Endogenous VEGF-A is responsible for mitogenic effects of MCP-1 on vascular smooth muscle cells

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Parenti, Astrid, Lydia Bellik, Laura Brogelli, Sandra Filippi, and Fabrizio Ledda. Endogenous VEGF-A is responsible for mitogenic effects of MCP-1 on vascular smooth muscle cells. Am J Physiol Heart Circ Physiol 286: H1978–H1984, 2004. First published December 23, 2003; 10.1152/ajpheart.00414.2003.—Vessel wall remodeling is a complex phenomenon in which the loss of differentiation of vascular smooth muscle cells (VSMCs) occurs. We investigated the role of rat macrophage chemoattractant protein (MCP)-1 on rat VSMC proliferation and migration to identify the mechanism(s) involved in this kind of activity. Exposure to very low concentrations (1–100 pg/ml) of rat MCP-1 induced a significant proliferation of cultured rat VSMCs assessed as cell duplication by the counting of total cells after exposure to test substances. MCP-1 stimulated VSMC proliferation and migration in a two-dimensional lateral sheet migration of adherent cells in culture. Endogenous vascular endothelial growth factor-A (VEGF-A) was responsible for the mitogenic activity of MCP-1, because neutralizing anti-VEGF-A antibody inhibited cell proliferation in response to MCP-1. On the contrary, neutralizing anti-fibroblast growth factor-2 and anti-platelet-derived growth factor-bb antibodies did not affect VSMC proliferation induced by MCP-1. RT-PCR and Western blot analyses showed an increased expression of either mRNA or VEGF-A protein after MCP-1 activation (10–100 pg/ml), whereas no fms-like tyrosine kinase (Flt)-1 receptor upregulation was observed. Because we have previously demonstrated that hypoxia (3% O2) can enhance VSMC proliferation induced by VEGF-A through Flt-1 receptor upregulation, the effects of hypoxia on the response of VSMCs to MCP-1 were investigated. Severe hypoxia (3% O2) potentiated the growth-promoting effect of MCP-1, which was able to significantly induce cell proliferation even at a concentration as low as 0.1 pg/ml. These findings demonstrate that low concentrations of rat MCP-1 can directly promote rat VSMC proliferation and migration through the autocrine production of VEGF-A.

METHODS

Animals. This investigation conforms with the Guide for the Care and Use of Laboratory Animals [published by National Institutes of Health (NIH Publication No. 85-23, Revised 1996)]. Male Wistar rats (body wt, 220–250 g) were purchased from Harlan (S. Pietro al Natisone, Italy).

Cell culture and proliferation. VSMCs were isolated from the thoracic aorta of male Wistar rats and cultured as previously described (24). Cell proliferation was quantified by the total cell number as previously reported (24). Briefly, 5 × 10³ cells in 5% bovine calf serum (BCS) were seeded onto 48-well plates and allowed to adhere overnight. Cells were then kept in starvation conditions (0.1% BCS) for 48 h, and media were successively replaced with 1% BCS medium (DMEM) that contained rat MCP-1 (1–100 pg/ml). After 96 h, cells were fixed with methanol and stained with Diff-Quick. Proliferation was evaluated as cell duplication by the counting of adherent cells in culture. Endogenous VEGF-A was responsible for the mitogenic activity of MCP-1, because neutralizing anti-VEGF-A antibody inhibited cell proliferation even at a concentration as low as 0.1 pg/ml. These findings demonstrate that low concentrations of rat MCP-1 can directly promote rat VSMC proliferation and migration through the autocrine production of VEGF-A.

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neuroprobes) were used for chemotaxis studies. Polyvinyl pyrrolidone-migration. Methanol-

Fbb, 5 ng/ml) was chosen as a positive control, and the effect of 1%

C for 4 h. Platelet-derived growth factor-bb (PDGF-

°

incubated at 37°C and 37

°

C, 30 s of annealing conditions at 55

°

C, and 30 s of extension at

°

94

°

C. The Flt-1 receptor was ampli-

of extension at 72

°

C. The Flt-1 receptor was ampli-

on 10%

als and quantitative evaluations were obtained as ratios between the

products.

Effects of MCP-1 on VSMC proliferation. Serum-starved rat VSMCs were stimulated with increasing concentrations of rat MCP-1 (0.1–100 pg/ml) and left in culture for 4 days. Proliferation was assessed as the total number of cells counted in each well. PDGFb (5 ng/ml) was chosen as a positive control owing to its well-known mitogenic effect on VSMCs. MCP-1 induced VSMC proliferation in concentrations as low as 1 pg/ml (27 ± 10% over basal proliferation, n = 5), as shown in Fig. 1. The maximum stimulating effect, which was observed at 100 pg/ml, amounted to 47 ± 6% over basal proliferation and ~80% of the effect obtained with PDGFb, which increased proliferation by ~83%. The increase in MCP-1 concentration to 1 ng/ml did not induce any additional stimulation of VSMC proliferation (data not shown).

Effects of MCP-1 on VSMC migration. The ability of MCP-1 to stimulate either chemotaxis of single cells in suspension or migration of cultured cells was assessed. It was observed that MCP-1, when used at the same concentrations tested on cell growth assays, slightly stimulated VSMC chemotaxis in a statistically insignificant manner (data not shown). Therefore, the effects of MCP-1 on anchorage-dependent cell movement and proliferation were investigated by using an experimental model that allows measurement of the combination of migration and proliferation of adherent cells in culture. Under control conditions (1% BCS), cell migration progressed up to 388 ± 24 μm from the starting line; this distance was almost tripled by the addition of PDGFb (912 ± 70 μm), which was taken as a positive control. MCP-1, at concentrations ranging from 1 to 100 pg/ml, stimulated the progression of a significant number of cells from the starting line toward the periphery with maximal effects observed at 10 pg/ml (Fig. 2, A and D). Under these conditions, the distance covered by the cell
sprouting from the edge increased by 43% over control unstimulated cells; this distance amounted to ~60% of the value recorded with PDGFbb. MCP-1 (10 pg/ml) also significantly increased the total number of cells compared with control unstimulated cells (Fig. 2, B and D), thus showing that proliferation plays a role in this experimental model.

Effects of anti-VEGF-A antibody on VSMC growth in culture. Because we have previously shown that rat VSMCs may be responsive to VEGF-A (25), and because it is known that VSMCs produce this growth factor (21, 32), we investigated whether a cooperation between MCP-1 and endogenous VEGF-A exists. The results of this study showed that the growth-promoting effect of MCP-1 on VSMCs was mediated by endogenous VEGF-A, because the addition of a neutralizing VEGF-A antibody (1 μg/ml) significantly inhibited VSMC proliferation induced by all of the MCP-1 concentrations, whereas nonimmune IgG was ineffective. Moreover, the antibody did not affect basal proliferation (Fig. 3A). The possible role of other endogenous mitogens such as FGF-2 and PDGFbb were then assessed, as it is known that these growth factors may stimulate VSMC growth in an autocrine manner (20, 24, 26). The neutralizing anti-FGF-2 antibody did not influence the proliferative effect of MCP-1 on VSMCs as shown in Fig. 3B.

In the same manner, the block of endogenous PDGFbb did not influence cell proliferation in response to MCP-1 (data not shown).

Effects of MCP-1 on VEGF-A and Flt-1 receptor production. We tested the hypothesis that MCP-1-induced VSMC proliferation was associated with VEGF-A upregulation. Serum-starved cells were stimulated with MCP-1 (10–100 pg/ml), and VEGF-A production was measured as either mRNA or protein. Immunoblotting of unstimulated rat VSMCs showed a double band for VEGF-A (nonreducing conditions) located at 46 kDa, which is higher than the human recombinant one as previously detected by Zheng et al. (36). The addition of MCP-1 (10–100 pg/ml) to starved VSMCs significantly increased VEGF-A production. The increase was maximal after 8 h of exposure and remained high after overnight incubation (Fig. 4).

To assess whether VEGF-A upregulation by MCP-1 reflected an increase in the steady-state levels of VEGF-A mRNA, differential RT-PCR analysis was performed. As shown in Fig. 5A, 6 h of exposure to MCP-1 (10–100 pg/ml) significantly increased all VEGF-A mRNA isoforms. The effect was particularly evident for the VEGF145 and VEGF165 isoforms, which are almost undetectable in unstimulated cells...
Because we previously demonstrated that VEGF-A stimulates VSMCs by acting through Flt-1 receptors (25), the expression of these receptors was also measured in response to MCP-1. MCP-1 did not increase mRNA Flt-1 expression either after a 6-h stimulation (Fig. 5A) or after overnight stimulation (data not shown).

**Effects of hypoxia on VSMC proliferation in response to MCP-1.** The data reported above show that MCP-1 proliferation is partially due to VEGF-A upregulation in VSMCs. Because we recently demonstrated that VEGF-A is able to increase VSMC mitogenesis after severe hypoxia through an upregulation of Flt-1 receptor (25), the effects of hypoxia on MCP-1-induced mitogenesis of VSMCs were investigated. When cells were exposed for 48 h to severe hypoxia (3% O2) and then stimulated with increasing concentrations of MCP-1, potentiation of VSMC proliferation was observed. In fact, the chemokine induced statistically significant cell proliferation under these experimental conditions at concentrations as low as 0.1 pg/ml (31 ± 8% over basal proliferation; n = 3; Fig. 6).

**DISCUSSION**

This study focuses on the still-debated problem of mitogenic activity of MCP-1 on VSMCs and demonstrates that this chemokine promotes in vitro proliferation of rat VSMCs at very low concentrations (picograms per milliliter) on the condition that species specificity is respected. In addition, it shows that direct activity of MCP-1 on VSMCs promotes VEGF production and that inactivation of this endogenous factor inhibits MCP-1-induced effects.

MCP-1 was previously shown by different authors to either stimulate or inhibit VSMC growth; these findings were obtained by using human MCP-1 on rat (12, 27) or rabbit (34).
VSMCs. More recently, Selzman et al. (31) have stressed the importance of species specificity for MCP-1 activity on VSMC proliferation in experiments where human recombinant MCP-1 was used to stimulate human VSMC growth. The discrepancy between the results obtained in previous studies and those observed in the present work are probably due to differences in experimental planning because species homology was respected in our study in which rat VSMCs were stimulated with rat MCP-1. A growth-promoting effect on rat VSMCs was previously observed by Porreca et al. (27) only in the presence of very high human-MCP-1 concentrations (>100 ng/ml). This observation suggests that differences may exist between rat and human CC chemokine receptor-2 (CCR2). Although human VSMCs were recently demonstrated to possess CCR2 receptors (11), no information has yet been reported regarding CCR2 receptors and rat VSMCs. Moreover, it has been suggested (29) that the receptor of human VSMCs may be different from that of monocytes. The low concentrations of MCP-1 found to be effective in our study seem to be physiologically relevant, because it was recently reported that the MCP-1 concentration increases to nearly 100 pg/ml in injured vessels (8).

VSMC proliferation and migration are key events during pathological vessel-wall remodeling. Expansive remodeling, with lumen enlargement, also occurs during arteriogenesis, which is a process that compensates for a persistent flow insufficiency by increasing collateral flow (5). MCP-1 is well known to be implicated in all of these processes, and in vivo studies (15) have shown that vessel walls express MCP-1 within 4 h of endothelium removal. MCP-1 is also secreted by VSMCs in atherosclerotic plaques as well as by endothelial cells and macrophages; this suggests that MCP-1 may contribute to the pathogenesis of atherosclerosis (30). Moreover, MCP-1 has been colocalized in both resident and inflammatory cells in plaque (17). In several vascular experimental models, antagonism of MCP-1 or its receptor CCR2 appears to inhibit lesion development (3, 8). Aside from its putative role in atherosclerosis, MCP-1 represents a potent stimulus for arteriogenesis; it has been demonstrated (13) that MCP-1 is able to increase collateral vessel development after arterial occlusion in experimental models in vivo. However, although MCP-1 has arteriogenic properties (28), its beneficial potential in the course of ischemia may be hampered by its inflammatory and profibrotic effects. MCP-1 activity is attributed to its effect on mononuclear cells, which are recruited at the site of injury where they become macrophages that are able to secrete numerous cytokines and growth factors (28). Nevertheless, on the basis of recent experimental findings including the present one, a direct, monocyte-independent effect of MCP-1 on VSMC growth can be suggested. In fact, present data demonstrate that MCP-1 is able to promote migration and/or proliferation of cultured VSMCs in a bidimensional lateral-sheet migration model (1, 23). Chemotaxis of VSMCs was previously found to be unaffected by MCP-1 (8). Our present data obtained with a modified Boyden chamber are in agreement with those of Furukawa et al. (8), in that no significant VSMC chemotaxis was detectable even in the presence of MCP-1 concentrations that are able to stimulate VSMC growth. However, it is noteworthy that we were able to demonstrate activity of MCP-1 on VSMCs by using an experimental model that allows measurement of the combination of migration and proliferation of adherent cells in culture. In fact, growth-arrested cells cultured in silicon gaskets responded to MCP-1 by forming a significant sprouting when the gasket was removed. This was a potent effect, because the maximum activity was produced by 10 pg/ml MCP-1. On the basis of these findings, we suggest that MCP-1 can directly activate VSMCs and thus induce both proliferation and migration; it can be speculated that this mechanism, in association with the well-known monocyte recruitment effect, is involved in vascular wall remodeling. However, the major finding of the present study consists of the original observation that the growth-promoting effect of MCP-1 is mediated by the production of endogenous VEGF-A. During vascular remodeling, VEGF-A is secreted by many cell types and is involved in the modulation of angiogenesis and re-endothelialization of injured vessels (7, 16). The strong and specific activity on endothelial cells displayed by VEGF-A has led to a proposal (19) that this growth factor can be considered a therapeutic agent for inducing angiogenesis in hypoxic tissues of patients suffering from coronary artery and peripheral vascular diseases. We recently demonstrated (25) that experimental conditions mimicking vascular injury increase the responsiveness of VSMCs to VEGF-A. Results from the present study clearly show that the proliferative effect of MCP-1 is partially dependent on endogenous VEGF-A production, because a VEGF-A-neutralizing antibody was able to significantly inhibit VSMC proliferation. In agreement with this observation, an upregulation of this growth factor, measured either as mRNA or protein, was observed in VSMCs exposed to MCP-1 at picogram-per-milliliter concentrations. On the contrary, an upregulation of Flt-1 was not observed, which thus excludes the possibility that MCP-1 effects may be linked to upregulation of VEGF receptors in VSMCs. Also the demonstration that significant cell stimulation was detected only in experiments on adherent cells in culture is in line with our hypothesis that the effects of MCP-1 are mediated by endogenous VEGF-A production. In fact, it can be expected that the 4-h incubation period of cell chemotaxis experiments may be inadequate to induce a significant VEGF-A upregulation. Conversely, a significant production of endogenous factor can be expected in the three-day

![Fig. 6. Effects of hypoxia on VSMC proliferation in response to MCP-1. Cells were starved (0.1% BCS) for 48 h under hypoxic conditions (3% O2) and then stimulated with MCP-1 under normoxic conditions. Proliferation is expressed as number of cells counted per well. Bars represent means ± SE of three experiments in triplicate. **P < 0.01; ***P < 0.001 vs. basal unstimulated cells.](http://ajpheart.physiology.org/)
observation period used in the experiments on two-dimen-
sional lateral-sheet migration of adherent cells in culture.

In the present study, the hypothesis that the proliferative
effects of MCP-1 may be mediated by endogenous FGF-2
and PDGFbb was also tested, because it is well known that
these growth factors have a prominent role in VSMC pro-
iferation (20, 24, 26). It was found that unlike endogenous
VEGF-A, neither FGF-2 nor PDGFbb were involved in the
growth-promoting effects of MCP-1, in that the neutralizing
anti-FGF-2 and anti-PDGFbb antibodies did not modify the
effects of the chemokine. Finally, potentiation of the cell
growth-promoting effects induced by MCP-1 was observed
in cells previously exposed to severe hypoxia. This finding
reinforces the conclusion reached in this study; we have
recently shown (25) that exposure to hypoxia induces up-
regulation of the VEGF receptor Flt-1 in VSMCs. Thus it is
conceivable that a condition that increases the cell respon-
siveness to VEGF-A could enhance the proliferative re-
sponse to an agent such as MCP-1, which acts through
VEGF-A secretion.

In conclusion, all of these observations support the hypoth-
esis that MCP-1 exerts direct monocyte-activity-independent
stimulating effects on VSMCs, and that these effects are
mediated by the induction of VEGF-A, which in turn is
responsible for an autocrine-paracrine stimulation of VSMC
function by interaction with VEGF-A receptors. Moreover, the
demonstration that MCP-1 potently activates VSMC prolifer-
ation and reorganization via endogenous VEGF-A production
adds to our knowledge on the role of the chemokine in vascular
remodeling and reinforces the hypothesis that autocrine regu-
lation of VSMC functions may be involved in pathological
processes characterized by vascular remodeling.

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