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Generation of lipid free radicals by adherent leukocytes from transplanted rat liver

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Abstract The production of free radicals in blood correlates with primary nonfunction of transplanted livers, but the source of the free radicals is unknown. The purpose of this study was to determine if adherent leukocytes in the transplanted liver are responsible for the radicals detected in blood. First, a new method to harvest adherent leukocytes from the liver without enzymatic digestion was developed and characterized by transplanting livers from ethanol-treated rats, which increases primary nonfunction, and from saline-treated controls. Free radicals were then detected in isolated leukocytes using the spin-trapping technique and electron spin resonance (ESR) spin spectroscopy. Livers were perfused with a balanced salt solution (200 ml), followed by a Ca^{2+}-free solution containing EGTA and heparin (400 ml). Perfusion with Ca^{2+}-free buffer removed greater than 90% of all adherent leukocytes from saline-treated livers and nearly 80% of all leukocytes from fatty livers without removing Kupffer cells. Transplanted fatty livers from rats given ethanol contained significantly more adherent leukocytes (5.0×10^7 cells/liver) than grafts from control donors (3.2×10^7 cells/liver) and almost double the number of adherent neutrophils and monocytes. Moreover, adherent white blood cells from transplanted livers produced the same three free radical species that have been detected previously in blood; however, cells from ethanol-treated livers produced about five times more radical adducts. These data show that adherent white blood cells produce free radicals that are important in the mechanism of primary graft nonfunction.

Key words Liver transplantation, rat, free radicals · Free radicals, liver transplantation, rat · Adherent leukocytes, free radicals

Introduction

Fatty livers taken from donors that have ingested ethanol or a high-fat diet have a higher risk of primary nonfunction and failure shortly after implantation [8]. Because of this increased risk, organs with 50% or greater fatty hepatocytes are usually not utilized by most transplant centers. Rather, they are discarded, wasting an organ that could otherwise save a patient. The mechanisms leading to failure of fatty liver grafts are not completely understood, but previous studies with a rat model of orthotopic liver transplantation have shown that a hypoxia-reperfusion injury occurs after cold storage upon implantation, leading to endothelial cell damage [5, 11, 12, 20, 21]. Furthermore, free radical adducts in blood, as detected by spin-trapping techniques and ESR spectroscopy, are increased almost immediately after reperfusion and correlate with primary nonfunction [6, 7, 13].

Free radicals, regardless of the source, can initiate lipid peroxidation and cause cell injury, yet the source(s)

of free radical adducts in the blood that are associated with graft failure have not been identified [4, 10]. There are many possible sources of free radicals, such as xanthine oxidase [23, 39] and activated Kupffer cells [5, 20, 21]. Additionally, using in vivo fluorescence microscopy and electron microscopy, several groups have shown a qualitative increase in the number of adherent leukocytes within minutes after reperfusion of transplanted livers that have a higher probability of failure because of ethanol treatment of the donor or prolonged cold storage [33, 34, 37]. Adherent, inflammatory white blood cells (e.g., neutrophils and monocytes) can be activated to undergo a respiratory burst, leading to the production of free radicals [29, 33]. Indeed, adherent white blood cells from the liver produce superoxide free radicals [42]. Radical scavengers have been shown to improve transplant outcome and decrease leukocyte adhesion after reperfusion [25]. Based on these data, it was hypothesized that adherent leukocytes may be responsible for production of free radical adducts that have been previously detected in blood and correlate with graft failure.

Previously, adherent infiltrating leukocytes have been isolated from the liver by enzymatic digestion to break down connective tissue [2, 14]. Leukocytes were then separated from other cells using discontinuous Percoll or Ficoll gradients. While this procedure yields leukocyte-rich preparations, it is expensive, time-consuming, and may alter the expression of surface molecules [32]. Therefore, one purpose of this study was to develop and characterize a new, simple, quick, and inexpensive method to isolate and quantify viable adherent leukocytes from the transplanted liver without the use of enzymatic digestion or density gradients. This novel procedure is based on the principle that adhesion of circulating leukocytes via integrins in the liver sinusoid is calcium-dependent [15, 38]. These cells were then counted and identified, and the hypothesis that isolated adherent white blood cells produced free radicals was tested using the spin-trapping technique and ESR spectroscopy. Preliminary accounts of these data have appeared elsewhere [31].

Materials and methods

Animals

Inbred, female Lewis rats (200–250 g) were used for all experiments to exclude immunological interference. Rats were allowed free access to chow diet and water. All rats were anesthetized with methoxyflurane prior to surgical procedures and received humane care in accordance with the "Principles of laboratory animal care" of the NIH and with the institutional guidelines of the University of North Carolina.

Cytologic quantitation of adherent leukocytes

After isolation of adherent cells, erythrocytes were lysed by incubation in distilled water for 20 s [3]. Leukocytes were resuspended in 10 ml of Hank's balanced salt solution (HBSS), 100 µl aliquots were added to 900 µl of HBSS containing 0.4 mM trypan blue, and viability was determined with a hemocytometer. Isolated leukocytes (~ 10^4 cells/ml) were diluted in HBSS and albumin (final concentration 0.1 %) and concentrated on a microscope slide using a centrifuge (Cytospin). Cells were fixed with 100 % methanol for 30 s, dried, and stained with modified Wright-Giemsa. Two hundred cells per liver were identified by nuclear shape and the number of different leukocytes was calculated.

Quantitation of adherent cells in the liver sinusoids

Livers were perfused with 250 ml HBSS containing Ca^{2+}, Mg^{2+}, and 1 U/ml heparin, followed by 400 ml HBSS without Ca^{2+} and Mg^{2+}, containing 1 mM EGTA and 1 U/ml heparin. Three liver sections were cut and fixed in 1 % formalin. The tissue was embedded in paraffin and sectioned for light microscopy. Sections were stained immunohistochemically with an antibody to ED-1, a specific marker of macrophages, to label Kupffer cells [9], and were counterstained with toludine blue to stain leukocytes [37]. Liver sections were viewed at 400X to count ED-1-positive cells and dark blue leukocytes in a 1-mm^2 section of tissue.

Transplantation

Twenty-four hours before the liver was explanted, rats were given ethanol (5 g/kg) or saline vehicle (control) intragastrically. Liver weights were similar in both groups. Liver explantation and transplantation were performed after cold storage as described by Kamada et al. [16] without reconnecting the artery. Briefly, livers were explanted, prepared, and stored in University of Wisconsin cold storage solution for 24 h at 0 °–4 °C and then rinsed with 3 ml of cold Ringer's solution before implantation. Organs were implanted by connecting the superior vena cava with a running suture, connecting the descending vena cava and portal vein with cuffs, and performing anastomosis of the bile duct with an intraluminal splint.

Spin-trapping of free radicals from adherent leukocytes and ESR spectroscopy

Isolated leukocytes (1.0×10^7 cells) were incubated at 37 °C for 30 min with 25 mM of α-phenyl N-*tert*-butylnitrone (PBN) in a total volume of 3 ml. This suspension was extracted with chloroform:methanol (2:1) and after phase separation and drying of the organic layer, the organic solvent was removed by vacuum distillation. The lipid residue was resuspended in chloroform (0.5 ml), bubbled with nitrogen, and subjected to ESR analysis. A Bruker ECS-106 ESR spectrophotometer, equipped with a TM110 cavity, was used for all analyses. The instrument was set to 20 mW microwave power, 1.0-G modulation amplitude, and an 80-G scan width. Data were collected with an IBM-compatible computer for analysis of hyperfine coupling constants by computer simulation [7, 13, 22]. The magnitude of the signal was measured at identical gains and expressed in arbitrary units (1 Unit = 1 mm chart paper).

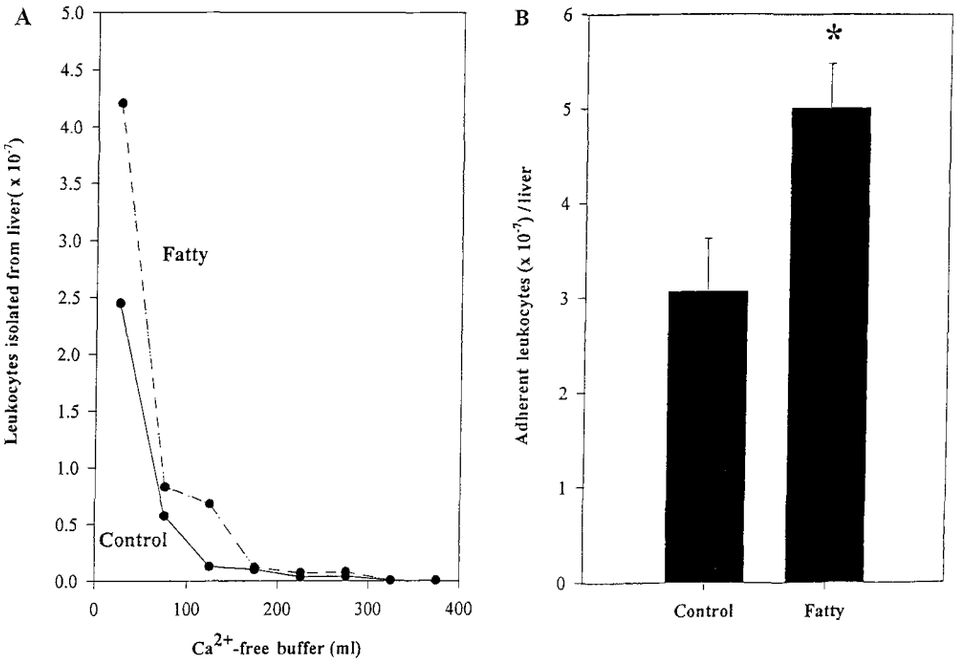

Fig. 1A, B Number of adherent leukocytes collected during perfusion with Ca^{2+}-free buffer. Effluent was collected sequentially in 50-ml samples. **A** After lysing the erythrocytes, leukocytes were counted in each aliquot. Data are from a typical experiment repeated four times (*solid line* control donor, *dashed line* fatty donor). **B** Pooled aliquots were also counted to determine the total number of adherent leukocytes in the liver. Viability was greater than 90% as determined by trypan blue exclusion. Data represent mean ± SEM ($n = 6$) $^*P < 0.05$ (Student's two-tailed t-test)

Statistics

All groups were compared using Student's two-tailed t-test or analysis of variance (ANOVA) with Bonferroni's posthoc test, as appropriate. Differences were considered significant when the P level was below 0.05.

Results

A new perfusion technique to isolate adherent leukocytes from transplanted livers

The first goal of this study was to develop and validate a simple, new method to isolate adherent leukocytes from transplanted livers. Livers from saline- and ethanol-treated rats were explanted, cold-stored for 24 h, and implanted as described in Materials and Methods. Thirty minutes after opening the portal vein and initiating reperfusion, the rat was reanesthetized and the portal vein was cannulated. The liver (8–12 g) was perfused with 250 ml of rinse buffer at rates of 30 ml/min and pressure less than 8 cm water (HBSS containing Ca^{2+}, Mg^{2+}, and 1 U/ml heparin) to remove free blood elements and prevent clotting. In preliminary experiments, it was determined that this volume was sufficient to wash out nonadherent blood cells and blanch the liver since fewer than 10^4 cells came out of the liver in the last 50 ml of effluent. Next, 400 ml of Ca^{2+}-free buffer (HBSS without Ca^{2+} and Mg^{2+}, containing 1 mM EGTA and 1 U/ml heparin) was perfused through the liver to chelate the calcium and remove adherent blood elements. Effluent was collected from the suprahepatic vena cava in 50-ml aliquots and centrifuged at 300 g for 7 min to pellet the cells. Contaminating erythrocytes were removed by hypotonic lysis and centrifugation at 500 g for 7 min. Leukocytes were then suspended in 50 ml of rinse buffer and centrifuged again. The cell pellet was resuspended in 10 ml of rinse buffer and counted. Viability was excellent as all cell preparations excluded trypan blue greater than 90%.

Regardless of the treatment of the donor rat, nearly 50% of the total adherent leukocytes isolated by this procedure were removed from the liver by perfusion with the first 50-ml aliquot of Ca^{2+}-free buffer (Fig. 1A). Subsequently, there was a gradual decrease in the number of cells recovered from the graft in each successive fraction. After perfusion with 400 ml of Ca^{2+}-free buffer, very few cells appeared in the effluent.

The cells from each aliquot were pooled and counted, as described in "Materials and methods". Figure 1B shows the number of adherent leukocytes obtained from saline or ethanol-treated donor grafts 30 min after implantation. The total number of adherent leukocytes isolated was almost two times greater from ethanol-treated fatty donors than from controls, demonstrating that leukocyte adherence is increased dramatically in livers by ethanol treatment.

To determine the percentage of adherent leukocytes removed by this isolation procedure, cells in the sinusoids were counted in liver sections before and after perfusion with 400 ml of Ca^{2+}-free buffer, stained immunohistochemically with ED-1 to identify Kupffer cells, and

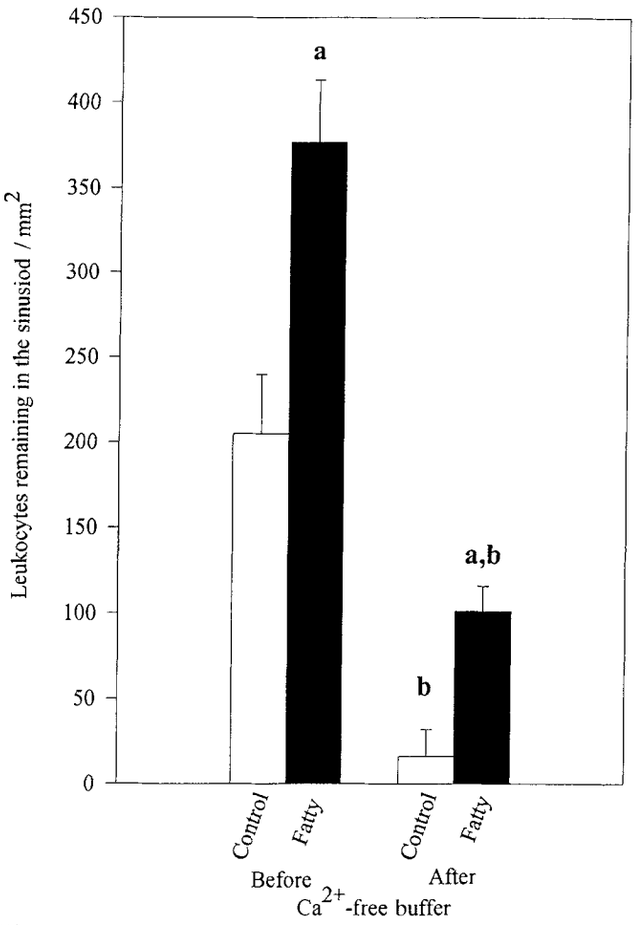

Fig. 2 Number of adherent leukocytes in control and fatty transplanted livers before and after perfusion with Ca^{2+}-free buffer. Cells in the sinusoids were counted in a 1-mm^2 area of tissue at 400 ×. Data represent mean ± SEM ($n = 4$). *a* Significantly different from control within each group. *b* Significantly different from values before Ca^{2+}-free buffer perfusion. $P < 0.05$ (ANOVA and Bonferroni's posthoc test)

counterstained with toludine blue to stain leukocytes (Fig. 2). After perfusion with rinse buffer alone, 205 ± 35 leukocytes/mm^2 tissue remained in the sinusoids of the saline-treated donor livers while there was a significant increase in adherent cells in the ethanol-treated grafts (377 ± 37 leukocytes/mm^2 tissue). After perfusion with 400 ml of Ca^{2+}-free buffer, the number of leukocytes in the sinusoids was reduced 80%–90% in grafts (Fig. 2). Furthermore, this procedure did not isolate Kupffer cells since the number of ED-1-positive cells was not significantly different before (83 ± 6 cells/mm^2 tissue) and after (74 ± 5 cells/mm^2 tissue) perfusion with the Ca^{2+}-free buffer.

To confirm that leukocytes tightly adhere in the liver only during inflammation and that Kupffer cells are not removed by this procedure, attempts were made to isolate adherent cells from saline- or ethanol-treated donor livers before and after 24 h of cold storage (groups 1–4, Table 1). In all cases, no cells (fewer than 0.02% of cells isolated from transplanted livers) were isolated from these control groups. This result shows that adherent cells isolated from the transplanted liver originate in the recipient and are not present before the graft is implanted. Second, the perfusion procedure does not isolate other liver cell types such as Kupffer, endothelial, or pit cells. Cells were only isolated from livers after transplantation (groups 5–6, Table 1).

Table 1 Treatment groups subjected to leukocyte isolation by perfusion

Donor treatment group	24-hour cold storage	Implantation
1. Saline	–	–
2. Ethanol	–	–
3. Saline	+	–
4. Ethanol	+	–
5. Saline	+	+
6. Ethanol	+	+

Table 2 Differential count of leukocytes isolated 30 min after transplantation of control and fatty livers. Values represent mean ± SEM ($n = 6$) * $P < 0.05$ (ANOVA and Bonferroni's post-hoc test)

Leukocyte type	Saline-treated control donor (× 10^{-7})	Ethanol-treated fatty donor (× 10^{-7})	Percent increase ethanol saline
Lymphocytes	2.39 ± 0.06	3.50 ± 0.11*	146
Neutrophils	0.25 ± 0.05	0.56 ± 0.06*	224
Monocytes	0.41 ± 0.02	0.88 ± 0.08*	215
Eosinophils	0.03 ± 0.01	0.09 ± 0.02	300

Quantitation of adherent leukocytes after transplantation of saline- and ethanol-treated donor livers

Cells were identified by nuclear shape, and percentages of each blood cell type were determined (Table 2). After 30 min of reperfusion of the transplanted liver, the number of adherent lymphocytes was 1.5 times greater and the numbers of neutrophils and monocytes were more than two times greater in grafts from ethanol-treated donors than from saline-treated control animals.

Production of free radical adducts from adherent leukocytes

Isolated white blood cells were incubated with PBN for 30 min and spin-trapped radical adducts were mea-

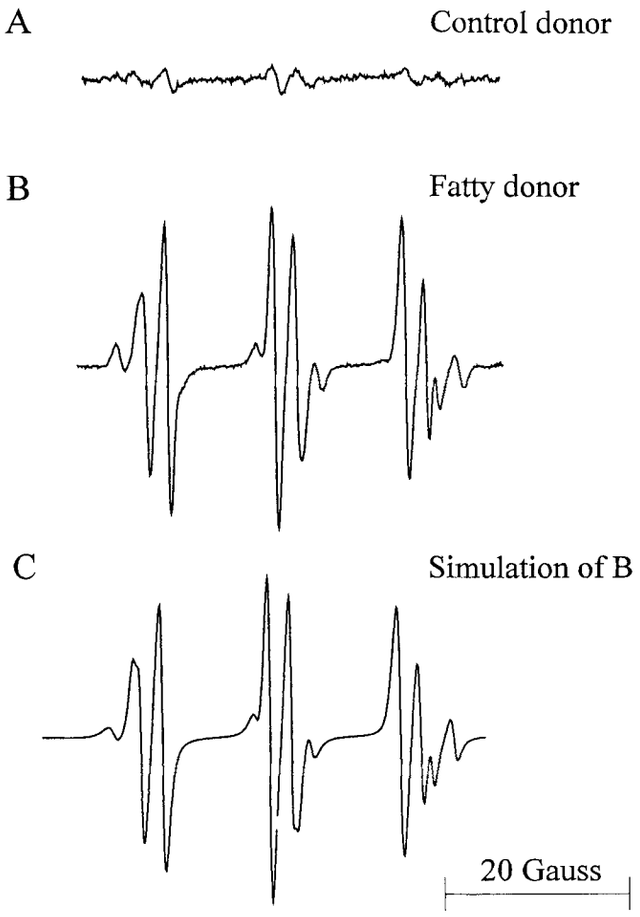

Fig. 3A–C ESR spectra of radical adducts from white blood cells isolated from control and fatty liver grafts 30 min after transplantation. Adherent white blood cells (1.0×10^7 cells/ml) isolated from a liver derived from **A** saline-treated or **B** ethanol-treated donors were incubated with PBN at 37 °C for 30 min. Representative ESR spectra of PBN-radical adducts in chloroform after chloroform:methanol (2:1) extraction of an experiment repeated six times. **C** A computer simulation of a radical spectra from an ethanol-treated rat

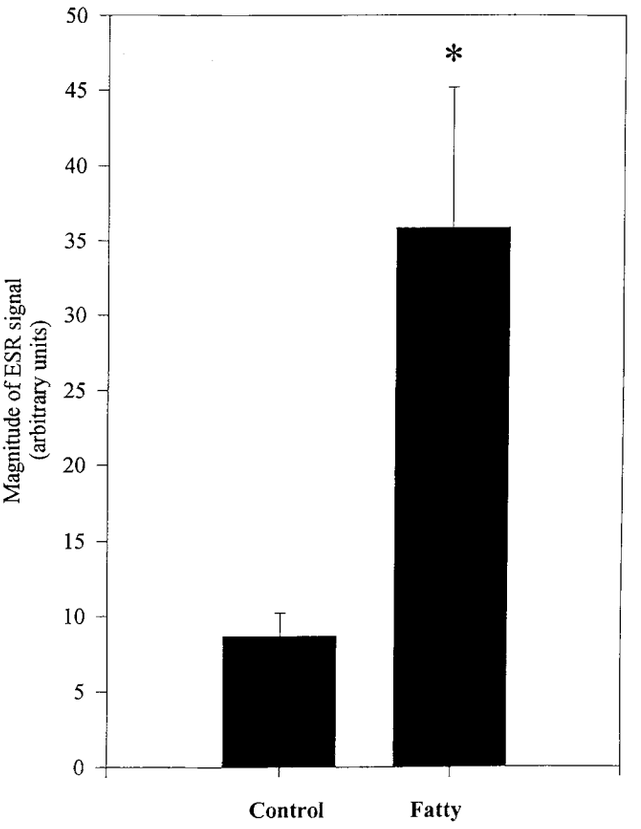

Fig. 4 Relative magnitudes of ESR spectra from adherent white blood cells. Conditions are as described in Fig. 3. Data represent mean ± SEM ($n = 5$–6) $^*P < 0.001$ (Student's two-tailed t-test)

sured by ESR (Fig. 3). A small PBN-radical adduct was detected in adherent leukocytes from saline-treated rats (Fig. 3 A). White blood cells isolated from ethanol-treated fatty grafts produced a six-line ESR spectrum (Fig. 3B) that was more than five times larger than that from adherent cells from saline-treated grafts (Fig. 4). Simulation (Fig. 3C) showed that these spectra are a composite of three radical species that are nearly identical to those previously isolated from whole blood (species I: $a^N = 15.1$ G and $a\beta^H = 4.9$ G, species II: $a^N = 14.8$ G and $a\beta^H = 3.3$ G, species III: $a^N = 14.1$ G and $a\beta^H = 2.2$ G) [13]. Radical adduct I and II had coupling constants characteristic of carbon-centered radicals while radical species III had coupling constants suggestive of an oxygen-centered radical adduct.

Discussion

Isolation of adherent leukocytes from the transplanted liver

Free radical adducts in blood and increased leukocyte adherence have been associated with primary nonfunction of fatty grafts. Since it is known that adherent leukocytes produce superoxide radicals [42], it was hypothesized that these adherent leukocytes produce radical adducts that have been previously detected in blood [13]. In order to study adherent leukocytes after liver transplantation, a new procedure was developed to quickly remove these cells from the liver. This protocol is based on the fact that adherence of leukocytes to endothelial cells in the sinusoid, which occurs largely through adhesion molecules, is Ca^{2+}-dependent [15, 38]. This new method was characterized by quantifying

the cells collected in the outflow during perfusion of transplanted livers from saline- and ethanol-treated donors with Ca^{2+}-free buffer. These experiments demonstrated that 400 ml of buffer was sufficient (Fig. 1A) to remove greater than 80% of adherent leukocytes from transplanted livers without isolating Kupffer cells, endothelial cells, or pit cells (Fig. 2). Therefore, it is concluded that a simple, new method has been developed that can be used effectively to recover the majority of adherent leukocytes from the liver without enzymatic digestion of the organ.

The isolation method developed here has several advantages over previous techniques using enzymatic digestion of the organ and isolation of the leukocytes from nonparenchymal cells by multiple centrifugation steps through density gradients [2]. First, adherent leukocytes are obtained after one centrifugation step and lysis of erythrocytes, avoiding time-consuming multiple centrifugation steps since longer isolation periods could adversely affect cell function. Moreover, it is likely that enzymatic digestion used in other methods alters leukocytes by cleaving surface receptor proteins. It is also known that Ficoll and Percoll alter the expression of integrins on the surface of leukocytes, which probably also affects functional characteristics [32]. Second, this procedure does not isolate other liver cell types, such as Kupffer, endothelial, or pit cells, although others have shown that pit cells can be removed from the liver with a similar method using high pressure [36]. Not only can this new technique be used to study the role of leukocytes in the failure of transplanted grafts, but it can also be applied easily to pathophysiological states where blood elements adhere to the sinusoids (e.g., allograft rejection, drug-induced hepatitis, fulminant hepatic failure, or warm ischemic liver injury) [17–19]. The only drawback to the procedure described here is that cells that have already undergone diapedesis into the tissue cannot be recovered; however, this represents only a small fraction of infiltrating cells during the early stages of inflammation (e.g., soon after liver transplantation).

Leukocyte adherence after transplantation of fatty grafts

After this new method was validated, it was used to study differences in the number of adherent leukocytes in the sinusoid after transplantation of livers from saline-treated controls and of fatty livers from acute, ethanol-treated donors. Even one large dose of ethanol causes an increase in triglycerides and lipids in the liver [35], leading to increased primary nonfunction rates [30, 39, 41]. In a previous study from this laboratory, graft survival after 24 h of cold storage was reduced from nearly 90% to about 30% when the donor animal was given one acute dose of ethanol 24 h prior to liver explantation [41]. It has been previously shown with electron and video microscopy that leukocytes adhere in the sinusoid as early as 20 min after implantation of the liver, yet the increase in leukocyte adherence was not quantified [24, 33, 37]. In this study, the total number of adherent leukocytes recovered after transplantation of livers from ethanol-treated fatty donors was doubled 30 min after transplantation compared to saline-treated controls (Fig. 1B). These findings further support the hypothesis that increased leukocyte adherence very early after implantation of fatty grafts is involved in the mechanisms of primary nonfunction and graft failure.

Interestingly, the percentage increase in inflammatory cell adherence (monocytes, neutrophils, and eosinophils) in fatty grafts was greater than the increase in lymphocytes (Table 2). The role of increased adherence of lymphocytes in the ethanol-treated graft is unclear; however, others have shown a correlation between early infiltration of lymphocytes into the graft and the development of rejection [1, 28]. These data are consistent with the hypothesis that grafts from donors that have previously consumed ethanol may have an increased risk of chronic rejection.

Since it is known that adherent neutrophils and monocytes can be activated to produce superoxide free radicals [42], isolated adherent leukocytes were incubated with the spin trap PBN to test the hypothesis that adherent white blood cells from the graft are responsible for the production of free radical adducts detected in blood after transplantation and that correlate with primary nonfunction [13]. Indeed, adherent leukocytes produced free radicals regardless of prior treatment of the graft (Fig. 3A, B) although the production was almost undetectable in cells from control, nonfatty grafts (Fig. 4). Adherent white blood cells from saline- or ethanol-treated transplanted livers produce three free radical species essentially identical to radicals that have been detected previously in whole blood immediately after transplantation [6, 7, 13]. The chemical structures of these radical adducts remain unknown; however, the coupling constants suggest that they are lipid radicals. It is possible that these lipid free radicals arise from superoxide free radicals through a lipid peroxidation cascade resulting in oxidation of lipids in the membrane of the white blood cell during ex vivo incubation. However, because white blood cells adhere to the endothelium in vivo, it is possible that these same free radicals are important in damage to endothelial cells [27], which occurs after reperfusion of transplanted livers [20]. This hypothesis is supported by the fact that reoxygenation leads to adherence and activation of inflammatory cells, which results in the production of superoxide and hypochlorous acid to initiate lipid peroxidation [4, 26, 33, 40, 42]. These data also show that increased white blood cell adherence correlates with primary nonfunction,

which occurs after ethanol ingestion by the donor. Moreover, free radical production by adherent cells correlates with primary nonfunction since patients receiving fatty livers have a higher mortality rate [41]. Overall, these data suggest that therapies that reduce free radical production or block the adherence of leukocytes immediately upon implantation would improve graft function and decrease the risk of primary nonfunction of transplanted fatty livers.

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