Lipopolysaccharide-Induced Cytokine and Chemokine Expression in Human Carotid Lesions

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Key Words
IL-1β, -6, -8, -10 · TNF-α · MCP-1 · IP-10 · RANTES · MIG · Atherosclerosis

Abstract
The release of cytokines and chemokines from activated immune-competent cells plays a crucial role in determining the pathology of the atherogenic progress. We investigated the effect of bacterial lipopolysaccharide (LPS) on cytokine/chemokine expression in carotid lesions and normal renal arteries. The lesions or renal arteries were incubated for 6 h at 37 °C in serum-free media treated with or without LPS. After LPS treatment, increased protein levels of IL-1β, IL-6, IL-8, IL-10, TNF-α and MCP-1 were observed in the culture medium from the lesions measured with cytometric bead array. We were able to detect the induction of IL-1β, IL-6, IL-8, IL-10, TNF-α and MCP-1 mRNA in the lesions after stimulation with LPS using real-time PCR. In renal arteries, LPS also induces mRNA expression of all chemokines and cytokines investigated with the exception of IL-6. However, LPS induces significantly higher levels of TNF-α, IL-1β and IL-10 mRNA in lesions compared to renal arteries. The results suggest that infectious agents are capable of enhancing the production of cytokines/chemokines in an already ongoing inflammatory process such as in atherosclerosis, and that low levels of circulating LPS may affect the levels of pro-inflammatory cytokines much more in atherosclerotic vessels than in normal vessels and may contribute to the development of the atherosclerotic lesion.

Introduction
The pathophysiology of atherosclerosis involves a triggered inflammatory process and activation of immune-competent cells. Characteristics for the atherogenic inflammatory process include monocyte, T-cell and mast cell recruitment from the bloodstream into the wall of the artery [1, 2]. Several epidemiological and animal studies have highlighted the importance of pro-inflammatory cytokines in atherogenesis [3, 4]. Infectious agents as potential participants in the inflammatory process of atherosclerosis are gaining acceptance in experimental science. Several epidemiological studies have postulated an association between infectious agents, like gram-negative bacteria, and chronic cardiovascular disease such as atherosclerosis [5, 6]. This has been demonstrated in animal models where the administration of lipopolysaccharide (LPS), a major pathogenic component of gram-negative bacteria cell wall, is found...
to increase the atherosclerotic lesion size [5]. However, the molecular mechanisms in which LPS exerts its action in the development of atherosclerosis are yet to be explored. Hence, we set to investigate the effect of the bacterial cell wall component (LPS) in an ongoing inflammatory process as in atherosclerosis.

**Materials and Methods**

**Patient Samples**

Carotid specimens from 12 patients were taken from plaques removed during carotid endarterectomy. All patients were operated on due to symptomatic carotid artery stenosis greater than 70%. The arterial specimens were washed immediately in phosphate-buffered saline to remove peripheral blood cells. All specimens contained advanced atherosclerotic lesions. Each arterial specimen was then divided into two sections, approximately 700 mg each. The tissue sections were then incubated separately for 6 h at 37°C in 2 ml Dulbecco’s modified Eagle’s medium/Ham’s F12 medium (Gibco, Rockville, Md., USA) containing 2% albumin (Pharmacia) with or without 100 ng LPS. Thereafter, the conditioned media were collected, aliquoted and stored in different vials than the tissue samples at −70°C until analysis. Six patients scheduled for nephrectomy were included, and biopsies from the renal artery were obtained preoperatively and placed in Dulbecco’s modified Eagle’s medium/Ham’s F12 medium (Gibco) enriched with human albumin 30 mg/ml (Biovitrum AB, Stockholm, Sweden). The renal arteries were divided into two equal pieces and incubated with 100 ng/ml LPS from *Escherichia coli*, O55:B5 (Sigma Chemical, St. Louis, Mo., USA) or left unstimulated at 37°C for 6 h. The renal arteries are histologically free of atherosclerosis.

**Total RNA Preparation**

Artery sections were frozen in liquid nitrogen and disrupted in a Mikro-Dismembrator II (B. Braun), and RNeasy Fibrous Tissue Mini Kit (Qiagen, USA) was used to isolate RNA according to the manufacturer’s instructions. To ensure proper results, the integrity and quantity of the isolated RNA were evaluated with the Agilent 2100 bioanalyzer using the RNA 6000 Nano Assay kit (Agilent Technologies). Recovered RNA was stored at −70°C until use.

**Reverse Transcription and PCR Amplification**

One microgram of total RNA was diluted in Rnase-free H2O to 20 μl for the synthesis of cDNA by reverse transcription using SuperScript™ II Rnase H− reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. To ensure proper results, the integrity and quantity of the isolated RNA were evaluated with the Agilent 2100 bioanalyzer using the RNA 6000 Nano Assay kit (Agilent Technologies). Recovered RNA was stored at −70°C until use.

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**Statistical Analysis**

The nonparametric Wilcoxon signed ranks test was used to assess differences between groups. Differences were accepted as significant at a level of p < 0.05.

**Results**

**Cytokine Protein Expression in Media from LPS-Treated Carotid Lesions**

Specimens from 6 patients were taken from plaques removed during carotid endarterectomy. All patients were operated on due to symptomatic carotid artery stenosis greater than 70%. Carotid lesions were incubated for 6 h at 37°C in serum-free medium. After incubation, culture media were collected and analysed using cytometric bead array to quantify protein levels of IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, TNF-α and IFN-γ. Cytokine protein expression was detectable in the medium, i.e. expressions of IL-6, IL-1β, IL-10 and TNF-α. The mean expression of IL-6 was highest, 9,000 pg/ml (range 1,900–28,600 pg/ml) followed by TNF-α 100 pg/ml (range 13–317 pg/ml), IL-1β 50 pg/ml (range 14–204 pg/ml) and IL-10 20 pg/ml (range 2–52 pg/ml). We were not able to detect IL-2, IL-4, IL-12 or IFN-γ in the culture media. LPS induced a 50- and 15-fold increase in the expressions

**IL-1β F-5'-CTG ATG GCC CTA AAC AGA TGA AG-3', IL-1β R-5'-GGT CGG AGA TTC GTA GCA GCT GGA T-3' and probe 5'-TTC CAG GAC GAC CTC TGC CTC C-3'; IL-6 F-5'-CGG GAA CGA AAG AGA AGT CTT TTG AGA AGG AGT TCA-3' and probe 5'-TCC CCT CCA GGA GCC CAG CT-3'; TNF-α F-5'-AGG CGG TGC TTG TCT CTC A-3'; TNF-α R-5'-GTT CGA GAA GAT GAT CTG ACT GCC-3' and probe 5'-CCA GAG GGA AGA AGT CCC CAG GGA C-3'.**

Gene expressions were normalized to β-actin gene expression with the primers β-actin F-5'-CTG GCT GAC CTC AAA CAT GAT CTG GTT-3' and the β-actin probe 5'-CCT GAA CCC CAA GGC CAA CCG-3'. The probes were labelled with FAM as reporter dye and TAMRA as quencher dye. The ABI Prism™ 7700 Sequence Detection System was used for RT-PCR amplifications. During amplification, thermal cycler conditions were as follows: each sample was analysed in duplicate for 2 min at 50°C, 10 min at 95°C, 15 s at 95°C and 1 min at 60°C. The PCR amplification was correlated against a standard curve.

Quantitative Determination of Cytokines and Chemokines in Serum-Free Culture Medium

The BD human chemokinome 1, human Th1/Th2 cytokine CBA kit II and human inflammation CBA kit; BD Biosciences) were used to quantitatively measure cytokine/chemokine expression levels in culture media. Fifty microlitres of conditioned medium were used and the assay was performed according to the manufacturer’s instructions and analysed on the FACSCalibur flow cytometer (Becton Dickinson).

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of TNF-α and IL-10, respectively, compared to untreated lesions (fig. 1), whereas IL-1β as well as IL-6 showed merely a 5- and 3-fold increase, respectively, in expression levels after LPS treatment (fig. 1). Even after treatment with LPS, we were not able to detect expressions of IL-2, IL-4, IL-12 or IFN-γ.

Chemokine Protein Expression in Media from Cultured Carotid Lesions Treated with LPS
Medium that contained atherosclerotic lesions showed expression of all the chemokines analysed in the study (IL-8, IP-10, MCP-1, MIG and RANTES). The average protein expression of MCP-1 was 5,300 pg/ml (range 2,600–13,300 pg/ml), an amount that is twice the quantity expressions of IL-8 (2,600 pg/ml, range 940–9,630 pg/ml) and MIG (2,400 pg/ml, range 700–7,400 pg/ml) and almost four times more than RANTES (1,300 pg/ml, range 142–5,720 pg/ml) and IP-10 (900 pg/ml, range 444–1,986 pg/ml).

LPS treatment induced a 5- and 2-fold increase, respectively, in the expressions of IL-8 and MCP-1. Despite a high expression level of RANTES, IP-10 and MIG in media from non-LPS-treated carotid lesions, we were not able to observe any significant elevation of them after LPS administration (fig. 2).

mRNA Expression of Cytokines and Chemokines in LPS-Stimulated Renal Arteries and Carotid Lesions
We compared the induction of cytokines and chemokines in atherosclerotic lesions after LPS stimulation with the response in normal artery, where we used renal artery obtained preoperatively from patients scheduled...
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for nephrectomy. We also used a new set of carotid lesions. As in carotid lesions, LPS significantly induced TNF-α (p < 0.05), IL-1β (p < 0.05), IL-8 (p < 0.001), IL-10 (p < 0.01) and MCP-1 (p < 0.05) mRNA expressions in normal renal artery. However, in contrast to carotid lesions, LPS did not induce IL-6 expression in normal renal artery (fig. 3b).

Furthermore, LPS induces significantly higher levels of TNF-α (p = 0.05), IL-1β (p = 0.001) and IL-10 (p < 0.01) mRNA in carotid artery lesions compared to normal renal artery (fig. 3).

Discussion

The pathophysiological progress of atherosclerosis includes activation of the immunologic response leading to an increase in production and release of cytokines and chemokines [7]. In the present study, we detected protein expressions of IL-1β, IL-6, IL-10, TNF-α, IL-8, IP-10, MCP-1, MIG and RANTES in culture media that had contained carotid lesion. These findings give clear indications of substantial activity of the immune competent cells found in the atherosclerotic lesions [8, 9]. To demonstrate the consistency of our findings, we found that cytokines/chemokines that showed highest protein expression were also most abundant in mRNA expressions. IL-1β, IL-6, IL-10 and TNF-α are cytokines that have been implicated in atherosclerosis [10]. Animal models of atherosclerosis have shown that IL-6 is expressed in the atherosclerotic plaque and that exogenous IL-6 administration enhanced fatty lesion development [11]. Subsequent studies confirmed these findings from immune histochemical studies where protein expressions of IL-6 are found in human atherosclerotic lesion [12, 13].

In this study, we have shown an overall high protein expression of the chemokines IL-8, IP-10, MIG, RANTES and MCP-1 compared to the levels of cytokines, with the exception of IL-6. This indicates that cells in human carotid plaques produce chemokines that may serve to recruit additional inflammatory cells into the plaque enhancing the inflammatory reaction that in turn may result in accelerated plaque development. There are a multitude of studies that have suggested a pathogenic role of IL-8 and MCP-1 in atherosclerosis by demonstrating their expression in areas of atherosclerotic lesions [14–16]. A more direct evidence for the role of MCP-1 was shown in animal models where combined MCP-1/LDLR knockout mice had 80% decrease in lesion size, 55% decrease in macrophage content in the plaques and less lipid accumulation throughout their aortas compared to control MCP-1+/+ /LDLR–/– mice [17]. However, less is said about the role of IP-10, MIG and RANTES in atherogenesis compared to the role of IL-8 and MCP-1 [18].

Fig. 3. mRNA expressions of TNF-α, IL-1β, IL-10 (a), IL-6, IL-8 and MCP-1 (b) in LPS-stimulated human renal arteries and carotid lesions. Significant induction of TNF-α (p < 0.01; p < 0.05), IL-1β (p < 0.01; p < 0.05), IL-10 (p < 0.01; p < 0.01), IL-8 (p < 0.01; p < 0.01) and MCP-1 (p < 0.01; p < 0.05) were observed in LPS-treated carotid lesions and renal arteries, respectively (a, b). Induction of IL-6 (p < 0.01) was only seen in carotid lesions and not in renal arteries (b). LPS significantly induced higher levels of TNF-α (p < 0.05), IL-1β (p < 0.01) and IL-10 (p < 0.01) mRNA in carotid lesions compared to renal arteries. a, b Data are expressed as relative values. All mRNA values are normalised to the expression of β-actin.
Studies have linked novel risk factors including infections as contributing factors in the pathogenesis of atherosclerosis [19]. The presence of micro-organisms such as *Chlamydia pneumoniae* and herpes viruses within atherosclerotic lesions has been documented and they may act as additional risk factors for the development and progression of atherosclerosis in experimental models [19–21].

The present study demonstrates that LPS administration promotes substantial activity within the atherosclerotic plaque, with release and expression of various cytokines such as TNF-α, IL-10 and chemokines such as IL-8 and MCP-1. In the normal renal artery, LPS also induces mRNA expression of all chemokines and cytokines investigated in this study, with the exception of IL-6. This is in line with the findings by Rice et al. [22] which demonstrated increased expressions of IL-8 and MCP-1 in human saphenous veins after exposure to endotoxin. However, the level of cytokines was higher in the carotid lesions compared to normal renal artery after LPS treatment. These results suggest that low levels of circulating endotoxins may affect the levels of pro-inflammatory cytokines much more in atherosclerotic blood vessels than in the normal vessels and may contribute to the development of atherosclerotic lesions.

The key role of cytokines/chemokines such as IL-1β, IL-8, IL-10, TNF-α and MCP-1 in plaque development has been confirmed in several studies both in human and in animal models [23]. Since LPS enhances their expression, we therefore suggest a pivotal role for LPS in plaque development. However, the mechanisms by which LPS exerts its action on the immunologic reaction are undoubtedly complex and multifactorial, differing probably between patients and environmental conditions. Nevertheless, in 2001, Medzhitov [24] suggested that initial recognition of microbes as they enter the body is based on the ability of the cells of the innate immune system such as macrophages to recognize common and conserved structural components of microbial origin by pattern recognition receptors, i.e. toll-like receptors (TLRs). Furthermore, studies of Edfeldt et al. [25] showed that TLRs are colocalized with NF-kB in endothelial and macrophage-rich areas in human atherosclerotic lesions, suggesting a possible TLR-NF-kB pathway that may accelerate immunologic activity within atherosclerotic plaque through the activation of endothelial cells and macrophages by bacterial endotoxin such as LPS; however, further studies on this postulation remain to be performed.

We speculate that the limited induction of IL-6 and all chemokines measured after LPS administration may be due to their already high expression prior to LPS exposure. However, a more complete assessment of the role of administered LPS and incubation times are to be considered. It is noteworthy to point out that according to our knowledge, this in vitro pilot study is the first attempt being made to quantitatively verify comparative quantities of cytokine and chemokine expression in culture media that had contained human carotid plaque either unstimulated or stimulated with LPS.

In summary, the present study demonstrates that LPS exposure on human carotid plaque and normal renal artery in vitro leads to induction of cytokines and chemokines. We suggest that endotoxin of gram-negative bacteria may accelerate an already ongoing inflammatory process as in atherosclerosis by the induction of proatherogenic cytokines, and it should be of importance to further investigate the contribution of pathogenic components such as LPS in the pathophysiological process of atherogenesis.

**Acknowledgments**

The study was supported by grants from the Swedish Health Care Sciences Postgraduate School (NFVO) Karolinska Institutet, Swedish Medical Research Council (K2002-71X-02042-36A) and the Swedish Heart Lung Foundation.
References


