Opposing Effects Mediated by the Chemokine Receptor CXCR2 on Myocardial Ischemia-Reperfusion Injury
Recruitment of Potentially Damaging Neutrophils and Direct Myocardial Protection

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Background—The timely reperfusion of ischemic myocardium limits infarction, but components of reperfusion, such as inflammation, may be injurious. The chemokine receptor CXCR2 mediates neutrophil chemotaxis. CXCR2 activation also inhibits hypoxia-induced death of isolated cardiac myocytes. This study assesses whether CXCR2 mediates protection in the intact heart and, if so, the magnitude of this protection relative to CXCR2-mediated chemotaxis of potentially damaging inflammatory cells.

Methods and Results—After ischemia-reperfusion in vivo, CXCR2−/− mice exhibited infarcts that were 50.5% smaller (P<0.05) with 44.3% fewer inflammatory cells (P<0.05) than wild type mice. These data suggest that in this model, CXCR2-mediated chemotaxis may be important in myocardial cell death. To isolate the role of CXCR2 specifically on blood cells, adoptive transfer experiments were performed. After ischemia-reperfusion, infarcts were 53.4% smaller (P<0.05) and contained 65.0% fewer inflammatory cells (P<0.05) in lethally irradiated wild type mice reconstituted with CXCR2−/− compared with wild type bone marrow. Thus, CXCR2 on blood cells is important in myocardial damage, most likely because of CXCR2-mediated chemotaxis. To unmask whether CXCR2 mediates direct myocardial protection in the intact heart, wild type and CXCR2−/− hearts were studied in the absence of blood using Langendorff preparations. In this case, infarcts were 19.7% larger in CXCR2−/− than wild type hearts (P<0.05), revealing a novel CXCR2-mediated cardioprotective effect.

Conclusions—CXCR2 exerts opposing effects on myocardial viability during ischemia-reperfusion with recruitment of damaging inflammatory cells predominant over direct tissue protection. (Circulation. 2003;108:2387-2392.)

Key Words: ischemia ■ reperfusion ■ chemokines ■ leukocytes

A large body of work over the past 3 decades has established that the restoration of blood flow limits myocardial damage and cardiac dysfunction after prolonged ischemia.1 Although its net benefit is clear, reperfusion itself may cause some cardiac myocyte death,2,3 a portion of which occurs by apoptosis.4 Reperfusion injury may be mediated through multiple mechanisms, including reactive oxygen species, complement activation, endothelial dysfunction, cytokines, and neutrophil infiltration.5,6 The precise mechanisms by which neutrophils kill cardiac myocytes are not well understood but may involve the release of proteolytic enzymes, reactive oxygen species, and arachidonic acid metabolites.6–10 Although the role of neutrophil infiltration in reperfusion injury is well established in many tissues,11–15 there is controversy as to its importance in the myocardium, some of which may be explained by species and model differences.6,7,16–20

Neutrophil recruitment is regulated by a complex array of signals,5 including activated complement and the CXC family chemokines interleukin-8 (IL-8), macrophage inflammatory protein-2 (MIP-2), cytokine-induced neutrophil chemoattractant (KC/Gro α), and lipopolysaccharide-induced CXC chemokine (LIX).21–24 The expression of these chemokines is induced by ischemia in a variety of tissues, including the myocardium.24–26 These molecules signal through the G protein–coupled receptor CXCR2, and IL-8 also activates CXCR1.21–23,27,28 These receptors are present on neutrophils. Marked defects in neutrophil recruitment result from genetic inactivation of CXCR229 or its inhibition by truncated ligands30 in the mouse, which lacks endogenous IL-8 and...
CXCR1. Thus, CXCR2 is critical for neutrophil recruitment in the mouse.

In addition to its role in neutrophil chemotaxis, activation of CXCR2 by MIP-2 has been shown to protect mouse hepatocytes from adenosivus- and acetaminophen-induced injury, an effect that is abrogated in mice lacking CXCR2. This protection is believed to be a direct effect on resident cells rather than a secondary consequence of alterations in the inflammatory response. We have recently shown that MIP-2 inhibits hypoxia-induced death in isolated cardiac myocytes. Interestingly, hypoxia also stimulates the expression of MIP-2 as well as CXCR2 in these cells. These data suggest that CXCR2 activation may result in 2 diametrically opposed effects on the myocardium during ischemia-reperfusion: death caused by the recruitment of leukocytes and survival because of direct cytoprotection. Both of these effects would be anticipated to be mediated through CXCR2, the receptor for MIP-2 and the other CXC chemokines involved in neutrophil recruitment.

The objective of this study was to dissect the relative importance of the chemotactic and cytoprotective effects on the myocardium mediated through CXCR2 during ischemia-reperfusion injury. To accomplish this, ischemia-reperfusion was studied in mice lacking CXCR2 in all cell types (including cardiac myocytes and leukocytes), in chimeric mice that we engineered to lack CXCR2 only in hematopoietic cells, and in CXCR2−/− isolated hearts in the absence of blood. The data demonstrate that CXCR2 activation results in both leukocyte recruitment and direct myocardial protection. Of these, leukocyte recruitment is the predominant effect.

Methods

Mouse In Vivo Ischemia-Reperfusion Model

Eight-week-old wild type or CXCR2−/− male mice on a BALB/c background were studied (Jackson Laboratories, Bar Harbor, Me). Mice were anesthetized initially with methoxyflurane (Pitman-Moore) and ventilated through a nose cone with a tidal volume of 0.2 mL at 120 breaths/min using a rodent respirator (model 683; Harvard Apparatus). Anesthesia was maintained with 2% isoflurane (Ohmeda PPD) delivered in 100% O2 through the ventilator. A thoracotomy Apparatus). Anesthesia was maintained with 2% isoflurane (Ohmeda PPD) delivered in 100% O2 through the ventilator. A thoracotomy was performed in the left third intercostal space, and the beating heart was isolated. An 8-0 Prolene suture was passed under the left coronary artery at the inferior edge of the left atrium and tied with a slipknot to produce occlusion. Air was then evacuated from the chest cavity and the chest closed in layers with the ends of the slip outside of the incision. The ventilator was then removed and normal respiration restored. After 45 minutes of ischemia, the left coronary artery occlusion was released by pulling on the slipknot. After 24 hours of reperfusion, animals were killed.

Evaluation of Risk Area and Infarct Size

The heart was excised and arrested in ice-cold phosphate-free Krebs-Henseleit buffer containing (in mmol/L) NaCl 118, KCl 5.3, CaCl2 2.0, MgSO4 1.2, EDTA 0.5, NaHCO3 25, pyruvate 0.5, and glucose 11, equilibrated with 95% O2/5% CO2, yielding a pH of 7.40. The aorta was then connected to a 23-gauge perfusion cannula, and retrograde perfusion of the Krebs-Henseleit buffer was performed at a constant pressure of 80 mm Hg at 37.5°C. The pericardium and extracardiac tissues were carefully trimmed off. The heart was then arrested with 70 mmol/L KCl, after which it was perfused with 1% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma) in 70 mmol/L KCl. TTC, which is reduced by mitochondrial reductases to a brick-red color, demarcates viable tissue. The absence of this color indicates tissue death by necrosis or apoptosis. After this, the original suture, which remained in place, was re-tied. The heart was then perfused with 5% phalothal blue dye (Heucotuch). The presence of phalothal blue indicates perfusion, and its absence indicates lack of perfusion. The left ventricle (LV) including the interventricular septum was next dissected away and placed in 10% neutral buffered formalin for 30 minutes. After the development of tissue turgor, the LV was sectioned into 5 to 7 slices, which were reimmersed in the formalin for 72 hours. The slices were then weighted, and both sides of each slice were photographed with a digital camera (Nikon, Coolpix). For each picture, LV area excluding chamber, area lacking phalothal blue staining (risk area), and area lacking TTC staining (infarct area) were determined by use of Image J (NIH Image). The measurements from both sides of each slice were averaged. For each slice, the ratios of risk area/LV and infarct area/LV were determined and multiplied by the weight of the slice. These numbers for each slice were then summed over all slices, divided by the total weight of all slices, and multiplied by 100 to yield the risk area/LV and infarct area/LV for that heart as a percentage. Last, the infarct area as a percentage of the risk area was calculated by dividing the infarct area/LV by the risk area/LV and multiplying by 100. Infarct size determinations were performed by a single individual who was blinded to genotype and treatment status.

Historical Assessment

Tissue blocks from the above hearts were paraffinized, cut into 5- to 10-µm sections, stained with hematoxylin and eosin, and examined by light microscopy. For each section, the number of inflammatory cells per mm2 was quantified by averaging the number of inflammatory cells in 16 randomly selected fields in the LV free wall (which includes the infarct in our model) and dividing by the area of each field (0.027 mm2). Three sections were assessed per heart.

Generation of Chimeric Mice

Six-week-old wild type BALB/c male mice received 9 Gy of γ-radiation administered as a split dose, after which 5×105 cells of wild type or CXCR2+/− bone marrow was injected through the tail vein. Twenty-four days later, mice were subjected to 45 minutes of ischemia/24 hours of reperfusion in vivo, and risk area and infarct size were determined as described above.

Mouse Langendorff Regional Ischemia-Reperfusion Model

Eight-week-old wild type or CXCR2−/− male mice on a BALB/c background were heparinized (100 U/IP) and killed 20 minutes later. The heart was excised and hung as a Langendorff preparation as described above except for the omission of KCl arrest. After 20 minutes of perfusion with the Krebs-Henseleit buffer at a constant pressure of 80 mm Hg at 37.5°C to equilibrate the heart, the left coronary artery was ligated at the level of the inferior edge of the left atrium for 45 minutes. The suture was then released and the heart reperfused for 2.5 hours. Sham-operated animals underwent the same procedure except that the suture was briefly passed under the left coronary without being tied. The hearts were then arrested with 70 mmol/L KCl injected through the side port of the perfusion system, and risk area and infarct size were measured as described above.

Statistics

All data are expressed as the mean±SEM. Differences between groups were determined with a 2-tailed Student’s t test or a 1-way ANOVA followed by a Newman-Keuls post hoc test. Probability values of P<0.05 were considered to be significant.

Results

CXCR2-Deficient Mice Exhibit Smaller Infarcts With Fewer Inflammatory Cells After Ischemia-Reperfusion In Vivo

To determine the effect of CXCR2-mediated signaling on infarct size, animals deficient in CXCR2 in all cells were
subjected to 45 minutes of ischemia followed by 24 hours of reperfusion in vivo. Despite similar risk areas (Figure 1A; wild type 48.5±2.3% [n=6], CXCR2−/− 51.7±3.5% [n=6], P=NS), the CXCR2−/− mice exhibited infarcts within the risk areas that were 50.5% smaller than wild type (Figure 1A; wild type 50.3±4.6% [n=6], CXCR2−/− 24.9±3.2% [n=6], P<0.05). Thus, the overall effect of CXCR2-mediated signaling contributes to the development of the infarct. A potential explanation for this result is that CXCR2-mediated recruitment of inflammatory cells influences infarct size to a greater extent than whatever direct effects CXCR2 activation has on inhibiting myocardial cell death. In keeping with this possibility, histological examination of heart sections showed 44.4% fewer inflammatory cells in CXCR2−/− hearts, consistent with less myocardial damage.

Selective Absence of CXCR2 on Bone Marrow–Derived Cells Decreases Infarct Size and Inflammatory Cell Infiltration After Ischemia-Reperfusion In Vivo

To isolate the effect of CXCR2-mediated inflammatory cell infiltration on infarct size, we generated chimeric mice that were either wild type in all cells or wild type in all cells except those derived from bone marrow, which were CXCR2−/−. These chimeras were generated by transplanting lethally irradiated wild type animals with either wild type or CXCR2−/− bone marrow cells. Twenty-four days later, these mice were subjected to 45 minutes of ischemia/24 hours of reperfusion in vivo. Despite similar risk areas (Figure 2A; wild type bone marrow 48.9±3.2% [n=8], CXCR2−/− bone marrow 45.3±2.8% [n=8], P=NS), mice receiving CXCR2−/− bone marrow exhibited infarcts within the risk areas that were 53.4% smaller than mice receiving wild type bone marrow (Figure 2A; wild type bone marrow 47.6±3.0% [n=8], CXCR2−/− bone marrow 22.2±4.0% [n=8], P<0.05).
Histological examination of heart sections showed 65.3% fewer inflammatory cells in recipients of CXCR2−/− compared with wild type bone marrow (Figure 2, B and C; wild type 1051±66 cells/mm² [n=3], CXCR2−/− 365±24 cells/mm² [n=3], P<0.05). These data demonstrate directly the importance of CXCR2 on bone marrow–derived cells for infarct development and reinforce the importance of inflammatory cell recruitment in this model.

Protective Effect of CXCR2 on the Myocardium During Ischemia-Reperfusion

On the basis of our experiments demonstrating that CXCR2 activation by MIP-2 protected isolated neonatal cardiac myocytes from hypoxia-induced death, we hypothesized that CXCR2 would mediate myocardial protection in the setting of the intact heart. To unmask this effect and assess its magnitude in the absence of neutrophil recruitment, 2 approaches were considered. Experiments in which lethally irradiated wild type or CXCR2−/− bone marrow were not performed because of concerns that the CXCR2−/− animals would be unable to withstand lethal irradiation. The alternative approach that we chose was to study hearts from wild type and CXCR2−/− mice in isolation of blood cells using buffer-perfused Langendorff preparations. Hearts of both genotypes were subjected ex vivo to 45 minutes of regional left coronary artery ischemia followed by 2.5 hours of reperfusion. Despite similar risk areas (Figure 3; wild type 54.4±2.9% [n=13], CXCR2−/−
MIP-2 inhibits hypoxia-induced death in these cells.26 This suggests that CXCR2 mediates both the infiltration of neutrophils and direct myocardial cytoprotection. Although our experiments unmasked a myocardial protective action of CXCR2 during ischemia-reperfusion in the intact heart, the net effect of CXCR2 signaling in the intact animal is death of myocardial cells. As noted, this is likely a result of the recruitment of inflammatory cells in this model. It will be important in future work to carefully delineate the signaling events downstream of CXCR2 in both cardiac myocytes and neutrophils. It is possible that differences in signaling between the 2 cell types may provide a means to selectively activate cardiac myocyte cytoprotection while inhibiting neutrophil chemotaxis, thereby limiting myocardial damage.

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