internal mammary and renal arteries obtained from patients undergoing coronary artery bypass surgery and nephrectomy, respectively. All human specimens were collected with the informed consent of the patients after permission by the local ethics committee was granted. In total, 25 atherosclerotic lesions (mRNA analysis n=13; protein detection n=12) and 19 healthy vessels (mRNA analysis n=12; protein detection n=7) were included in the present study.

Cell Culture
Human umbilical vein endothelial cells (HUVECs; Clonetics, Bio-Whittaker) maintained at 37°C, 5% CO2 in endothelial growth medium-2 (EGM-2) Clonetics; Bio Whittaker) were stimulated in EGM-2 with different concentrations of synthetic LL-37 for 6 hours and 24 hours. Human monocytes were isolated from peripheral blood from 2 donors, cultured in DMEM containing 10% FCS, 2 mmol/L l-glutamine, and 1 mmol/L sodium pyruvate, and maintained at 37°C, 5% CO2. Human macrophages were generated by stimulating monocytes with 500 U/mL granulocyte/macrophage colony-stimulating factor (GM-CSF; Peprotech EC) for 7 days.

C. pneumoniae Infection and Infectivity Assay
C. pneumoniae isolate Kajaani 63 was used in the present study. The infectivity, as measured by inclusion forming units (IFU) of bacterial preparation, was determined in HL cells (a human epithelial cell line conventionally used in C. pneumoniae cultivation). Aliquots of bacteria were incubated with different concentrations of LL-37 for various times and diluted 10- to 200-fold before they were used to infect overnight duplicate cultures of confluent HL cells. Alternatively, infected HL cells were coincubated with different concentrations of LL-37. Cells were incubated at 35°C for 72 hours in 5% CO2, thereafter stained with a fluorescein isothiocyanate–conjugated Chlamydia-specific monoclonal antibody (Bio-Rad), and IFU of C. pneumoniae were quantified by fluorescence microscopy.

C. pneumoniae Infection of Human Monocytes and Macrophages
Monocytes and macrophage cultures were infected with C. pneumoniae by centrifugation for 1 hour, 500g at 35°C. A multiplicity of infection of 3 was used. At different time points after infection, cells were washed with PBS and then lysed in RNA extraction buffer.

Real-Time RT-PCR
Total RNA isolated from human specimens and cells was reverse-transcribed to cDNA before real-time PCR using primers and probes for human LL-37,29 intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein 1 (MCP-1), cyclooxygenase 2, cyclophilin A, and GAPDH.

cDNA Array Analysis
A focused cDNA array (GEArray Q Series Human Cardiovascular Disease Gene Array II; Atherosclerosis, SuperArray Inc.) was used to analyze the effect of LL-37 on 96 atherosclerosis-related genes in human endothelial cells.

Immunohistochemistry and Immunofluorescence
Acetone-fixed cryostat sections were preincubated for 30 minutes with 5% normal serum. After incubation with primary antibody at 4°C overnight, sections were incubated with biotinylated goat-anti rabbit 7.5 μg/mL or horse anti-mouse IgG 7.5 μg/mL, followed by avidin-biotin peroxidase complex and developed with diaminobenzidine (all from Vector Laboratories).

Double-staining was performed using immunofluorescence. After preincubation with 5% normal serum in 50 mmol/L NH4Cl for 30 minutes, sections were incubated with primary antibodies overnight at 4°C, followed by goat anti-rabbit biotin or goat anti-mouse Texas Red 10 μg/mL. Subsequently, 1 μg/mL Oregon green streptavidin (Molecular Probes) was incubated for 30 minutes at room temperature when appropriate. 4,6-diamidino-2-phenylindole dihydrochloride (Sigma) was used to stain cell nuclei in the tissue.

Western Blot Analysis
Tissue homogenates or cell lysates were separated by SDS-PAGE using 10% to 20% Tris-Tricine Ready Gels (Bio-Rad Laboratories)
for LL-37 and 7.5% Tris-HCl gel for ICAM-1, and transferred to polyvinylidene difluoride membranes as described previously. Immunoreactivity was detected using rabbit anti-human LL-37 followed by goat anti-rabbit conjugated with horseradish peroxidase (DakoCytomation) or mouse anti-human ICAM-1 (Santa Cruz Biotechnology) followed by rabbit anti-mouse conjugated with horseradish peroxidase (DakoCytomation).

**Enzyme-Linked Immunosorbent Assay**
Concentrations of human MCP-1 were determined in supernatants from HUVECs stimulated with LL-37 for 24 hours by DuoSet ELISA (R & D Systems Inc.) following manufacturer instructions.

**Statistical Analysis**
Results are reported as mean±SEM using Student t test to assess differences between treatments in the in vitro cell culture experiments. The Mann–Whitney nonparametric test was used for comparing mRNA levels of LL-37 in human specimens because the data did not seem normally distributed. Differences were considered significant at \( P \leq 0.05 \).

**Results**

**High Levels of hCAP18/LL-37 mRNA Expression in Human Atherosclerotic Lesions**

mRNA levels of hCAP18/LL-37 were detected in 12 normal arteries and 13 atherosclerotic lesions by real-time RT-PCR. Despite modestly elevated LL-37 mRNA detected in 2 normal arterial samples, there was a 6-fold increase in the mRNA expression in atherosclerotic specimens compared with the normal vessels (Figure 1A).

**LL-37 Is Present in Inflammatory Cells Within Human Atherosclerotic Lesions**

Cellular localization of LL-37 in lesions was investigated by immunohistochemical staining using a peptide-specific anti-body. LL-37 was detected in the intima of all atherosclerotic lesions examined (n=12). In contrast, few LL-37–positive cells could be found in the wall of normal vessels of internal mammary arteries (Figure 1Ba). By double-label immunofluorescence, LL-37 colocalized predominantly with cells expressing CD163 (macrophages) but only occasionally with cells expressing CD3 (T lymphocytes; Figure 2). Interestingly, LL-37 was also found in some endothelial cells in 4 of 12 examined endarterectomy specimens (Figures 1B and 2). Furthermore, \( \alpha \)-smooth muscle actin–positive cells stained for LL-37 neither in atherosclerotic nor in normal vessels (data not shown).

**Both the Proform and Mature Form of LL-37 Exist Inside Atherosclerotic Lesions**

hCAP18/LL-37 is an 18-kDa protein stored as an inactive proform. LL-37, the bioactive peptide, is cleaved from its C-terminal segment by proteinase 3. To assess the presence of processed LL-37 in atheroma, 6 lesions and 4 normal arteries were analyzed by Western blot using a specific antibody against LL-37. Both the active peptide LL-37 (4.5 kDa) and the proprotein hCAP18 (18 kDa) were detected in all examined atheromatous specimens (Figure 3). The hCAP18 proprotein was also found in 2 of 4 normal arteries, but only trace amounts of LL-37 were identified in 1 of 4 such specimens, the other being negative (Figure 3).

**LL-37 Induces MCP-1 and ICAM-1 mRNA Expression in HUVECs**

The proposed function of LL-37 as an inflammatory stimulus led us to investigate the gene expression profile of HUVECs stimulated with 2 \( \mu \text{mol/L} \) LL-37. The transcription profile 6

![Figure 2](http://atvb.ahajournals.org/download/1553/Figure2.png)

**Figure 2.** Cellular localization of LL-37 in atherosclerotic lesion. Immunofluorescent staining for LL-37 (green) and cell markers (red) on atherosclerotic lesions. Double-positive cells are yellow in the overlay panel. Cell nuclei are stained blue with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). Original magnification \( \times 200 \) and \( \times 400 \). CD163 (macrophages), von Willebrand factor (vWF; endothelial cells) and CD3 (T cells).
hours after LL-37 stimulation was screened using an atherosclerosis-specific CDNA array containing 96 atherosclerosis-related genes. By comparing LL-37–stimulated HUVECs with untreated control cells, we found that LL-37 induced a limited set of inflammatory genes, including MCP-1, ICAM-1, and cyclooxygenase 2 (data not shown). To validate these findings, expression of MCP-1 and ICAM-1 was further analyzed by real-time RT-PCR. Stimulation of HUVECs with LL-37 for 24 hours resulted in a dose-dependent induction of MCP-1 and ICAM-1 in HUVECs (Figures 4A and 5A). MCP-1 was also measured in culture supernatants of LL-37–stimulated endothelial cells by ELISA. Incubation of endothelial cells with LL-37 leads to a dose-dependent increase in MCP-1 secretion (Figure 4B). By means of Western blot, we could demonstrate that LL-37 also induces ICAM-1 protein in a dose-dependent manner (Figure 5B).

**C pneumoniae Infection and Its Effect on LL-37 Levels**

By means of real-time RT-PCR, hCAP18/LL-37 mRNA expression was studied in human peripheral blood monocytes. The present results suggest that primary monocytes expressed very low levels of hCAP18/LL-37 in vitro. In comparison, GM-CSF–differentiated macrophages displayed a 10- and 24-fold increase in level of the gene transcripts (Figure 6A). Certain microorganisms influence the expression of hCAP18/LL-37, possibly as part of a virulence strategy. Because *C pneumoniae* may be present in the atherosclerotic lesion, we investigated whether *C pneumoniae* infection affects the expression of the antimicrobial peptide. Assessment of hCAP18/LL-37 mRNA revealed a transient downregulation in human monocytes and macrophages after the infection (Figure 6B and 6C).

**C pneumoniae Is Resistant to LL-37**

The effect of LL-37 on the infectivity and replication of *C pneumoniae* was determined by directly incubating the peptide with *C pneumoniae* for 2.5 hours and 24 hours. LL-37 did not alter the infectivity of the bacteria in HL cells in the doses between 2.5 and 40 μmol/L (data not shown). To investigate whether LL-37 could influence the replicating form of *C pneumoniae*, already infected cells were incubated with different doses of LL-37 for 72 hours. Our results demonstrated no effect of LL-37 on *C pneumoniae* replication by doses up to 10 μmol/L (data not shown). In control experiments, LL-37 exhibited strong antimicrobial activity against *Neisseria gonorrhoeae* with a minimal bactericidal concentration of 0.8 μmol/L.

**Discussion**

The present data show, for the first time, that LL-37, an antimicrobial cathelicidin, is produced in the human atherosclerotic lesion. Cathelicidins are key components of innate immunity, and LL-37 was found in infiltrating macrophages as well as in some endothelial cells of the lesions, suggesting that it is upregulated as part of the inflammatory process of atherosclerosis. Both mRNA and protein for its proform, as well as active LL-37 peptide, were detected in surgical specimens, indicating that it is produced by lesion cells.

Apart from its antimicrobial effect, LL-37 has also been shown to induce the expression of some inflammatory genes. In agreement with this, we show that LL-37 is an inducer of inflammation when acting on endothelial cells. A dose-dependent upregulation of ICAM-1 and MCP-1 transcripts was observed in endothelial cells after stimulation with LL-37 for 24 hours. This tendency was confirmed for MCP-1 when determining protein levels in supernatants from stimulated endothelial cells and for ICAM-1 in cell lysates by Western blot analysis. The stimulatory effects of LL-37 on endothelial cells occur within the range of concentration (2 to 15 μmol/L) detected in the bronchoalveolar lavage fluid from infants with systemic or pulmonary inflammation.
suggest that LL-37 on its own is a modest inducer of inflammation in endothelial cells. It is possible that LL-37, in conjunction with other inflammatory stimuli such as cytokines, may amplify the immune response of the diseased vessel (eg, by inducing the expression of adhesion molecules and chemokines). These results suggest that besides its antimicrobial activities, LL-37 may act as an immune modulator in atherosclerosis.

Infectious agents including C pneumoniae, herpes simplex virus, cytomegalovirus, and Helicobacter pylori have been associated with cardiovascular disease. Despite some patients with atherosclerosis being seropositive for H pylori, this bacterium has not been isolated from any atherosclerotic plaques. Given the presence of abundant LL-37 in atheroma lesions and its strong bactericidal effect against this bacterium, it seems plausible that H pylori may not be able to persist in atherosclerotic lesions. Unlike H pylori, C pneumoniae has been isolated from lesions. Similar to recent findings, data of the present study show that LL-37 did not alter either infectivity or replication of C pneumoniae. Thus, our finding may reveal a new paradigm of C pneumoniae infection; by resistance to LL-37 (and perhaps other innate immune effectors), C pneumoniae has found a way to escape attack by innate immunity. Therefore, it may reside persistently in the atherosclerotic lesion.

LL-37 is primarily produced by macrophages in atherosclerotic lesions. Whereas some bacteria influence the expression of LL-37, C pneumoniae infection does not induce the gene in human monocytes and macrophages. On the other hand, expression of LL-37 is evidently upregulated in the GM-CSF–primed human monocytes. Thus, cell differentiation is likely an essential mechanism regulating LL-37 expression in macrophages.

LL-37 may also have an angiogenic role by acting directly on endothelial cells to induce neovascularization. Neovascularization can promote atherosclerosis and lead to lesion growth through mechanisms that are not fully resolved but might involve increased recruitment of leukocytes into the lesion. However, we did not see LL-37 staining in microvessels of the atherosclerotic lesions, and it remains uncertain whether LL-37 may contribute to new vessel formation at these sites.

The presence of LL-37 in human atherosclerotic lesions together with previous findings locating α-defensins in the plaque indicates that antimicrobial peptides may play a role in the pathogenesis of the disease. It is possible that LL-37, either directly or by inducing cell surface adhesion molecules
and chemokines, promotes the recruitment of leukocytes to the site of infection in the vessel wall, thus enhancing innate immunity and modulating the local inflammatory response of diseased vessels.

Together, our data demonstrate that the human antimicrobial peptide LL-37 is expressed in atherosclerotic lesions mainly in macrophages but may also be expressed in endothelial cells and other immune cells such as T cells. LL-37 functions as an immune modulator inducing expression of inflammatory genes including adhesion molecules and chemokines in human endothelial cells. C pneumoniae, a bacterium that has been found in atherosclerotic plaques withstands the antimicrobial activity of LL-37. Our findings offer new insights into the function of innate immunity in atherosclerosis.

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Disclosures
None.

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