

# Induction of adhesion molecule expression in liver ischaemia–reperfusion injury is associated with impaired hepatic parenchymal microcirculation

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**Background:** Activated neutrophils may be important mediators in liver ischaemia–reperfusion injury (I/R). Adhesion of leucocytes to the endothelial cell surface is a result of activation of cell adhesion molecules. The aim of this study was to investigate the effect of I/R on the hepatic microcirculation (HM) and intercellular adhesion molecule (ICAM) 1 expression.

**Methods:** Four groups of six Sprague–Dawley rats underwent laparotomy for liver exposure. Group 1 acted as controls, and groups 2–4 underwent partial liver ischaemia for 30, 45 and 60 min respectively followed by reperfusion for 60 min. Flow in the HM was measured by laser Doppler flowmetry. Liver biopsies were taken at the end of the reperfusion period. ICAM-1 expression was assessed by immunohistochemistry (graded 0–3).

**Results:** Mean flow in the HM was significantly reduced with I/R (mean(s.e.m.) red cell flux 140(21), 52(3) and 43(2) with 30, 45 and 60 min ischaemia compared with control 230(17); all  $P < 0.001$ ). ICAM-1 expression was significantly induced (mean(s.e.m.) 1.30(0.21), 2.50(0.22) and 2.80(0.17) with 30, 45 and 60 min ischaemia *versus* control 0.50(0.22); all  $P < 0.001$ ).

**Conclusion:** I/R produced a significant upregulation of ICAM-1 expression which correlated with impaired flow in the HM.

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## Introduction

Ischaemia–reperfusion (I/R) injury remains a crucial problem in liver transplantation<sup>1</sup>. Cell damage following ischaemia is biphasic, with injury being initiated during ischaemia and exacerbated during reperfusion<sup>2,3</sup>. During ischaemia the cell is deprived of the energy needed to maintain ionic gradients and homeostasis. Failure of enzyme systems leads to cell death<sup>4</sup>. The pathological changes resulting from ischaemia can be delayed but not prevented by hypothermia<sup>5</sup> and this may introduce new damaging factors on reperfusion<sup>6,7</sup>.

Reperfusion is a prerequisite for recovery from ischaemic injury and has two beneficial consequences for ischaemic tissue, restoring the energy supply and removing toxic metabolites. These toxic metabolites, however, return to the systemic circulation and may produce serious

metabolic effects distant from the local tissue injury<sup>8</sup>. Reperfusion may salvage cells damaged by ischaemia, but some injuries are irreversible<sup>9,10</sup>. The pathophysiology of reperfusion injury is complex and involves free radical-mediated lipid peroxidation, arachidonic acid metabolites, complement, cytokines and the tissue accumulation of neutrophils<sup>11–13</sup>. Sequestration of neutrophils is central to the later phases of tissue damage<sup>14</sup>. Reperfusion injury is mediated by the interaction of free radicals, endothelial factors and neutrophils<sup>15,16</sup>. Three types of reperfusion injury have been postulated<sup>17,18</sup>. Molecular events inside both parenchymal and endothelial cells of the organ concerned, such as mitochondrial disruption, free radical production and calcium redistribution, might be critical<sup>3,19</sup>. Reperfusion injury may be caused by endothelial cell swelling, interstitial oedema or neutrophil

plugging, any of which could limit the reinstatement of normal blood flow to the ischaemic area, by affecting the microcirculation. This phenomenon has been investigated in detail<sup>20,21</sup>. Finally, reperfusion injury may be caused by the infiltration of activated neutrophils, which can initiate an inflammatory response and cause further tissue destruction<sup>16,22,23</sup>. This mechanism involves direct interaction between activated neutrophils and hepatic sinusoidal endothelial cells (SECs) as well as indirect communication between different cellular components, both releasing and responding to a variety of potent mediators such as cytokines, tumour necrosis factor and oxygen-derived free radicals<sup>24,25</sup>.

Leucocyte recruitment from the bloodstream involves tethering to the vessel wall, rolling along the endothelium and firm adhesion before migration out of the vasculature<sup>26,27</sup>. This sequence of events is mediated by a number of different adhesion molecules expressed on the cell membranes of both SECs and leucocytes<sup>28,29</sup>. Firm adhesion of neutrophils is mediated by  $\beta_2$ -integrins (CD11/CD18) binding to the endothelial ligand intercellular adhesion molecule (ICAM) 1<sup>30–32</sup>. ICAM-1 is expressed at a low level in endothelial cells, but is absent from most cells in normal tissues. Local inflammatory reaction results in a rapid increase in ICAM-1 expression in endothelial cells and induction of ICAM-1 in epithelial and mesenchymal cells<sup>33</sup>. As the inflammatory response progresses, upregulation of ICAM-1 contributes to the recruitment of additional leucocytes, including monocytes, eosinophils and lymphocytes<sup>27,34</sup>.

ICAM-1 expression is not measurable by immunohistochemical methods in normal rat<sup>28</sup> or human<sup>35</sup> liver. Following I/R injury ICAM-1 expression on SECs is upregulated as are other inflammatory mechanisms<sup>28,36</sup>. Recent studies have used laser Doppler flowmetry (LDF) to measure hepatic parenchymal blood flow<sup>37,38</sup>. This method is simpler, more direct and less invasive than the hydrogen gas clearance method or <sup>133</sup>Xe portal vein scintigraphy.

The aim of this study was to investigate the effect of liver I/R injury on ICAM-1 expression and blood flow in the hepatic microcirculation (HM) measured by LDF.

## Materials and methods

The study was conducted under a license granted by the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986.

Male Sprague–Dawley rats weighing 200–250 g were used. The animals had free access to a standard diet and tap water before the experiment. All experiments were performed under general anaesthesia using intraperitoneal

urethane (Sigma, Gillingham, UK) 0.7 ml per 100 g bodyweight in non-recovery procedures. The relationship between hepatic blood flow and adhesion molecule expression with I/R injury was investigated by LDF in the rat hepatic lobar I/R model.

## Experimental protocol

Four groups of six animals were used. Group 1 (controls) underwent sham operation. Groups 2–4 underwent hepatic ischaemia for 30, 45 or 60 min respectively, followed by a 60-min reperfusion period.

Animals were placed in the supine position on a heating pad (Harvard Apparatus, Edinburgh, UK) for maintenance of body temperature between 36 and 37°C. The arterial oxygen saturation and heart rate were monitored continuously by a pulse oximeter (Biox 3740™; Ohmeda, Louisville, Kentucky, USA). Polyethylene catheters were inserted into the femoral artery for monitoring mean arterial blood pressure and into the femoral vein for saline infusion (10 ml per kg per h) to compensate for intraoperative fluid loss. Laparotomy was carried out via a midline incision and ligamentous attachments to the liver were divided. The hepatoduodenal ligament was dissected. An atraumatic microaneurysm clip was used to induce lobar ischaemia in the median and left lobes by clamping the hepatic artery and portal vein branches. This method produces ischaemia to the left and median lobes of the liver, leaving the blood supply to the right and the caudate lobes uninterrupted<sup>39</sup>. At the end of the ischaemic period, the vascular clip was removed and reperfusion was allowed for 60 min in all groups. During I/R, the abdomen was covered with a plastic wrap to prevent fluid evaporation. At the end of the experiment animals were killed by exsanguination.

Liver biopsies taken from the left (ischaemic) and right (non-ischaemic) lobes of the liver at laparotomy (baseline) and at the end of reperfusion were assessed for ICAM-1 expression. ICAM-1 was demonstrated on duplicate sections by the indirect immunoperoxidase technique<sup>40</sup>. The staining intensity on SECs and hepatocytes was scored semiquantitatively as reported previously<sup>36,41–43</sup>. ICAM-1 expression was graded from 0 to 3; 0 represented no stain (no ICAM-1 expression), and 1–3 mild, moderate and intense staining respectively<sup>35,44</sup>.

HM was assessed using a commercially available laser Doppler flowmeter (DRT4; Moor Instruments, Axminster, UK)<sup>45</sup>. A surface multiemitter/detector integrating laser Doppler probe (P9; Moor Instruments) was used. The probe was placed on the surface of the left lobe of the liver. The Doppler signal varied linearly with the product of the total number of moving red blood cells in a measured

volume and the mean velocity of these cells<sup>45,46</sup>; this product is termed perfusion units or blood cell flux. LDF measurements at the relevant time points were collected as a mean of data collected over 1 min. The LDF output signal was fed into a portable computer equipped with software to give a real-time display, and record and analyse the blood flow pattern and values (Moor Instruments). Blood flow results were expressed in arbitrary flux units.

The output from the LDF and pulse oximeter were fed into a commercial analogue-to-digital data acquisition system (Maclab; AD Instrument, Hastings, UK). The data were acquired at a sampling rate of 1 Hz. The LDF measurement was continuous with a reading averaged over each 15-min interval throughout the study.

### Statistical analysis

Values are presented as mean(s.e.m.). Analysis of covariance was used to compare perfusion between sham and graded I/R groups. This allowed for any bias in the perfusion measurements caused by other haemodynamic data such as blood pressure or heart rate. ANOVA and Student's *t* test with Bonferroni's adjustment for multiple comparisons were used for statistical analysis between the groups. Spearman's test was used to examine the correlation between ICAM-1 expression and HM.  $P < 0.050$  was considered statistically significant.

### Results

There were no procedure-related deaths, and no differences in heart rate, body temperature and oxygen saturation between groups. The inter-section variability for ICAM-1 expression was less than 5 per cent.

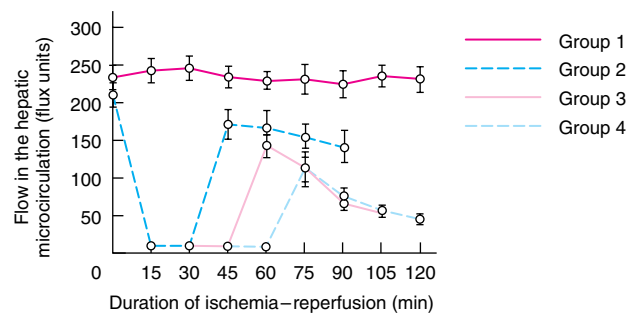
Liver sections from sham-operated controls (group 1) showed no ICAM-1 expression on SECs or hepatocytes either at the beginning or end of the procedure. In group 2 the staining intensity in the SECs on the ischaemic side of the liver increased substantially (moderate to intense ICAM-1 expression), whereas hepatocytes showed no staining. In group 3 SECs showed intense ICAM-1 expression and hepatocytes moderate expression. In group 4 both SECs and hepatocytes showed intense ICAM-1 expression. Results are summarized in *Table 1*. ICAM-1 expression in the left lobe of biopsies taken after reperfusion was significantly greater in the I/R groups than the sham-operated group ( $P < 0.001$ , ANOVA).

ICAM-1 expression in the non-ischaemic right lobe was greater after ischaemia for 30, 45 and 60 min followed by 60 min of reperfusion than that in the control group

**Table 1** Intercellular adhesion molecule 1 expression at baseline and after reperfusion

Ischaemia/reperfusion period	Baseline	After reperfusion	<i>P</i>
Group 1	0.17(0.17)	0.50(0.22)	0.641
Group 2	0.17(0.17)	1.30(0.21)	< 0.001
Group 3	0.33(0.21)	2.50(0.22)	< 0.001
Group 4	0.17(0.17)	2.80(0.17)	< 0.001

Values are mean(s.e.m.). Student's *t* test.



**Fig. 1** Effect of ischaemia–reperfusion injury on the hepatic microcirculation. Values are mean(s.e.m.)

( $P = 0.012$ , ANOVA). ICAM-1 expression in the non-ischaemic lobe was markedly less than that in the ischaemic lobe and no significant differences were noted between groups 2–4.

In the sham-operated group there was no change in HM measurements over the study interval (*Fig. 1*). In the I/R groups flow in the HM was close to zero during ischaemia and increased rapidly following reperfusion, but did not return to baseline values. In group 2, there was a 32 per cent drop in HM at the end of the reperfusion period, which was significantly lower than the baseline measurement ( $P < 0.001$ ). In groups 3 and 4 the HM was reduced by 75 and 79 per cent respectively ( $P < 0.001$  versus baseline). HM measurements in the I/R groups were significantly lower than those in the sham-operated group ( $P < 0.001$ , one-way ANOVA) (*Fig. 1*).

ICAM-1 expression was inversely related to flow in the HM ( $P < 0.001$ , Spearman correlation  $r = 0.74$ ).

### Discussion

Migration through the endothelium represents an essential step in the trafficking of inflammatory cells from blood into the hepatic parenchyma<sup>27,47</sup>. The primary interaction between leucocytes and stimulated endothelial cells occurs through the binding of the adhesive integrin complex of leucocyte to ICAM-1<sup>24,48</sup>.

In this study a lobar hepatic I/R model was used, in which blood flow to the left and median lobes of the liver was occluded while maintaining flow to the right lobes. In this model the blood pressure and acid–base balance were unaltered during the ischaemic interval and a severe ischaemic insult could be produced without inducing mesenteric venous congestion. The local effects of ischaemia and reperfusion in the liver could be determined without systemic instability<sup>39,49</sup>.

ICAM-1 expression was measured by an immunohistochemical technique. Several other methods have been used, including measurement of *ICAM-1* mRNA by northern analysis and western blotting for detection of protein<sup>35,50</sup>. These molecular methods provide quantitative or semi-quantitative information on ICAM-1 expression. Immunohistochemistry has the advantage of being able to identify the site of adhesion molecule expression within a complex tissue.

ICAM-1 expression was induced by I/R injury in this model, as demonstrated previously<sup>27,51</sup>. ICAM-1 expression was also induced in the non-ischaemic lobe, albeit at a lower level, suggesting that systemic mediators of ICAM-1 induction are released by activated Kupffer cells and SECs during I/R<sup>27</sup>.

Leucocyte adherence in postsinusoidal venules is mediated by ICAM-1; this is mainly responsible for the manifestation of hepatocellular dysfunction and tissue injury<sup>36</sup>. I/R injury induces leucocyte stasis in hepatic sinusoids<sup>16</sup>, associated with perfusion failure of sinusoids as demonstrated by intravital fluorescence microscopy<sup>52</sup>. In the present study LDF was used to continuously measure blood flow in the HM without interfering with the circulation itself<sup>7</sup>. In the rat, the interlobular and intralobular distribution of portal venous blood flow is homogeneous, and LDF on the surface of the liver gives specific information about average blood flow in the HM<sup>53</sup>. LDF measurements on the liver surface have been shown to correlate well with the parenchymal microcirculation<sup>43</sup>. LDF revealed a failure of parenchymal perfusion during the ischaemic period. On restoring the blood flow, the parenchymal perfusion recovered but did not return to normal. This reperfusion failure increased with ischaemic time. Impairment of the HM was associated with significant upregulation of ICAM-1 expression. A direct correlation between the microvascular component of I/R and induction of adhesion molecule expression on hepatic sinusoids was demonstrated.

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