Contribution of Monocytes/Macrophages to Compensatory Neovascularization

The Drilling of Metalloelastase-Positive Tunnels in Ischemic Myocardium

Nicanor I. Moldovan, Pascal J. Goldschmidt-Clermont, Jan Parker-Thornburg, Steven D. Shapiro, Pappachan E. Kolattukudy

Abstract—In a transgenic model of ischemic cardiomyopathy in which monocytes are attracted to the myocardium by the targeted overexpression of monocyte chemoattractant protein-1 (MCP-1), we have observed the presence of endothelial NO synthase and platelet endothelial cell adhesion molecule-1–negative tunnels, occasionally containing blood-derived cells, that probe the cardiac tissue. Immunohistochemical data show that monocytes/macrophages (MCs/MPhs) drills tunnels using the broad-spectrum mouse macrophage metalloelastase. 5-Bromo-2′-deoxyuridine incorporation and neo-endothelial markers present in the microvasculature of MCP-1 mouse hearts suggest an active angiogenic process. Further studies will be required to establish that the MC-/Mph-drilled tunnels evolve to become capillaries, connected to the existing vessels and colonized by circulating endothelial cell progenitors. This possibility is supported by the availability of these cells, which is demonstrated by cell tagging with β-galactosidase placed under an active endothelial Tie-2 promoter. This phenomenon might represent another mechanism, in addition to the secretion of the angiogenic factors, by which MCs/MPhs may participate in the elaboration of new blood vessels in adult tissues. (Circ Res. 2000;87:378-384.)

Key Words: monocytes ■ metalloelastase ■ angiogenesis ■ ischemic heart disease

Angiogenesis refers to a process in which new capillaries extend from existing vessels to carry blood to ischemic tissues. Proliferation and migration of endothelial cells (ECs) are instrumental to the genesis of sprouting vessels. Under the control of multiple factors, ECs are induced to form capillary tubes that are connected to existing vessels and expand distally to tissues that are in need of additional blood supply. For example, the growth of tumors is exquisitely dependent on the supply of blood, which requires angiogenesis within the cancerous tissue. In turn, the progression of the angiogenic process is tightly controlled by the production of both angiogenic factors, such as basic fibroblast growth factor or vascular endothelial growth factor, and angiostatic factors, such as tissue inhibitors of metalloproteinases, angiostatin, or endostatin. A steady-state balance is established between agonistic and antagonistic factors, which defines the angiogenic activity, and consequently, the alteration of this balance may have an impact on ischemic tissue recovery or on tumor growth.

However, it has recently been shown that some rapidly growing tumors may rely on a different mechanism of blood supply, called “vascular mimicry,” which consists in formation of periodic acid-Schiff (PAS)–positive, endothelium-free “tunnels” apparently drilled within the tumoral matrix by the cancer cells themselves. This endothelial disguise may go so far that the epithelial tumor cell line T24/83 was for a long time mistaken for an EC line, ECV 304.

In this study we present data that suggest a similar genesis of alternative microcirculation formation occurring in the ischemic myocardium, in which the native capillary network collapsed as a consequence of transgenically induced upstream occlusion due to thrombosis or other pathological processes and in which monocytes/macrophages (MCs/MPhs) are attracted by chemotaxis. In the myocardium, a dense matrix of extracellular proteins connects the cardiomyocytes (CMs) to form a compact tissue that is capable of generating force and sustaining substantial transmural pressures during systole. Chronic ischemia of the myocardium resulting from epicardial coronary atherosclerosis is associated, in a subset of patients, with the enlargement of existing arterioles (arteriogenesis) and the sprouting of new capil-
laries (angiogenesis, in its “classical” meaning)\textsuperscript{1,3} to generate a network of collateral vessels. The presence of such collaterals can markedly reduce damage to the myocardium that is produced by acute or chronic ischemia.

In such a setting, and because of the unique properties of the myocardial tissue, researchers had anticipated that the process of formation of new blood vessels might have particular characteristics, including the contribution of MCs/MPhs,\textsuperscript{12,13} and induction of adult-type vasculogenesis based on circulating precursor ECs.\textsuperscript{14} Data presented here suggest that in this instance, in addition to their secretion of endothelial growth factors,\textsuperscript{15} the inflammatory cells may contribute to a compensatory microcirculation with their ability to “tunnelize” the ischemic regions. The conduits thus created can facilitate the access of other blood components, setting up a tubular network prone to the re-establishment of a collateral microvascular system.

Materials and Methods

Animals

The construction and characterization of the transgenic mouse model expressing monocyte chemoattractant protein-1 (MCP-1) selectively in the heart, under the myosin heavy chain promoter $\alpha$, has been described previously.\textsuperscript{16} Homozygous (4- to 6-month-old) and heterozygous (8- to 10-month-old) MCP-1 mice or LacZ-Tie-2–expressing transgenic mice (The Jackson Laboratory) and age-matched wild-type controls were euthanized with CO$_2$. The hearts were collected, either cryofixed or fixed in buffered 10% formalin or in methanol, and embedded in paraffin for sectioning and for further histological and immunohistochemical staining. All protocols were approved by the Ohio State University Institutional Animal Care and Use Committee.

Histology and Ultrastructural Analysis

Paraffin sections were stained with hematoxylin and eosin (HE), Weigert’s elastic van Gieson (EVG), or PAS reagent with hematoxylin counterstaining. For electron microscopy, the tissues were fixed with 3% glutaraldehyde in 0.1 mol/L phosphate buffer supplemented with 10 mmol/L sucrose for 3 hours, postfixed in 1% osmium tetroxide, dehydrated in graded ethanol, and embedded in Spurr resin. Ultramicrotome sections were counterstained with 2% uranyl acetate and Reynolds’ lead citrate and examined with a Phillips CM-12 electron microscope operated at 60 kV.

Immunohistochemistry

Immunostaining was done on paraffin sections by using the following antibodies: anti–smooth muscle $\alpha$-actin (clone 1A4) from Sigma; anti-Thy-1, anti–platelet endothelial cell adhesion molecule-1 (PECAM-1), anti–endothelial NO synthase (eNOS), and anti-Mac-3 from PharMingen; and rabbit anti-mouse MC/MPh metalloelastase (MME), which was prepared as described.\textsuperscript{17} The detection system for these antibodies comprised Vectastain ABC-peroxidase and avidin/biotin blocking kits from Vector Laboratories. The sections were counterstained with hematoxylin. For the assessment of cell proliferation, mice were implanted with osmotic minipumps (Alza Corp) releasing 5-bromo-2’-deoxyuridine (BrdU, Sigma) at a rate of 25 mg/kg per day for 10 days. Paraffin sections obtained from the labeled hearts were immunostained for BrdU incorporation using a commercial kit (Zymed Laboratories).

Exposure of MCP-1 Hearts to Circulating, Labeled Endothelial Progenitor Cells

To perfuse chronically the MC-tunneled tissues with mouse blood containing labeled progenitor ECs,\textsuperscript{18} the MCP-1 hearts were transplanted heterotopically\textsuperscript{19} to syngeneic recipients transgenically modified to express $\beta$-galactosidase under the control of the endothelial marker Tie-2.\textsuperscript{20} The data presented were obtained from a nonrejected (beating) MCP-1 heart, which was harvested 5 days after transplantation, included in OCT, cryosectioned, and stained for $\beta$-galactosidase activity.

Results

MME-Lined Tunnels Found in MCP-1 Hearts

In a previous report,\textsuperscript{16} we demonstrated the targeted expression of MCP-1 in the myocardium of transgenic mice under the control of the myosin heavy chain promoter. We have also provided data indicating that expression of the MCP-1 gene under the control of this cardiac-selective promoter induced the progressive accumulation of MCs within myocardium. The MCP-1 secretion and the subsequent MC infiltration remained asymptomatic during the early life of most of the animals. However, in the homozygous mice, the targeted expression of MCP-1 eventually produced the deterioration of cardiac function, with myocardial enlargement, heart failure, and premature death (N.I. Moldovan et al, unpublished results, 1998). The detailed pathogenesis of this cardiomyopathy is currently under investigation.

With the advancement of the cardiomyopathic process, we observed on microscopic sections a multitude of tunnels within the myocardium of MCP-1 mice, which had not been recognized in our previous study of this model (Figures 1, 3, and 4). Therefore, we addressed the hypothesis that MCs/ MPhs might be using potent metalloproteinases to infiltrate the myocardial tissue. The broad-spectrum mouse metalloelastase MME\textsuperscript{21} was immunodetected as being associated with MCs/MPhs and lining empty cylindrical spaces (tunnels) found in longitudinal and transversal sections of MCP-1 myocardium (Figures 1A and 1C through 1G). A particular feature, present in 2 of 6 examined specimens, was a higher intensity of the immunostaining in the subendarcardial myocardium (Figure 1A), suggesting a likely, although indirect, association between tunneling and ischemia in this model. MME positivity was not present in the endocardium of control, wild-type littermates (Figure 1B).

MME is a proteinase specific to MCs/MPhs\textsuperscript{21} and, together with the Mac-3 antigen, was used here to confirm the MC/MPh phenotype of tunnel-forming, infiltrating mononuclear cells (Figure 1H). MME staining was also present in the avascular tissue of cardiac valves (Figure 1F) but was absent within native microvessels (Figure 1G). These observations suggest that the tunnels and the capillaries are distinct entities.

The concurrent finding of extravascular structures staining for MME and of MME-positive MCs/MPhs (Figures 1C, 1E, and 4A) within these tunnels strongly suggest, although not definitively prove, the implication of MCs/MPhs in the process of tunnel formation. Indeed, MCs/MPhs displaying migratory, elongated morphology could be detected within these tunnels (Figures 1E and 1H).

Ischemia of the MCP-1 Hearts

Cross sections of failing MCP-1 mouse hearts revealed both intact epicardial coronary vessels (Figure 2B) and vessels affected by an occlusive vasculopathy (Figure 2A), with destruction of the elastic lamina and thrombotic complications. Moreover, many small arterioles were occluded, either by cellular (Figure 2C) or thrombotic (not shown) elements.
Downstream from the obstructed and thrombosed coronary arteries within the hearts of MCP-1 mice, signs of ischemic cardiomyopathic changes could be observed. Although the recruitment of MCs/Mphs to the myocardium per se could have contributed to the myocardial damage, we have accumulated evidence that loss of functional blood vessels may also have contributed to the cardiomyopathic process. We detected typical contraction bands (Figure 2D), whereas ultrastructural analysis of MCP-1 hearts by electron microscopy demonstrated swollen mitochondria with electron-dense deposits (Figure 2E), myofibril breakdown, and Z-band disorganization within CMs (Figure 2F). Myocytolysis with extensive CM loss was detected as well (Figures 1H, 3G, and 4F). These changes were consistent with the expected findings for an ischemic cardiomyopathy model.22,23

Blood Elements Are Found in Tunnels, Which May Contribute to Neovascularization
A detailed histological examination of MCP-1 hearts confirmed the presence of a network of tunnels of fairly uniform diameter, usually devoid of ECs (Figure 3A). PAS staining of MCP-1 hearts revealed PAS-positive structures of which the distribution was similar to that of MME-positive tunnels (Figure 3C), as well as occasional PAS-positive mononuclear cells (Figure 3C, arrowhead). Well-organized tunnel-like structures (PAS- and MME-positive), the detection of which is possibly facilitated by an increased incidence of edema, were found exclusively in MCP-1–expressing hearts and were largely absent from the hearts of nontransgenic littermates (Figures 3B and 3D), ruling out the possibility of processing artifacts.

The possible existence of 2 distinct microvascular structures in MCP-1 hearts was also suggested by immunostaining for 2 endothelial markers, eNOS and PECAM-1. Erythrocyte-filled, eNOS-negative tunnels were detected in MCP-1 hearts along with eNOS-positive microvessels (Figure 3E). In the wild-type control mice, all erythrocytes were confined to a high-density network of eNOS-positive capillaries (Figure 3F). This result was confirmed by the distribution of PECAM-1 immunoreactivity, which was found to be almost
absent in the tunnel containing MCP-1 hearts (Figure 3G), whereas PECAM-1 was present in the wild-type controls at comparable levels with eNOS immunostaining (Figure 3H).

Some nonendothelialized tunnels contained blood components, such as MCs (Figures 1E and 4A) and erythrocytes (Figures 3A, 3E, and 4C), whereas other tunnels were apparently colonized by EC-like cells (Figures 4A, 4B, and 4E). This finding is consistent with, although it does not prove, a process in which MC-/Mph-drilled tunnels can evolve to become neocapillaries if connected by a still-unknown mechanism to the microcirculatory system.

To collect additional support for this hypothesis, we stained paraffin sections from MCP-1 hearts with an antibody to Thy-1, a marker of hematopoietic stem cells, which is believed to be expressed also on ECs during adult neovascularization. Clusters of small vessels and incidental mononuclear cells were positive for Thy-1 staining (Figure 4F). In other microscopic fields, we found a patchy staining for Thy-1 within the contour of a single capillary (N.I. Moldovan, unpublished data, 2000).

An active angiogenic process was also indicated by the finding that many cells with EC morphology lining the tunnels displayed signs of DNA synthesis as assessed by BrdU incorporation (Figure 4B). The endothelial nature of the cells found within the tunnels was further supported by ultrastructural analysis, which revealed the presence of large vacuoles (Figure 4E), presumably the precursors of capillary lumens, as shown for sprouting ECs.

Moreover, when transplanted in recipients transgenically expressing β-galactosidase under an active endothelial-specific Tie-2 promoter (Figure 4D), MCP-1 hearts displayed a sparse infiltration with mononuclear, β-galactosidase-positive, presumably precursor ECs, both in capillaries and in tunnel-like structures (Figure 4C).

**Discussion**

In this study, we describe a network of MME-positive tubular structures (tunnels) penetrating the extracellular matrix and associated with the presence of MCs/Mphs in the myocardium of mice topically expressing MCP-1. As will be detailed below, the definition of a tunnel is not limited to a tubular “empty space,” but a tunnel is also characterized by the distribution of the nuclei of colonizing cells, MME immunostaining, and endothelial markers.

We have accumulated the following evidence that the infiltrating mononuclear cells produced these tunnels using the proteolytic activity localized at their surface: (1) MME-positive cells were found in tunnels, (2) the cross-sectional size of tunnels is in the range of diameters of mononuclear cells, (3) regions where MCs/Mphs are present display increased MME immunostaining, and (4) the finding of empty MME-positive tunnels suggests that they are produced by migrating MCs/Mphs.

MME, the mouse homologue of human matrix metalloprotease 12, is a broad-spectrum metalloprotease specifically produced by mononuclear phagocytic cells. Its expression correlates with MC/Mph activation status. MME is differentially expressed and clustered around vascular structures during experimental angiogenesis in the mouse. MME was
shown to be necessary for the penetration of basement membranes by MCs/Mφs in vivo and in vitro and for degradation of extracellular matrix proteins. Although direct evidence is lacking, correlative data suggest that tissue activity of metalloelastases is downregulated in some instances by tissue inhibitors of metalloproteinases and possibly stimulated by urokinase-type plasminogen activator. Both of these regulators are known to be modulated by ischemic conditions.

Therefore, the preferential immunostaining for MME found within the subendocardial region of MCP-1 hearts supports the possibility of an ischemia-driven activation of MCs/Mφs and, consequently, an MME-based proteolytic activity. In view of the report that MME production is not affected by MCP-1 levels, our finding that MME activity and MC-/Mφ-based tunneling are associated with the advanced stages of ischemic cardiomyopathy in this model might suggest that ischemia itself, or other factors related to ischemia, may contribute to the observed tunneling, although a more direct role for MCP-1 in this process cannot be ruled out.

Activation of MCs/Mφs increases their ability to invade the extracellular matrices in vitro as well, producing lasting tunnels similar to those described here. Moreover, phorbol 12-myristate 13-acetate, an MC/Mφ activator responsible for this effect, is also known as an inducer of MME.

Previous studies suggested that MME might be antiangiogenic, because of its ability to form angiostatin from plasminogen in vitro and to form endostatin from collagen XVIII. If this were the case, one would expect that the inhibition of MME would stimulate angiogenesis. In fact, MME displays an exquisite sensitivity to downregulation by dexamethasone, but contrary to expectations, this effect actually parallels a reduction, not the stimulation of angiogenic process in a murine wound-healing model in vivo. Moreover, the mere presence of an MME antigen lining the tunnels, presumably representing MME molecules or fragments thereof (supposedly shed during MC/Mφ migration), does not necessarily imply that the enzyme is still active. Actually, the limited and fairly uniform diameter of tunnels argues that MME activity is tightly regulated by local inhibitors.

In longitudinal, oblique, and transversal cross sections (as required in a critique of the tumor-associated PAS-positive “channels,” which were recently described as a form of “vascular mimicry”), we found PAS-staining material lin-
ing some of the endothelium-free tunnels, which might contribute to the retention and possibly to the differentiation of circulating EC progenitor cells.

In tunnels found in MCP-1 hearts, we could detect blood products such as erythrocytes and EC-like (ie, attached to tunnels’ walls) cells. This observation raises the intriguing possibility that some MC-/Mph-produced tunnels, if connected to a blood supply, can be colonized in a later stage by circulating precursor ECs displaying heightened mitotic activity.\(^\text{18}\) We were able to confirm the availability of circulating precursor ECs by Tie-2 cell tagging of the infiltrate of the MCP-1 myocardium and by the presence of immature ECs, by electron microscopy.

We consider it improbable that the infiltrating, Tie-2–driven, β-galactosidase–positive ECs could have migrated within the transplanted myocardium as a result of a sprouting process, given the distance from the sutures between the recipient’s β-galactosidase–positive endothelia and the actual places where we observed the tagged cells in the transplanted heart, as well as their individualized, single-cell localization. Furthermore, it is known that the re-endothelialization of grafts proceeds through the “fallout” of blood-derived, proliferating, BrdU-positive endothelial precursors.\(^\text{38}\) However, we do not rule out that blue cells originated from the recipient vessels and renested in the graft capillaries.

Discontinuity (“spottiness”) of the covering with ECs along a given tunnel, and of the labeling for markers of neoangiogenesis within and among microvessels (Figure 4), makes the sprouting an unlikely mechanism of re-endothelialization in our model of ischemic heart failure. One would expect ECs occurring by sprouting to be in contact with one another (contiguous) and of similar phenotype. New endothelial covering of former de-endothelialized capillaries may be ruled out as well on the basis of the MME positivity of the tunnels. Therefore, our findings can be better interpreted within the new paradigm of adult vasculogenesis\(^\text{14,18}\) on the basis of the dissemination of circulating precursor ECs. In this respect, the data reported in this study may contribute to understanding the mechanism of engrafting of circulating endothelial precursors within the ischemic tissue.

One may consider the presence of erythrocytes within endothelium-free tunnels in MCP-1 hearts as a manifestation of the leakiness of defective or immature capillaries. However, to our knowledge this type of leakiness was convincingly demonstrated mostly in the microcirculation of some tumors.\(^\text{39}\) Otherwise, what is generally considered “leakiness” of new capillaries refers mostly to an increased molecular permeability to solutes, which might be the effect of vascular endothelial growth factor (also known as the “vascular permeability factor”) on the transcellular diffusion of macromolecules rather than creation of large interendothelial junctional defects.\(^\text{40,41}\)

The limited amount of blood we found in tunnels may be explained either by the variable extension of their connection to the bona fide blood conduits or by the postmortem washing out

Figure 4. New mechanism of neovascularization in MCP-1 hearts. A, EC-like cells in an MME-positive tunnel cut in longitudinal section (arrow); a transversal section through a tunnel is also displayed (arrowhead). B, BrdU incorporation and mitotic figure (double arrow) within an elongated nucleus lining a tunnel. Long distance between adjacent nuclei (marked with single arrow vs double arrows) makes this structure likely to be a tunnel “seeded” with precursor ECs rather than a capillary (the same consideration applies to Figures 3G and 4A). C, β-Galactosidase–positive (Tie-2–expressing) mononuclear cells, found in an MCP-1 heart after perfusion with blood containing β-galactosidase–tagged, circulating endothelial precursors, by transplantation in a LacZ-Tie-2 mouse. Capillaries in this cryosection are detected by the presence of erythrocytes (arrows), whereas the empty space containing one of the β-galactosidase (Tie-2)–positive cells appears to be a tunnel. D, β-Galactosidase (Tie-2)–positive vessels on a cryosection from the heart of the recipient mouse (wild type for MCP-1 expression). E, Electron micrograph of cells filling a tunnel in the space between 2 CMs (arrows). Note the large flat vacuoles, suggestive of the immature lumen of sprouting or precursor ECs. F, Immunoperoxidase staining for Thy-1, a marker of activated/adult neovascular, in a selected group of capillaries in the epicardial region of an MCP-1 heart. Magnifications: A and B, ×720; C, ×360; D, ×240; E, ×2500; F, ×300.

Moldovan et al Monocyte-Drilled Vascular Tunnels 383
of blood. However, in an in vitro model, we have been able to demonstrate the formation of tunnels by MCs/Mphs in extracellular matrices, the stimulating effect of MCP-1 on this process, and the ability of these tunnels to be filled by blood-derived erythrocytes (N.I. Moldovan, unpublished data, 2000).

In conclusion, with the availability of a transgenic model in which the development of a dilated ischemic cardiomyopathy can be reliably reproduced by target expression of MCP-1 in the myocardium, we made a series of observations that are shedding light on a possible novel mechanism of neovascularogenesis as it takes place in the ischemic myocardium and on the role of MCs/Mphs in this process, indicating new strategic targets for therapeutic angiogenesis.

Acknowledgments

This work was supported by NIH Grants GM53236 and HL52315 (to P.J.G.-C.) and by an award from the Scleroderma Research Foundation. This work was supported by NIH Grants GM53236 and HL52315 (to P.J.G.-C.); by an award from the American Heart Association (Established Investigator award to P.J.G.-C.); by an award from the American Heart Association, Ohio Valley Affiliate (to N.I.M.); and by the Scleroderma Research Foundation.

References

Contribution of Monocytes/Macrophages to Compensatory Neovascularization: The Drilling of Metalloelastase-Positive Tunnels in Ischemic Myocardium
Nicanor I. Moldovan, Pascal J. Goldschmidt-Clermont, Jan Parker-Thornburg, Steven D. Shapiro and Pappachan E. Kolattukudy

_Circ Res._ 2000;87:378-384
doi: 10.1161/01.RES.87.5.378

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/87/5/378

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/