INHIBITION OF FREE RADICAL GENERATION AND IMPROVED SURVIVAL BY PROTECTION OF THE HEPATIC MICROVASCULAR ENDOTHELIUM BY TARGETED ERYTHROCYTES IN ORTHOTOPIC RAT LIVER TRANSPLANTATION

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The capacity of specifically targeted erythrocytes to inhibit free radical-mediated injury to the endothelial cell after cold preservation, and improve liver function was studied in two experimental models: An isolated perfused rat liver (IPRL) system and syngeneic orthotopic rat liver transplantation. In the IPRL model, livers were preserved in University of Wisconsin solution for 24 h at 4°C. At the end of the preservation period, livers were flushed with lactated Ringer's (control), immunoorthoerythrocytes (IES), or blank intact erythrocytes prior to warm reperfusion for 2 h using an assanguinous Krebs-Henseleit buffer. Production of superoxide (O₂⁻) anion during warm reperfusion in the IES-treated liver was reduced by 65% as compared with controls (P<0.001) and by 74% (P<0.001) when compared with blank erythrocyte-treated livers. Endothelial cell preservation, as assessed by levels of purine nucleoside phosphorylase (PNP), was much better in the IES-treated group (P<0.001) when compared with untreated livers.

Hepatocellular preservation was markedly improved in the IES-treated livers.

In the syngeneic liver transplantation model, livers were preserved in UW solution for 24 h at 4°C. Prior to implantation, livers were flushed with 5 ml of cold lactated Ringer's or immunoorthoerythrocytes. Survival after three weeks was 60% in the IES-treated group and 30% in the untreated group. Survival in the IES-treated group was not significantly different from a control (no preservation) group.

IES-treated livers in both models demonstrated better endothelial cell integrity and ultimate liver function. IES treatment therefore appears to protect the hepatic microvascular endothelial cell from reperfusion injury and could prove to be an easy reproducible method of donor organ preservation after cold preservation.

Liver transplantation is now the therapy of choice for end-stage liver disease (1). Reperfusion injury following preservation is caused by toxic oxygen radicals (free radicals) localized at the microvascular endothelial cell (2, 3) and has deleterious biochemical and immunological consequences for the entire organ (4, 5). Free radical scavengers have been used in the past with mixed success (6, 7), unless they are delivered intracellularly, such as by liposomes (8, 9). Through the use of liposomes— or, for more specific targeting— immunoliposomes,
(10) an increase in intracellular concentration of antioxidants can be achieved with subsequent inhibition of free radical generation.\(^4\) Intact red blood cells can prevent damage to the endothelial cell by hydrogen peroxide (H\(_2\)O\(_2\)) (11, 12), presumably due to their high levels of endogenous antioxidants (13). Targeting of these erythrocytes should in theory be able to inhibit intracellular superoxide generation following reperfusion after cold preservation. In our experiments specific targeting was accomplished by the conjugation of antibodies to factor VIII (endothelial cell–specific antigens) to the surface of freshly washed erythrocytes (14) (immunoerythrocytes [IES]).\(^*\) We tested the capacity of these IES to protect the endothelial cell in two experimental models: isolated perfused rat liver (IPRL) and orthotopic liver transplantation.

**MATERIALS AND METHODS**

**Preparation of erythrocytes.** Immunoerythrocytes were prepared by conjugating factor VIII antibodies, (ICN, Immunobiologicals No. 68-112) using chromium chloride as a conjugating agent (15). This method resulted in an average specific binding of 15%, as measured by \(^{125}\)I protein-A. Blank erythrocytes were processed in a similar manner, except for the conjugation of antibodies to their surface.

**IPRL.** Livers harvested from male Lewis rats (250–300 g) were preserved for 24 h in University of Wisconsin lactobionate solution at 4°C (16). At the end of the preservation period, the livers were flushed in a retrograde fashion through the infrahepatic IVC with 5 ml of cold lactated Ringer's (control n=12), IES solution (hematocrit 35–40%, n=10), or intact nonimmune erythrocytes (n=12). Warm reperfusion was carried out for 2 h at 37°C with an asanguinous Krebs-Henseleit buffer containing 2% bovine serum albumin.

**OLTX.** Syngenic transplants were carried out using male Lewis rats (200–250 g) as donors (200–250 g) and recipients (250–300 g). Livers were preserved in UW solution for 24 h at 4°C. Immediately prior to implantation, livers were flushed in a retrograde fashion (17) with 5 ml of cold lactated Ringer's (n=10) or IES solution (n=10). Orthotopic liver transplantation was carried out according to the method of Miyata (18). Three-week survival in these two groups was compared with that of a control group where transplants were carried out immediately after harvest.

**Assays.** Endothelial cell preservation was assessed by measuring effluent levels of purine nucleoside phosphorylase (PNP) (19). Free radical generation was analyzed by measuring levels of superoxide anion (20). Hepatocyte preservation was assessed by measuring transaminase levels (16) using a Technicon RA 500 autoanalyzer (Technicon Instruments, Tarrytown, NY).

**Histology.** Analysis of tissue by light microscopy was carried out by a staff pathologist in a blinded fashion.

**Statistical analysis.** Data are expressed as means ± SD. The statistical significance of difference between group means was analyzed by the Student's \(t\) test. The alpha level has been adjusted for multiple comparisons using Bonferroni's adjustment (21). The alpha level per comparison was \(P<0.01\).

**RESULTS**

**IPRL: superoxide generation.** Reperfusion following preservation resulted in a burst of superoxide generation (Fig. 1) in all the three groups. Levels of superoxide anion at the end of 30 min of reperfusion were 0.67±0.31, 1.06±0.59, and 0.91±0.47 nmol cytochrome c reduced/ml in untreated, IES-treated, and blank erythrocyte–treated livers, respectively. As reperfusion continued, superoxide levels in untreated and blank erythrocyte–treated livers steadily increased to 1.32±0.39 and 1.75±0.33 nmol cytochrome c reduced/ml, respectively. These levels were significantly higher (\(P<0.001\) for both) than those observed in IES-treated livers (0.47±0.24 nmol cyto. c red./ml).

**PNP levels.** A steady rise in PNP levels was observed in all three groups, indicating increasing leakage of the cytosolic enzyme. The rate of leakage was most marked in control untreated livers. The PNP value at the end of 2 h of reperfusion in these livers was 182.17±79.34 mu/ml, which was significantly higher (\(P<0.001\)) than that observed in IES-treated livers of 84.80±26.03 milliunits/ml. The amount of PNP produced by the blank erythrocyte–treated livers was 113.0±29.09 milliunits/ml, which was significantly lower than (\(P<0.01\)) that observed in untreated livers. PNP values at the end of reperfusion were not statistically different in the two erythrocyte–treated groups (Fig. 2).

**FIGURE 1.** Reperfusion following preservation results in a burst of superoxide generation in all the three groups. As reperfusion progresses, superoxide generation is significantly reduced in the IES-treated livers when compared with the control untreated (\(P<0.001\)) or blank erythrocyte–treated livers (\(P<0.0001\)).

**FIGURE 2.** Reperfusion following cold preservation was associated with a rise in PNP levels in all the three groups. Endothelial cell function was best preserved in the IES-treated livers where PNP levels were significantly lower (\(P<0.001\)) than those in control untreated livers.

\(^*\)Rao P, Makowka L, Walsh T, et al. Immunoerythrocytes inhibit superoxide generation and protect the hepatic microvascular endothelial cell from reperfusion injury following warm ischemia (submitted for publication).

\(^*\)Abbreviations: IES, immunoerythrocytes; IPRL, isolated perfused rat liver; PNP, purine nucleoside phosphorylase.
SGOT and SGPT. In the untreated livers reperfusion was associated with a significant leakage of both transaminases into the effluent. SGOT and SGPT values at the end of 2 h were, respectively, 1056.42±650.45 and 1082.58±773.08 IU/ml. These values were significantly higher than those observed in IES-treated livers: 319.7±146.05 (P<0.005) and 319.6±253.12 (P<0.01) IU/ml. Transaminase levels in IES-treated livers were similar to those observed in blank erythrocyte-treated livers: 485.42±381.70 and 507.17±499.91 IU/ml, respectively (Figs. 3 and 4).

Bile production. Mean rates of bile production at the end of 30 min in untreated, IES-treated, and blank erythrocyte-treated livers were, respectively, 0.56±0.19, 0.51±0.19, and 0.37±0.16 ml/min/g of tissue. As the perfusion progressed, bile production decreased 42% in the control (untreated) (P<0.01) and 33% in the blank erythrocyte-treated livers (P<0.0001), as compared with a 4% decline at the end of the reperfusion in IES-treated livers (Fig. 5).

OLTX. Eleven of fourteen animals (78.57%) survived 3 weeks after OLTX in the control, nonpreserved group. This dropped significantly to 30% (3 of 10) (P<0.001) for the rats that received livers not treated after 24 h of cold preservation. Treatment of livers with IES prior to reperfusion resulted in a increase of the survival to 60% (6 of 10), which was higher than the untreated UW preserved group and similar to the survival obtained in fresh nonpreserved livers (Fig. 6).

Histology. IES treated livers in both experiments appeared normal on gross examination and morphologically on light microscopy (Fig. 7).

DISCUSSION

Reperfusion following cold preservation results in significant injury to the microvascular endothelial cell, as evidenced by an increasing leakage of purine nucleoside phosphorylase. This is in keeping with the finding of Caldwell Kenkell et al. (22), and...
Thurman et al. (1988) (23), who also demonstrated increasing injury to the microvascular endothelial cell on reperfusion following cold ischemia. Contrary to McKeown's finding (24), this injury was manifest on warm reperfusion rather than during cold ischemia. (Fig. 2). Toxic oxygen radicals liberated at reperfusion have been implicated in the injury to the endothelial cell (25, 26). In our experiments, warm reperfusion following cold preservation resulted in a significant burst of superoxide generation (Fig. 1) in both the nontreated and treated livers early in reperfusion, followed by a steady decrease in the IES-treated livers. The lag period of superoxide inhibition in the IES-treated livers can be expected, as endocytosis necessary for the delivery of the antioxidants contained within the IES will occur after the liver has warmed to 37°C. Indeed, at low temperature, almost 90% of bound immunoliposomes can be washed away (10). As reperfusion progresses, however, a significant inhibition of superoxide generation is achieved in the IES-treated livers. This was not observed in blank erythrocyte-treated livers, which is in keeping with the findings of Tsan and White, who observed that intact erythrocytes could inhibit the generation of H₂O₂ outside the cell but were unable to inhibit the intracellular generation of superoxide. This indicates a role for the intracellular delivery of antioxidants by IES in the amelioration of free radical–induced endothelial cell injury.

Reperfusion injury to the microcirculation eventually results in hepatocellular injury (19, 24). This is confirmed by the significantly elevated levels of SGOT and SGPT in the effluent of control untreated livers (Figs. 3 and 4). Protection of the microvascular endothelial cell by immunoeerythrocytes appears to protect the hepatocyte as evidenced by significantly lower levels of transaminase leakage in these livers. A limited amount of protection to the hepatocyte was also accorded by treatment with blank erythrocytes. Bile production is considered as an index of ATP generation (27, 34)—hence postoperative organ viability. Reperfusion injury to the endothelial cell results in a significant decrease in bile production in control untreated livers. Treatment with immunoeerythrocytes appears to inhibit this injury and maintain optimal liver function, as reflected by the maintenance of rate of bile production by these livers.

To test the clinical applicability of the IES we evaluated the protective capacity of the immunoeerythrocytes in an orthotopic liver transplantation model. Rats that received livers that were preserved for 24 h in cold UW solution demonstrated a three-week survival rate of 30%. Most of the recipients died early in the posttransplant period of graft nonfunction. Loading the graft with immunoeerythrocytes just prior to implantation increased survival two-fold to 60%, which was similar to that observed in fresh nonpreserved livers. Treatment with IES appears to have a role in preserving organ viability following cold preservation in UW solution. Livers appeared normal on gross examination and light microscopy. Immunoeerythrocyte treatment did not result in sludging of sinusoids in the perfusion or the transplant experiment, while the untreated preserved livers often swelled considerably.

The vascular endothelium is a primary target for rejection reactions (35, 36), and T lymphocytes play a major role in cell mediated allograft rejection (37). It has been demonstrated in the nonpreserved group. In particular though the sinusoids and perisinusoidal cells have normal appearing architecture with nuclei intact and with minimal if any debris within the lumen of the microvasculature (H & E; original magnification: ×164).
that antioxidant therapy can inhibit the proliferation of T lymphocytes, probably through an inhibition of interleukin-2 receptor expression (38, 40). Our experiments demonstrate that pretreatment of rat livers with immunoerythrocytes targeted to the microvascular endothelial cell can prevent oxidant-induced reperfusion injury—and, therefore, potentially T lymphocytes proliferation. In the clinical situation donor organ pretreatment with immunoerythrocytes could prove to be an easy, recipient-specific method to protect the vascular endothelium from post-ischemic reperfusion injury.

REFERENCES