ROLE OF ICAM-1 IN CHRONIC HEPATIC ALLOGRAFT REJECTION IN THE RAT

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Abstract

The pathogenesis of hepatic allograft rejection remains unclear. We aimed to clarify the early role of intercellular adhesion molecule-1 (ICAM-1)-mediated cell recruitment in chronic hepatic rejection. Liver transplantation was performed in Lewis to Lewis rats (isograft controls) and Lewis to Brown Norway rats (allograft rejection group). The allografted rats were treated with either ICAM-1 antisense oligonucleotides (10mg/kg/day x 6 days, i.p.) or a control preparation (either ICAM-1 missense oligonucleotide or normal saline). Hepatic leukocyte recruitment in vivo was studied on day 6 using intravital microscopy. Liver histology, biochemistry and survival rates were also examined. Leukocyte adhesion in terminal hepatic venules was significantly increased in the rejection group compared to isograft controls. Antisense ICAM-1 in the allografted group effectively reduced leukocyte adhesion. Histology and liver chemistry were less deranged in the antisense-treated groups compared to control-treated allografted rats. In the allograft groups, survival was significantly prolonged in the antisense-treated rats (42.3±1.2 days) compared to the controls (25.2±2.7 days). These results showed that early leukocyte recruitment in the hepatic microvasculature of rejecting allografts is ICAM-1-dependent, and suggest that impacting on early cell recruitment can significantly ameliorate chronic rejection.
Introduction

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 immunologic 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). Intercellular adhesion molecule-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, enhances recognition of antigen-presenting cells to T lymphocytes, formation of lymphocyte germinal centres and enhancing 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 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 mice.
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, as 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 hour 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 hours before surgery.

*Donor operation.* The donor rat was anesthetized with sodium pentobarbital (50 mg/kg, i.p.). 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 gastric vein. The inferior vena cava (IVC) was cleared from the connective
tissues and dissected down to the level of 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 10ml of cold (4°C) saline containing 50 units of heparin through the aorta. The
portal vein, supra- and infra-hepatic IVC were divided. The liver was then removed from the
donor into a 4°C saline bath.

Cuff preparation. The cuff for portal vein (PE-240) and IVC (PE-280) consisted of a 0.3-cm
length of polyethylene tubing. The cuff preparation of both vessels was performed in an iced
saline bath. With a clamp holding the tubing edge, a pair of fine forceps was used to pass the
portal vein 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 (1ml) was injected intravenously to compensate for blood loss. The IVC and
portal vein were crossed-clamped with microvascular clips and clamping time being 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 anatomical position
immediately. The suprahepatic IVC was first to be anastomosed end-to-end by 7-O Prolene
continuous stitches. The portal vein and infrahepatic IVC were connected to their corresponding cuffs by 5-O silk ties. 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-O silk. The portal vein clamping time of all animals did not exceed 15 minutes. Immediately after the operation, penicillin G (100,000 units/kg, i.m.) and gentamicin (1mg/kg, i.p.) were administered to the recipient rat. All animals were allowed free access to water after consciousness was regained. Diet was resumed 12 hours after the operation.

ICAM-1 antisense and missense treatment. ICAM-1 antisense oligonucleotides (ISIS 9125, donated by Isis Pharmaceuticals, Carlsbad, CA) were administered to the rats intraperitoneally at 10mg/kg/day for 6 days after transplantation. These doses were previously demonstrated to be effective in blocking ICAM-1 expression in rat kidney allografts (28). ICAM-1 scrambled or missense oligonucleotide (ISIS 13944, Isis Pharmaceuticals) at the same dose and duration of treatment, was used in some experiments as a control probe.

Originally, we had intended to use a murine monoclonal anti-ICAM-1 antibody (1A29, Pharmacia Upjohn, Kalamazoo, MI) as a complementary protocol to confirm the effects of eliminating ICAM-1 activity in our preparation. We used an intravenous dose of 1mg/kg/day x 6 days, previously shown to be effective (7,12). However, while the effects on leukocyte rolling and adhesion in the allografted rats treated with the anti-ICAM-1 mAb (data not shown) were similar to the effects of antisense ICAM-1 oligonucleotides, we found an unexpected decrease in survival (11.8 ± 0.9 days) with the mAb. Because of concerns about potential complement-
activating properties of the intact 1A29 mAb, we then elected to concentrate only on the antisense ICAM-1 oligonucleotide as a means to eliminate ICAM-1 effects.

**Liver Biochemistry.** Blood was collected by cardiac puncture for the liver biochemistry study. All serum samples were frozen at -20°C for a maximum period of 2 weeks before analysis. Alanine aminotransferase (ALT) activity and total bilirubin levels were measured using commercially available Sigma diagnostic kits.

**Histology.** Hepatic histological examination was performed at days 10 and 20 after transplantation. Liver sections of 8-µm thickness were stained with hematoxylin & eosin (H&E) and examined under light microscopy. All slides were coded and scored in a blinded fashion for inflammation and damage (none, mild, moderate and severe) by an experienced hepatic histopathologist (S. Urbanski).

**ICAM-1 Immunohistochemistry staining.** Immunohistochemistry was performed to ensure that the antisense regimen prevented ICAM-1 expression. An indirect immunoperoxidase technique was applied to 8-µm-thick sections of normal livers, rejecting livers and rejecting livers treated with ICAM-1 antisense oligonucleotides, on the sixth postoperative day. Liver tissues were fixed in paraformaldehyde overnight after resection from the recipient. The tissues were then rinsed in 20% sucrose in phosphate-buffer. Cryostat sections were cut on the following day and stored at -20°C before staining. After rehydration, liver samples were incubated for 30 minutes with 5% normal horse serum to block non-specific protein interactions. Sections were then treated with the primary ICAM-1 antibody (1A29) in appropriate dilution for 90 minutes. Endogenous peroxidase activity was blocked by 0.3% H₂O₂. The biotinylated horse anti-mouse
IgG secondary antibody and avidin-biotin-complex (ABC) (Vector Labs, Burlingame, CA.) were applied for 60 minutes and 30 minutes respectively. The tissues were then incubated with diaminobenzidine (DAB) solution for 5 minutes and counterstained with hematoxylin. Controls were obtained by omitting the primary antibody, substituted by antibody diluent alone. Staining was studied under light microscopy.

**Immunohistochemistry stain for ICAM-1 antisense oligonucleotide:** The method for immunohistochemical staining for the ISIS phosphorothioated oligonucleotides has previously been described (4). In brief, liver tissue samples were fixed in 10% Neutral Buffered Formalin for 24 hours and then prepared for histological analysis. Deparaffinized sections were stained for ISIS 9125 using a two-step HRP immunohistochemistry technique. After a hydrogen peroxide quench of 10 minutes with DAKO Blocking Solution (Carpenteria, CA) the slides were rinsed with 3 changes of PBS and then pre-treated with DAKO Proteinase K solution for 15 minutes. This was followed directly by a 45 minute incubation of primary antibody (2E1-B5 from Berkeley Antibody Company, Berkeley, CA). 2E1-B5 is an IgG1 antibody that specifically recognizes a CG or TCG motif in phosphorothioate oligonucleotides. The sections were rinsed with 3 changes of PBS and then incubated with Zymed Anti-IgG1 isospecific HRP conjugated secondary (San Francisco, CA) for 30 minutes. Again sections were rinsed with 3 changes of PBS and DAB was applied for 5 minutes. 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 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 hours before the experiment. They were anesthetized with pentobarbital sodium (50mg/kg, i.p.). The right jugular vein was cannulated for administration of the leukocyte-labeling fluorescent dye rhodamine 6G. A midline and a subcostal incision were made for exposure. Hepatic ligaments were freed. Neovascularizations from the stomach, duodenum, diaphragm, omentum and retroperitoneal tissues were carefully ligated and cleared from the liver capsule. Hemostasis was achieved by gauze packaging and cauterization. The intestine was covered with warm moist gauze. The left lobe of liver was then exteriorized and placed onto a 30°-tilt Plexiglass microscopic stage. The liver surface was covered by polypropylene wrap to hold the organ in position and prevent evaporation. Animals were maintained at 37°C using an infrared heat lamp, with rectal temperature monitored by an electrothermometer.

Leukocyte parameters. All animals received rhodamine 6G intravenously (0.3 mg/kg) to label leukocytes as previously described (30). Rhodamine 6G-associated fluorescence was visualized by epi-illumination at 510-560 nm, using a 590 nm emission filter. An intravital microscope (Optiphot-2; Nikon Inc., Mississauga, Canada) with a 40x water immersion lens (40/0.55 WI; Nikon, Tokyo, Japan) and a 10x eyepiece were used to observe the microcirculatory cellular events 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 monitor and the images were recorded for playback analysis using a videocassette recorder. Leukocytes were considered adherent to the hepatic venular endothelium if they remained
stationary for a period of time >30 seconds. 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 intravital 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 ± SEM. Differences between the groups were assessed by one-way analysis of variance (ANOVA) followed by a Bonferroni correction for multiple comparisons. Statistical significance was set at p<0.05. Statistical analysis for survival rates was performed using the Kaplan-Meier method.

Results

Liver Biochemistry. Serum alanine aminotransferase (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 Figure 1 (top panel). The isografted group had a statistically insignificant elevation of ALT levels compared to the unoperated controls. All the allografted groups (whether treated with saline or missense ICAM-1 control preparations or the ICAM-1 antisense treatment) had significantly higher enzyme levels compared to the isografted controls. Serum bilirubin levels of the 5 groups of animals are presented in Figure 1 (bottom panel). 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 normalisation 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 figure 2. The first panel (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) 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 to figure 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: A representative slide from an ISIS 9125-treated allograft liver and a saline-treated allograft, 24hr after the last dose, is shown in figure 3. Positive staining for the 9125 compound was visible in Kupffer cells and sporadic sinusoidal endothelial cells (3B). Hepatocytes did not show significant staining. The saline-treated allograft livers showed no detectable staining (3A).

ICAM-1 Immunohistochemistry staining. Representative ICAM-1 immunohistochemistry stains are shown in figure 4: A and B show zone 3 (centrilobular) views of an isograft control (A) and
an ICAM-1 antisense-treated allograft, while 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 isograft control liver (Figure 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 (Figure 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, and is more prominent in areas with cellular infiltration (Figure 4C). Bile ductular ICAM-1 immunohistochemical staining was variable; some ductules had scant staining (such as the ductule visible in the center 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 (Figure 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 to the rejecting allografts, and generally showed appearances similar to isograft controls.

*Leukocyte recruitment.* Leukocyte rolling flux and adhesion on postsinusoidal venules are presented in Figure 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 3-fold increase in rolling flux was observed in the saline- or ICAM-1 missense oligonucleotide-treated allografted animals.

Compared to unoperated controls, a 3-fold increase in leukocyte adhesion in postsinusoidal venules was noted in the isografted rats, whereas more than a 7-fold 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 24h 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 to 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 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 MHC 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) since rejection within this purebred strain was never observed, while 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, since all the major blood vessels supplying the liver were transiently clamped for vascular anastomosis. Vollmar *et al.* showed that this leukocyte-mediated manifestation of post-ischemic hepatic damage was ICAM-1-dependent and exhibited in the first few hours after the operation (29). As 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 hours (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. one month post-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 post-transplant 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 to 2 years 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 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, as 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 in spite of 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 to the allografted rejecting livers (figure 3). Speculating based on 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 due to the later time point (24hr) after dosing at which the specimens were obtained. Previous studies in the rat liver have demonstrated that hepatocytes show early (within 2 hr) 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 as 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 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 VCAM-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 since 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 obliteratorive 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 a) migrate out of the bloodstream (a process proven to be highly dependent on ICAM-1); and b) 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 a 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 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 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 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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Figure 1. Serum alanine aminotransferase (top) and bilirubin (bottom) levels of unoperated, isografted (Lewis to Lewis), saline-treated allografted (Lewis to Brown Norway) and allografted rats treated with ICAM-1 missense oligonucleotides (Allo+MS) or antisense oligonucleotides (Allo+AS) on the sixth post-operative day. (n=6 in each group). * significantly different from isograft controls, p<0.05. † significantly different from Allo+MS group, p<0.05.
Figure 2. Liver histology in isografted, allografted rejecting and allografted ICAM-1 antisense-treated rats. A) Isograft control (Lewis to Lewis) showing normal histology (x40, H&E stain). B) Rejecting allograft (Lewis to Brown Norway) at day 10 showing moderately severe damage. Extensive necrosis is present, and 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 to B. There is moderate portal and periportal inflammation, and only occasional lobular necrotic hepatocytes.
Figure 3: Immunohistochemistry staining for ISIS 9125 (ICAM-1 antisense oligonucleotide). A) 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.
Figure 4. ICAM-1 immunohistochemistry staining in liver grafts. A) A zone 3 view of an isograft control shows weak diffuse staining in sinusoidal lining cells including the terminal hepatic venule (center) whereas hepatocytes are negative for ICAM-1. B) The zone 3 of an allograft treated with ICAM-1 antisense oligonucleotides shows weak diffuse staining in sinusoidal endothelium including terminal hepatic venules (center), very similar to slide A. C) Portal areas (zone 1) of an untreated rejecting allograft demonstrates positive staining in hepatocytes, sinusoids and portal venular endothelium. Leukocytes are seen adhering to the vessel walls. D) A zone 1 view of an ICAM-1 antisense oligonucleotide-treated allograft shows weak staining on sinusoidal endothelium and lack of significant staining on the portal venule (left center) and bile ductular epithelium (right center).
Figure 5. Leukocyte adhesion (top) and rolling flux (bottom) in the 5 groups of rats. Leukocyte adhesion in the postsinusoidal venules was significantly increased in the allografts treated with saline (Allograft) or ICAM-1 missense oligonucleotides (Allo+MS), but treatment with ICAM-1 antisense oligonucleotides (Allo+AS) reduced adherent cells to isograft levels. Rolling flux was also increased in the allograft groups, but was not affected by the treatment with ICAM-1 antisense oligonucleotides. n=6 in each group. * significantly different from isograft, p<0.05. † significantly different from Allo+MS group, p<0.05.