Monocyte chemotactic protein-1 directly induces human vascular smooth muscle proliferation

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THE INFLAMMATORY RESPONSE to vascular injury has evolved into a unifying theme in the pathogenesis of intimal hyperplasia (19, 22). Both direct (angioplasty) and indirect (nicotine) insults promote endothelial cell damage and dysfunction. The wounded lining of the vessel thus serves as a nidus for adherence of circulating platelets, monocytes, and lymphocytes with subsequent release of multiple cytokines, chemokines, and growth factors. Vascular smooth muscle cells (VSMC) respond to these mitogens by proliferating, migrating into the intima, and secreting matrix products (12). Monocyte chemotactic protein-1 (MCP-1) is one member of a large family of endogenous chemokines that recruits circulating monocytes to areas of vessel injury (17, 18). On adherence, monocytes transmigrate into the vessel wall where they phenotypically transform into macrophages. In addition to acting as reservoirs for other cytokines and growth factors, macrophages ingest cholesterol and oxidize lipids to assist in the development and instability of atheromatous plaques. In several vascular experimental models, antagonism of MCP-1 or its receptor, CCR2, appears to inhibit lesion development (2, 5, 7, 8). Cumulatively, these investigators target the influence of MCP-1 on mononuclear cell recruitment as the effector of vascular protection.

Whereas its chemotactic properties are well recognized, we sought to investigate the influence of MCP-1 on another aspect of vascular injury, specifically VSMC proliferation. Indeed, VSMC not only secrete MCP-1 (29) but also express the CCR2 receptor (9). To our knowledge, three studies examined the effect of MCP-1 on VSMC proliferation in vitro. These studies utilized models of rat VSMC and offer conflicting conclusions: MCP-1 either is a mitogen (14), has no effect (30), or inhibits VSMC proliferation (10). The purpose of the present study was to determine the direct influence of MCP-1 on human VSMC proliferation.

MATERIALS AND METHODS

Cells isolation and culture. Human VSMC were isolated from the thoracic aorta and iliac vessels of transplant donors as previously described (21). Procurement of human VSMC was approved by the Colorado Multiple Institutional Review Board (no. 96-161). Purity of isolation was demonstrated by the typical “hill and valley” morphology revealed by phase-contrast microscopy with uniform phalloidin staining for F-actin and α-smooth muscle actin (Sigma Immunochemicals; St. Louis, MO). Routine staining for von Willebrand factor and CD14 (Sigma) consistently demonstrated the lack of endothelial cell or macrophage contamination, respectively. VSMC were grown to confluence in a tissue culture flask in a 37°C, 5% CO2 incubator with “complete media.” Complete media consisted of 5% fetal bovine serum (Summit Biotechnology, Ft. Collins, CO), 5% human cord serum (graciously
incubated with 200 µl of 0.05% trypsin for 5 min at 37°C. After the trypsin was deactivated with 50 µl of fetal bovine serum, cells were aspirated into tubes and centrifuged at 1,100 rpm for 5 min. The supernatant was decanted, and cells were resuspended in 1 ml of PBS. VSMC were then counted by using a Coulter model ZM analyzer (Coulter; Hialeah, FL). Each experiment was done in duplicate on three separate occasions.

Immunohistochemistry. VSMC were plated with complete media on Lab-Tek Chamber Slides (Nalge Nunc International; Naperville, IL). Twenty four hours later, serum-free medium was substituted to allow growth arrest. Forty-eight hours after stimulation, VSMC were washed twice with PBS (Sigma) and incubated with 200 µl of 0.05% trypsin for 5 min at 37°C. After the trypsin was deactivated with 50 µl of fetal bovine serum, cells were aspirated into tubes and centrifuged at 1,100 rpm for 5 min. The supernatant was decanted, and cells were resuspended in 1 ml of PBS. VSMC were then counted by using a Coulter model ZM analyzer (Coulter; Hialeah, FL). Each experiment was done in duplicate on three separate occasions.

Cell counting. VSMC were plated at a density of 20,000 cells/well on 24-well plates with complete media for 24 h. Media were substituted with serum-free media for 48 h to allow synchronous growth arrest. Forty-eight hours after stimulation, VSMC were washed twice with PBS (Sigma) and incubated with 200 µl of 0.05% trypsin for 5 min at 37°C. After the trypsin was deactivated with 50 µl of fetal bovine serum, cells were aspirated into tubes and centrifuged at 1,100 rpm for 5 min. The supernatant was decanted, and cells were resuspended in 1 ml of PBS. VSMC were then counted by using a Coulter model ZM analyzer (Coulter; Hialeah, FL). Each experiment was done in duplicate on three separate occasions.

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PDGF (10 ng/ml) (25) to demonstrate the relative potency of MCP-1 as a direct proliferative agent. To find the optimal dose of MCP-1, we performed dose-response experiments by using BrdU uptake as a marker of DNA synthesis. As shown in Fig. 1, MCP-1-induced concentration-dependent BrdU uptake. As expected, PDGF stimulated VSMC proliferation compared with control (1.9 ± 0.21 vs. 1.00 ± 0.01, P < 0.05). Compared with control VSMC, MCP-1-induced VSMC proliferation in concentrations as low as 0.1 ng/ml (1.40 ± 0.14, P < 0.05 vs. control). Maximal MCP-1 stimulation was observed at 1 ng/ml (1.65 ± 0.16, P < 0.05 vs. control). Higher doses of MCP-1 appeared to have no influence on VSMC proliferation. Quantitatively, PDGF did promote more vigorous BrdU uptake than VSMC stimulated with MCP-1; however, this difference was not significant. From these results, a concentration of 1 ng/ml of MCP-1 was used in subsequent experiments.

Whereas MCP-1-induced BrdU uptake, we sought to validate this assay of VSMC proliferation by also directly counting cell numbers following stimulation (Fig. 2). Compared with control, MCP-1-induced VSMC proliferation was easily detected at 0.1 ng/ml (1.65 ± 0.16, P < 0.05 vs. control). Higher doses of MCP-1 appeared to have no influence on VSMC proliferation. Quantitatively, PDGF did promote more vigorous BrdU uptake than VSMC stimulated with MCP-1; however, this difference was not significant. From these results, a concentration of 1 ng/ml of MCP-1 was used in subsequent experiments.

MCP-1 and intracellular signaling. We next sought to examine the influence of MCP-1 on two intracellular signaling pathways that are known to be important to VSMC proliferation. To assess the influence of MCP-1 on NF-κB, we stimulated VSMC and measured NF-κB activity by using an enzyme-linked immunosorbent assay (Fig. 5). We utilized a newer immunoassay that is 10-fold more sensitive than the traditional electromobility shift assay to quantitate activation of NF-κB (15). MCP-1-induced VSMC proliferation with PDGF had a vigorous NF-κB response compared with control cells (0.41 ± 0.32 vs. 0.09 ± 0.01 mean λ/sample, P < 0.05). This effect was similar to that seen with a classic NF-κB stimulus, LPS (100 ng/ml). Interestingly, MCP-1 had no effect on nuclear translocation of the NF-κB p65 subunit (0.12 ± 0.02). These results represent experiments performed on two separate VSMC donors, in duplicate through two separate passages.

To interrogate the importance of phosphotidylinositol 3-kinase (PI3 kinase) signaling to MCP-1-induced
VSMC proliferation, we treated VSMC with a specific PI3 kinase antagonist, LY-294002 (Santa Cruz Biotechnology) and measured BrdU uptake (Fig. 6). As previously demonstrated, MCP-1 increased BrdU uptake compared with control (0.25 ± 0.03 vs. 0.44 ± 0.06 \( P < 0.05 \)). When treated with LY-294002, MCP-1 invoked BrdU uptake only to the level of control (0.21 ± 0.02). When given to control cells, LY-294002 did not appear to affect BrdU uptake (0.24 ± 0.03 \( \lambda \)).

**DISCUSSION**

Three studies have previously investigated the influence of MCP-1 on VSMC proliferation in vitro, and they offer disparate conclusions. Ikeda and colleagues (10) demonstrated dose-dependent inhibition of \(^{3}H\)thymidine uptake in rat aortic VSMC after exposure to MCP-1. They reported maximal inhibition at a dose of 100 ng/ml; no difference was identified at our ideal dose of 1 ng/ml. Direct cell counting was performed, but an inhibitory effect was not detected until 4 days of culture. Additional experiments suggested that their observations were not related to VSMC release of prostaglandins or nitric oxide. Porreca and colleagues (14) similarly evaluated the influence of MCP-1 on rat aortic VSMC proliferation. Compared with Ikeda’s group, these investigators altered culture conditions: longer preincubation, longer phase of cell quiescence, and lower cell density. They demonstrated a dose-dependent increase in VSMC proliferation as assessed by both \(^{3}H\)thymidine uptake and cell numbers. A maximal effect was observed at 100–200 ng/ml. Most recently, Watanabe and colleagues (30) investigated the interaction between MCP-1 and serotonin on rabbit aortic VSMC proliferation. These investigators demonstrated no effect of MCP-1 (dose range 25–200 ng/ml) on VSMC proliferation as assessed by \(^{3}H\)thymidine uptake. MCP-1 did, however, augment the mitogenic influence of serotonin.
In the present study, we demonstrate that MCP-1 induces human VSMC proliferation as assessed by BrdU uptake and cell counting. From previously conflicting reports, our results must be interpreted with several caveats. In their discussion, Porreca and colleagues (14) mention the importance of specific culture criteria in distinguishing their findings from Ikeda’s study. Indeed, we performed our experiments after a 48-h period of growth arrest. Care was also taken to seed VSMC to avoid confluency before stimulation. Certainly, variations in culture technique might affect our observations. Importantly, our model examined human VSMC. All of the above-mentioned studies utilized human MCP-1 to stimulate rat or rabbit aortic VSMC. Quite possibly, differing results may reflect unique interactions between human protein and other species’ chemokine receptors. Finally, Porreca and colleagues (14) identified their optimal dose of MCP-1 at either 100 or 200 ng/ml. We observed maximal VSMC stimulation at 1 ng/ml while still noting a difference in growth at a dose as low as 100 pg/ml. We can only speculate the relevance of these doses. In native, non-injured vessels, MCP-1 expression is very low (5, 13). Two hours after balloon injury in rat carotid arteries, MCP-1 concentrations increase to nearly 130 pg/mg protein (5). Extrapolating from these observations, the doses identified in our experiments appear to be relevant.

We implicate several different mechanisms for MCP-1-induced VSMC proliferation. Whereas we did not perform flow cytometric studies, Porreca and colleagues (14) previously reported that MCP-1 promoted a twofold increase in the percentage of VSMC in S-phase, with a corresponding decrease of cells in the G0/G1 phase. Our study extends these observations by examining specific mitotic marker proteins. Immunohistochemical and Western blot immunoassays suggest that MCP-1 directly influences the cell cycle. PCNA is a 36-kDa polypeptide, which is only expressed in the nuclei of cells that are actively proliferating. Cyclin A is only one of a myriad of molecules that assist in regulating the cell cycle (27). It is especially important in governing the G1/S transition and the S phase of mitosis (6). Whereas we demonstrate that MCP-1 induces cyclin A protein expression, we acknowledge that there are likely several other cell cycle proteins that may be involved.

Previous studies have suggested that MCP-1 activates VSMC calcium-dependent protein kinase C and mitogen-activated protein kinase pathways (14, 30). We looked at two additional intracellular signaling intermediates as candidate targets for MCP-1. Classically, NF-κB exist in the cytoplasm as a heterodimer of its two subunits, p50 and p65, and its inhibitory protein, IkB (26). On activation, IkB is degraded, allowing...
the p65 subunit to migrate to the nucleus and bind its target DNA. We have previously demonstrated a causal relationship between cytokine and growth factor-induced VSMC proliferation and activation of NF-κB (23, 24). As such, we were intrigued to observe that MCP-1 did not increase p65 translocation into stimulated VSMC nuclei. To our knowledge, there are no reports that implicate NF-κB as a transduction intermediate in MCP-1 and its receptor signaling.

When activated, the PI3 kinase pathway is important in mitogen-induced cellular proliferation, quite possibly by promoting entry into S phase of the cell cycle (4, 16). Whereas MCP-1 gene expression is mediated by PI3 kinase (1), MCP-1 activation of PI3 kinase cycle (4, 16). Whereas MCP-1 gene expression is mediated by transient activation of PI3 kinase in mice that overexpress human apolipoprotein B. J Clin Invest 103: 773–778, 1999.


