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Original Article

MMPs 2 and 9 are essential for coronary collateral growth and are prominently regulated by p38 MAPK

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ABSTRACT

Transient, repetitive ischemia (RI) stimulates coronary collateral growth (CCG) in normal, healthy (SD) rats, which requires p38 MAPK activation. In contrast, RI does not induce CCG in the metabolic syndrome (JCR) rats, which is associated with lack of p38 MAPK activation. The functional consequences of p38 MAPK activation in CCG remain unknown. Theoretically, effective collateral growth would require extracellular matrix remodeling; however, direct assessment as well as identification of proteases responsible for this degradation are lacking. In this study, we investigated the role of p38 MAPK in the regulation of matrix metalloproteinases 2 and 9 (MMPs 2 and 9) and their requirement for CCG in SD vs. JCR rats. The rats underwent the RI protocol (8 LAD occlusions, 40 s each, every 20 min, in 8 h cycles for 0, 3, 6, or 9 days). MMP expression was measured in the ischemic, collateral-dependent zone (CZ) and the normal zone (NZ) by Western blot, and MMP activity by zymography. Expression and activation of MMP 2 and 9 were significantly increased (~3.5 fold) on day 3 of RI in the CZ of SD rats. In vivo p38 MAPK inhibition completely blocked RI-induced MMP 2 and 9 expression and activation. MMP activation correlated with increased degradation of components of the basement membrane and the vascular elastic laminae: elastin (~3 fold), laminin (~3 fold) and type IV collagen (~2 fold). This was blocked by MMP 2 and 9 inhibition, which also abolished RI-induced CCG. In contrast, in JCR rats, RI did not induce expression or activation of MMP 2 or 9 and there was no associated degradation of elastin, laminin or type IV collagen. In conclusion, MMP 2 and 9 activation is essential for CCG and is mediated, in part, by p38 MAPK. Furthermore, compromised CCG in the metabolic syndrome may be partially due to the lack of p38 MAPK-dependent activation of MMP 2 and 9 and resultant decreased extracellular matrix degradation.

1. Introduction

Myocardial ischemia–reperfusion injury is a well-known phenomenon resulting from prolonged periods of ischemia followed by re-oxygenation. However, short, repetitive periods of ischemia followed by reperfusion can lead to adaptive responses and render the myocardium tolerant to longer periods of ischemia, in part through promoting collateral development [1]. Stable angina pectoris is a consequence of significant coronary artery constriction, and is characterized by transient periods of ischemia, upon increased myocardial metabolic demand followed by reperfusion at rest. Coronary collateral growth (CCG) is an adaptive response to transient, repetitive myocardial ischemia (RI). Clinically, patients with stable angina have a decreased incidence of fatal myocardial infarction, which is associated with better developed collateral networks [2]. In contrast, CCG has been shown to be severely impaired in patients suffering from type II diabetes [3] and the metabolic syndrome [4]. Likewise, CCG is impaired in our metabolic syndrome rat model (JCR:LA-cp or JCR) [5]. The JCR rat is obese, dyslipidemic (low HDL, high LDL, VLDL, and triglycerides) [5], insulin resistant with impaired glucose tolerance [6], and hypertensive [5], and thus, mimics the complex pathology of the human metabolic syndrome.

The process of CCG involves endothelial and vascular smooth muscle cell (VSMC) proliferation and migration, as well as extracellular matrix (ECM) remodeling. The early phase of collateral growth is associated with inward remodeling, in which cells migrate across the internal elastic lamina and the basement membrane, into the lumen of the pre-existing native collaterals. This is followed by outward remodeling in which cells migrate across the external elastic lamina into the vascular adventitia and the surrounding myocardium, thus allowing for vessel expansion and significant increases in blood flow [7–9]. Consequently, reorganization of the ECM, including ECM degradation, is a presumed integral part of collateral remodeling. However,

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direct measurements of this process during collateral growth have never been reported.

ECM degradation requires matrix metalloproteinases (MMPs), zinc-dependent endopeptidases capable of degrading extracellular matrix proteins. MMPs can be separated based on substrate specificity into interstitial collageneases (MMPs 1, 8 and 13), broad specificity MMPs (MMPs 3 and 7), metalloelastases (MMP 12), membrane-bound MMPs (MMP 14 (MT1-MMP) and MMP 17), and gelatinases (MMP 2 and 9). MMP 2 and 9 have been shown to degrade type IV collagen, laminin and elastin, the primary components of the vascular basement membrane and the internal and external elastic laminae, in vitro [10–13]. They are known to play a role in cell proliferation, migration, differentiation, angiogenesis associated with cancer metasthesis, neointima formation following vascular injury and aneurysm formation and rupture [14–16]. Although degradation of the basement membrane and the vascular elastic laminae is a common aspect shared between these processes and collateral remodeling, they are not identical, and conclusions drawn from these studies do not uniformly apply to collateral growth. Increased MMP 2 and 9 expression has been associated with collateral growth, but the results are not entirely in agreement. In one study, during the early, inward remodeling phase in growing coronary collaterals, the neointima showed high expression of MMPs 2 and 9 while mature collaterals expressed low levels of these MMPs [17]. On the other hand, MMP 2 but not MMP 9 expression and activity were increased in mesenteric collateral vessels [18]. Importantly, a conclusive requirement for MMP 2 and 9 activation in CCG has not been shown. Furthermore, it is unknown whether MMP 2 and/or 9 regulation is altered in the metabolic syndrome, where CCG is impaired.

MMPs are regulated at the level of both expression and activation. Several signaling pathways have been shown to regulate MMP expression and/or activation. Among these are the mitogen activated protein kinases (MAPKs). MAPKs can be divided into the extracellular signal-regulated kinases (ERK1/2), p38 MAPK, and c-Jun N-terminal kinase (JNK) [19]. The ERK1/2 pathway has been shown to regulate expression and activation of many MMPs including MMP 9 [20]. p38 MAPK, when activated during inflammation or the innate immune response, lead to the activation of MMP 9 [21], and a single study implicates p38 MAPK in the regulation of MMP 9 activation in cultured airway smooth muscle cells [22]. However, it is not known which signaling pathways may regulate MMP expression and/or activation in collateral growth.

We have previously shown that transient p38 MAPK activation on day 3 of RI is required for CCG in the normal healthy rat model where inhibition of p38 MAPK resulted in ~60% reduction in RI-induced CCG [23]. In addition, we have shown that RI-induced CCG was severely compromised in the metabolic syndrome JCR rat model, and that this correlated with lack of RI-induced p38 MAPK activation [24]. However, the functional consequence of p38 MAPK activation in collateral growth remained unknown. Therefore the goals of this study were to determine: 1) whether RI-induced activation of p38 MAPK regulated the development of coronary collaterals through the activation of MMP 2 and 9 and the degradation of their ECM substrates, 2) whether MMP 2 and 9 were required for this ECM degradation and CCG, and 3) whether MMP 2 and/or 9 expression and/or activity were altered in the metabolic syndrome.

2. Materials and methods

2.1. Rat model of coronary collateral growth/RI

Male, 10–12 week old Sprague–Dawley (SD) (300–350 g) or obese JCR:LA-cp rats (JCR) (650–700 g) were used for chronic implantation of a pneumatic occluder over the left anterior descending coronary artery (LAD), as described previously [25]. The RI protocol for rats consisted of 8 40s occlusions, one every 20 min over 2 h 20 min followed by a period of "rest" for 5 h 40 min. This 8 h cycle was repeated three times per day for 0–9 days. The specific inhibitors of p38 MAPK, SB203580 (3.2 mg/kg/day, Calbiochem), and of MMP 2 and 9, SB-3CT (25 mg/kg/day, Enzo Life Sciences), were delivered once/day on days 2 and 3 of the RI protocol i.v. via a jugular vein catheter implanted at the time of LAD occluder implantation. All surgical procedures were performed in accordance with the Animal Welfare Act and are approved by the IACUC of the University of South Alabama.

Myocardial and collateral-dependent blood flow measurements were performed as described previously [26]. Color microspheres (5×10^5, 15 μM diameter) labeled with gold (at day 0 of RI (initial surgery)) or samarium (at day 10 of RI) were injected into the left ventricle (LV) during LAD occlusion. Arterial reference blood samples (carotid) and heart tissue from the normal, non-ischemic zone (NZ) and the collateral-dependent, ischemic zone (CZ), were collected, weighed and sent to BioPal (Worcester, MA) for analysis. Blood flows to the normal (NZ) and collateral-dependent (CZ) zones (ml/min/g) were calculated from the formula: Blood Flow = [(radioactive counts in myocardial tissue) × (blood reference withdrawal rate)/(radioactive count in blood reference)]/(weight of myocardial tissue). Blood flows were measured in the following groups of animals: SD sham, SD sham + SB-3CT, SD RI and SD RI + SB-3CT. Results were expressed as the CZ/NZ flow ratio at day 10 of RI. All experiments were n = 5. Results were analyzed by 2-way ANOVA followed by Bonferroni correction. $p<0.05$ determined statistical significance.

2.2. Western blot analysis

Hearts were excised, and the LAD-dependent, collateral-dependent zone (CZ) was separated from the non-ischemic normal zone (NZ) then snap-frozen in liquid nitrogen before homogenization in lysis buffer containing 0.1% SDS and 1% Triton as previously described [25]. Equal amounts of protein (30 or 50 μg) were separated by SDS-PAGE, and transferred to Hybound-ECL nitrocellulose membranes. Anti-MMP 2 (Anaspec), anti-MMP 9 (Milipore), phospho-specific anti-p38 MAPK and anti-MK2 (Cell Signaling) were used for Western blot analysis. Bands were visualized by enhanced chemiluminescence (Amersham) and quantified using Un-Scan-It Image software (Silk Scientific Corporation). Experiments were n = 5 animals per group, and analyzed by two-way ANOVA followed by the Bonferroni post-hoc analysis. $p<0.05$ determined statistical significance.

2.3. Gelatin zymography

Hearts were excised, the CZ separated from the NZ, and snap-frozen in liquid nitrogen before homogenization in lysis buffer containing 4% SDS and 25% glycerol, followed by incubation at 37 °C for 20 min. Equal amounts of protein (30 μg) were separated by SDS-PAGE gels containing 1 mg/ml gelatin (Bio-Rad 161–1167). The gels were then incubated in renaturing buffer (2.5% X-100) twice for 20 min and washed after each incubation period with ddH_2O, then incubated in the developing buffer (50 mM Tris–HCl, pH 7.6, 0.2 M NaCl, 5 mM $CaCl_2$, and 0.02% BriJ 35) overnight at 37 °C in a shaking water bath. After being rinsed with ddH_2O, gels were stained with Coomassie brilliant blue for 30 min to 1 h then partially de-stained using a 10% acetic acid/20% methanol/70% ddH2O solution for 30–45 min. Gels were scanned using HP Scanjet G4050 and bands quantified using Un-Scan-It Image software. Experiments were n = 5 animals per group, and analyzed by two-way ANOVA followed by the Bonferroni post-hoc analysis. $p<0.05$ determined statistical significance.

2.4. ECM degradation

Type IV collagen, laminin and elastin degradation in tissue lysates was evaluated according to similar protocols [27–29]. Hearts were excised, and the CZ was separated from the NZ then snap-frozen in liquid nitrogen before homogenization in lysis buffer containing 0.1% SDS