MCP-1 Contributes to Arteriovenous Fistula Failure

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Dysfunction of hemodialysis vascular access, an endemic problem among patients on maintenance hemodialysis, contributes substantially to the morbidity and mortality that afflict this patient population.1–5 Indeed, such dysfunction accounts for some 20% of hospitalization of these patients and accrues approximately one billion dollars in health care costs per year.5 Even the most favored vascular access, the arteriovenous fistula (AVF), exhibits remarkably high rates of primary nonfunction, dysfunction, and eventual failure: up to 50% of AVFs do not mature to the point that they can sustain effective hemodialysis (primary nonfunction), and of the remainder that do mature, the primary unassisted patency is decreased to 75% after 2 years.5 A fundamental pathologic lesion that contributes to the pathogenesis of primary or secondary failure of an AVF is neointimal hyperplasia, which thickens the venous wall, narrows the luminal area, and predisposes to intravascular thrombosis.1–5 In view of the far-ranging, adverse consequences resulting from dysfunction or failure of a hemodialysis AVF, there is increasing emphasis on the elucidation of mechanisms that give rise to venous neointimal hyperplasia in an AVF and the delineation of strategies that may interrupt its occurrence.1–5

Monocyte chemoattractant protein-1 (MCP-1; chemokine C-C motif ligand 2 [CCL2]), a member of the C-C chemokine family, is broadly incriminated in the pathogenesis of atherosclerosis and other vascular diseases.6–10 Upregulation of MCP-1 is also incriminated in assorted nephropathies and inflammatory processes in other organs and tissues.6–10 This involvement of MCP-1 in vascular and other diseases reflects, among others, the following effects of MCP-1: potent chemotaxis of monocytes/macrophages; activation and migration of endothelial cells; promotion of proliferation and migration of smooth muscle cells; and the induction of tissue factor and other procoagulant effects.6–10

This study examined whether MCP-1 is induced in a murine model of an AVF and the functional significance of such induction. One week after establishing an AVF in mice, the venous limb exhibited increased expression of MCP-1 mRNA and protein, along with increased activity of the transcription factors NF-κB and AP-1 (Figure 1). Genetic deficiency of MCP-1 proved markedly protective in this murine model, reflected by increased fistula patency 6 weeks after its formation, decreased venous wall thickness, and increased luminal area. An early effect of MCP-1 deficiency was the attenuation of the marked induction of CCL5 (RANTES) that occurred in this model, a chemokine recently recognized as a critical participant in vascular injury. Finally, in a rat model of an arteriovenous fistula, we localized expression of MCP-1 to the endothelium, proliferating smooth muscle cells and infiltrating leukocytes. In summary, marked upregulation of MCP-1 occurs in the venous segment of an arteriovenous fistula in rodents, and this vasculopathic chemokine contributes to failure of the fistula.

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Expression by ELISA, NF-κB was determined by quantitative real-time reverse transcriptase-PCR, MCP-1 protein

Figure 1. Expression of MCP-1 and relevant MCP-1 transcription factors 1 week after establishing an AVF in mice. In the venous segment of the AVF and in the contralateral, nonoperated, jugular vein (Control), measurements of (A) MCP-1 mRNA (Control: n = 7; AVF: n = 9), (B) MCP-1 protein (Control: n = 4; AVF: n = 8), (C) NF-κB (n = 6 in both groups), and (D) AP-1 (n = 6 in both groups) were undertaken. MCP-1 mRNA expression was determined by quantitative real-time reverse transcriptase-PCR, MCP-1 protein expression by ELISA, NF-κB activation by a chemiluminescence-based assay kit, and AP-1 activation by a colorimetric assay kit. For MCP-1 protein analysis by ELISA, four control veins and two AVF veins were pooled for each determination. For all other analyses, one vein was used for each determination.

1+/+ mice (Figure 2). At this time point, the thickness of the venous wall was decreased in MCP-1−/− mice and was accompanied by increased luminal area compared with corresponding indices in the AVF in MCP-1+/+ mice (Figure 2).

To determine a possible molecular basis for these changes, assorted genes were examined 1 week after creating the AVF in MCP-1+/+ mice and MCP-1−/− mice. In this murine model, two genes have been previously shown to be vaso-protective, namely, HO-1 and BMP-7; as shown in Figure 3, no differential changes in expression in either of these genes could be identified as the basis for the protective effects of MCP-1 deficiency. However, CCL5 (RANTES [Regulated upon Activation, Normal T-cell Expressed and Secreted]) was markedly induced in the AVF in MCP-1+/+ mice, and such induction was substantially attenuated in MCP-1−/− mice with an AVF. Expression of the following mRNAs in the venous limb of the AVF in MCP-1+/+ mice and MCP-1−/− mice was not significantly different: PDGF-A, PDGF-B, TGF-β1, osteopontin, IL-6, IL-10, matrix metalloproteinase-2, matrix metalloproteinase-9, and tissue factor (data not shown).

Because of the observed upregulation of MCP-1 and its contribution to the failure of the AVF, we attempted to localize by immunohistochemistry the cellular expression of MCP-1 in the venous segment of the murine AVF; we were unable to consistently obtain specific immunohistochemical staining and to delineate the cellular expression of MCP-1. In view of the recognized difficulty in the localization of tissue expression of MCP-1 in the mouse using currently available antibodies (we are unable to find immunohistochemical studies of MCP-1 in the venous vasculature in the mouse in PubMed), we used a rat model of an AVF, which we have recently shown to exhibit increased MCP-1 mRNA expression at 1 and 4 weeks after the creation of the AVF.13 In this rat AVF model and at 5 weeks after the creation of the AVF, we confirmed increased expression of MCP-1 mRNA (3.9 ± 0.5 versus 12.7 ± 1.9 standardized units; P < 0.05, n = 9 for each group), and we now show that this is accompanied by increased amounts of MCP-1 protein as determined by ELISA (612 ± 75 versus 1472 ± 161 pg/mg protein; P < 0.05, n = 6 and n = 9, respectively). Immunohistochemical localization of MCP-1 in this AVF model showed marked induction of MCP-1 in the endothelium, smooth muscle cells, and leukocytes in the neointima in the AVF (Figure 4).

In aggregate, our findings are the first to show that upregulation of MCP-1 in the venous vasculature contributes to the dysfunction and failure of an AVF, thereby identifying a new molecular determinant for the failure of an AVF. Our findings are relevant to dysfunction and failure of hemodialysis AVFs because of the following clinical observations: plasma levels of MCP-1 are increased in patients with chronic kidney disease and inversely correlate with the decline of GFR;14 in patients on maintenance hemodialysis, plasma levels of MCP-1, as for IL-6 and plasminogen activator inhibitor-1, are risk factors for dysfunction of hemodialysis AVFs15–17; and in hemodialysis patients, certain polymorphisms in the MCP-1 gene that lead to higher plasma levels of MCP-1 seem to be risk factors for cardiovascular disease.18

Findings in other experimental models are germane to, and consistent with, these observations. First, the upregulation of MCP-1 mRNA we observed in the venous segment of the murine AVF is a molecular signature we also detected in the venous segment of the AVF in two different models in the rat, namely an aorto-caval AVF model created by needle puncture of these central vessels19 and a femoral AVF model fashioned by surgical anastomosis of these peripheral vessels.13 MCP-1 mRNA expression is regulated by hemodynamic stress,20 and it is thus tempting to speculate that such consistent upregulation of MCP-1 in the venous vasculature of an AVF is hemody-
namically mediated. Second, in the rat femoral AVF model, we also noted that the administration of N\(^\text{G}\)-nitro-L-arginine methyl ester worsened neointimal hyperplasia and concomitantly increased MCP-1 mRNA expression in the venous segment.\(^{13}\) Third, in the murine model of an AVF, we showed that genetic deficiency of \(\text{HO-1}\) led to an exacerbation of neointimal hyperplasia and early closure rate and was associated with increased expression of MCP-1 mRNA.\(^{11}\) Fourth, in studies involving the engraftment of venous segments in the arterial circuit, anti-MCP-1 gene therapy reduced neointima formation.\(^{21}\) This study is the first to localize the site of increased expression of MCP-1 in the venous segment of an AVF, to identify induction of MCP-1 in a murine AVF model, and, using mutant mice unable to express MCP-1, to directly show that MCP-1 critically contributes to failure of an AVF.

Our studies also provide the first demonstration that CCL5 (RANTES, a potent chemotactic agent for T cells and monocytes) is strikingly upregulated in the venous segment of an AVF and that such upregulation is attenuated in the AVF in MCP-1\(^{-/-}\) mice. These findings are germane to the recent recognition of the importance of CCL5/chemokine C-C motif receptor 5 (CCR5) in neointimal hyperplasia in atherosclerosis and other vasculopathies.\(^{22-26}\) For example, neointimal hyperplasia in models of arterial injury and atherosclerosis is decreased by antagonists of CCL5/CCR5 signaling\(^{25}\) and reduced in mice that do not express CCR5\(^{22,24}\); additionally, YB-1 knockdown strategies targeted to reduce CCL5 expression reduce neointimal hyperplasia in experimental atherosclerosis.\(^{23}\) CCL5/CCR5 signaling may also contribute to cardiovascular diseases in uremia and attendant mortality in this patient population: for example, polymorphisms of the CCR5 gene leading to deletion of CCR5 are accompanied by decreased mortality in dialysis patients concomitantly expressing increased levels of inflammatory markers, such as C-reactive protein, that may be predictive of cardiovascular diseases.\(^{27}\)

In summary, our findings identify expression of MCP-1 in an AVF as a determinant of dysfunction and failure of AVFs. Failure of an AVF is thus driven by increased expression of a chemokine of fundamental importance in cardiovascular diseases, the latter occurring with heightened frequency in chronic kidney disease.\(^{6-10}\) That upregulation of MCP-1 not only represents a mechanism for cardiovascular disease but also accounts for the failure of an AVF suggests that hemodialysis AVF dysfunction may reflect, in part, the overarching propensity for cardiovascular disease imposed by the uremic milieu; in this regard, examining the role of MCP-1 in AVF failure in the uremic setting would be of interest. Finally, substantial efforts are currently underway to develop clinically applicable strategies that interrupt either the actions of MCP-1 or cell signaling emanating from CCR2, the receptor for MCP-1.\(^{10,28,29}\) We suggest that the study of the effects of these agents on the functionality of the murine AVF would be an important step in exploring the clinical applicability of these findings.

**CONCISE METHODS**

**Rodent AVF Models**

All studies were approved by the Institutional Animal Care and Use Committee of Mayo Clinic and performed in accordance with National Institutes of Health guidelines. As used in our previous study, we constructed an AVF...
in C57BL/6j mice (10 to 12 weeks) using an end-to-side anastomosis of the right common carotid artery and jugular vein. This AVF model was also constructed in 20- to 30-week-old MCP-1−/−/− mice (B6.129S4-Ccl2tm1Rol/J; The Jackson Laboratory, Bar Harbor, ME) and age- and sex-matched control mice. In additional studies, a rat AVF was used, constructed by an end-to-side anastomosis of the left femoral artery and vein in male Sprague-Dawley rats (225 to 300 g; Harlan, Indianapolis, IN), as described previously.

Transcription Factor Binding Assays
Whole cell extracts were prepared from venous tissue using an extraction kit (catalog no. 40010; Active Motif, Carlsbad, CA). NF-κB and AP-1 binding was assessed using the TransAM p65 Chemi Transcription Factor Assay Kit and the TransAM c-Jun Colorimetric Transcription Factor Assay Kit (catalog nos. 40097 and 46096; Active Motif), respectively, according to the manufacturer’s instructions.

Measurement of MCP-1 by ELISA in Rat and Mouse AVF Venous Limbs
Rat and mouse venous homogenates were prepared in lysis buffer: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20, 1 mM PMSE, and a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). After centrifugation at 10,000 × g, MCP-1 levels were measured in the supernatants using ELISA kits (OptEIA, catalog nos. 555130 and 555260; BD Biosciences, San Diego, CA) according to the manufacturer’s instructions.

Localization of MCP-1 by Immunohistochemical Staining
Formalin-fixed, paraffin-embedded tissue sections were deparaffinized, and antigen retrieval was conducted using 1 mM EDTA, pH 8.0, in a 98°C steamer for 30 minutes. Slides were treated with 3% H2O2 to inactivate endogenous peroxidase, followed by incubation with Rodent Block R (catalog no. RBR962; Biocare Medical, Concord, CA). Rabbit polyclonal anti-MCP-1 antibody (catalog no. 500-P76; PeproTech, Rocky Hill, NJ) was applied for 60 minutes at room temperature, followed by visualization using Rabbit on Rodent Horseradish Peroxidase (catalog no. RMR622; Biocare Medical) with diaminobenzidine substrate and counterstaining with hematoxylin.

mRNA Expression by Quantitative Real-Time Reverse Transcriptase-PCR
Total RNA was extracted from venous tissue using the TRIzol method (Invitrogen, Carlsbad, CA) and further purified with an RNaseasy Mini Kit (Qiagen, Valencia, CA), according to each manufacturer’s protocol. As in our previous study, cDNA was synthesized in reverse transcription reactions (Transcriptor First Strand cDNA Synthesis Kit, Roche, Indianapolis, IN) and was subsequently used in quantitative real-time PCR analysis, using probes and primers obtained as assay sets for each target mRNA (TaqMan Gene Expression Assays; Applied Biosystems, Foster City, CA). Expression of 18S rRNA was used for standardization of the expression of each target gene.

Statistical Analysis
Results are expressed as mean ± SE and considered statistically significant for P < 0.05; t tests and Mann-Whitney tests for parametric and nonparametric data, respectively, were used as appropriate.

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DISCLOSURES
None.

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AVF, whereas endothelial cells express MCP-1 in the sham-operated vein. All sections are detected in endothelial cells (E), smooth muscle cells (SM), and leukocytes (L) in the sham-operated vein (A) and the venous segment of the AVF (B) and with an MCP-1 after establishing the AVF. Studies were performed with nonimmune serum for the localize expression of MCP-1 in the venous segment of the femoral AVF in the rat 5 weeks after the formation of the AVF. Immunohistochemistry studies were performed to Figure 4. MCP-1 expression by immunohistochemistry in the femoral AVF in the rat 5 weeks after the formation of the AVF. Immunohistochemistry studies were performed to localize expression of MCP-1 in the venous segment of the femoral AVF in the rat 5 weeks after establishing the AVF. Studies were performed with nonimmune serum for the sham-operated vein (A) and the venous segment of the AVF (B) and with an MCP-1 antibody for the sham-operated vein (C) and the venous segment of the AVF (D). MCP-1 is detected in endothelial cells (E), smooth muscle cells (SM), and leukocytes (L) in the AVF, whereas endothelial cells express MCP-1 in the sham-operated vein. All sections are shown at a magnification of 400x.