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Cardioprotective effect of interleukin-10 in murine myocardial ischemia-reperfusion

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Abstract

We investigated the cardioprotective effects of rat interleukin-10 in a murine model of myocardial ischemia-reperfusion (20 min ischemia, 24 h reperfusion). Interleukin-10 (100 μg/rat) administered 15 min prior to reperfusion, significantly ($P < 0.01$) attenuated myocardial injury compared to rats receiving only 0.9% saline as a vehicle, as indicated by a reduced loss of myocardial creatine kinase from the ischemic-reperfused myocardium. Cardiac myeloperoxidase activity was also significantly ($P < 0.01$) attenuated by interleukin-10 within the ischemic-reperfused region compared to vehicle treated rats. To further investigate the mechanism of interleukin-10 we observed the in vitro adherence of neutrophil to rat vascular endothelium. Interleukin-10 treatment significantly ($P < 0.05$) attenuated neutrophil adherence to rat superior mesenteric artery endothelium stimulated with interleukin-1β. Thus, interleukin-10 demonstrated significant cardioprotective effects as evidenced by a decrease in myocardial creatine kinase loss as well as an inhibition of neutrophil accumulation within the myocardium. It appears as though interleukin-10 mediates its effects, at least in part, by inhibiting leukocyte–endothelial interactions. © 1997 Elsevier Science B.V.

Keywords: Creatine kinase activity; Neutrophil adherence; Myeloperoxidase activity; Interleukin-1β; Neutrophil accumulation

1. Introduction

Early reperfusion following myocardial ischemia reestablishes blood flow and curtails tissue hypoxia. However, early reperfusion has been shown to initiate an inflammatory response which results in additional injury and a concomitant decrease in viable myocardium (Werns and Lucchesi, 1988; Tsao et al., 1990). Substantial evidence now exists implicating polymorphonuclear leukocytes (neutrophils), in the progression of ischemia-reperfusion injury. Activated neutrophils mediate injury by adhering to the vascular endothelium and many of these extravasate through the endothelium, and finally infiltrate into reperfused tissues (Albertine et al., 1994; Entman and Smith, 1994) where they release a number of cytotoxic substances including oxygen-derived free radicals, hydrolytic enzymes, as well as cytokines (Weiss, 1989). Numerous cytokines have been identified as originating from neutrophils. Such cytokines include interleukin-1β (Marucha et al., 1990), interleukin-6 (Cicco et al., 1990), interleukin-8 (Cassatella et al., 1992), and tumor necrosis factor-α (Wei et al., 1993).

Since neutrophils have been implicated as a primary mediator of reperfusion injury, and because several cytokines have been identified as originating from neutrophils, much attention has focused on the pro- and anti-inflammatory properties of cytokines in the pathogenesis of ischemia-reperfusion injury. One such cytokine that has demonstrated an ability to suppress local and systemic inflammatory responses is interleukin-10, which was first identified as cytokine synthesis inhibiting factor (Fiorentino et al., 1989). The anti-inflammatory properties of interleukin-10 appear to be broad based. Interleukin-10 has been shown to inhibit the production of interleukin-1α, interleukin-1β, interleukin-6, interleukin-8, and tumor necrosis factor-α from monocytes/macrophages (Bogdan et al., 1991; Fiorentino et al., 1991). Similarly, interleukin-10 inhibits lymphocyte production of granulocyte–macrophage colony stimulating factor, interferon-γ, lymphotoxin, interleukin-2, and interleukin-3 (Fiorentino et al., 1991, 1989), as well as interleukin-8 from neutrophils (Cassatella et al., 1993; Wang et al., 1994).

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Interleukin-1β markedly upregulates endothelial cell adhesiveness for neutrophils, monocytes, basophils, and eosinophils (Bevilacqua et al., 1985; Bochner et al., 1988). Interleukin-1β is believed to mediate these adhesive responses in several ways including the upregulation of intercellular adhesion molecule-1 (Pober et al., 1986a), vascular cell adhesion molecule-1 (Rice et al., 1988), and E-selectin (Pober et al., 1986b), as well as by stimulating the release of platelet activating factor (Bussolino et al., 1986) and pro-inflammatory cytokines (Sironi et al., 1989). The firm adherence as well as the transendothelial migration of neutrophils is mediated in large part by the immunoglobulin family of adhesion molecules including intercellular adhesion molecule-1, an 80 to 90 kDa single chain glycoprotein. Constitutively expressed intercellular adhesion molecule-1 is involved in the early stages of leukocyte–endothelial interaction, however its contribution becomes more prominent approximately 4 h following reperfusion, due to the fact that the intercellular adhesion molecule-1 is maximally upregulated at this time (Pober et al., 1986a; Weyrich et al., 1995). A monoclonal antibody directed against intercellular adhesion molecule-1 has been shown to be effective in curtailing myocardial ischemia-reperfusion injury in cats (Ma et al., 1992).

Thus, the present investigation was conducted in order to determine whether a single dose of rat interleukin-10 is able to protect against myocardial injury in a well established in vivo murine model of myocardial ischemia (20 min) and reperfusion (24 h). A second goal was to observe the effects of rat interleukin-10 on leukocyte–endothelium interactions on rat vascular endothelium at concentrations similar to those observed in the in vivo model.

2. Materials and methods

2.1. Experimental protocol

Male Sprague–Dawley rats weighing 185–215 g were anesthetized with ethyl ether prior to surgery. A thoracotomy was performed at the fifth intercostal space. Ischemia was produced by exteriorizing the heart and placing a 4–0 silk slip knot around the left coronary artery approximately 2–3 mm from its origin. The rat heart possesses minimal collateral circulation and this procedure results in a uniform degree of myocardial injury in each animal. After the closure of the slip knot the heart was immediately retracted into the thoracic cavity, air was evacuated from the thorax, and the chest wall was closed with a purse-string suture. The procedure was performed without supporting respiration since the surgical procedure compromised only one lung leaving the remaining lung fully functional. The entire procedure was completed within 2–3 min and this time was consistent for all rats. Five minutes after ischemia, a single s.c. injection of either interleukin-10 or an equivalent volume of 0.9% saline (i.e., 1.0 ml) was given to each rat. Following 20 min of ischemia, the slip knot was released thereby initiating reperfusion. Sham-operated control rats were subjected to the same surgical procedures except that the suture placed around the left coronary artery was not tied.

Rats were randomly assigned to one of three major groups based on a predetermined schedule. Assignments were made to one of three groups as follows: (1) sham myocardial ischemia rats receiving interleukin-10 (100 μg/rat) or vehicle (pH 7.4, 0.9% saline), (2) myocardial ischemia-reperfusion rats receiving vehicle, and (3) myocardial ischemia-reperfusion rats receiving interleukin-10 (25 or 100 μg/rat). Interleukin-10 or its vehicle was given s.c. 15 min prior to reperfusion.

Twenty-four hours following reperfusion rats were again anesthetized with ether, and their hearts were excised and placed in ice-cold Krebs–Henseleit buffer consisting of (in mmol): 118 NaCl, 4.75 KCl, 2.54 $CaCl_2 \cdot 2H_2O$, 1.19 KH_2PO_4, 1.19 $MgSO_4 \cdot 7H_2O$, 12.5 $NaHCO_3$, and 10.0 glucose. The intraventricular septum and left ventricular free wall were dissected free and placed in cold 0.25 mol sucrose (1:10, wt/vol) containing 1 mmol EDTA and 1 mmol 2-mercaptoethanol. Samples were homogenized with a Polytron (PCU-2) homogenizer and centrifuged at 36 000 $\times g$ at 4°C for 30 min. Supernatants were decanted and analyzed for creatine kinase and myeloperoxidase activities.

2.2. Determination of cardiac creatine kinase and myeloperoxidase activity

It has previously been demonstrated that the myocardial washout of creatine kinase from the injured left ventricle is a useful index of myocardial damage following myocardial ischemia (Kjekhus and Sobel, 1970; Buerke et al., 1995). In addition, Hock et al. (1985) reported that peak creatine kinase loss occurs 24 h after the onset of myocardial ischemia. Therefore, in order to assess myocardial injury, we compared creatine kinase activity between the ischemic-reperfused left ventricular free wall and the control non-ischemic intraventricular septum utilizing the method of Rosalki (1967). The rate of NADH formation, measured spectrophotometrically at 340 nm, is directly proportional to the creatine kinase activity in the sample (Sigma, St. Louis, MO). In addition to creatine kinase activity, each sample was assayed for total protein concentration by the biuret method of Gornall et al. (1949). Myocardial creatine kinase loss was calculated by subtracting the intraventricular septum creatine kinase activity from the left ventricular free wall creatine kinase activity and expressing this difference in international units per 100 mg of protein.

Myeloperoxidase is an enzyme occurring almost exclusively in neutrophils (Mullane et al., 1985). Thus, an increase in myeloperoxidase activity in supernatants of the homogenized myocardium indicates accumulation of neutrophils within this tissue (Mullane et al., 1985). Myeloper-

oxidase activity was determined spectrophotometrically in supernatants of homogenized myocardium utilizing the method of Bradley et al. (1982) as modified by Mullane et al. (1985). One unit of myeloperoxidase activity is defined as that quantity of enzyme hydrolyzing 1 mmol of peroxide per min at 25°C. Myeloperoxidase accumulation was calculated by subtracting the intraventricular septum myeloperoxidase activity from the left ventricular free wall myeloperoxidase activity and expressing this difference in units per 100 mg of wet tissue weight. All creatine kinase, myeloperoxidase, and protein assays were conducted in a blind fashion without knowledge of the group to which the rat was assigned.

2.3. Neutrophil adherence to superior mesenteric artery endothelium

Rats received a 10 ml i.p. injection of 0.5% glycogen (Sigma Chemical, St. Louis, MO), which stimulates the emigration of neutrophils from the vascular compartment into the peritoneal cavity. Eighteen hours following the injection, neutrophils were collected by peritoneal lavage in phosphate buffered saline and centrifuged at 3000 rpm at 4°C for 10 min. The supernatant was discarded and neutrophils were again washed in phosphate buffered saline. Isolated neutrophils were then labeled with a Zynaxis PKH-2 cell linker (Zynaxis Cell Science, prepared for Sigma Immunochemicals, Malvern, PA) based on the procedure of Yuan and Fleming (1990). Two ml of diluent and 10 ml dye were added to a loose cell pellet containing approximately 40 million cells. Following a seven min incubation period, 200 ml of 0.2% bovine serum albumin were added to stop the reaction and 2 ml phosphate buffered saline were added to underlay the suspension. The mixture was then centrifuged for 10 min at 1800 rpm. The cells were resuspended in phosphate buffered saline, counted, and utilized in adherence procedures.

The superior mesenteric arteries were removed from donor rats, placed in warmed Krebs–Henseleit buffer, and cleaned of all external adipose and loose connective tissue. Artery segments were then sectioned into 2–3 mm rings, opened, and placed into wells containing 2 ml Krebs–Henseleit buffer. Opened superior mesenteric artery rings were incubated with interleukin-1β (10 ng/ml) for 220 min at 37°C in order to induce the surface expression of intercellular adhesion molecule-1 on the endothelial surface. Rat interleukin-10 (5 or 10 μg/ml) was added 3 min prior to the addition of interleukin-1β. These concentrations of interleukin-10 were selected since they approximate in vivo circulating concentrations based on estimated blood volume and on a single dose of 100 μg interleukin-10. After the 220 min incubation period, segments were removed and placed in fresh Krebs–Henseleit buffer. Superior mesentery artery segments were then incubated with labeled neutrophils (10^6 cells) alone or with labeled neutrophils and an anti-intercellular adhesion molecule-1 monoclonal antibody for 20 min at 37°C. Following the 20 min incubation period, sections were washed in Krebs–Henseleit buffer and placed endothelial side up on glass microscope slides. Neutrophils adhering to the endothelium were counted using epifluorescence microscopy (Nikon Diaphot, Nikon, Garden City, NY). Five different fields of each endothelial surface were counted and the results expressed as adherent neutrophils/mm^2 of endothelial surface.

2.4. Statistical analysis

All values in the text and figures are presented as means ± S.E.M. All data were subjected to analysis of variance to determine if significant differences occurred between groups. Following a significant *F*-ratio, a Fisher's *t*-test post-hoc analysis was conducted to determine where the significant differences occurred. All differences were considered significant at $P \leq 0.05$.

3. Results

3.1. Effect of interleukin-10 on myocardial injury following ischemia-reperfusion

In order to determine the extent of myocardial injury following ischemia-reperfusion, we measured creatine kinase activity in homogenates of ischemic (left ventricular free wall) and non-ischemic (intraventricular septum) myocardium after 24 h of reperfusion. No significant differences were observed between sham myocardial ischemia-reperfusion rats receiving 100 μg interleukin-10 and sham myocardial ischemia-reperfusion rats receiving 0.9% saline (Fig. 1). Both groups demonstrated a small negative creatine kinase loss indicating greater creatine kinase activity

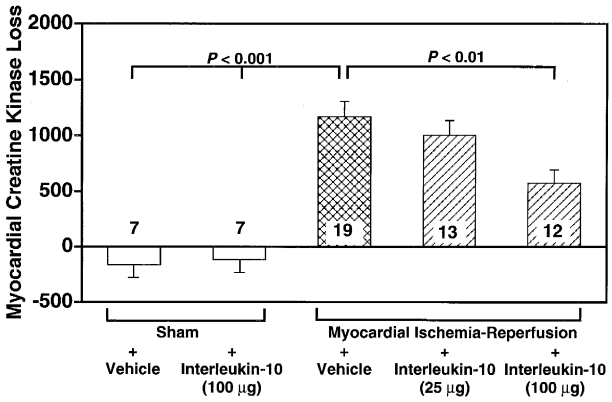

Fig. 1. Effect of single dose administration of rat interleukin-10 on myocardial injury after reperfusion of ischemic myocardium. Differences in myocardial creatine kinase activity of the left ventricular free wall and intraventricular septum are expressed in international units per 100 mg protein. Sham myocardial ischemia rats and myocardial ischemia-reperfusion rats received either interleukin-10 (25 or 100 μg) or 0.9% saline subcutaneously 15 min prior to reperfusion. Values are means ± S.E.M., and numbers at the bottom of each bar represent the number of rats in each group.

in the left ventricular free wall than in the intraventricular septum. In contrast, rats subjected to coronary occlusion and reperfusion given only the vehicle showed a significant ($P < 0.001$) loss of creatine kinase activity from the left ventricular free wall, indicating a substantial degree of myocardial injury. Subcutaneous administration of 25 μg interleukin-10 decreased left ventricular free wall myocardial creatine kinase loss from a control value of 1171 ± 135 IU/g protein to 1008 ± 128 IU/g protein (not significant), whereas 100 μg interleukin-10 decreased left ventricular free wall myocardial creatine kinase loss to 576 ± 120 IU/g protein ($P < 0.01$). These data clearly demonstrate that a single dose of 100 μg interleukin-10 given 15 min prior to reperfusion significantly attenuates myocardial necrotic injury.

3.2. Neutrophil accumulation in the ischemic-reperfused myocardium

Numerous investigations have demonstrated the critical role neutrophils play in the development and progression of reperfusion injury (Albertine et al., 1994; Buerke et al., 1994; Entman and Smith, 1994). Therefore, we measured myeloperoxidase activity in the ischemic area (i.e., left ventricular free wall) as an index of neutrophil accumulation. Sham-operated control rats receiving interleukin-10 (100 μg) showed only small myeloperoxidase differences between left ventricular free wall and intraventricular septum, which were similar to those observed in sham-operated control rats given 0.9% saline (Fig. 2). Conversely, myocardial ischemia-reperfusion rats receiving only the vehicle exhibited a substantial increase in myeloperoxidase activity in the ischemic left ventricular free wall with virtually no change in the intraventricular septum, thus indicating a significant increase in neutrophil accumulation within the ischemic-reperfused region of the myocardium. Subcutaneous administration of 100 μg interleukin-10 15 min prior to reperfusion substantially attenuated the increase in myeloperoxidase activity within the ischemic-reperfused left ventricular free wall, decreasing myeloperoxidase accumulation from a control value of 1.58 ± 0.22 to 0.46 ± 0.13 IU/g heart weight ($P < 0.01$). In contrast, administration of 25 μg interleukin-10 failed to attenuate the increase in myeloperoxidase activity within the left ventricular free wall, there being no significant difference in myeloperoxidase activity compared to vehicle-treated myocardial ischemia-reperfusion rats.

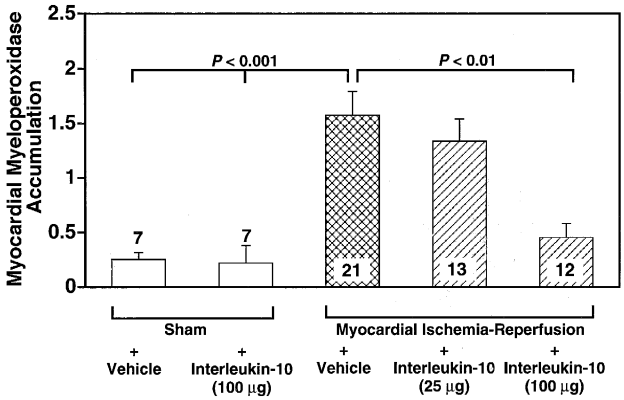

Fig. 2. Effect of single dose administration of rat interleukin-10 on cardiac myeloperoxidase activity calculated as the difference in left ventricular free wall and intraventricular septum myeloperoxidase activity expressed as units per 100 mg tissue wet weight. Sham myocardial ischemia rats and myocardial ischemia-reperfusion rats received either interleukin-10 (25 or 100 μg) or 0.9% saline subcutaneously 15 min prior to reperfusion. Values are means $\pm$ S.E.M., and numbers at the bottom of each bar represent the number of rats in each group.

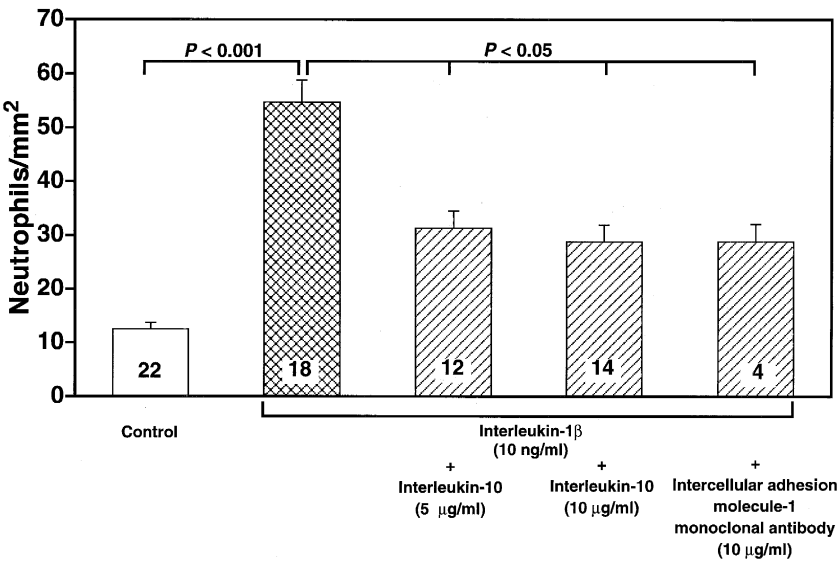

Fig. 3. Effect of in vitro addition of rat interleukin-10 on neutrophil adherence to rat superior mesenteric artery endothelium stimulated with interleukin-1β. Data are expressed as numbers of adherent neutrophils per mm^2 endothelial surface area. Values are means $\pm$ S.E.M., and the number at the bottom of each bar represents the number of rats in each group.

3.3. Neutrophil adherence to rat superior mesenteric artery endothelium

We also observed the effects of interleukin-10 on autologous neutrophil adherence to rat superior mesenteric artery endothelial cells (Fig. 3). Few neutrophils adhered to unstimulated superior mesenteric artery endothelium, suggesting that the isolation of neutrophils as well as superior mesenteric artery segments failed to significantly stimulate either cell type. We stimulated rat superior mesenteric artery endothelium with 10 ng/ml interleukin-1β. Stimulation of rat superior mesenteric artery endothelium by interleukin-1β resulted in a significant ($P < 0.001$) increase in neutrophil adherence to the endothelium, indicating that the endothelium was not injured by the isolation procedures. Incubation of superior mesenteric artery segments with 5 and 10 μg/ml interleukin-10 significantly ($P < 0.05$) reduced adherence to interleukin-1β stimulated segments from 54.7 ± 4.1 neutrophils/mm^2 to 31.3 ± 3.2 and 28.8 ± 3.1 neutrophils/mm^2, respectively. Interleukin-1β stimulated rat superior mesenteric artery segments incubated with 20 μg/ml intercellular adhesion molecule-1 monoclonal antibody demonstrated a response similar to interleukin-10 (5 μg/ml) treated segments with adherence reduced to 28.8 ± 3.2 neutrophils/mm^2 ($P < 0.05$) when compared to interleukin-1β stimulation alone.

4. Discussion

The data obtained in this investigation suggest a cardioprotective effect of interleukin-10 in a well established murine model of myocardial ischemia-reperfusion. The observed cardioprotective effect was characterized by a reduction in cardiac creatine kinase loss, a reduction in cardiac myeloperoxidase accumulation, and an inhibition of neutrophil adherence to interleukin-1β stimulated vascular endothelium. These results suggest that interleukin-10 maintains myocardial cell integrity following ischemia-reperfusion induced injury in rats. Inhibition of the interaction of neutrophils with the endothelium could substantially decrease the exposure of the myocardium to a number of neutrophil-derived substances including hydrogen peroxide, superoxide anion, hydroxyl radical, elastase, and pro-inflammatory cytokines which lead to myocardial necrosis (Buerke et al., 1994).

The attenuation of myocardial myeloperoxidase accumulation in interleukin-10 treated myocardial ischemia-reperfusion rats suggests that the effects of interleukin-10 are mediated in part by inhibiting leukocyte–endothelial interactions. Therefore, we investigated the effects of interleukin-10 on leukocyte adherence to interleukin-1β activated endothelium in vitro. Our findings are consistent with other findings (Bevilacqua et al., 1985; Bochner et al., 1988; Smith et al., 1988) which have shown a significant attenuation of intercellular adhesion molecule-1 dependent leukocyte adherence to interleukin-1β stimulated endothelium.

Several mechanisms of action have been proposed for the cardioprotective effects of interleukin-10. Originally interleukin-10 was described as a cytokine synthesis inhibiting factor (Fiorentino et al., 1989) based on its ability to inhibit the synthesis and release of a number of pro-inflammatory cytokines including interleukin-1, interleukin-6, interleukin-8, and tumor necrosis factor-α (Bogdan et al., 1991; Fiorentino et al., 1991) from lymphocytes and monocytes. However, until recently, little was known regarding the cytokine inhibitory effects of interleukin-10 on neutrophils. Wang et al. (1994) demonstrated that interleukin-10 markedly inhibited the release of interleukin-1α, interleukin-1β, interleukin-6, interleukin-8, and tumor necrosis factor-α in isolated neutrophils. Subsequent analysis of the specific effects of interleukin-10 on interleukin-8 production from neutrophils showed a concentration dependent inhibition involving a reduction in cytokine messenger RNA expression. Cassatella et al. (1993) showed a similar reduction in cytokine synthesis by stimulated neutrophils at time periods of 4 h or longer. It is believed that interleukin-10 acts by degrading cytokine messenger RNA, by inhibiting messenger RNA transcription, or by both mechanisms. In comparison to monocytes, neutrophils produce relatively small quantities of pro-inflammatory cytokines including interleukin-1β, interleukin-8, and tumor necrosis factor-α. However, under in vivo conditions, neutrophils may be the primary source of pro-inflammatory cytokines in ischemia-reperfusion since they consistently comprise about 50–60% of the total circulating leukocyte count compared with less than 10% for monocytes, and that a larger percentage of neutrophils are localized at the site of inflammation.

In addition to its ability to inhibit the production of pro-inflammatory cytokines, interleukin-10 appears to attenuate the adherence of neutrophils to the endothelium. Perritti et al. (1995) reported no alterations in neutrophil function or in the upregulation of neutrophil adhesion molecules stimulated with interleukin-10 in vitro. They did however, suggest that the inhibition of neutrophil adherence and migration induced by interleukin-1β may be a secondary response to the inhibition of interleukin-8, which acts as a chemoattractant for neutrophils. In addition, interleukin-10 may exert its anti-adherent effect by stimulating the production of interleukin-1 receptor antagonist in neutrophils. The interleukin-1 receptor antagonist belongs to the interleukin-1 receptor family, binds to the same cellular receptors, yet does not mediate any of the effects of interleukin-1. The release of interleukin-1 receptor antagonist by neutrophils has been shown to be stimulated by granulocyte/macrophage colony stimulating factor, tumor necrosis factor-α, and lipopolysaccharide (McColl et al., 1992; Cassatella et al., 1994). Although another anti-inflammatory cytokine, interleukin-4, has been shown to increase interleukin-1 receptor antagonist messenger RNA

expression, interleukin-10 appears to influence interleukin-1 receptor antagonist by prolonging the half-life of the interleukin-1 receptor antagonist messenger RNA (Cassatella et al., 1994). This may be due to reduced expression of a nuclease which selectively degrades interleukin-1 receptor antagonist messenger RNA or increases the expression of a co-factor that decreases the vulnerability of interleukin-1 receptor antagonist messenger RNA to degradation by the nuclease (Cassatella et al., 1994). This mechanism appears to be of primary importance in our in vitro adherence studies in which neutrophil adherence is inhibited by interleukin-10 even when interleukin-1 is added to the incubation medium.

Activated neutrophils are capable of mediating considerable injury on inflamed tissue by the release of oxygen-derived free radicals including hydrogen peroxide, superoxide anion, and hydroxyl radicals. Although the results are far from clear (Bogdan et al., 1991; Cassatella et al., 1993; Chaves et al., 1996), there appears to be preliminary evidence suggesting that interleukin-10 may inhibit the generation of free radicals from neutrophils. Chaves et al. (1996) reported a significant attenuation in free radical generation in granulocytes perhaps via a cyclic AMP mechanism.

Our data clearly demonstrate that interleukin-10 provides cardioprotection following myocardial ischemia-reperfusion in vivo, and that this protective effect appears to be mediated at least in part by inhibiting leukocyte–endothelial cell interaction. This may be related to the finding that interleukin-10 inhibits the release of pro-inflammatory cytokines, enhances the effects of endogenous interleukin-1 receptor antagonist, and inhibits the release of oxygen-derived free radicals.

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