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IL-10 Attenuates Hepatic I/R Injury and Promotes Hepatocyte Proliferation

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Background. **One of the most important determinants of the outcome of hepatic ischemia and reperfusion (I/R) injury is the onset of the inflammatory response. Interleukin-10 (IL-10) is a potent anti-inflammatory cytokine. It inhibits the production of interleukin-6 (IL-6), which however, also is involved in priming hepatocyte proliferation. The aim of this study was to examine the protective effects and the influence on the regenerative response of exogenous as well as endogenous IL-10 in a rat model of hepatic I/R injury.**

Materials and methods. **Seventy percent Liver I/R was induced in male Wistar rats for 60 min followed by 24 h reperfusion. One group underwent a midline laparotomy with recombinant rat (rr)IL-10 administration (SHAM + IL-10). The other groups underwent 60 min ischemia with administration of saline (I/R + saline), rrIL-10 [at two different time-points, i.e., I/R + IL-10pre(ischemia) and I/R + IL-10end(ischemia)] or anti-rat IL-10 antibody (I/R + antiIL-10).**

Results. **Parenchymal damage, as assessed by plasma alanine aminotransferase and aspartate aminotransferase, was significantly reduced by rrIL-10 and by endogenous IL-10 ($P < 0.05$). Also, rrIL-10 significantly reduced IL-6 production and the accumulation of neutrophils in liver and lung tissue, as measured by myeloperoxidase activity. Necrosis and apoptosis were significantly reduced and hepatocyte proliferation was stimulated by rrIL-10.**

Conclusions. **RrIL-10 and, to a lesser extent, endogenous IL-10, attenuate damage and inflammation, while rrIL-10 also promotes proliferation after hepatic I/R injury in rats. Therefore, rrIL-10 has potential use to prevent I/R injury and to promote liver regeneration after partial liver resection with temporary inflow occlusion.**

Key Words: **liver; ischemia; reperfusion; interleukin-10; proliferation; apoptosis.**

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INTRODUCTION

Hepatic inflow occlusion (Pringle's maneuver) can be applied to reduce blood loss during liver resection. This will result in ischemic liver injury, which is aggravated by restoration of oxygenated blood flow (reperfusion), depending on the duration of ischemia. One of the most important determinants of the outcome of hepatic, postischemic reperfusion (I/R) injury is the onset of the inflammatory response. This inflammatory response is caused by the activation of Kupffer cells, which constitutes the main source of reactive oxygen species (ROS) formation during the initial reperfusion period [1, 2]. ROS cause injury to the hepatocytes and endothelial cells, potentially resulting in microcirculatory failure [2–4]. Kupffer cell activation also leads to the production of proinflammatory cytokines such as tumor necrosis factor (TNF)-α and IL-6, as well as chemokines, which are mainly responsible for the induction of neutrophil sequestration in the liver [5, 6]. This causes further tissue injury several hours after the initiation of reperfusion [4]. Tissue injury can lead to necrosis or apoptosis, ultimately resulting in functional loss.

The effect of IL-10 administration has been studied in various models of I/R injury [7–10]. In most of these studies, IL-10 appears to have an attenuating effect on organ damage caused by I/R, due to anti-inflammatory properties. The anti-inflammatory action of IL-10 is thought to occur via inhibition of nuclear factor κB (NFκB) in CD3+ and CD4+ T cells [11]. Furthermore, IL-10 stimulates stat3 signaling, which plays a key functional role in inhibition of macrophage activation [12]. This results in potent inhibition of the production of TNF, IL-1α and β, IL-6, IL-10 itself, and other proinflammatory cytokines by activated monocytes, macrophages, neutrophils, and CD4+ T cells [13–15]. Furthermore, IL-10 suppresses the expression of adhesion molecules [16].

Besides mediating the inflammatory response, TNF-α and IL-6 are also involved in priming hepatocyte prolif-

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eration when functional liver mass is lost [17]. It is also known that IL-6 has anti-apoptotic activity by maintaining adequate levels of intracellular anti-apoptotic proteins [18]. This leads to the question whether IL-10, by inhibiting production of IL-6, also inhibits priming of proliferation after major loss of functional parenchymal cells due to I/R injury.

This study tested the hypothesis that because of its anti-inflammatory action, IL-10 administration reduces hepatic injury in a rat model of hepatic I/R. In addition, the role of endogenous IL-10 and of IL-6 was examined in relation with necrosis and apoptosis on the one hand, and proliferation on the other hand.

MATERIALS AND METHODS

Animals

This study was approved by the Animal Experiment Committee of the Academic Medical Center, University of Amsterdam, The Netherlands. Male Wistar rats (Harlan, Horst, The Netherlands), weighing 320 to 340 g were used. All rats were allowed to adapt to the laboratory environment for seven days with free access to water and standard laboratory chow (Hope Farms, Woerden, The Netherlands). Rats were housed under standard environmental conditions with a 12 h light-dark cycle. Before use in experiments, rats were fasted overnight with free access to water.

Experimental Design

Thirty-three rats were randomly appointed to five experimental groups. Group 1 received 50 μg/kg rrIL-10 (Cell Sciences, Norwood, MA) and underwent a midline laparotomy (SHAM + IL-10, $n = 5$). Group 2 to 5 underwent 60 min ischemia. Group 2 received saline solution prior to ischemia (I/R + saline, $n = 7$), group 3 received 500 μg/kg rabbit (polyclonal) anti-rat IL-10 antibody (Biosource Int., Camarillo, CA) preischemically to inhibit endogenous IL-10 (I/R + antiIL-10, $n = 7$), group 4 received 50 μg/kg rrIL-10 prior to ischemia (I/R + IL-10pre, $n = 7$) and group 5 received 50 μg/kg rrIL-10 at the end of the ischemic period (I/R + IL-10end, $n = 7$). The chosen dose of 50 μg/kg rrIL-10 was obtained from literature [7, 19]. A reperfusion period of 24 h was applied in all animals.

Anesthesia

All animals were anesthetized via inhalation of a mixture of O_2:N_2O (1:1 L/min) and isoflurane 3 to 4% (Florene; Abbott Laboratories Ltd., Queensborough, Kent, United Kingdom). After endotracheal intubation, rats were ventilated (Zoovent ventilator; Instruvet, Amerongen, The Netherlands) and anesthesia was maintained with a mixture of O_2:N_2O (1:1 L/min) and isoflurane 2 to 3%. Tidal volume was adjusted according to end-tidal CO_2 levels [20]. A polyethylene catheter (Ø 0.9 mm; Braun, Melsungen, Germany) was introduced into the right carotid artery and tunneled subcutaneously to the back of the rats for assessment of hemodynamic parameters during operation as well as for withdrawal of blood samples. Arterial blood pressure was maintained at approximately preoperative levels by adjustment of isoflurane levels. The animals were kept in supine position on a heating pad and rectal temperature was maintained at 37°C with the use of a heating lamp [21, 22].

Surgical Procedure

A midline laparotomy was performed and the afferent vessels to the median and left lateral lobes of the liver were exposed. An atraumatic vascular clip was applied to these vessels to induce partial hepatic ischemia (70%) for 60 min, after which the clip was removed and subsequent reperfusion initiated. After surgical closure of the abdomen, rats were allowed to recover and were provided with water and food. After 24 h of reperfusion the rats were anesthetized with above mentioned anesthesia. A polyethylene catheter (Ø 0.4 mm) was inserted into the distal bile duct and bile was collected during 15 min. The rats were subsequently sacrificed under anesthesia, and liver biopsies were taken, frozen in liquid nitrogen and stored at −80°C or fixed in 4% (w/v) formaldehyde for further analysis.

Hepatocellular Damage

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined in heparinized plasma by routine spectrophotometry using α-ketoglutaric acid and pyridoxal phosphate (General Clinical Chemistry Laboratory, AMC, The Netherlands).

Inflammatory Response

Plasma IL-6 and IL-10 concentrations were assessed using commercial ELISA kits (Pierce Endogen, Rockford, IL). Manufacturer's instructions were followed. Samples were assessed in duplicate and concentrations were calculated using a standard curve.

Myeloperoxidase (MPO) activity, an index of neutrophil infiltration, was measured in liver and lung homogenates as described by Krawisz *et al.* with some modifications [23]. Briefly, tissue samples were homogenized (Heidolph, Diax 900, Berlin, Germany) in 5 mM phosphate buffer (pH 6.0). After a sample was taken from the homogenate for protein content determination (BCA protein assay, Pierce Endogen, Rockford, IL), the sample was centrifuged for 10 min at 12,000 g and 4°C. The pellet was homogenized in 0.5% hexadecyltrimethyl ammonium bromide (HTAB) (Sigma Chemicals, Munich, Germany) and 10 mM EDTA in 50 mM phosphate buffer (pH 6.0). Homogenates were freeze-thawed three times, pottered 10 times (Bellco 20; RW 20.n IKA Labortechnik, Staufen, Germany), sonicated for 10 s and centrifuged at 10,000 rpm at 4°C for 10 min. The supernatants were collected and assayed for MPO activity by addition of 0.167 mg/mL *o*-dianisidine dihydrochloride (Sigma Chemicals) and 0.001% H_2O_2 (Sigma Chemicals) in 50 mM sodium phosphate buffer (pH 6.0). The change in absorbance was measured spectrophotometrically (Victor^2 Wallec 1420; Perkin-Elmer Life Sciences, Boston, MA) at 450 nm during 10 min at 37°C. One Unit is defined as the amount of enzyme necessary to produce a change in absorbance of 1.0 per min. The MPO activity is expressed as Units per mg protein.

Proliferation and Apoptosis

Liver biopsies of the middle and left lateral liver lobes taken 24 h after ischemia were analyzed. For caspase-3 immunohistochemistry, sections were incubated with cleaved caspase-3 antibody (9661, dilution 1:200; Cell Signaling Technology, Inc., Beverly, MA) for 1 h at room temperature. Hereafter, sections were exposed to secondary antibody (dilution 1:1, Sigma Chemicals) and peroxidase activity was detected by diaminobezidine and counterstained with hematoxylin. Cleaved caspase-3 positive cells were counted by two independent observers in a blinded fashion and expressed as number of positive cells per 30 microscopic fields (40×).

A MIB-5 antibody was used to determine hepatocyte proliferation. MIB-5 is a rat equivalent of human Ki-67 antibody, which detects all active parts of the cell cycle (G1, S, G2, and mitosis) and shows a strong positive correlation with proliferating antigen expression, bromodeoxyuridine incorporation, and thymidine incorporation [24]. Briefly, after formaldehyde fixation and paraffin-embedding, 4 μm sections were deparaffinized and immersed in citric acid pH 6.0, preheated, and boiled (2 bar, 120°C, 20 min) in a pressure cooker. Sections were then incubated with a MIB-5 antibody (dilution 1:50; DAKO Cytomation, Glostrup, Denmark) for 60 min. After incubation with a secondary antibody (dilution 1:1, Poly-HRP; Invitrogen,