Research Article

The Serine Protease Plasmin Triggers Expression of the CC-Chemokine Ligand 20 in Dendritic Cells via Akt/NF-κB-Dependent Pathways

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The number of dendritic cells is increased in advanced atherosclerotic lesions. In addition, plasmin, which might stimulate dendritic cells, is generated in atherosclerotic lesions. Here, we investigated cytokine and chemokine induction by plasmin in human dendritic cells. In human atherosclerotic vessel sections, plasmin colocalized with dendritic cells and the CC-chemokine ligand 20 (CCL20, MIP-3α), which is important for homing of lymphocytes and dendritic cells to sites of inflammation.

Stimulation of human dendritic cells with plasmin, but not with catalytically inactivated plasmin, induced transcriptional regulation of CCL20. By contrast, proinflammatory cytokines such as TNF-α, IL-1α, and IL-1β were not induced. The plasmin-mediated CCL20 expression was preceded by activation of Akt and MAP kinases followed by activation of the transcription factor NF-κB as shown by phosphorylation of its inhibitor IκBα, by nuclear localization of p65, its phosphorylation, and binding to NF-κB consensus sequences. The plasmin-induced CCL20 expression was dependent on Akt- and ERK1/2-mediated phosphorylation of IκBα on Ser32/36 and of p65 on Ser276, whereas p38 MAPK appeared to be dispensable. Thus, plasmin triggers release of the chemokine CCL20 from dendritic cells, which might facilitate accumulation of CCR6+ immune cells in areas of plasmin generation such as inflamed tissues including atherosclerotic lesions.

1. Introduction

The serine protease plasmin is mainly recognized for its central role in fibrinolysis. In addition, however, plasmin may also be generated at inflammatory sites from ubiquitously distributed plasminogen [1]. Indeed, generation of plasmin has been shown in a number of chronic inflammatory conditions including arthritis and atherosclerosis [1]. Specifically in unstable atherosclerotic lesions, plasminogen and plasmin appear to be associated with clinical complications [2–5]. Local plasmin generation at sites of inflammation might aggravate inflammatory processes by triggering proinflammatory effects. In vitro, plasmin is capable of stimulating lipid mediator release and of eliciting chemotaxis of human monocytes [6, 7]. In addition, plasmin is a potent inducer of proinflammatory cytokines in human macrophages [8] and monocytes, where it also causes expression of procoagulant tissue factor [9].

Dendritic cells play a crucial role in innate and adaptive immune responses [10]. Dendritic cells are crucial for immune diseases including rheumatoid arthritis, where they accumulate in synovial tissue and activate T cells [11]. Likewise, the number of dendritic cells is strongly increased in advanced atherosclerotic lesions, where they colocalize with T cells [12–14]. Dendritic cells induce differentiation of T cells into different T-cell subsets through direct interaction with T-cell receptors and the release of cytokines. Dendritic cells are heterogeneous in their origin and their ability to activate either tolerogenic or immunogenic T-cell responses [15]. A distinct dendritic cell type is monocyte-derived dendritic cells, which arise in the course of inflammation [15, 16]. We have recently shown that plasmin is a potent chemoattractant for immature dendritic cells, and that it activates dendritic cells to produce interleukin-12 (IL-12) and to promote polarization of CD4+ T cells towards the interferon-γ (IFN-γ-) producing, proinflammatory Th1 phenotype [17].

Chemokines orchestrate the homing of lymphocytes and dendritic cells to lymphoid tissues as well as the recruitment
of leukocytes to sites of infection or tissue damage [18]. As a result, chemokines play crucial roles in the pathogenesis of diseases that are characterized by inflammatory cell accumulation, such as atherosclerosis [12, 14, 18, 19].

CCL20 (also known as liver- and activation-regulated chemokine, LARC, or macrophage inflammatory protein-3α, MIP-3α) is a CC-type chemokine, which activates chemokine receptor CCR6 and therefore plays an important role in homing CCR6+ lymphocytes and dendritic cells into secondary lymphoid organs and to sites of inflammation [20]. Memory T lymphocytes, naïve and memory B cells, Langerhans cells, and subsets of immature dendritic cells all express CCR6 [20] and migrate to sites of CCL20 expression, for example, in atherosclerosis [20], inflammatory bowel disease [21], arthritis [19], chronic obstructive pulmonary disease [22], psoriasis [23], and tumor tissues [24]. Accordingly, an important role for CCL20 has been postulated in atherosclerosis, skin, and mucosal immunity, in rheumatoid arthritis, and in cancer [20].

Immunohistochemical colocalization of immature dendritic cells and CCL20 indicates a link between the accumulation of immature dendritic cells and the local production of CCL20 by epithelial and tumor cells [20, 22]. Indeed, subsets of immature dendritic cells express CCR6 and are able to migrate towards CCL20 [25]. However, dendritic cells might also be a source of CCL20 on their own. Thus, CCL20 secretion can be induced in dendritic cells either by stimulation with LPS [26] or extracellular nucleotides [27].

Here, we investigated whether plasmin might affect the expression of cytokines by human monocyte-derived dendritic cells and whether this might occur in human atherosclerotic lesions.

2. Materials and Methods

2.1. Materials. Antibodies used are phospho-IκBα, phospho-ERK1/2, phospho-p38, phospho-Akt (Ser473), phospho-p65 Ser276, and phospho-p65 Ser536, and phospho-p65 transcription factor ELISA was from Active Motif (Carlsbad, CA).

2.2. Methods

2.2.1. Immunohistochemical Staining. Sections of surgical specimens from human abdominal aorta from 3 patients were stained with antibodies recognizing plasmin, CCL20, or the DC marker S100, which is exclusively expressed by dendritic cells in the arterial wall [28, 29]. For immunohistochemical double staining, HRP- and AP-conjugated secondary antibodies were visualized by DAB, Fast Red, or AEC substrates (PicTure kit, Invitrogen). The images were digitally recorded with an Axioskop microscope and a Sony MC-3249 CCD camera using Visupac 22.1 software (Carl Zeiss, Göttingen, Germany) [17, 30]. The study was approved by the University Ethics Review Board (approval reference number 114/10) and complied with the principles of the Declaration of Helsinki.

2.2.2. Cell Preparation and Differentiation. Immature dendritic cells were differentiated from human monocytes obtained from buffy coats with 1000 U/mL GM-CSF and 25 ng/mL IL-4 for 6 days in RPMI 1640 containing 10% FCS [30]. The differentiation was confirmed by flow cytometric analysis of HLA-DR, CD80, CD86, and CD1a using a FACScan (BD Biosciences, Franklin Lakes, NJ). Dendritic cells were used on day 6 of differentiation. The cells (1 × 10^6 cells/mL) were kept for 12 h in AIM-V medium (Invitrogen, Carlsbad, CA) without cytokines and FCS before treatment with plasmin. In some experiments, the dendritic cells were treated with catalytically inactivated plasmin, equivalent to 0.143 CTA U/mL of native plasmin. Catalytically inactivated plasmin (VPLCK plasmin) was prepared as described [31].

2.2.3. Analysis of mRNA Expression. mRNA was isolated from dendritic cells stimulated with plasmin or equivalent amounts of active site-blocked plasmin (VPLCK plasmin) [9, 30] and analyzed by RT-PCR and quantitative real-time PCR. Primer pairs for CCL20 were sense 5′-GAATAAGGCGAAGAGTTTAT-3′, antisense 5′-TTATTTGACAGTCCGAGG-3′ [32]; GAPDH served as control [33]. The identity of the PCR products was confirmed by direct sequencing (Abi Prism 310, Applied
Biosystems, Foster City, CA). Quantitative PCR was performed using real-time PCR system (7300 Real-Time PCR, Applied Biosystems), and the relative gene expression was determined by normalizing to GAPDH using the $\Delta\Delta C_T$ method.

### 2.2.4. Analysis of Protein Expression

Protein expression was analyzed by western immunoblotting, proteome profiler array, ELISA, and flow cytometry [30, 34]. Dendritic cells were kept in AIM-V medium for 12 h prior to stimulation. For the analysis of phosphorylated IκBα and p65, whole cell lysates were analyzed by western immunoblotting [9]. CCL20 secretion was measured by ELISA (R&D Systems) in supernatants of dendritic cells stimulated for 24 h with plasmin or the positive control LPS (0.5 μg/mL). For flow cytometric analysis, dendritic cells were pretreated with 1 μg/mL brefeldin A (Sigma) for 4 h prior to analysis to prevent release of CCL20 from the cells. Dendritic cells were fixed with paraformaldehyde, permeabilized with 0.5% saponin, stained with antibodies against CCL20 or control IgG and analyzed by FACScan (BD Biosciences). TNF-α, IL-1α, and IL-1β were analyzed by proteome profiler array (R&D Systems) in the supernatants of dendritic cells stimulated with plasmin (0.143 CTA U/mL) or LPS (0.5 μg/mL) for 60 min [9]. Results are expressed as fold activation compared to the control samples.

### 2.2.6. Statistical Analysis

Data shown represent mean ± SEM where applicable. Statistical significances were calculated with the Newman-Keuls test. Differences were considered significant for $P < 0.05$.

### 3. Results

#### 3.1. Plasmin and Dendritic Cells Colocalize with CCL20 in the Human Atherosclerotic Vessel Wall

Immunohistochemical analysis of sections from atherosclerotic tissue specimens obtained from human abdominal aorta confirmed that plasmin is abundant in the atherosclerotic vessel wall, where it colocalizes with clusters of dendritic cells (Figures 1(a) and 1(b)). In addition, these immunohistochemical studies revealed that plasmin and dendritic cells are in close proximity to CCL20 (Figures 1(a), 1(c), and 1(d)) suggesting that dendritic cells might be activated by locally generated...
Figure 2: Plasmin induces time- and concentration-dependent expression of CCL20 mRNA in dendritic cells. (a) Plasmin does not induce release of the proinflammatory cytokines TNF-α, IL-1α, and IL-1β. At day 6, dendritic cells were left untreated or stimulated with plasmin (0.143 CTA U/mL) for 24 h. Release of cytokines in the culture media was analyzed with the proteome profiler array. (b) Plasmin induces time-dependent expression of CCL20 mRNA in dendritic cells. Dendritic cells were treated with plasmin (0.143 CTA U/mL) or the positive control LPS (0.5 μg/mL) for the indicated time. mRNA was isolated and subjected to RT-PCR (b) and real time qPCR (c) analysis using CCL20-specific primers; GAPDH served as control. Results are mean ± SEM of 3 experiments. (d) Dendritic cells were treated for 6 h with the indicated concentrations of plasmin, and CCL20 mRNA expression was analyzed by RT-PCR. (e) Proteolytic activity of plasmin is required for the induction of CCL20 mRNA expression. Dendritic cells were stimulated either with plasmin (0.143 CTA U/mL) or the equivalent amount of catalytically inactivated plasmin (VPLCK plasmin) for 6 h. CCL20 mRNA expression was analyzed by RT-PCR. All data are representative of at least 3 independent experiments.

3.2. Plasmin Induces CCL20 mRNA Expression in Dendritic Cells. To address the possible generation of cytokines and chemokines by plasmin-activated dendritic cells, we stimulated monocyte-derived dendritic cells with plasmin in vitro. Analysis of the supernatants of such cells revealed that in contrast to human monocytes [9] and macrophages [8], dendritic cells do not release proinflammatory cytokines, such as TNF-α, IL-1α and β (Figure 2(a)), or IL-16 (LCF), nor did they release chemokines such as CXCL10 (IP-10), CXCL11 (I-TAC), CXCL12 (SDF-1), CCL1 (I-309), CCL2 (MCP-1), or CCL5 (RANTES). Control dendritic cells produced CXCL8 (IL-8) and small amounts of CXCL1 (GRO), but the release of these chemotactic cytokines remained unaffected by plasmin treatment (data not shown). However, stimulation of dendritic cells with human plasmin (0.143 CTA U/mL) elicited a time-dependent increase of CCL20 mRNA expression as analyzed by RT-PCR.
(Figure 2(b)) and real-time qPCR (Figure 2(c)). The maximum of the CCL20 mRNA expression was observed 6 h after stimulation with either plasmin (0.143 CTA U/mL) or the positive control LPS (0.5 μg/mL). The stimulatory effect of plasmin was concentration dependent with a maximum at 0.143–0.43 CTA U/mL (Figure 2(d)).

Previous studies had suggested that the proteolytic activity of plasmin might be required for cell activation [7, 8, 31, 36]. To test whether this is also true for the plasmin-induced activation of human dendritic cells, we generated catalytically inactivated plasmin (VPLCK plasmin) [9]. In contrast to active plasmin, catalytically inactivated plasmin did not trigger any CCL20 induction in dendritic cells (Figure 2(e)) indicating that the plasmin-mediated dendritic cell activation depends on a proteolytic signaling mechanism.

3.3. Plasmin Induces Release of CCL20 in Dendritic Cells.

The transcription of CCL20 mRNA by plasmin was followed by a concentration-dependent release of CCL20 with a maximum at 0.143 CTA U/mL (Figure 3(a)). Similar to the mRNA expression levels, higher plasmin concentrations did not further increase the amount of secreted CCL20. The positive control LPS (0.5 μg/mL) induced release of higher amounts of CCL20 (792.9 ± 129.9 pg/mL, n = 5) compared to 0.143 CTA U/mL plasmin (132.6 ± 26.1 pg/mL, P < 0.01, n = 8); control cells released 35.8 ± 10.4 pg/mL CCL20.

Consistently, flow cytometric analysis of the CCL20-expressing cells revealed that about 41% of the dendritic cells treated with plasmin expressed CCL20 within 24 h after treatment (Figure 3(b)). Thus, plasmin triggers production of chemotactic CCL20 by human dendritic cells.

3.4. Plasmin Elicits Activation of Akt, ERK1/2 and p38 MAP Kinases, and NF-κB Signaling.

Expression of cytokines and chemokines is regulated primarily at the level of transcription. The promoter region of CCL20 is known to contain an NF-κB consensus sequence indicating that the expression of CCL20 might be regulated by NF-κB [37]. In addition, it has been previously shown that NF-κB can be activated by Akt-dependent IκBa kinase phosphorylation [38, 39], and Akt mediates an IL-17A-induced expression of CCL20 in human airway epithelial cells [40]. Moreover, ERK1/2 and p38 MAP kinases have been implicated in the regulation of the NF-κB activation via MSK1/2 activation and the phosphorylation of p65 [39, 41].

Taking into account that NF-κB is involved in the expression of various proinflammatory genes including chemokines [42], and that plasmin in turn activates NF-κB in monocytes and macrophages [8, 9, 43], we investigated whether plasmin might activate Akt, MAP kinases, and NF-κB in dendritic cells.

Western immunoblot analysis of plasmin-stimulated dendritic cells indicated that plasmin triggers a rapid phosphorylation of Akt, ERK1/2, and p38 MAP kinases (Figure 4(a)). In addition, the phosphorylation of IκBa was increased with a maximum response at 15–30 min after stimulation (Figure 4(b)) indicating activation of NF-κB.

Phosphorylation of IκBα by IκB kinases is a prerequisite for IκB ubiquitination and degradation required for the release of p65 and other NF-κB subunits, and their subsequent nuclear translocation and NF-κB-dependent gene induction [42]. Among different NF-κB subunits, the p50/p65 heterodimer is the most abundant. Only the p65 subunit of the p50/p65 heterodimer contains a domain initiating transcriptional activation essential for the expression of the NF-κB-dependent genes [41]. In addition, p65 overexpression significantly increased the CCL20 mRNA expression in HeLa cells stimulated with TNF-α [44]. Therefore, we analyzed activation of p65 in the nuclear extracts of dendritic cells that had been stimulated for 1 h with either plasmin or the positive control LPS (0.5 μg/mL). Plasmin induced a significant increase in the p65 NF-κB activity (2.00 ± 0.27-fold compared to control, P < 0.05) (Figure 4(c)); LPS induced a higher NF-κB activity (8.20 ± 0.59-fold, P < 0.01), which is consistent with the higher amounts of CCL20 released by the LPS-stimulated dendritic cells (Figure 3(a)).

3.5. Plasmin Induces CCL20 Expression in Dendritic Cells through Akt- and ERK1/2 MAPK-Dependent NF-κB Activation.

To analyze the role of Akt and MAPK in the plasmin-induced CCL20 expression, dendritic cells were pretreated with pharmacological inhibitors of Akt (Akt inhibitor VIII) [45], MEK/ERK1/2 (U0126), p38 (SB203580) [46], and NF-κB (AKβBA) [35, 47–49] before addition of plasmin. AKβBA is an NF-κB inhibitor targeting IκB kinases (IKK) thereby inhibiting NF-κB-dependent signaling in monocytes [48] and tumor cells [35]. In preliminary tests, we ensured that the used concentrations induced specific inhibition of the respective pathways, yet did not impair cell viability. The Akt inhibitor VIII, the MEK/ERK1/2 inhibitor U0126, and the IκB kinase inhibitor AKβBA, but not the p38 MAPK inhibitor SB203580, abolished the plasmin-induced expression of CCL20 mRNA and CCL20 protein release (Figures 5(a) and 5(b)) indicating that plasmin-activated activation of Akt, ERK1/2, and NF-κB is indispensable for the CCL20 expression.

To address whether the plasmin-induced activation of Akt and ERK1/2 would be located upstream of the NF-κB activation, we analyzed protein phosphorylation in the presence of the inhibitors. Inhibition of either Akt or ERK1/2 impaired the plasmin-induced IκBα phosphorylation and the phosphorylation of p65 at Ser276, whereas the phosphorylation of p65 at Ser536 remained unaffected (Figure 6). These data indicate that Akt and ERK1/2 activation is indispensable for the plasmin-induced NF-κB activation and the subsequent expression of CCL20.

4. Discussion

The serine protease plasmin is activated under physiological and pathological conditions. Plasmin is locally generated during tissue damage or thrombus formation, but also in the context of contact activation during inflammatory processes [1, 50–53]. It has been shown that the plasminogen activator uPA and its receptor are present on the surface of
Figure 3: Plasmin elicits CCL20 protein expression in dendritic cells. (a) Plasmin induces a concentration-dependent release of CCL20. Dendritic cells were stimulated with various concentrations of plasmin or LPS (0.5 μg/mL) for 24 h before being analyzed by ELISA. The results are mean ± SEM of 8 experiments, *P < 0.05, **P < 0.01 versus control. (b) Flow cytometric analysis of CCL20 expression by dendritic cells. Dendritic cells were either unstimulated or stimulated with plasmin (0.143 CTA U/mL) for 24 h. Brefeldin A was added to the cell culture 4 h before the end of the incubation, and the cells were fixed, permeabilized, stained, and analyzed by flow cytometry. Representative data of 3 independent experiments are shown.

Figure 4: Plasmin activates Akt, MAPK, and NF-κB signaling in dendritic cells. (a) Time-dependent phosphorylation of Akt (Ser473) and MAP kinases in dendritic cells after stimulation with plasmin (0.143 CTA U/mL). Dendritic cells were stimulated with plasmin, and whole cell lysates were analyzed by western immunoblotting; actin served as loading control. Representative data of three experiments are shown. (b) Time-dependent phosphorylation of IκBα (Ser32/Ser36) in dendritic cells after stimulation with plasmin (0.143 CTA U/mL). Representative data of three experiments are shown. (c) Activation of p65 NF-κB as analyzed with the NF-κB TransAM ELISA. Nuclear extracts were obtained from dendritic cells stimulated with plasmin (0.143 CTA U/mL) or LPS (0.5 μg/mL) for 1 h. The results are mean ± SEM of 3 independent experiments, *P < 0.05, **P < 0.01 versus control.
CCL20 is constitutively expressed by lymphoid and nonlymphoid tissue, where it contributes to homeostatic functions and immunity [20]. Thus, mucosa-associated lymphoid tissues and different tumors constitutively express CCL20 [20]. Under inflammatory conditions, CCL20 can be rapidly induced by proinflammatory cytokines, bacterial and viral infections of epithelial cell, keratinocytes, fibroblasts, or endothelial cells [20, 27, 40, 55]. Recent studies have shown that neutrophils produce CCL20 in response to treatment with LPS or TNF-α [56]. Human monocytes express CCL20 when activated with LPS, extracellular nucleotides [20, 27], or under hypoxic conditions [37]. Similarly, dendritic cells can produce CCL20 when stimulated with LPS, CD40L [26], or extracellular nucleotides [27], but not TNF-α [26]. Here, we show for the first time that plasmin elicits CCL20 expression in dendritic cells. The plasmin-induced expression of CCL20 is very rapid and is not dependent on the release of proinflammatory TNF-α. Moreover, we show that plasmin does not induce expression of TNF-α by dendritic cells, and TNF-α does not induce expression of CCL20 in dendritic cells [26]. Similar to the plasmin-induced activation of monocytes and macrophages [8, 9, 31], the proteolytic activity of plasmin is essential for the induction of the CCL20 expression in dendritic cells.

Chemokines are regulated primarily at the level of gene transcription. The CCL20 promoter region contains binding sites for different transcription factors such as activator protein-1 (AP-1) and AP-2, CAAT/enhancer-binding protein (CEBP) and CREB.
activity of p65 via phosphorylation at Ser276 [59]. ERK1/2 gene expression via histone 3 phosphorylation creating a mediated MSK activation might also contribute to enhanced activation that MSK1/2 can be potent activators of CREB, whose activity, in turn, is essential shown to activate nuclear kinases MSK1/2 [39, 41], which are transcriptional activators.

The role of Akt in the plasmin-induced NF-κB activation is more complex. The ability of Akt to regulate NF-κB activity might occur through the phosphorylation of IκB kinase, which in turn phosphorylates IκB and allows the release of NF-κB [38], and/or by stimulating transactivation of the p65 subunit by IκB kinase-dependent phosphorylation of p65 on Ser536 [60, 61]. However, the later process is p38 dependent. Consistent with the fact that plasmin-activated dendritic cells did not utilize the p38 MAPK pathway to induce CCL20, we did not observe any effects of p38 inhibition on p65 phosphorylation. However, the Akt inhibition impaired the plasmin-induced IκBα and p65 Ser276 phosphorylation, indicating the Akt-dependent activation of IKK. We have previously shown that plasmin-induced ERK1/2 activation in dendritic cells is Akt dependent [17]. Therefore, Akt might induce the p65 Ser276 phosphorylation via ERK1/2. The activation pathway triggered in dendritic cells by plasmin is different to the IL-17A-induced CCL20 expression in human airway epithelial cells, which is Akt and NF-κB dependent, although both pathways act independently [40]. The plasmin-induced expression of CCL20 in dendritic cells also differed from that in a transformed T-cell line, where Akt inhibition resulted in reduced phosphorylation of p65 on Ser536, whereas the IκBα phosphorylation remained unaffected [62]. Akt might also positively regulate the NF-κB activity through GSK3β inhibition. GSK3β regulates the phosphorylation and function of certain transcriptional coactivators, such as C/EBP and β-catenin, and some transcriptional repressors [40]. Therefore, it is possible that plasmin-induced PI3K/Akt/GSK3β pathway is involved in the modulation of transcriptional activators and/or repressors, which might contribute to the plasminduced expression of CCL20.

In summary, the present study demonstrates that plasmin and dendritic cells colocalize with CCL20 in human atherosclerotic vessels. We also show that plasmin is a potent activator of dendritic cells triggering CCL20 expression by the coordinated activation of Akt, ERK1/2, and NF-κB signaling pathways. Hence, by activating dendritic cells to produce CCL20, locally generated plasmin might control the composition of the cellular infiltrate and modulate inflammatory and immune reactions in atherosclerotic lesions. By contrast, such effects might be rather unlikely during conditions of fibrinolysis, where plasmin in the plasma phase would be spatially separated from inflammatory dendritic cells and rapidly bound to fibrin or quickly inactivated by plasmin inhibitors such as α2-antiplasmin and α2-macroglobulin [1].

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