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Interleukin-1 potently contributes to 25-hydroxycholesterol-induced synergistic cytokine production in smooth muscle cell-monocyte interactions

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ABSTRACT

Objectives: Inflammation is essential for atherogenesis. Cholesterol, a cardiovascular risk factor, may activate inflammation in the vessel wall during this process. Cytokine-mediated interactions of human monocytes with vascular smooth muscle cells (SMCs) may perpetuate this process. **Methods**: We investigated the capacity of the cholesterol metabolite 25-hydroxycholesterol to induce inflammatory mediators in cocultures of freshly isolated monocytes with SMCs. We determined the role of interleukin-(IL)-1 in this interaction using qPCR, bioassays, ELISA and western blot. Cocultures with SMC to monocyte ratios from 1:4 to 1:20 were tested. **Results**: In separate SMC and monocyte cultures (monocultures) 25-hydroxycholesterol only poorly activated IL-1, IL-6 and MCP-1 production, whereas LPS stimulated much higher cytokine levels than unstimulated cultures. In contrast, cocultures of SMCs and monocytes stimulated with 25-hydroxycholesterol produced hundredfold higher cytokine levels than the corresponding monocultures. Blocking experiments with IL-1-receptor antagonist showed that IL-1 decisively contributed to the 25-hydroxycholesterol-induced synergistic IL-6 and MCP-1 production. The presence of intracellular IL-1β precursor, released mature IL-1β, and caspase-1 p10 indicated that the inflammasome was involved in this process. Determination of IL-1-mRNA in Transwell experiments indicated that the monocytes are the major source of IL-1, which subsequently activates the SMCs, the primary source of IL-6 in the coculture. **Conclusion**: Taken together, these interactions between local vessel wall cells and invading monocytes may multiply cholesterol-triggered inflammation in the vessel wall, and IL-1 may play a key role in this process. The data also indicate that lower cholesterol levels than expected from monocultures may suffice to initiate inflammation in the tissue.

1. Introduction

Inflammatory processes potently contribute to atherogenesis [1,2]. Cytokines are key mediators of inflammatory processes involved in this disease [1–6]. Among many other functions cytokines act as chemoattractants and are essential for the transmigration of monocytes into the vessel wall and the mediation of cell-cell interactions. Leukocytes, such as patrolling monocytes [7]

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capturing the vessel wall, can interact with local cells, such as vascular smooth muscle cells (SMCs), thus perpetuating the production of inflammatory mediators in the vessel wall (compare [6,8]).

We have previously shown that interleukin-6 (IL-6) and monocyte-chemoattractant protein-1 (MCP-1) production are synergistically enhanced upon LPS-stimulated SMC-monocyte interactions [9] and that this tremendous amount of IL-6 production is reduced by statin-treatment [10]. However, endotoxin is not essential for atherogenesis. In contrast, oxysterols produced *in vivo* from endogenous cholesterol [11] are central risk factors for atherosclerosis and may be involved in the induction of inflammation in this disease [12]. Recently, cholesterol-oxysterol-rich fractions derived from human plaque have been shown to induce cytokine expression, in contrast to the triglyceride fraction isolated in parallel [13]. Oxysterols are present in early atherosclerosis, they can induce cytokine expression [14], and cholesterol crystals have been shown to activate the inflammasome [15,16]. However, the capacity of oxysterols to escalate cytokine-mediated interactions between monocytes and vascular cells has not yet been investigated.

Researchers have suggested that in mice, the production of monocyte-derived interleukin-1 (IL-1) after inflammasome activation may activate endothelial cell (EC) or SMC functions [17]. In experiments using human vascular ECs or SMCs IL-1 was a highly potent activator of cytokine production [18–20]. Furthermore, we have shown in an LPS-stimulated coculture model with human monocytes and SMCs that IL-1 plays a critical role in the induction of inflammatory mediator production [9]. Therefore, we hypothesized that IL-1 may also represent a central activator in the coculture model upon stimulation with 25-hydroxycholesterol, which has not yet been addressed. IL-1 has been demonstrated to play an important role in cardiovascular diseases through experiments with IL-1-receptor- or IL-1-deficient mice [21–24]. Based on the potential contribution of IL-1 to the pathogenesis of atherogenesis, its role in disease has been examined in various clinical trials [25,26].

Therefore, in the present study we investigated the capacity of 25-hydroxycholesterol crystals to activate cytokine production in the established coculture model and analyzed the involvement of IL-1 in the 25-hydroxycholesterol-stimulated cocultures. We show that 25-hydroxycholesterol potently induced synergistic cytokine production in the SMC-monocyte coculture, whereas it activated cytokine production by the separately cultured cells to a much lower degree. We also show that monocyte-derived IL-1 potently contributed to the IL-6 and MCP-1 production induced by 25-hydroxycholesterol in the cocultures. These data indicate that, in the presence of cellular interactions, cholesterol may escalate inflammatory processes more potently than expected, which may be important for the atherogenic process.

2. Materials and methods

The isolation of human vascular smooth muscle cells [9,19,27], and monocytes [28] is described in the Supplement, along with the standard measurement methods such as ELISA, IL-6 bioassay [29], western blot, and realtime PCR.

2.1. Coculture design

As previously described [9,10], each coculture experiment consisted of separately cultured SMCs (SMC-monoculture), separately cultured freshly isolated mononuclear cells or monocytes (MNC/monocyte-monoculture), as well as the coculture, containing both cell types at the same conditions as the monocultures. To identify the cell source of cytokines in the coculture, in addition to the standard coculture, cocultures were performed in parallel in Transwell systems (Nunc; Schwerte, Germany). SMCs (10,000 SMC/cm^2) were seeded first onto the 6-well culture plate and the monocytes (200,000 monocytes/cm^2) were seeded into the Transwell inserts.

2.2. Substances

Cells were stimulated with lipopolysaccharide (LPS) of *Salmonella enterica* serovar Friedenau kindly provided by Prof. Dr. H. Brade (FZ-Borstel, Germany) and 25-hydroxycholesterol (Sigma Aldrich, Taufkirchen, Germany). An LAL-assay did not find LPS contamination in the 25-hydroxycholesterol used for stimulation. Recombinant interleukin-1 receptor antagonist (IL-1ra), IL-1α, IL-1β and tumor necrosis factor-α (TNF) were purchased from CellConcepts (Umkirch, Germany). The stimulus concentrations are presented in the respective legends.

2.3. Statistical methods

ELISA and IL-6-bioassay measurements were performed in triplicate or quadruplicate. The resulting values were used to calculate the mean and the standard deviation. Significances were calculated by Mann–Whitney *U*-test or ANOVA with post hoc analysis by LSD, as described in the respective legends, using SPSS. Correlations were calculated in SPSS using a Spearman analysis.

3. Results

3.1. Interaction of 25-hydroxycholesterol-stimulated SMCs and MNCs enhances the synergistic production of interleukin-6 and monocyte-chemoattractant protein-1

This work analyzed the inflammatory capacity of 25-hydroxycholesterol in a previously established coculture system [9,10]. In contrast to LPS stimulation, the IL-6 levels in 25-hydroxycholesterol-stimulated MNC monocultures (Fig. 1A; hatched columns) and SMC monocultures (white columns) were not substantially higher than the respective unstimulated cultures. In contrast, the IL-6 production in the 25-hydroxycholesterol-stimulated cocultures (black columns) was synergistically enhanced (18.1-fold) when compared to the monocultures. Summarizing 12 experiments, the synergism in the 25-hydroxycholesterol-stimulated cocultures was 2.4-fold higher than in the LPS-stimulated cocultures (Supplementary Table I). The ELISA and the IL-6 bioassay data shown in Fig. 1A were very well correlated ($\rho = 0.979$; $p = 4.3 \times 10^{-6}$; Spearman analysis). The western blot shown in Fig. 1B indicated that the supernatants of the stimulated cocultures contained the expected, differentially glycosylated forms of IL-6. Both the western blot band intensity (Fig. 1C) and the ELISA data (Fig. 1D) for the same samples demonstrated a large increase in IL-6 production in the cocultures upon LPS-and 25-hydroxycholesterol stimulation in comparison to the SMC and monocyte monocultures. Similarly to protein and activity measurements, LPS and 25-hydroxycholesterol stimulation also increased the IL-6 transcription in the stimulated cocultures (Supplementary Figs. 1 and 2). Thus, the expression and functional data supported each other and clearly noted the extraordinary cytokine level in the LPS- and 25-hydroxycholesterol-stimulated cocultures. In addition, 25-hydroxycholesterol also activated synergistic monocyte-chemoattractant protein-1 (MCP-1) production (Supplement Table II).