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Expression of monocyte chemotactic protein-3 mRNA in rat vascular smooth muscle cells and in carotid artery after balloon angioplasty

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Abstract

Monocyte chemotactic protein-3 (MCP-3) is a CC chemokine that functions in chemoattraction and activation of monocytes, T lymphocytes, eosinophils, basophils, natural killer cells and dendritic cells. The activation of the target cells by MCP-3 is via specific chemokine receptors CCR2 and CCR3, of which CCR2 is shared with MCP-1. MCP-1 and CCR2 have been implicated in vascular diseases including atherosclerosis and restenosis, that are known to be involved in inflammation (accumulation of T lymphocytes and monocytes) and smooth muscle cell (SMC) activation (proliferation, migration and matrix deposition). To investigate a potential role of MCP-3 in vascular injury, the present work examined its mRNA expression in rat aortic SMCs stimulated with various inflammatory stimuli including LPS, TNF-α, IL-1β, IFN-γ and TGF-β. A time- and concentration-dependant induction of MCP-3 mRNA in SMCs was observed by means of Northern analysis. A strikingly similar expression profile was observed for MCP-3 and MCP-1 mRNA in SMCs. Furthermore, MCP-3 mRNA expression was induced in rat carotid artery after balloon angioplasty. A significant induction in MCP-3 mRNA was observed in the carotid artery at 6 h (41-fold increase over control, $P < 0.001$), 1 day (13-fold increase, $P < 0.001$) and 3 days (6-fold increase, $P < 0.01$) after balloon angioplasty as quantitated by reverse transcription and polymerase chain reaction. These data provide evidence for the cytokine-induced expression of MCP-3 in SMCs and in carotid artery after balloon angioplasty, suggesting a potential role of MCP-3 in the pathogenesis of restenosis and atherosclerosis.

Keywords: Chemokine; Inflammation; Monocyte chemotactic protein-3; Restenosis; Smooth muscle cell

1. Introduction

Monocyte chemotactic protein (MCP)-3 is a member of the CC or β-chemokine subfamily that includes MCP-1, macrophage inflammatory protein (MIP)-1 and RANTES. The gene encoding for MCP-3 has been isolated in various species including human, mouse and rat [1–3]. The amino acid sequence of MCP-3 consists of 97 residues in human and 95 in mouse and rat, and shares considerable sequencing identity (>60% between rodents and human) or similarity (>86%) [2,3]. MCP-3 is chemotactic preferentially for monocytes, lymphocytes, eosinophils, basophils, natural killer cells and dendritic cells [4,5]. The receptors for MCP-3 have been characterized, which include CCR2 and CCR3 [5]. In particular, CCR2 is known to be shared with

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MCP-1, a chemokine that has been proposed to play an important role in the establishment and progression of atherosclerosis since its mRNA and protein are induced in human atherosclerotic lesions [6,7]. Recently, Boring et al. [8] demonstrated that the selective absence of CCR2 could markedly decrease atherosclerotic lesions in apoE null mice, suggesting the active involvement of CCR2 and MCP-1, and possibly MCP-3, in atherogenesis.

Vascular smooth muscle cell (SMC) is a predominant cell type in atherosclerotic lesions and in neointima after balloon angioplasty [9,10]. SMCs can be activated and induced for proliferation, migration, and production of extracellular matrix by various growth factors and cytokines. SMCs have also been demonstrated to be involved in inflammatory reactions in vitro and in vivo (during vascular injury). SMCs can be induced to produce various inflammatory cytokines (including chemokines) and growth factors, which in turn can react on T lymphocytes, macrophages and SMCs. For example, interleukin (IL)-8 and interferon-inducible protein (IP)-10 have been demonstrated for their ability of chemokine production and actions in SMCs for their migration, proliferation and inflammatory responses [11,12]. The demonstration of functional expression of chemokine receptors (such as CCR1 and CCR2) on human vascular SMCs [13] further supports the possibility that SMCs can react to chemokines, especially during vascular injury.

To investigate a potential role of MCP-3 in vascular pathogenesis, in the present study we examined the expression of MCP-3 mRNA in cultured rat aortic smooth muscle cells (RASMCs) in response to various inflammatory mediators (e.g., lipopolysaccharide (LPS) tumor necrosis factor (TNF)-α, IL-1β, interferon (IFN)-γ and transforming growth factor (TGF)-β) and growth factors (e.g., platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF)). These factors are known to play an important role in SMC activation and in particular during vascular injuries [9,10,14]. Furthermore, we evaluated MCP-3 mRNA expression in vascular disease process using a well characterized animal model of balloon angioplasty of carotid artery.

2. Materials and methods

2.1. Cell cultures

RASMCs were isolated and cultured as described in detail previously [15]. Briefly, RASMCs were isolated from medial explants of the thoracic aorta of male Sprague–Dawley rats (300–350 g) (Charles River, Raleigh, NC) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and gentamicin (50 μg/ml). SMCs were allowed to grow out from the tissue which was consequently removed. After confluence was reached, cells were harvested by brief trypsination and subcultured in T-75 flasks. The purity of SMCs was evaluated by staining the cells with monoclonal antibodies to SMC α-actin (from hybridoma cells, clone asm-1; Boehringer Mannheim) [15]. More than 96% of cells exhibited immunoreactivity for SMC α-actin. RASMC under 6 passages was used in the present study.

RASMCs were cultured in T-150 flasks, grown to confluence and serum-deprived for 48 h, then stimulated with various concentration and time of LPS, IFN-γ, TNF-α, IL-1β and TGF-β1 (purchased from Genzyme, Cambridge, MA or from Gibco BRL, Grand Island, NY) as indicated in the figure legends. Similarly, the serum-deprived RASMCs were stimulated with various concentrations (10^{-10}–10^{-8} mol/l) of PDGF, base FGF (bFGF) and EGF for 0, 1, 3, 6, 14 and 24 h.

2.2. Left common carotid artery balloon angioplasty

Left common carotid artery balloon angioplasty was performed on male Sprague–Dawley rats as described previously [15]. Briefly, following an anterior midline incision, the left external carotid artery was identified and cleared of adherent tissue, allowing the insertion of a 2-F Fogarty arterial embolectomy catheter (Baxter Healthcare, Santa Ana, CA). The catheter was guided a fixed distance (5 cm) down the common carotid arteries to a point such that the tip of the catheter was proximal to the aortic arch where the balloon was inflated and withdrawn

back to its point of insertion. This procedure was performed a total of three times after which the catheter was removed and a suture was tied around the external carotid artery to prevent exsanguination. Finally the wound was closed using 9-mm Autoclips (Clay Adams, Parsippany, NJ). Throughout the surgical procedure, body temperature was maintained at 37 ± 1°C using a K-20-F water blanket (American Hamilton, Cincinnati, OH). All surgical interventions were performed in accordance with the guidelines of the Animal Care and Use Committee, SmithKline Beecham and the American Association for Laboratory Animal Care.

Left common carotid arteries were isolated from rats immediately following exsanguination under sodium pentobarbital anesthesia (65 mg kg^{-1}, i.p.). Vessels that included the areas devoid of endothelium and containing endothelium were removed at the following time points: 0 (control), 6 h, 1, 3, 7 and 14 days after surgery. Once isolated, vessels were immediately frozen in liquid N_2 and stored at −70°C for RNA preparation.

2.3. Isolation of RNA

For RNA preparation, carotid arteries were homogenized, whereas cultured SMCs were directly lysed, in an acid guanidinium thiocyanate solution and extracted with phenol and chloroform as described previously [12].

2.4. Northern blot analysis

RNA samples extracted from cultured RASMCs or from carotid artery after balloon angioplasty were resolved by electrophoresis, transferred onto a GeneScreen Plus membrane (DuPont–New England Nuclear, Boston, MA), and subjected for Northern hybridization as described in detail previously [12]. For Northern analysis, cDNA fragments of rat MCP-3 [3], MCP-1 [16] and ribosomal protein L32 were isolated from electrophoresis and uniformly labeled with [α-^{32}P]dATP (3000 Ci/mmol, Amersham) using a random-priming DNA labeling kit (Boehringer Mannheim). The rpL32 gene is constantly expressed in SMCs and in carotid artery after balloon angioplasty [15] and therefore was used to normalize the differences of the samples loaded in each lane.

2.5. Reverse transcription and polymerase chain reaction

For reverse transcription (RT), total cellular RNA (3 μg/each sample) isolated from 0, 6 h, 1, 3, 7 and 14 days following carotid artery balloon angioplasty was reverse transcribed in the presence of 200 U of RNase H^- SuperScript II reverse transcriptase (Gibco BRL) and 1 μg of oligo$(dT)_{12-18}$ primer at 37°C for 60 min according to manufacturer's specification. The resultant cDNA products were phenol–chloroform extracted and ethanol precipitated. The cDNA pellets were then dried under speed vacuum, resuspended in 120 μl of TE (10 mM Tris–HCl and 1 mM EDTA, pH 7.5) and stored at −20°C until required for PCR amplification.

Polymerase chain reaction (PCR) primers for rat MCP-1 and rpL32 were described previously [12] and the primers for rat MCP-3 are 5′-CATGGAAGTCTGTGCTGAAG-3′ and 5′-TGAAACTTCAGTAGTCATACA-3′, for the upstream and downstream, respectively, corresponding to bases of 295–314 and 769–789 of the mRNA [3]. For quantitative purposes, MCP-3 or MCP-1 cDNA was co-amplified with rpL32 cDNA, an internal control, and the linear amplification was determined as described previously [12]. The following conditions were chosen as standard for PCR reactions in a volume of 50 μl: 100 ng RNA (for RT), 2.5 units of TaqAmpli polymerase (Perkin–Elmer Cetus), 30 cycles of amplification in the presence of 1×10^6 cpm (10 ng) labeled antisense primer for MCP-3 or MCP-1 and 5×10^4 cpm for rpL32 antisense primer together with 100 ng of each non-radioactive sense and antisense primers. The amplification was performed using a thermocycler (Perkin–Elmer Cetus) as described previously [12]. Briefly, the initial PCR amplification was performed as follows: denaturation, 3 min at 94°C; annealing, 1 min at 54°C; extension, 3 min at 72°C. Subsequent cycles of PCR were performed using the following conditions: denaturation, 15 s at 94°C; annealing, 20 s at 54°C; extension, 1 min at 72°C.

The PCR product (10 μl each sample) was electrophoresed using a 6% polyacrylamide gel. The gel was dried and subjected to autoradiography at room temperature for overnight. The identity of amplified cDNA products was confirmed by DNA sequence

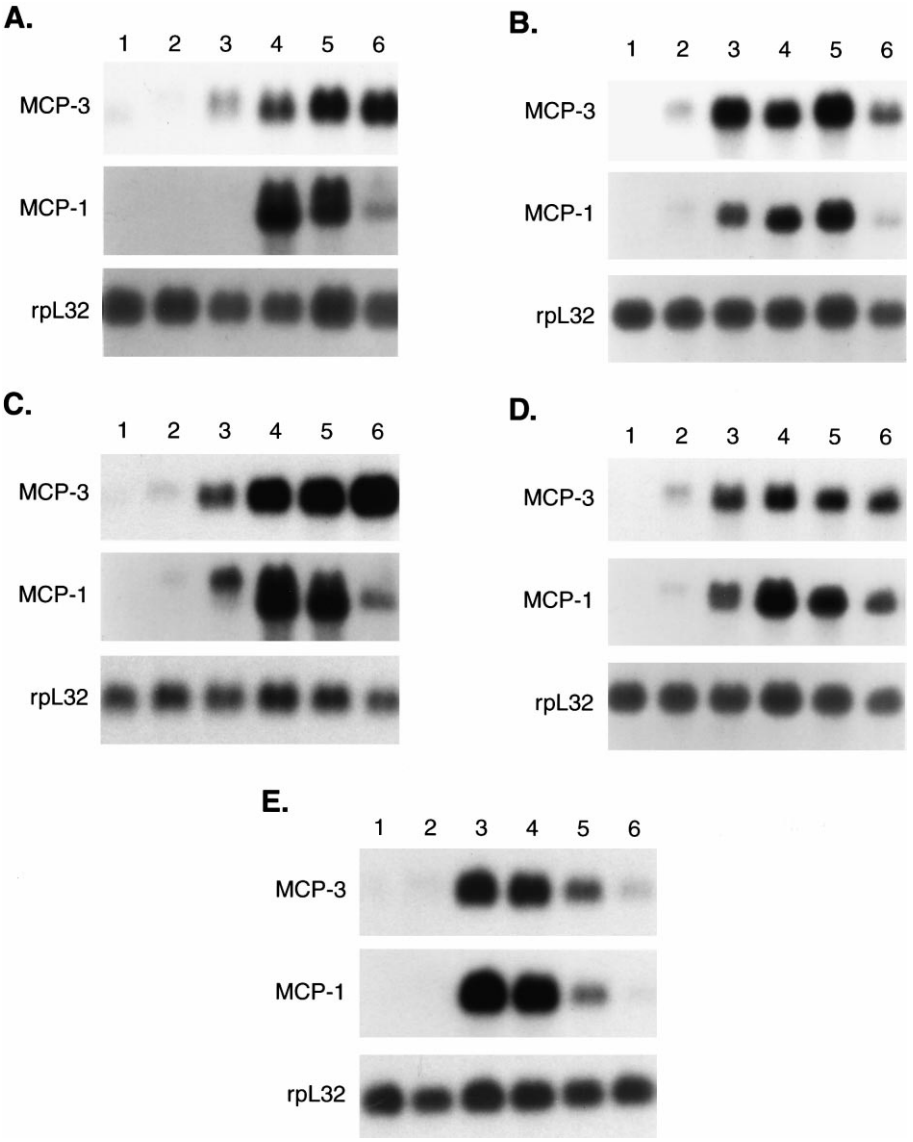

Fig. 1. Northern analysis of time-dependent expression of MCP-3 mRNA in LPS-, IFN-γ-, IL-1β-, TNF-α- and TGF-β1-stimulated RASMCs. RASMCs were grown to subconfluence in T-75 flasks, and made quiescent with serum-free medium for 48 h, then stimulated with 200 ng/ml LPS (A), 100 U/ml IFN-γ (B), 1 nM IL-1β (C) and 5 U/ml TNF-α (D) for 0, 0.5, 1, 3, 8 and 24 h for lanes 1, 2, 3, 4, 5 and 6, respectively, or treated with TGF-β1 (E) for 0, 1, 3, 6, 14 and 24 h in order. Total cellular RNA (20 μg/lane) was resolved by electrophoresis, transferred to a nylon membrane, and hybridized to the indicated cDNA probe.

analysis. The relative band intensities were measured using a PhosphorImager with an ImageQuant software package (Molecular Dynamics, Sunnyvale, CA). The signals of the MCP-3 cDNA were expressed as the relative ratio to those of rpL32 cDNA in each co-amplified sample.

2.6. Statistical analysis

Statistical comparisons were made by analysis of variance (ANOVA; Fisher's protected least squares difference) and values were considered to be significant when $P < 0.05$.

3. Results

3.1. MCP-3 mRNA expression in cultured RASMCs in response to LPS, IFN-γ, IL-1β, TNF-α and TGF-β stimulation

Unstimulated, serum-deprived cultured RASMC expressed only a very low or undetectable level of MCP-3 mRNA (Figs. 1 and 2). In the presence of LPS, IFN-γ, IL-1β, TNF-α and TGF-β, the expression of MCP-3 mRNA in SMCs was markedly increased in a concentration- and time-dependent manner. Fig. 1 illustrates a representative study of three independent experiments ($n = 3$) for the temporal expression of MCP-3 mRNA in RASMCs stimulated with LPS, IFN-γ, IL-1β, TNF-α or TGF-β (Fig. 1). LPS and IL-1β induced a consistent expression of MCP-3 mRNA and reached a highest level at 24 h after stimulation, whereas the induced expression of MCP-3 mRNA by IFN-γ, TNF-α and TGF-β was decreased at 24 h (Fig. 1). Overall, the expression profile of MCP-3 mRNA is similar to that of MCP-1 in these cytokine stimulations, except that MCP-1 mRNA expression was decreased at 24 h in LPS and IL-1β treated cells (Fig. 1). Fig. 2 illustrates a representative blot for MCP-3 mRNA expression in RASMCs stimulated at low and high concentration of cytokines for 3 and 8 h. These data also allowed us to make a direct comparison for the relative levels of MCP-3 mRNA induction in response to various stimuli.

3.2. Effect of growth factors on MCP-3 mRNA expression in RASMCs

To determine whether growth factors can affects MCP-3 mRNA expression in SMCs, cultured RASMCs were stimulated with various concentrations of PDGF, bFGF and EGF for 30 min–24 h and evaluated by Northern hybridization. Very low and inconsistent levels of MCP-3 mRNA expression was observed (data not shown) in these growth factor-stimulated RASMCs, while a strong induction was observed in these samples for positive controls (c-*fos* and osteopontin) as demonstrated previously [15].

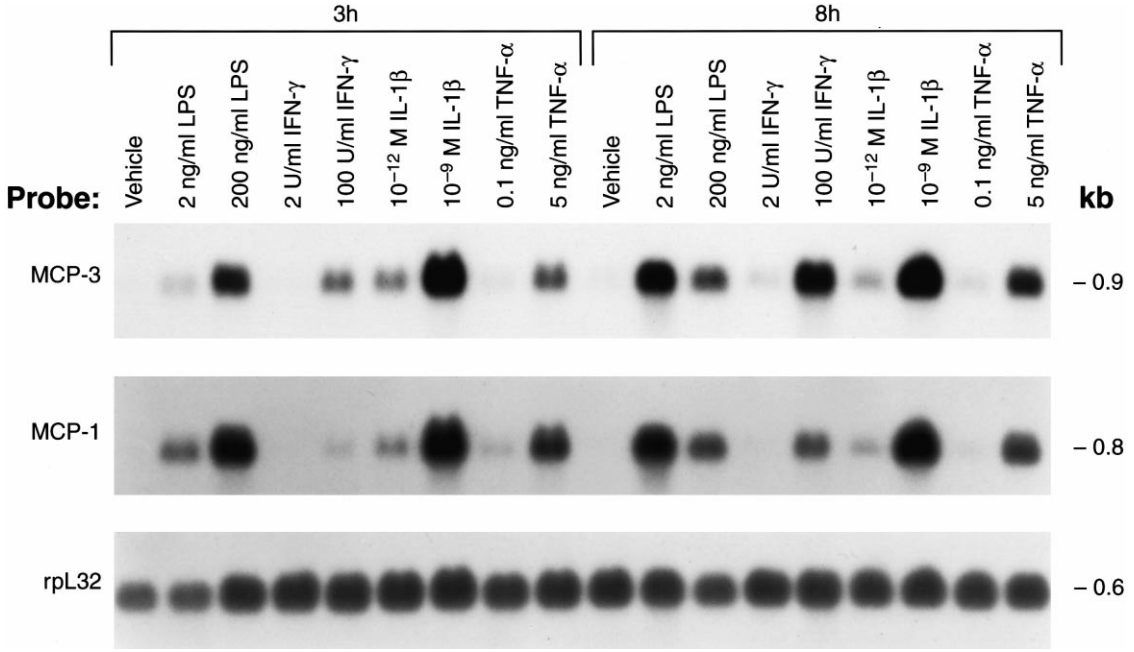

Fig. 2. Concentration-dependent induction of MCP-3 mRNA in LPS-, IFN-γ-, IL-1β- and TNF-α-stimulated RASMCs. RASMCs were deprived of serum for 48 h and stimulated with various stimuli for the indicated concentration for 3 and 8 h, and analyzed by Northern hybridization.

3.3. MCP-3 mRNA expression in rat carotid artery following balloon angioplasty

Since a similar expression profile of MCP-3 and MCP-1 was observed in RASMCs in response to cytokine stimulation and induced expression of MCP-1 mRNA was demonstrated previously following balloon angioplasty [12], in the present study we examined the MCP-3 mRNA expression in rat carotid artery following balloon angioplasty using semi-quantitative RT–PCR technique (Fig. 3A). The data, after normalizing to rpL32 mRNA, are depicted in Fig. 3B. A very low level of MCP-3 mRNA expression was observed in normal carotid artery. Similar to MCP-1, maximal MCP-3 mRNA induction was observed immediately after balloon angioplasty, i.e., at 6 h, with 41-fold increase of the mean value over control, $P < 0.001$. The significant induction in MCP-3 mRNA in carotid artery was observed at 1 day (13-fold increase, $P < 0.001$) and sustained up to 3 days (6-fold increase, $P < 0.01$). The MCP-3 induction after balloon angioplasty was further confirmed by Northern analysis (Fig. 4). As shown in Fig. 4, only a very low level of MCP-3 expression was observed at time 0, but the signal was peaked at 6 h and then diminished at 3 days after balloon injury. This expression profile is strikingly similar to the PCR data.

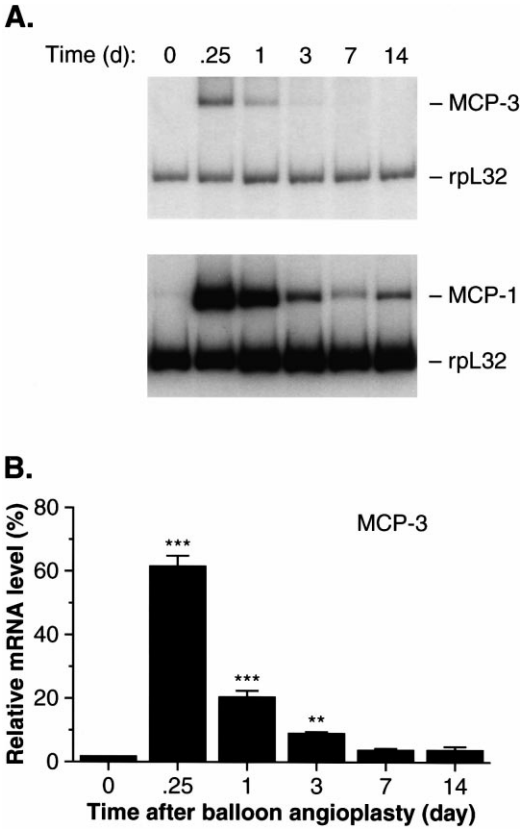

Fig. 3. Time-course study of MCP-3 mRNA expression in rat carotid artery following balloon angioplasty. Semi-quantitative RT–PCR was used to analyze the expression of MCP-3 mRNA in rat carotid artery after balloon angioplasty as described in detail in Section 2. (A) Representative autoradiographs of the PCR products (10 μl/lane) for MCP-3 and MCP-1, respectively, co-amplified with rpL32 using the standard conditions. (B) Quantitative data of MCP-3 mRNA expression in rat carotid artery following balloon angioplasty. The quantitation was carried out using PhosphorImager analysis, the ratio of MCP-3 to rpL32 was determined based upon each co-amplified sample, and the relative levels (illustrated as the ratios) of MCP-3 mRNA are depicted. Data are presented as the mean ± standard errors of five separate samples/experiments and each sample was pooled from three animals (i.e., 15 animals for each time point). $^{**}P < 0.01$, $^{***}P < 0.001$, compared to the controls (time = 0).

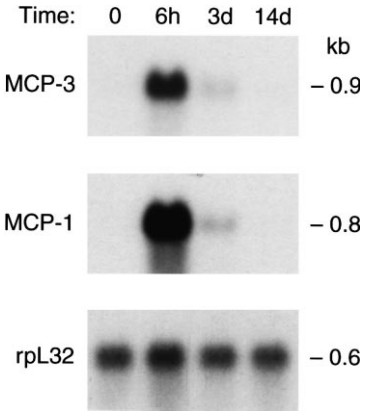

Fig. 4. Northern analysis of MCP-3 mRNA expression in rat carotid artery following balloon angioplasty. Total cellular RNA was prepared from the pooled samples (10 animals for each time point at 0, 0.25, 3 and 14 days following angioplasty) and 10 μg per lane was resolved by electrophoresis and subjected for Northern analysis as described in Fig. 1. The exposure time for the autoradiograms was 3, 2 and 2 days for the MCP-3, MCP-1 and rpL32 probes, respectively.

4. Discussion

Chemokines are proinflammatory cytokines that are traditionally known to play an important role in leukocyte chemoattraction and activation. Additional functions of chemokines have recently been

demonstrated. For example, HIV-1 infection of target cells has been shown to interact through specific chemokine receptors such as CCR3 and CCR5 [17]. Recently, chemokines have been implicated in the pathogenesis of vascular injury including atherosclerosis and restenosis. Several chemokines including MCP-1, IL-8 and RANTES have been demonstrated for their induced expression in atherosclerotic vessels [6,7,18,19]. Some chemokines such as MCP-1 and IP-10 are upregulated in neointimal formation [12,20]. On the other hand, functional chemokine receptors have been identified on cell surfaces of T lymphocytes and monocytes [5], as well as on vascular SMCs [13]. In particular, the absence of CCR2 (the receptor for MCP-1 and -3) was found to be critical in the development of atherosclerotic lesions [8], suggesting that its corresponding ligands are likely participating vascular diseases/injuries.

MCP-3 is known to share some key biological features with MCP-1; however, its expression and potential function in SMCs and vascular pathology have not been explored. To investigate the potential role of MCP-3 in vascular injury, the present study examined the expression of MCP-3 mRNA in vascular SMCs in response to various inflammatory mediators that are involved in vascular disease process [9,10,14]. Our data demonstrated that MCP-3 mRNA expression can be markedly induced in SMCs by these inflammatory cytokines and in the carotid artery after balloon angioplasty. The expression profile of MCP-3 under these conditions parallels to that of MCP-1, suggesting that both CC chemokines may play an important role in vascular injury such as atherosclerosis and restenosis. While both chemokines induced in these experimental conditions, they may play distinct roles in vascular injury since they have unique preferences to chemokine receptors, i.e., both chemokines react to CCR2 but only MCP-3 for CCR3. In addition, the different time-course expression was observed for MCP-1 and MCP-3 in SMCs in response to LPS and IL-1β stimulation (Fig. 1). Previous studies revealed a similar but distinct expression profile of these two cytokines in monocytes, with a more restricted cellular expression for MCP-3. For example, MCP-1 is highly expressed in fibroblasts in response to IL-1 and TNF stimulation but MCP-3 only at very low level [1].

The time-course studies revealed that MCP-3 mRNA expression in carotid artery after balloon angioplasty represents a first wave of injury response (0–3 days), with the characteristic of medial SMCs proliferation [10]. At this early time point, FGF, PDGF, TGF-β and angiotensin II are thought to play an important role. Similar temporal expression file was observed for MCP-1 mRNA after balloon angioplasty in rat [12,21]. While the exact biological function of MCP-3 and MCP-1 induction following vascular injury remains to be further investigated, recent data using neutralizing antibodies against MCP-1 suggest that MCP-1 may contribute to the accumulation of macrophages and the early increase of vascular SMCs in the intima after balloon angioplasty [21]. These data are in agreement with the previous localization and potential role of monocytes/macrophages in the vascular tissue after balloon angioplasty [22,23] in addition to the fact that SMC proliferation and migration are classically thought to be the major cellular response for restenosis. The induced expression of MCP-3 mRNA after vascular injury suggests that this chemokine may play a similar role in macrophage accumulation and SMC activation in the injured vascular tissue. It also should be pointed out that the possibility of the increased levels of MCP-3 mRNA to be translated into active protein after balloon injury remains to be demonstrated. It is also important to define the cellular sources of MCP-3 after vascular injury in order to elucidate the role of MCP-3 following balloon angioplasty.

In summary, the present study demonstrated MCP-3 mRNA expression in vascular SMCs in response to inflammatory cytokines and in carotid artery after balloon angioplasty, suggesting that MCP-3, in addition to MCP-1, may play an active role in the accumulation of monocytes/macrophages and in the SMC activation after vascular injury. Further studies remain to be carried out in order to validate the exact role of this chemokine in the neointima formation.

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