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Original article

De-novo collateral formation following acute myocardial infarction: Dependence on CCR2^{+} bone marrow cells

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ABSTRACT

Wide variation exists in the extent (number and diameter) of native pre-existing collaterals in tissues of different strains of mice, with supportive indirect evidence recently appearing for humans. This variation is a major determinant of the wide variation in severity of tissue injury in occlusive vascular disease. Whether such genetic-dependent variation also exists in the heart is unknown because no model exists for study of mouse coronary collaterals. Also owing to methodological limitations, it is not known if ischemia can induce new coronary collaterals to form ("neo-collaterals") *versus* remodeling of pre-existing ones. The present study sought to develop a model to study coronary collaterals in mice, determine whether neo-collateral formation occurs, and investigate the responsible mechanisms. Four strains with known rank-ordered differences in collateral extent in brain and skeletal muscle were studied: C57BLKS>C57BL/6>A/J>BALB/c. Unexpectedly, these and 5 additional strains lacked native coronary collaterals. However after ligation, neo-collaterals formed rapidly within 1-to-2 days, reaching their maximum extent in ≤7 days. Rank-order for neo-collateral formation differed from the above: C57BL/6>BALB/c>C57BLKS>A/J. Collateral network conductance, infarct volume^{-1}, and contractile function followed this same rank-order. Neo-collateral formation and collateral conductance were reduced and infarct volume increased in MCP1$^{-/-}$ and CCR2$^{-/-}$ mice. Bone-marrow transplant rescued collateral formation in CCR2$^{-/-}$ mice. Involvement of fractalkine ➔ CX_3CR1 signaling and endothelial cell proliferation were also identified. This study introduces a model for investigating the coronary collateral circulation in mice, demonstrates that neo-collaterals form rapidly after coronary occlusion, and finds that MCP ➔ CCR2-mediated recruitment of myeloid cells is required for this process.

1. Introduction

Most tissues have a native collateral circulation composed of infrequent arteriole-to-arteriole anastomoses that cross-connect the crowns of adjacent arterial trees [1–6]. The pressure drop that occurs across them after a sudden occlusion in the trunk of one of the trees causes blood to flow across the collateral network. By providing an alternative route for flow, collaterals can significantly lessen ischemic injury depending on their number and diameter, i.e., "extent". In animal models of sustained arterial obstruction, collaterals in the brain and lower extremities remodel (enlarge) their anatomic lumen diameter by shear stress-induced and, depending on the tissue, hypoxia-induced processes that require days-to-weeks for completion [1–7]. The ability of remodeling to reduce final infarct volume depends on the collateral extent at baseline before obstruction, the volume of ischemic penumbra or border zone surrounding the necrotic core, the tolerance of the tissue to ischemia, how rapid remodeling occurs, and how much collateral diameters increase. Collateral remodeling in heart, where these vessels are difficult to image directly, is believed to underlie the progressive increase with time in collateral-dependent flow in animal models of and patients with acute myocardial infarction (MI), coronary artery disease (CAD) and acute coronary syndrome [1–6].

Indirect measures of collateral-dependent blood flow suggest that native collateral extent in heart, brain and lower extremities varies greatly among "healthy" humans, i.e., in the absence of CAD [4] or peripheral artery disease [8] or when assessed in the hyper-acute phase of ischemic stroke [9,10], i.e., conditions where remodeling—which can also vary among individuals [1–7,11–15]—has not yet taken place. This variation at baseline has been suggested to be a major determinant of the wide variation in the severity of tissue injury when arterial

Abbreviations: αSMA, α-smooth muscle actin; B6, C57BL/6 mouse strain; BLKS, C57BLKS mouse strain; BMC, bone marrow-derived cell; CAD, atherosclerotic coronary artery disease; CCR2, chemokine CC motif receptor-2; CX_3CR1, chemokine CXC motif receptor-1; CCL, chemokine CC motif ligand; EdU, 5-ethynyl-2′-deoxyuridine; EC, endothelial cell; EPC, endothelial progenitor cell; LADX, left anterior descending coronary artery ligation; dp/dt +/−, derivative of left ventricular pressure rise/fall; LVED/SP, left ventricular end-diastolic/systolic pressure; MI, myocardial infarction; MCP1, monocyte chemoattractant protein-1; MSC, mesenchymal stem/stromal cell; SDF1, stromal-derived factor-1; SMC, smooth muscle cell; TTC, 2,3,5-triphenyltetrazolium chloride; WT, wildtype.

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obstruction develops [4,5,7,9–15]. Consistent with these studies, among patients in which thrombolytic treatment failed to elicit reperfusion during the acute phase of MI, those with "good/high" collateralization had 35% smaller infarct volumes [16]. Similarly, in patients with CAD, those with good collaterals had a 36% lower mortality risk than those with poor collaterals [4]. The cause of this variation in collateral circulation is unknown. However, recent studies in mice have found that differences in genetic background may be a major factor [5,7,12–15]. Collateral extent in brain varied 46-fold among 21 strains of mice [14], with large variation also present in skeletal muscle and intestine examined in several of the same strains [11–13]. The differences correlated with wide differences in tissue injury in models of acute and chronic arterial obstruction in brain (e.g., 30-fold variation in infarct volume [7]) and lower extremities [12,13]. Approximately 85 percent of the variation in both collateral extent and tissue injury was recently linked to a polymorphic genetic locus (*Dce1*), which when introgressed from a strain with abundant collaterals into one with poor collaterals, completely rescued the collateral deficit and functional outcome attributable to the locus [15]. This locus is thus a critical link in the pathway that directs collaterogenesis, i.e., formation of native collaterals. In mouse brain [17,18] and human heart [19] collaterogenesis occurs during development after the general arterial–venous circulation has formed, and in mouse determines collateral extent in the adult.

Whether such genetic variation in native collaterals also exists in heart to explain the above findings in humans [4,16] is unknown because of absence of methods to study the coronary collateral circulation in mice—the mammalian species most amenable to genetic manipulation. This presumably is because of the complex three-dimensional arrangement of the coronary circulation and difficulty in distinguishing collaterals from other vessels (problems not confined to mice), phasic contraction of the heart which in mouse exceeds 550 beats per minute, small size of the mouse heart, and difficulty in studying its coronary circulation. It is also not known if occlusive disease can induce new coronary collaterals to form. It has generally been assumed [1] (but not without exception [3]) that only remodeling of native collaterals occurs after arterial stenosis or occlusion. However, this assumption remains uncertain because of limitations in resolution of previous angiographic methods: native collaterals can be as small as the smallest arterioles in a tissue (~6–8 microns in diameter), are thousand-fold less abundant than nearby arterioles (e.g., in skeletal muscle [11]), and can only be anatomically differentiated from the latter by identifying their anastomotic connections. Once collaterals have remodeled after arterial occlusion, their many-fold larger diameter and increased tortuosity make them somewhat easier to identify. However, the number then detected would reflect native collaterals that have remodeled and—if in fact it occurs—*de novo* formation of additional ones ("neo-collaterals" [5]). And neither native collaterals nor neo-collaterals could be distinguished from each other based on diameter, since the amount of remodeling of native collaterals would depend on their baseline diameter [7,13] (the primary determinant of shear stress) and the vigor of the pathways controlling the remodeling process [1–6], while the final diameter of any neo-collaterals that formed would reflect the process of *de novo* collateral formation.

To address the above questions, we first optimized methods to study the coronary collateral circulation in mice, namely high resolution three-dimensional angiography, measurement of conductance of the collateral network, and histology on single identified collaterals. An unexpected finding followed: mice lack a native collateral circulation in heart even though they have collaterals in their other tissues. This surprising outcome, however, allowed us to unambiguously ask whether new collaterals form after arterial obstruction. Patent neo-collaterals indeed appeared rapidly within 1-to-2 days after acute MI and achieved their maximal number and diameter within 7 days. Neo-collateral formation varied with genetic background. Strains with greater formation had greater collateral conductance, smaller final infarct volumes, and better recovery of contractile function. However, the strain-specific pattern differed from that seen for differences in extent of the native collateral circulation in brain and other tissues of the same strains [7,12,13], indicating that different mechanisms direct collaterogenesis in the embryo and neo-collateral formation in the ischemic adult heart. In addition, we found that bone-marrow-derived myeloid cells and MCP1 ➔ CCR2, and to a lesser degree fractalkine ➔ CX_3CR1 signaling, were required. This study provides a model to study new collateral formation after acute myocardial infarction that may identify novel targets for treatment of ischemic disease.

2. Methods

See the online Data Supplement for additional details.

2.1. Animals

Mice, guinea pigs and rats were male and 3–5 months-old, except in the following experiments: inbred strains of mice on day-1 after ligation; measurement of retrograde fill time and infarct volume; bone marrow transplants; CCR2 and CX_3CR1 marker mice. These used ~equal numbers of male and female mice.

2.2. Coronary ligation, micro-angiography, morphometry, collateral conductance

The left anterior descending coronary artery was ligated 3 mm below the left atrial margin (LADX) to produce a small infarction of 10–20% of left ventricle-plus-septum (LVS) wall volume, thus minimizing stimuli for compensatory hypertrophy and vascular growth that otherwise occurs in the normal (remote) myocardium following a large MI [3]. Approximately 99% survival occurred. Proximal ligation to produce a larger infarction (~45% of LV volume) was also examined in a separate group of B6 mice, wherein ~50% survived without evidence of heart failure as reported previously [20]. Immediately or specified days after LADX, mice received one or more of the following after administration of heparin, maximal dilation with papavarine and nitroprusside, and fixation with paraformaldehyde to prevent any subsequent constriction ("hep-dil-fix"): 1) arterial angiography following infusion of Microfil^{R} and optical clearing to determine the number and lumen diameter of neo-collaterals within the border zone connecting distal branches of the LAD to adjacent circumflex, right coronary and septal arterial trees; 2) measurement of either the time^{-1} required to backfill the LAD tree to the point of ligation with Microfil or microsphere trapping (both administered at a constant inflow pressure) to determine the relative "conductance" or blood flow, respectively, of the nascent collateral network; 3) perfusion of Evans blue-in-phosphate buffered saline (PBS) to determine the territory of the LAD tree below the ligation.

2.3. Infarct volume, dp/dt analysis, histology, cell proliferation, apoptosis, bone marrow transplantation

Infarct volume was measured using 2,3,5-triphenyltetrazolium chloride staining (TTC) and cardiac function using dp/dt analysis. Histology was performed for neo-collaterals that were identified after hep-dil-fix, filling and clearing by: 1) their cross-connecting the LAD and adjacent arterial trees, and 2) retention of Microfil in their lumen following dissection of the tissue micro-block containing the collateral and sectioning at 8 microns. To determine cell proliferation, EdU (5-ethynyl-2′-deoxyuridine) was injected on day-2 and day-4 after LADX, followed by dissection of neo-collateral-containing tissue blocks on day-7. Bone marrow transplant was performed to generate EGFP;B6-transgenic mice and 4 reciprocal CCR2$^{-/-}$;C57BL/6 (B6) chimeras.