Published in final edited form as:

Angiogenesis. 2010 September ; 13(3): 219–226. doi:10.1007/s10456-010-9179-8.

MCP-1 promotes mural cell recruitment during angiogenesis in the aortic ring model

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Abstract

Rings of rat or mouse aorta embedded in collagen gels produce angiogenic outgrowths in response to the injury of the dissection procedure. Aortic outgrowths are composed of branching endothelial tubes and surrounding mural cells. Mural cells emerge following endothelial sprouting and gradually increase during the maturation of the neovessels. Treatment of aortic cultures with angiopoietin-1 (Ang-1), an angiogenic factor implicated in vascular maturation and remodeling, stimulates the mural cell recruitment process. Ang-1 induces expression of many cytokines and chemokines including monocyte chemoattractant protein-1 (MCP-1). Inhibition of p38 MAP kinase, a signaling molecule required for mural cell recruitment, blocks Ang1-induced MCP-1 expression. Recombinant MCP-1 dose-dependently increases mural cell number while an anti-MCP-1 blocking antibody reduces it. In addition, antibody mediated neutralization of MCP-1 abrogates the stimulatory effect of Ang-1 on mural cell recruitment. Aortic rings from genetically modified mice deficient in MCP-1 or its receptor CCR2 have fewer mural cells than controls. MCP-1 deficiency also impairs the mural cell recruitment activity of Ang-1. Our studies indicate that spontaneous and Ang1-induced mural cell recruitment in the aortic ring of model of angiogenesis are in part mediated by MCP-1. These results implicate MCP-1 as one of the mediators of mural cell recruitment in the aortic ring model, and suggest that chemokine pathways may contribute to the assembly of the vessel wall during the angiogenesis response to injury.

Keywords

Chemokines; Collagen; Neovascularization; Pericytes

Introduction

Angiogenesis, the formation of blood vessels, is a complex process characterized by endothelial sprouting, proliferation, capillary tube formation, and branching morphogenesis. Once formed, vessel outgrowths undergo extensive reorganization and remodeling. Some neovessels are

pruned and reabsorbed whereas others differentiate into fully functional blood conduits. During the maturation stage, neovessels are invested by a discontinuous layer of cells known as mural cells. These periendothelial cells are believed to originate from mesenchymal progenitor cells [1] or dedifferentiated smooth muscle cells [2]. After they have been recruited to the neovasculature, mural cells regulate the migration, proliferation and survival of endothelial cells, and the integrity of the vessel wall [3–5].

Mural cells are recruited through paracrine mechanisms orchestrated by sprouting endothelial cells and the periendothelial mesenchyme. Among the molecules implicated in mural cell recruitment, Angiopoietin-1 (Ang-1) and its receptor Tie-2 have emerged as critical regulators of the vascular maturation and remodeling that follows angiogenesis. Genetic disruption of Ang-1 [6] or Tie-2 [7–9] causes lethal defects in cardiovascular morphogenesis. The vasculature of Ang-1 $-/-$ or Tie-2 $-/-$ embryos contains a markedly reduced number of periendothelial cells and fails to mature into an arborizing network of small and large vessels. The lack of proper mural cell recruitment is believed to play an important role in the inability of Ang-1/Tie-2 defective vessels to mature.

Ang-1 promotes mural cell recruitment also during postnatal angiogenesis [10], but the underlying mechanisms of the Ang-1 mediated assembly of the vessel wall are incompletely understood. The prevailing hypothesis is that Ang-1 produced by perivascular mesenchymal cells stimulates production of mural cell chemokines and growth factors by endothelial cells. For example Ang-1 induces expression in endothelial cells of heparin binding epidermal growth factor (HB-EGF) and hepatocyte growth factor (HGF) both of which have the capacity to promote the recruitment of smooth muscle cells [11,12]. Ang-1 can also promote proinflammatory responses which may contribute to the perivascular recruitment of mesenchymal cells and mural cells [13,14].

The process of mural cell recruitment can be studied *ex vivo* by culturing rings of rat or mouse aorta in three-dimensional collagen gels. In this system aortic rings produce branching microvessels in response to the injury of the dissection procedure. Neovessels formed in aortic cultures are composed of an inner core of endothelial cells and an outer layer of mural cells. The number of mural cells in the aortic ring model can be increased by treating the cultures with recombinant Ang-1 [15]. Spontaneous as well as Ang-1-mediated mural cell recruitment can be blocked with inhibitors of p38 MAPK—a signaling molecule characteristically activated by cytokines and growth factors including Ang-1—and enhanced by transducing the cultures with MKK6 an upstream activator of p38 [16].

Microarray analysis demonstrates that aortic rings treated with Ang-1 overexpress many inflammatory cytokines and chemokines [17]. Among these, monocyte chemoattractant protein-1 (MCP-1/CCL2), a chemokine produced by different cell types including endothelial and smooth muscle cells [18], promotes the influx of monocytes, T-cells, and dendritic cells to sites of inflammation. MCP-1 also stimulates the migration, proliferation and invasiveness of mural cells [19–23] and has been implicated as an endogenous promoter of medial thickening in arterial hypertension [24,25] and neointima formation in atherosclerosis [26–29].

Reports on MCP-1 and smooth muscle cells focus primarily on arterial vessels, but there are no studies on the function of this chemokine in mural cell recruitment during angiogenesis. To fill this gap we investigated the role of MCP-1 and its receptor CCR2 in the aortic ring model. Our results indicate that spontaneous and Ang-1-induced mural cell recruitment are in part mediated by MCP-1 and can be blocked by interfering with the MCP-1/CCR2 system. These findings suggest that inflammatory pathways triggered by Ang-1 stimulation contribute to the morphogenesis and maturation of neovessels during the angiogenic response of the vessel wall to injury.

Methods

Preparation and treatment of ex vivo aortic ring cultures

All animal procedures were performed with approval from the Veterans Administration Puget Sound Health Care System institutional animal care and use committee and according to NIH guidelines. Thoracic aortas were dissected from CO₂ euthanized 1–2 month-old male Fischer 344 rats (Harlan, Indianapolis, IN) and mice deficient for MCP-1 or CCR2 (The Jackson Laboratory, Bar Harbor, ME) or age matched C57BL/6 controls. Aortas were cleaned of fibroadipose tissue and blood, and serially cross-sectioned into 1–2 mm rings as described [30]. Rat or mouse aortic rings were embedded in collagen gels cast in 4-well dishes and cultured as described [30–32]. All aortic rings were cultured in serum-free medium (EBM: Lonza, Walkersville, MD) with or without reagents of interest. Recombinant human Ang-1 (R&D Systems, Minneapolis, MN) and rat MCP-1 (BD Pharmingen, Franklin Lakes, NJ) were dissolved in PBS +0.1% BSA. Ang-1 was used at 100 ng/ml with 5 ng/ml poly-His monoclonal linker antibody (R&D Systems). Control cultures received poly-His antibody alone. For MCP-1 neutralization experiments, azide-free anti-MCP-1 hamster monoclonal antibody (BD Pharmingen) was added to the medium at a final concentration of 50 µg/ml. Control cultures received an equivalent concentration of non-immune hamster IgG. For p38 MAPK inhibition, SB203580 (Calbiochem-EMD Biosciences, San Diego, CA) was dissolved in dimethylsulfoxide, (DMSO, Sigma, St. Louis, MO) and added to the culture medium at a final concentration of 1 µg/ml. Control cultures received an equivalent amount of DMSO.

Measurement of angiogenesis

The angiogenic response of living aortic cultures was measured under bright field microscopy by counting the number of neovessels over time [30]. Images of live or formalin fixed cultures were captured with an Olympus MagnaFire digital camera (Olympus American, Melville, NY) mounted on a IX-71 Olympus microscope.

Measurement of mural cell number

Mural cells were identified by their distinct morphology and periendothelial location using a 20× objective and phase-contrast optics, as described [2,15,16]. Mural cell recruitment was measured by counting mural cells in 40 vessel segments per treatment group, each made of quadruplicate cultures. Mural cell number was divided by the vessel length which was measured with a reticular eyepiece calibrated with a Swift micrometer. Measured vessel segments ranged from 200 to 500 µm in length. Mural cell density was expressed as number of cells per 100 µm vessel length.

Immunoperoxidase histochemistry

Mural cell differentiation was demonstrated by immuno-staining whole mount preparations of aortic cultures with an anti-NG2 proteoglycan mouse monoclonal antibody (Millipore, Bellerica, MA), as reported [31]. Macrophages of aortic explants were immunostained with a monoclonal mouse anti-CD68 antibody (AbD Serotech, Raleigh, NC), as reported [33]. Nonimmune mouse IgG was used as negative control.

Quantitative reverse transcriptase polymerase chain reaction

Total RNA was isolated from rat aortic rings after snap freezing in liquid nitrogen and manual pulverization. Total RNA was extracted from 5 to 6 collagen cultures from each treatment group with the RNeasy Micro kit (Qiagen, Valencia, CA) and cDNA templates for real-time PCR were synthesized by reverse transcription (RT) as described [17]. Duplicate reactions were carried out lacking the Superscript enzyme to act as negative controls. To examine the relative expression of MCP-1, the two-step Quantitative reverse transcriptase polymerase chain

reaction (qRT-PCR) SYBR Green method was used (Applied Biosystems, Foster City, CA). 1/50 of the RT reaction was used as template in qRT-PCR reactions containing oligonucleotide primers (Invitrogen) MCP-1: 5'- TCACGCTTC TGGGCCTGTTG -3' and 5'- CAGCCGACTCATTGGGATCA TC -3'; β -Actin: 5'-GGGAAATCGT GCGTGACATT-3' and 5'-GCGGCAGTGGCCATCTC-3'. Melt curve analysis demonstrated that each primer set produced a single product. Relative quantification was carried out on an ABI 7000 thermal cycler (Applied Biosystems) as described [17]. Each PCR reaction was carried out at least in triplicate. qRT-PCR data were analyzed with Prizm software (Applied Biosystems) and relative ratios of fluorescent intensities of products from each treatment group were calculated by using the $2^{-\Delta\Delta C_t}$ method [34]. The mRNA expression levels of each sample were normalized to the levels of β -Actin expression measured in the same sample.

Statistical analysis

Experiments with aortic ring cultures were repeated at least three times. Quantitative data on angiogenesis and mural cell recruitment were evaluated with Prizm software (Graph Pad, Inc. San Diego, CA) and analyzed for differences among experimental groups with Student's unpaired two tailed *t*-tests. Statistical significance between groups was set at $P < 0.05$.

Results

Ang-1 treatment stimulates MCP-1 expression through p38 MAPK

We previously found that mural cell recruitment in the aortic ring model is promoted by Ang-1 [15] and blocked by inhibitors of p38 MAPK [16], a signaling molecule activated by Ang-1. Microarray studies showed that Ang-1 induces expression of MCP-1, a chemokine implicated in mural cell proliferation [17]. To evaluate if p38 MAPK and MCP-1 shared the same pathway, we studied MCP-1 gene expression on Ang-1-treated aortic rings cultured with or without the p38 MAPK inhibitor SB203580. Blockade of p38 MAPK completely abrogated Ang-1-induced overexpression of MCP-1 and significantly reduced expression of MCP-1 in unstimulated control cultures (Fig. 1). Taken together with work by others indicating that MCP-1 has the capacity to promote smooth muscle cell proliferation, our observations suggested that this chemokine might be involved in the recruitment of mural cells during angiogenesis, and serve as mediator of the stimulatory effect of Ang-1 on this process.

Exogenous MCP-1 stimulates mural cell recruitment in aortic ring cultures

To evaluate the effect of MCP-1 on mural cell recruitment, angiogenic cultures of rat aorta were treated with increasing doses of this chemokine. Vessels formed in MCP-1-treated cultures appeared thicker than untreated control vessels. Examination of cultures under phase contrast at high magnification demonstrated that the increased thickness of MCP-1-treated vessels was due to periendothelial accumulation of mural cells. The mural cell stimulatory effect of MCP-1 was dose dependent (Fig. 2). The mural cell nature of the recruited cells was confirmed by staining the cultures for the mural cell marker NG2. MCP-1 also modestly stimulated angiogenesis in cultures treated with the highest tested dose (1 μ g/ml).

To investigate the possible role of resident aortic macrophages in mural cell recruitment, we repeated the MCP-1 stimulation experiment with macrophage-depleted aortic rings [33]. Since depletion of adventitial macrophages renders the aortic rings quiescent, angiogenesis in these cultures was stimulated with exogenous VEGF [35]. Macrophage loss did not affect the capacity of MCP-1 to promote mural cell recruitment, as MCP-1 treatment in these cultures doubled the number of mural cells associated with neovessels ($N = 40$; $P < 0.001$).

Blockade of endogenous MCP-1 reduces mural cell number in control and Ang-1-stimulated cultures

To evaluate the role of endogenous MCP-1 in mural cell recruitment, control and Ang-1-treated rat aortic cultures were treated with an MCP-1 blocking antibody. Nonimmune IgG was used as negative control. Ang-1 significantly stimulated angiogenesis and mural cell recruitment. Antibody-mediated blockade of MCP-1 had no effect on the angiogenic response but reduced mural cell number to 84% of control and completely abrogated the Ang-1 stimulatory effect on the mural cells (Fig. 3).

Genetic disruption of MCP-1 or the MCP-1 receptor CCR2 impairs recruitment of mural cells in aortic ring cultures

To further define the contribution of endogenous MCP-1 to the recruitment of mural cells by sprouting endothelial cells, we examined this process in cultures of aortic rings from genetically modified mice with disrupted MCP-1 or the MCP-1 receptor CCR2. Vessels sprouted in cultures with defective MCP-1/CCR2 system had a significantly lower number of mural cells. Cell numbers were reduced to 40% of control in MCP-1 deficient cultures and to 60% in cultures with disrupted CCR2 (Fig. 4). There was no significant difference in angiogenic sprouting of the neovessels between normal aortic cultures and MCP-1 or CCR2 deficient cultures (data not shown).

Ang-1 stimulates mural cell recruitment in aortic cultures from control but not MCP-1 deficient mice

To evaluate if MCP-1 gene disruption affected the ability of Ang-1 to promote mural cell recruitment, aortic cultures from MCP-1 deficient mice or normal controls were treated with Ang-1 and mural cells were counted. Ang-1 significantly increased the number of mural cells in normal aortic cultures. Ang-1, however, was unable to enhance mural cell recruitment in MCP-1 deficient cultures (Fig. 5).

Discussion

Results presented in this paper indicate that the MCP-1/CCR2 system is implicated in mural cell recruitment during angiogenesis in the aortic ring model and functions as a mediator of the stimulatory effect of Ang-1 on this process. This interpretation is based on the following findings. (1) Ang-1 upregulates MCP-1 expression in aortic ring cultures; (2) Functional blockade of p38 MAPK, a signaling molecule required for mural cell recruitment [16], abrogates Ang-1-induced expression of MCP-1; (3) Recombinant MCP-1 increases mural cell recruitment during aortic angiogenesis; (4) Neutralization of endogenous MCP-1 reduces mural cell number in control cultures and blocks the promoting effect of Ang-1 on mural cell recruitment; (5) Genetic disruption of MCP-1 or its receptor CCR2 impairs mural cell recruitment in aortic ring cultures; (6) Ang-1 fails to stimulate mural cell recruitment in aortic cultures from MCP-1 deficient mice.

Our studies define a novel mechanism by which mural cells are recruited during angiogenesis. The finding that MCP-1 promotes assembly of the vessel wall corroborates our previous observation that mural cell recruitment can be blocked by inhibitors of p38 MAPK, a signaling molecule characteristically involved in the production of cytokines and chemokines during immune responses [36]. Our experiments with the p38 MAPK inhibitor SB203580 identified MCP-1 as a highly sensitive downstream target of p38 MAPK inhibition, as previously reported by others [37]. The involvement of MCP-1 in mural cell recruitment confirms and expands our recent observation that the angiogenic response of aortic rings is associated with widespread activation of the aortic immune system [17,33]. Our study is in keeping with previous reports

that MCP-1 mediates mural cell recruitment during TGF- β -induced angiogenesis [38] and promotes the thickening of the vessel wall in different pathologic conditions [19–23].

Since macrophages, a primary target of MCP-1 [39], play an important role in the angiogenic response of the aortic rings [33] and have been implicated in arteriogenesis and mural cell assembly, we performed additional experiments with macrophage depleted aortic rings. Aortic rings spontaneously lose adventitial macrophages and become quiescent when kept in floating cultures for 10–14 days prior to collagen embedding [33]. Angiogenesis and mural cell recruitment can be reactivated in this modified assay by treating the rings with exogenous VEGF [35]. In spite of macrophage loss, MCP-1 effectively stimulated mural cell recruitment in cultures of macrophage depleted aortic rings as observed in cultures of freshly cut aortic rings. This finding suggests that MCP-1 promotes mural cell recruitment through a macrophage independent mechanism. Since the MCP-1 receptor CCR2 is expressed in both mural and endothelial cells [20,40], exogenously added MCP-1 may promote mural cell recruitment directly or by inducing production of mural cell recruitment factors by endothelial cells.

Our results implicate MCP-1 as a mediator of the Ang-1 stimulatory effect on mural cell recruitment. The demonstration that inhibition of p38 MAPK, a molecule involved in Ang-1/Tie2 signaling, blocks both Ang-1-mediated MCP-1 production and mural cell recruitment [16] suggests that MCP-1 operates downstream of Ang-1 to stimulate mural cell assembly during angiogenesis. This finding is corroborated by the additional observation that Ang-1 fails to promote mural cell recruitment in aortic cultures from genetically modified mice with disrupted MCP-1. Previous studies have shown that isolated human umbilical vein endothelial cells treated with Ang-1 or virally transduced to overexpress Ang-1 produce HGF and HB-EGF which in turn promote migration of smooth muscle cells in a chemotaxis chamber assay [11,12]. Although limited to isolated cells, these studies suggest that Ang-1 induced mural cell recruitment may involve several mediators and vary depending on endothelial cell types, animal species and experimental conditions. It is also possible that some of these mediators share a common pathway. For example HGF has been reported to induce MCP-1 expression in synovial fibroblasts [41]. In addition, Ang-1 may stimulate directly the migration of Tie2+ mural progenitor cells, without the involvement of chemical mediators from the Tie2+ endothelium [15,42].

Antibody mediated neutralization of endogenous MCP-1 in rat aortic cultures not treated with Ang-1 significantly reduced mural cell recruitment by 16% whereas disruption of the MCP-1 and CCR2 genes in mouse aortic cultures was more effective, causing 50–60% reduction in mural cell number. These differences may be due to the diverse genetic backgrounds of rats and mice, the potency of the antibody, and/or the greater efficacy the gene disruption approach in eliminating the contribution of the MCP-1/CCR2 system. The incomplete depletion of mural cells by our MCP-1 neutralization methods indicates that mural cell recruitment is regulated by a redundant system of paracrine stimuli. Studies with genetically modified mice have demonstrated that disruption the PDGFB or PDGFR- β genes causes a site-specific reduction of mural cells [43] and indicate that mural cell recruitment involves PDGF-B-dependent and -independent mechanisms. In addition to Ang-1, HB-EGF, HGF, and PDGFB, paracrine stimuli for mural cell recruitment may involve sphingosine 1-phosphate [44], placenta growth factor [45–47], and VEGF [48].

The source of MCP-1 in aortic cultures remains to be determined. Molecular analysis of cells isolated from the rat aorta indicates that the endothelium is a primary source of MCP-1 (unpublished observations). More studies are, however, needed to localize the expression of MCP-1 and CCR2 in the aortic outgrowths at different stages of angiogenesis.

Interestingly, the rate of vascular regression in aortic cultures was not influenced by the degree of mural cell coverage of the neovessels. This is in contrast with *in vivo* studies indicating that mural cells have a stabilizing effect on the neovasculature [5]. One possible explanation is that the vasostabilizing properties of mural cells are dependent on hemodynamic factors and circulating plasma proteins that are missing in our *ex vivo* culture system.

In summary our studies demonstrate that mural cell recruitment during angiogenesis in the aortic ring model is in part mediated by MCP-1 and indicate the MCP-1/CCR2 receptor system cooperates with Ang-1 in promoting the assembly of the vessel wall in developing neovessels. More studies are needed to extend this observation to other models of angiogenesis and the more complex environment of the live animal. It will be of interest to investigate whether MCP-1-mediated mural cell recruitment is limited to reactive/inflammatory angiogenesis, as in the aortic ring model, or also contributes to developmental and tumor angiogenesis. Finally, systematic studies with isolated endothelial and mural cell types will be needed to further define the role of MCP-1 in the mechanisms that regulate vessel wall assembly in the aortic ring outgrowths and other neovasculatures from different anatomic sites.

Acknowledgments

Supported by the National Institute of Health (HL52585; R.F.N.) and the Medical Research Service, Department of Veterans Affairs (R.F.N.).

Abbreviations

Ang-1	Angiopoietin-1
bFGF	Basic fibroblast growth factor
EBM	Endothelial basal medium
HB-EGF	Heparin binding-endothelial growth factor
HGF	Hepatocyte growth factor
MCP-1	Monocyte chemotactic protein-1
VEGF	Vascular endothelial growth factor

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Fig. 1.

Ang-1 induced expression of MCP-1 is abrogated by blocking p38 MAPK. qRT-PCR showed that aortic rings treated with 100 ng/ml Ang-1 upregulated expression of MCP-1. Treatment with the p38 MAPK inhibitor SB203580 (1 μ g/ml) blocked the Ang-1 stimulatory effect and reduced MCP-1 expression in control cultures ($N = 4$, ** = $P < 0.01$)

Fig. 2.

MCP-1 stimulates mural cell recruitment in aortic ring cultures. Representative micrographs of neovessels from control (**a**) and MCP-1-treated (**b**, 1 µg/ml) collagen gel cultures of rat aorta show a higher number of mural cells (*arrows*) in cultures treated with MCP-1.

Immunoperoxidase staining demonstrates expression of the mural cell marker NG2 in recruited cells of control (**c**) and MCP-1-treated (**d**) cultures (*scale bar* = 50 microns). MCP-1 modestly stimulates angiogenesis when used at high dose (1 µg/ml; **e**, *line graph*, $N = 4$). Counts of mural cells shows dose dependent effect of MCP-1 on mural cell recruitment (**f**, *bar graph*, $N = 40$, ** = $P < 0.01$)

Fig. 3.

Neutralization of endogenous MCP-1 impairs mural cell recruitment but has no effect on angiogenesis. **a** Treatment of rat aortic cultures with anti-MCP-1 antibody (α MCP-1) significantly reduces the number of mural cells in control cultures and blocks the Ang-1 stimulatory effect on mural cell recruitment (*bar graph*; $N = 40$, $* = P < 0.05$; $*** = P < 0.001$). **b** The anti-MCP-1 antibody has no effect on the proangiogenic activity of Ang-1 (*line graph*; $N = 4$, $*** = P < 0.001$)

Fig. 4.

Recruitment of mural cells during angiogenesis is impaired in mouse aortic cultures with defective MCP-1/CCR2 system. Representative micrographs of control (a), MCP-1 deficient (b) and CCR2 deficient (c) mouse aortic cultures show fewer mural cells (*arrows*) in neovessels with disrupted MCP-1/CCR2 system. Mural cell counts demonstrate impaired recruitment of these cells in aortic cultures from CCR2- to MCP-1-deficient mice (d; $N = 48$; *** = $P < 0.001$)

Fig. 5.

The stimulatory effect of Ang-1 on mural cell recruitment is abrogated in aortic cultures from MCP-1 deficient mice. Mural cell counts demonstrate that Ang-1 is unable to induce mural cell recruitment in aortic cultures from MCP-1 deficient mice compared to normal control cultures ($N = 40$, *** = $P < 0.001$)