Role of ICAM-1 in chronic hepatic allograft rejection in the rat

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LIVER TRANSPLANTATION IS NOW AN ACCEPTED MODE OF THERAPY IN END-STAGE AND ACUTE FULMINANT HEPATIC FAILURE. A COMMON CAUSE OF GRAFT LOSS IN LIVER TRANSPLANTATION REMAINS IMMUNLOGIC REJECTION OF THE ALLOGRAFT. REJECTION PATHOGENESIS IS STILL INCOMPLETELY CLARIFIED. IN PARTICULAR, THE MECHANISMS AND CONSEQUENCES OF EARLY CELLULAR INFILTRATION INTO THE ALLOGRAFT REMAIN UNCLEAR.

In liver transplants, as in the case of other solid organ transplants, adhesion molecules play an integral role in cellular infiltration, activation, and the binding of effector cells (leukocytes) to the target tissues (bile duct epithelium and hepatic endothelial cells). Interleukin-1 (ICAM-1) has been shown to be of particular importance for the recognition of donor cells by recipient T lymphocytes (24, 25). ICAM-1 promotes localization of leukocytes to the area of inflammation and enhances recognition of antigen-presenting cells to T lymphocytes, formation of lymphocyte germinal centers, and natural killer cell response (3, 9, 17, 21). The expression of ICAM-1 on bile ducts, endothelium, and perivenular hepatocytes has been shown to be greater in patients with acute hepatic rejection than in those with stable grafts (1, 26). This expression was even more significant in those progressing to chronic rejection (1). In the rat acute rejection model, ICAM-1 is induced on sinusoidal and portal vascular endothelial cells within 2 days of transplantation, with its expression increasing with the progression of rejection (20).

Direct evidence for the involvement of ICAM-1 and leukocyte function-associated antigen-1 (LFA-1) adhesion molecules in allograft rejection was provided by the survival study by Isobe et al. (14), in which treatment with anti-ICAM-1 and anti-LFA-1 monoclonal antibodies for 6 days in the mouse heterotopic cardiac transplant model resulted in an indefinite survival between fully incompatible mouse strains. Harihara et al. (11) reported a prolongation of rat hepatic allograft survival from 11 to 24 days after a single dose of antibodies to ICAM-1 and LFA-1 just before the anhepatic phase. Similar results were observed by other groups (7). However, the exact mechanism of the protective effect of neutralizing these adhesion molecules remains unclear, because they have been shown to affect not only cell recruitment but also cell reactivity. For example, immunoneutralization of ICAM-1 does not reduce cell recruitment but dramatically decreases oxidant production in liver reperfusion injury (8).

Therefore, the purpose of the present study was to investigate the role of ICAM-1 in the early stages of leukocyte recruitment in the transplanted liver and to determine if this affected the course of later chronic rejection. Intravital microscopy was used to visualize
leukocyte function at early time points in the rejection process, and ICAM-1 antisense oligonucleotides were used to inhibit the effects of ICAM-1 in a rat hepatic allograft rejection model.

MATERIALS AND METHODS

Orthotopic liver transplantation. All animals were housed in an environmentally controlled vivarium with a 12/12-h light/dark cycle and access to rat chow and water ad libitum. The protocol was approved by the Animal Care Committee of the University of Calgary according to the guidelines of the Canadian Council on Animal Care. Liver transplantation was performed according to the method described by Kamada and Calne (15) with minor modifications (5, 12). Food but not water was withdrawn from the cages 12 h before surgery.

Donor operation. The donor rat was anesthetized with sodium pentobarbital (50 mg/kg ip). A midline abdominal incision was made, and all ligamentous attachments around the liver were divided. The left phrenic, right adrenal, and right renal veins were ligated. The portal vein was divided from the left hepatic vein. The portal vein and celiac trunk were cleared from all branches. The common bile duct and celiac trunk were cleared to the level of the left renal vein. The hepatic artery and celiac trunk were cleared from the connective tissues and dissected down to the level of the left renal vein. The hepatic artery and celiac trunk were cleared from all branches. The common bile duct was divided, and a 6-mm length of PE-50 polyethylene tubing was inserted and secured into the proximal end. The animal was injected intravenously with heparinized saline. The IVC and portal vein were then clamped. The liver was flushed with 10 ml of cold (4°C) saline containing 50 units of heparin through the aorta. The portal vein and supra- and infraportal patic vein were divided. The liver was then removed from the donor into a 4°C saline bath.

Cuff preparation. The cuffs for portal vein (PE-240) and IVC (PE-280) consisted of 0.3-cm lengths of polyethylene tubing. The cuff preparation of both vessels was performed in an iced saline bath. With a clamp holding the tubing edge, the portal vein was passed through the lumen of the tube, everted over it, and secured with a circumferential silk suture. The same principle was applied to the IVC.

Recipient operation. The recipient was anesthetized with inhaled halothane. A midline abdominal incision was made. The left phrenic and right adrenal veins were ligated. The hepatic artery was ligated and divided. The bile duct was tied close to the liver and also divided. Isotonic saline (1 ml) was injected intravenously to compensate for blood loss. The IVC and portal vein were cross-clamped with microvascular clips, and clamping time was noted. The suprahepatic IVC was also clamped with a teaspoon clamp. The vessels were divided, and the recipient liver was removed. The donor liver was then placed in the anatomic position immediately. The suprahepatic IVC was first to be anastomosed end-to-end by 7-0 Prolene continuous stitches. The portal vein and infraportal IVC were connected to their corresponding cuffs by 5-0 silk stitches. The vascular clamps were released after the connections were secured. Arterialization was performed by diverting blood from recipient’s right renal artery into the graft via the donor’s celiac trunk. The bile duct was bridged by a PE-50 catheter. The abdominal incision was closed with 3-0 silk. The abdominal incision was closed with 3-0 silk. The IVC were connected to their corresponding cuffs by 5-0 silk Prolene continuous stitches. The portal vein and infrahepatic IVC were divided. The liver was then removed from the suprahepatic IVC was also divided by 7-0 Prolene continuous stitches. The portal vein and infraportal IVC were connected to their corresponding cuffs by 5-0 silk stitches. The vascular clamps were released after the connections were secured. Arterialization was performed by diverting blood from recipient’s right renal artery into the graft via the donor’s celiac trunk. The bile duct was bridged by a PE-50 catheter. The abdominal incision was closed with 3-0 silk. The abdominal incision was closed with 3-0 silk.
quench of 10 min with DAKO blocking solution (DAKO, Carpenteria, CA), the slides were rinsed with three changes of PBS and then pretreated with DAKO proteinase K solution for 15 min. This was followed directly by a 45-min incubation of primary antibody (2E1-B5; Berkeley Antibody, Berkeley, CA); 2E1-B5 is an IgG1 antibody that specifically recognizes a CG or TCG motif in phosphorothioate oligonucleotides. The sections were rinsed with three changes of PBS and then incubated with Zymed anti-IgG1 isospecific horse-radish peroxidase-conjugated secondary antibody (Zymed, San Francisco, CA) for 30 min. Again, sections were rinsed with three changes of PBS and diaminobenzidine was applied for 5 min. Finally, slides were rinsed in distilled water, counterstained with Gill's hematoxylin, dehydrated, cleared, and mounted in synthetic resin. Sections were then evaluated and photographed to document localization of the oligonucleotide expression.

Intravital microscopy. Hepatic microvasculature of transplanted animals was studied on day 6 postoperatively by using intravital microscopy. This method of studying the microvasculature of internal organs in vivo was based on the method of McCuskey (18), with modifications as previously described (30). All animals were fasted for 12 h before the experiment. They were anesthetized with pentobarbital sodium (50 mg/kg ip). The right jugular vein was cannulated for administration of the leukocyte-labeling fluorescent dye rhodamine 6G. A midline and a subcostal incision was made on the liver surface. A silicon-intensified fluorescent camera (C-2400–08; Hamamatsu Photonics, Hamamatsu City, Japan) mounted on the microscope projected the image onto a monochrome video display (C-2400–08; Hamamatsu Photonics, Hamamatsu City, Japan) mounted on the microscope projected the image onto a monochrome video display (C-2400–08; Hamamatsu Photonics, Hamamatsu City, Japan) mounted on the microscope projected the image onto a monochrome video display (C-2400–08; Hamamatsu Photonics, Hamamatsu City, Japan) mounted on the microscope projected the image onto a monochrome video display (C-2400–08; Hamamatsu Photonics, Hamamatsu City, Japan) mounted on the microscope projected the image onto a monochrome video display. Leukocytes were considered adherent to the hepatic venular endothelium if they remained stationary for a period of time >30 s. Rolling leukocytes were defined as those moving with a definite rotational motion at a velocity at least 30% less than that of erythrocytes within a given vessel. After the liver was isolated and placed under the intravitral microscope, the centrilobular zones (zone 3) were located. In a single animal, 10–15 acinar zones with postsinusoidal venules were studied.

Statistical analysis. All values were reported as means ± SE. Differences between the groups were assessed by one-way ANOVA followed by a Bonferroni correction for multiple comparisons. Statistical significance was set at P < 0.05. Statistical analysis for survival rates was performed by using the Kaplan-Meier method.

RESULTS

Liver biochemistry. Serum ALT levels of unoperated, isografted (Lewis to Lewis), allografted (Lewis to Brown Norway), saline-treated, and ICAM-1 missense and antisense oligonucleotide-treated allografted groups are presented in Fig. 1A. The isografted group had a statistically insignificant elevation of ALT levels compared with the unoperated controls. All of the allograft groups (whether treated with saline or missense ICAM-1 control preparations or the ICAM-1 antisense treatment) had significantly higher enzyme levels compared with the isografted controls. Serum bilirubin levels of the five groups of animals are presented in Fig. 1B. The allografted animals undergoing rejection (saline- and ICAM-1 missense-treated controls) had significantly higher bilirubin levels than the isograft controls. Treatment with ICAM-1 antisense in the allografted group resulted in normalization of serum bilirubin levels.

Liver histology. Histological grading of damage at day 10 in the allograft control group was rated as “severe” in 4 of 4 samples, whereas the ICAM-1 antisense-treated group showed “moderate” damage in 4 of 5 and severe in 1. The differences between the allograft and ICAM-1 groups were significant (P < 0.05, Fisher’s Exact test). Representative histological sections of an isograft control, rejecting allograft, and ICAM-1 antisense-treated allograft are shown in Fig. 2. Figure 2A demonstrates an isografted control liver (Lewis-to-
Lewis transplant). The hepatic architecture is well maintained, and necroinflammatory changes are absent. Figure 2B shows a saline-treated rejecting allograft (Lewis to Brown Norway transplant) 10 days after transplantation. There is prominent portal, periportal, and perivenular necrosis, with marked patchy edema. Extensive mononuclear inflammatory infiltrate is seen in the portal spaces with lobular expansion. The lobular architecture is distorted. Figure 2C shows an ICAM-1 antisense-treated rejecting allograft at day 10. Compared with Fig. 2B, there is much less damage. A moderate mononuclear inflammatory infiltrate is observed in the portal areas, with isolated bile duct destruction. No confluent necrosis is visible, and there is only isolated lobular hepatocyte dropout.

**Immunohistochemistry stain for ICAM-1 antisense oligonucleotide.** Representative slides from an ISIS 9125-treated allograft liver and a saline-treated allograft 24 h after the last dose are shown in Fig. 3. Positive staining for the 9125 compound was visible in Kupffer cells and sporadic sinusoidal endothelial cells (Fig. 3B). Hepatocytes did not show significant staining. The saline-treated allograft livers showed no detectable staining (Fig. 3A).

**ICAM-1 immunohistochemistry staining.** Representative ICAM-1 immunohistochemistry stains are shown in Fig. 4. A and B show zone 3 (centrilobular) views of an isograft control (A) and an ICAM-1 antisense-treated allograft (B), whereas C and D show zone 1 (portal area) views of a saline-treated rejecting allograft (C) and an ICAM-1 antisense-treated allograft (D). A weak background staining of ICAM-1 was noted in the hepatic sinusoidal endothelial cells, including the terminal hepatic venular endothelium of the liver, and there is only isolated lobular hepatocyte dropout.

**Fig. 2. Liver histology in isografted, allografted rejecting, and allografted ICAM-1 antisense-treated rats. A: isograft control (Lewis to Lewis) showing normal histology (×40, hematoxylin and eosin stain). B: rejecting allograft (Lewis to Brown Norway) at day 10 showing moderately severe damage. Extensive necrosis is present, as well as portal and periportal mononuclear infiltration and edema. The lobular architecture is moderately distorted. C: allograft, treated with ICAM-1 antisense oligonucleotides, at day 10 showing much less damage compared with B. There is moderate portal and periportal inflammation and only occasional lobular necrotic hepatocytes.**

**Fig. 3. Immunohistochemistry staining for ISIS 9125 (ICAM-1 antisense oligonucleotide). A: saline-treated allograft shows no detectable staining. B: ISIS 9125-treated allograft shows positive staining in Kupffer cells and sporadic sinusoidal endothelial cells. Hepatocytes show no staining.**
isograft control liver (Fig. 4A). A similar degree of weak staining in the sinusoids, with scant terminal hepatic venular staining, is seen in the allografted liver treated with ICAM-1 antisense (Fig. 4B). The appearances of A and B are very similar. Zone 1 in the isograft controls also showed a similar appearance, with diffuse weak sinusoidal endothelial staining, including weak portal venular endothelial staining.

The portal areas (zone 1) of the saline-treated rejecting allograft show noticeable ICAM-1 staining in the hepatocytes, sinusoids, and portal venules that is more prominent in areas with cellular infiltration (Fig. 4C). Bile ductular ICAM-1 immunohistochemical staining was variable; some ductules had scant staining (such as the ductule visible in the middle right of the field), whereas others were more heavily stained. A portal area in an ICAM-1 antisense-treated allograft shows sinusoidal staining but scant staining in the venular endothelium and bile ductule (Fig. 4D), suggesting that the antisense compound blocked ICAM-1 expression to some degree but not completely. Treatment of allografts with the missense ICAM-1 oligonucleotide control (photo not shown) had no discernible effect; this group was indistinguishable from saline-treated rejecting allografts.

To summarize, isograft controls showed diffuse weak sinusoidal and venular (postsinusoidal and portal) endothelial staining for ICAM-1. Rejecting saline- and missense ICAM-1-treated allografts showed heavy sinusoidal, portal, and postsinusoidal venular staining; bile ductular epithelial staining was variable, and hepatocyte staining was discernible. Allografts treated with ICAM-1 antisense oligonucleotide showed decreased staining of all cell types compared with the rejecting allografts and generally had appearances similar to isograft controls.
Leukocyte recruitment. Leukocyte rolling flux and adhesion on postsinusoidal venules are presented in Fig. 5. A baseline rolling flux of 2–3 cells/min in the unoperated animals was noted. In the isografted rats, rolling flux was not significantly different from the unoperated animals. A threefold increase in rolling flux was observed in the saline- or ICAM-1 missense oligonucleotides treated allografted animals. Compared with unoperated controls, a threefold increase in leukocyte adhesion in postsinusoidal venules was noted in the isografted rats, whereas a more than sevenfold increase was observed in the allografted animals. Treatment with ICAM-1 antisense oligonucleotides reduced the number of adherent cells to the control isograft level.

Survival rates. Animals that died within the first 24 h after surgery (due to operative complications such as massive hemorrhage or intestinal gangrene) were excluded from the survival study. Isografted recipients survived for more than a year, i.e., normal survival for these strains. Saline-treated allografted rats had a mean survival of 25.2 ± 2.7 days, whereas allografted animals treated with ICAM-1 antisense oligonucleotides had a significantly prolonged mean survival of 42.3 ± 1.2 days (P < 0.05 compared with the saline-treated allograft group). Necropsies, including cultures to rule out infection, were routinely performed on all animals that died. Patency of all cuffs and vascular anastomoses was also verified. In some cases, the exact cause of death remained unclear because widespread autolysis precluded any firm conclusions being reached. Insofar as we could determine given these limitations, the major cause of death in all allografted groups appeared to be chronic rejection with liver failure. These animals had gross jaundice and ascites.

DISCUSSION

We examined the importance of ICAM-1 in chronic liver allograft rejection by using the rat orthotopic transplant model. The Lewis-to-Brown Norway combination used in the present study enabled us to investigate the pathophysiology of this irreversible condition frequently encountered after human liver transplantation. With the availability of purebred strains and well-defined major histocompatibility complex phenotypes, the recipient histopathology, graft survival, liver enzymes, bilirubin levels, and postmortem findings were experimentally reproducible and correlated well with the human condition of allograft rejection. The use of immunosuppressants was not necessary in the rat isograft controls (Lewis to Lewis) because rejection within this purebred strain was never observed, whereas a progressive rejection was always encountered in the Lewis-to-Brown Norway allograft combination, with a consistent graft survival time.

Some degree of ischemia-reperfusion injury after liver transplantation was inevitable, because all of the major blood vessels supplying the liver were transiently clamped for vascular anastomosis. Vollmar et al. (29) showed that this leukocyte-mediated manifestation of postschemic hepatic damage was ICAM-1 dependent and exhibited in the first few hours after the operation. Because the aim of the present study was focused on the early microvascular events of rejection, animals were examined by intravital microscopy on postoperative day 6. Previous work has demonstrated that reperfusion-induced microvascular injury returns to control values within 48 h (16), suggesting that the leukocyte recruitment process in our study was primarily due to graft rejection. On the other hand, we did not extend our study to compare the hepatic microcirculation at a very late time point (e.g., 1 mo after transplant) when most of the leukocyte-mediated destruction would have been irreversible. The ongoing development of a thick, opaque liver capsule after transplantation also limited the intravital study to the first week.

One may argue that posttransplant microcirculation of the liver could be affected by the systemic and splanchnic hyperemia. In fact, several studies in humans have demonstrated a persistent hyperdynamic
circulation up to 1–2 yr after successful grafting (10, 13). In a previous study with this rat model, we have also demonstrated a significantly higher cardiac output, lower systemic vascular resistance, higher hepatic arterial flow, and splanchnic hyperemia by using the radioactive microsphere method (31). In the present study, this increased afferent circulation to the liver did not alter leukocyte rolling flux in the postsinusoidal venules of the isograft. This is important, because in vitro increased shear forces can indeed affect rolling flux. The increased flow through the hepatic microvasculature could conceivably reduce cell adhesion by imparting increased hydrodynamic dispersal forces at the leukocyte-endothelial interface. In other words, the increased adhesion during transplant therefore occurs despite increased hemodynamic flow characteristics.

The ICAM-1 immunohistochemical studies showed minimal expression in sinusoidal and venular endothelial cells in the isografted control rat livers, in agreement with previous studies on human liver (26). We acknowledge the limitations of immunohistochemistry as a crude quantitative method. However, bearing this in mind, it still appears that the ICAM-1 antisense–treated allografts had less ICAM-1 expression in various hepatic cell types compared with the allografted rejecting livers (Fig. 3). Speculating on the basis of our results on whether bile duct epithelial cells show increased ICAM-1 expression during rejection is hazardous given the limitations of immunohistochemistry. Moreover, the ICAM-1 expression pattern during acute and chronic rejection has been well documented by previous studies and was not the main purpose of the present study. Our goal in the immunohistochemistry studies was to show that the ISIS 9125 oligonucleotide was able to penetrate into the liver and ultimately reduce ICAM-1 expression in different cell types during rejection. We believe that aim was achieved with our immunohistochemistry results. The pattern of ISIS 9125 localization in the rejecting rat livers was generally consistent with previous studies of small phosphorothioated oligonucleotides (2, 4). We found only sinusoidal cellular uptake in Kupffer cells and endothelial cells. The lack of hepatocyte uptake of ISIS 9125 is probably attributable to the later time point (24 h) after dosing at which the specimens were obtained. Previous studies in the rat liver have demonstrated that hepatocytes show early (within 2 h) uptake, whereas sinusoidal cells show more prolonged uptake patterns (2, 4).

In the rejecting allografts, the endothelial lining of postsinusoidal and portal venules were heavily stained with ICAM-1, corresponding to the increased leukocyte adhesion in these microvessels observed by intravital microscopy on day 6. This represented a relatively early cellular recruitment in a chronic rejection model. The early cellular infiltration around the postsinusoidal or terminal hepatic venules (acinar zone 3) may be clinically significant, because it is becoming clear that an important mechanism of chronic allograft rejection is infiltration and consequent hepatocyte necrosis in this area (22). This study for the first time demonstrated a reduced leukocyte adhesion in the hepatic postsinusoidal venules of rejecting liver allografts after treatment with ICAM-1 antisense oligonucleotides, suggesting a possible role of these antisense compounds in blocking recruitment of white blood cells and hence reducing leukocyte-mediated injury. However, the incomplete inhibition of adhesion by ICAM-1 antisense suggests that either alternative adhesion pathways that do not require ICAM-1, such as vascular cell adhesion molecule-1, induce leukocyte recruitment into the liver microvasculature during rejection or that ICAM-1 was not entirely inhibited.

In the present study, we showed that liver enzymes remained high in the allografted rats even after treatment with ICAM-1 antisense oligonucleotides. This suggests that hepatocellular injury persists despite blockade of the ICAM-1 adhesion pathway. This is not surprising because the pathogenesis of allograft rejection is multifactorial, some of which is independent of ICAM-1. Humoral responses, ischemia, low-grade infection, and Kupffer cell-mediated injury may perhaps contribute to the deterioration in liver function during rejection.

Intrahepatic bile ducts are known to be the major targets for immune destruction during chronic rejection. The gradual development of cholestatic jaundice with rising levels of serum bilirubin are clinical manifestations of the process. Since the sole blood supply to the intrahepatic bile ducts is derived from the hepatic artery, an arterialized model is preferable and was employed in the present chronic rejection study. Intrahepatic bile duct loss is predominantly due to direct lymphocytotoxic attack, although ischemia due to oblitative vasculopathy may also be a cofactor in the pathogenesis of bile duct injury (19, 22, 23). Before a leukocyte can destroy the biliary system, it must (1) migrate out of the bloodstream (a process proven to be highly dependent on ICAM-1) and (2) attach to the bile duct epithelial cells. Whether the second step is also directly ICAM-1 mediated is unknown. However, as emigration is entirely dependent on adhesion, then modulation of ICAM-1-induced adhesion would indirectly affect emigration. Expression of ICAM-1 on bile duct epithelium and close proximity of the lymphocytes to the bile ducts during rejection strongly suggest that it is an ICAM-1-mediated process. In the present rejection model, bile duct injury and cholestasis were confirmed by histology and hyperbilirubinemia. The role of ICAM-1 in the leukocyte-mediated destruction of the biliary tree was demonstrated by normalization of the bilirubin level after effective blockade of this adhesion molecule with antisense oligonucleotides.

Exactly how the early intervention with ICAM-1 antisense and subsequent reduction in leukocyte-induced inflammation leads to later improvement in chronic allograft rejection remains unclear. An interesting study in a murine skin graft model showed that ICAM-1 antibody treatment induces T-lymphocyte hyporesponsiveness and thus prolongs graft survival (6). Rats significantly benefited from the ICAM-1 antisense oligonucleotides, with a prolonged mean survival
from 25 to 42 days. However, despite the near doubling of survival times, the treated rats continued to die of chronic rejection. This may reflect the relatively short duration of the antisense treatment, whereas rejection in these models is an ongoing process. Interestingly, in the mouse heterotopic heart transplant model, a 7-day treatment of ICAM-1 antisense oligonucleotides in combination with anti-LFA-1 monoclonal antibody resulted in essentially normal survival (>150 days) (27). This suggests that blockade of ICAM-1 and its ligand LFA-1 simultaneously may produce a much more potent and effective synergistic in vivo immunosuppressive action.

In summary, the present study demonstrated the importance of the ICAM-1 pathway in early leukocyte recruitment in the destruction of hepatic allografts. Prolonged survival with improvement in cholestasis after 6-day treatment with ICAM-1 antisense oligonucleotides suggested a protective role of anti-ICAM-1 therapy in the rejecting liver. Antisense oligonucleotides to ICAM-1 show promise as a therapeutic agent to block experimental liver allograft rejection.

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REFERENCES