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DIFFERENTIAL INDUCTION OF mRNA FOR ICAM-1 AND SELECTINS IN HEPATOCYTES, KUPFFER CELLS AND ENDOTHELIAL CELLS DURING ENDOTOXEMIA

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Intercellular adhesion molecule-1 (ICAM-1) and selectins (E- and P-selectin) mRNAs were determined in individual liver cell types by Northern blot analysis before and after injection of endotoxin. A constitutive expression of ICAM-1 mRNA was found in endothelial cells and Kupffer cells but not in hepatocytes. All three cell types showed upregulation of ICAM-1 mRNA after endotoxin. No constitutive selectin expression could be detected in any liver cell, but endotoxin induced massive synthesis of E- and P-selectin mRNA in endothelial cells and Kupffer cells. The differential expression of cellular adhesion molecules in the liver is consistent with the involvement of selectins in neutrophil rolling in the vasculature and ICAM-1 in transendothelial migration and adherence to parenchymal cells.

Cellular adhesion molecules, expressed in a variety of organs, are important for leukocyte accumulation at sites of inflammation (1). Based on experiments to study neutrophil-endothelial cell interactions in dynamic systems, i.e. using flow chambers *in vitro* (2,3) or intravital microscopy *in vivo* (4,5), the selectin family (L-, P- and E-selectin) was identified as being responsible for the initial contact between neutrophils and the vessel wall ("rolling phenomenon"). During this rolling process, the neutrophil is exposed locally to activating factors, which induce the shedding of L-selectin and the upregulation of the β_2 integrin Mac-1 (CD11b/CD18) within minutes (6). The increased expression of Mac-1 leads to the firm adherence of the neutrophil to endothelial cells via its counterreceptor, (ICAM-1) (7). The subsequent transendothelial migration of the neutrophil out of the vasculature is strongly

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Abbreviations: ICAM-1, intercellular adhesion molecule-1; CAM, cellular adhesion molecule; TNF-α, tumor necrosis factor-α; IL-1, interleukin-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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dependent on LFA-1 (CD11a/CD18) and Mac-1 on the neutrophil as well as ICAM-1 on endothelial cells (8). Neutrophil-induced injury to parenchymal cells, e.g. myocytes, also requires adherence, which is dependent on the upregulation of Mac-1 on neutrophils and ICAM-1 on the target cell (9).

The liver can be a target for neutrophil-induced injury during hemorrhagic shock (10), endotoxin shock (11), and ischemia-reperfusion injury (12). An increase in ICAM-1 mRNA and protein in the liver was shown for endotoxin shock (13) and hepatic ischemia-reperfusion injury (14,15). Monoclonal ICAM-1 antibodies protected against endotoxin-induced liver injury (13) and hepatic ischemia-reperfusion injury (14). Furthermore, antibodies against the α-chain (CD11b) and the β-chain (CD18) of Mac-1, also prevented liver injury during endotoxin shock (11,16) or ischemia-reperfusion (16,17). The role of selectins is less well studied in inflammatory liver injury. High doses of endotoxin induced a moderate increase of P-selectin mRNA in the liver (18). Although a lower dose of endotoxin failed to upregulate E-selectin mRNA in the liver (19), a significant increase of hepatic E-selectin expression was found during severe sepsis in baboons (20). Inflammatory liver diseases in humans are characterized by upregulation of E- and P-selectin (21) and in particular ICAM-1 (21-25). These data demonstrate the importance of different CAMs for leukocyte-mediated injury in the liver. Further investigations are necessary to elucidate the molecular mechanism of CAM gene expression. However, complex tissues such as the liver consist of several different cell types, e.g. parenchymal cells, Kupffer cells and endothelial cells. An adhesion molecule gene transcribed only in one cell type, especially if this cell type is in the minority, may not be detected in an RNA extract of the whole liver. To study the differential transcription of ICAM-1 and selectin genes in parenchymal versus nonparenchymal cells in rat liver *in vivo*, animals were treated with endotoxin and endothelial cells, Kupffer cells and hepatocytes were isolated from the liver and tested for changes of CAM mRNA expression by Northern blot analysis. Our data show an increase of ICAM-1 and selectin mRNAs in the nonparenchymal cell fraction, in particular endothelial cells, and an induction of only ICAM-1 in hepatocytes.

MATERIALS AND METHODS

Animals. Male Fischer rats (240 - 290 g body wt.) were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). The animals had free access to food (certified rodent diet # 5002C; PMI™ Feeds Inc., Richmond, IN) and water. The animals were treated with an intravenous injection of 0.5 mg/kg *Salmonella enteritidis* endotoxin

(Sigma Chemicals Co., St. Louis, MO) or 1 ml/kg saline under light ether anesthesia. After 1 or 5 h, the animals were anesthetized with pentobarbital and used for isolation of liver cells. Hepatocytes were isolated after collagenase digestion, hepatic endothelial cells and Kupffer cells were separated by centrifugal elutriation as described in detail (14,16,17,26,27). Each cell fraction was washed repeatedly with Hanks' balanced salt solution and was ≥95% pure as assessed by morphology and peroxidase staining (26). Cell viability for each cell fraction was above 90% for hepatocytes and above 95% for Kupffer cells and endothelial cells as determined by trypan blue exclusion. Immediately after isolation, RNA was isolated from the individual cell fractions as described below. In some experiments, RNA was isolated from the intact liver.

Preparation of cDNA Probes. cDNA clones encoding rat P-selectin and E-selectin were isolated by homology cloning (18). A cDNA clone encoding rat ICAM-1 (28) was kindly provided by Dr. T. Horiuchi (Daiichi Pharmaceutical Co., Japan). For use in hybridization protocols, DNA fragments were prepared from these cDNA clones by PCR (29). Briefly, gene-specific oligonucleotides were designed to generate fragments of approximately 500 bp in length from P-selectin and ICAM-1 cDNA clones and 1.6 Kb in length from the E-selectin cDNA clone. The following oligonucleotide pairs were used (5'oligo/3'oligo; each sequence as 5' to 3'): P-selectin: CGACTTGACTGTCACTCA/ ACAAGTGAGATACACAG encompassing sequences encoding the cytoplasmic domain and 3' untranslated region; E-selectin: TTACTACTGGATTGGAATCAG/ TGTTTCTGATTGTTTTGAACTTA encompassing sequences encoding the EGF-like, complement regulatory-like repeats, the transmembrane and cytoplasmic domains; ICAM-1: AGGTGTGATATCCGGTAGA/CCTTCTAAGTGGTTGGAACA encompassing the 3' untranslated region. The probe for the metabolic enzyme GAPDH was a 1.2 kb *Pst*I insert fragment from a plasmid containing the rat GAPDH cDNA. Purified fragments were radiolabeled with [α^{32}P]dCTP using a random hexanucleotide primer kit (Stratagene, La Jolla, CA) to a specific activity of 10^9 dpm/μg.

Northern Blot Analysis. Total cellular RNA was isolated from liver tissue according to a method described by Chomczynski and Sacchi (30). Freshly excised liver sections or individual liver cell fractions were homogenized in RNAZOL (Tel-Test Inc., Friendswood, TX), and extracted with guanidine thiocyanate-phenol-chloroform. RNA was collected by overnight precipitation at 4 °C with isopropanol. RNA pellets were washed twice with ice-cold 75% ethanol (v/v), vacuum dried and then dissolved in a small volume of 1 mM EDTA (pH 7.0). Isolated RNA was stored at -70 °C. RNA was quantified spectrophotometrically and equal amounts of RNA samples were electrophoresed on denaturing agarose-formaldehyde gels and transferred to Gene Screen Plus hybridization membranes (NEN Research Products, Boston, MA) using the capillary elution technique (31). RNA was cross-linked by baking the membranes at 80 °C for 2 h under vacuum. Transferred membranes were pre-hybridized with RAPID-Hyb buffer (Amersham, Arlington Heights, IL) at 65 °C for 2 h and then hybridized with labeled probes overnight at 65 °C. Membranes were washed in 1xSSC (0.15 M sodium chloride, 0.015M sodium citrate, pH 7.0) containing 0.1% SDS for 15 min at room temperature. Membranes were washed twice at 55 °C in 0.2xSSC containing 0.1% SDS for 30 min. The washed blots were exposed to Hyperfilm MP X-ray film (Amersham) at -80°C.

Statistics. All data are given as mean ± SE. Comparisons between multiple groups were performed with one way analysis of variance followed by Bonferroni t test. $P < 0.05$ was considered significant.

RESULTS

Total RNA isolated from control livers and probed for ICAM-1 and selectins showed only a minor baseline expression of ICAM-1 mRNA (Figure 1). Injection of 0.5 mg/kg *Salmonella enteritidis* endotoxin increased ICAM-1 gene transcription during the following 5 h, however, mRNA for E- or P-selectin was not detected (Figure 1). Even prolonged exposure of the gel did not reveal any selectin mRNA at the whole organ level. To investigate potential differences regarding the expression of adhesion molecules on parenchymal cells versus sinusoidal lining cells, hepatocytes, endothelial cells and Kupffer cells were isolated by collagenase digestion and centrifugal elutriation. In the hepatocyte fraction, no expression of ICAM-1 mRNA was detected, however, a significant upregulation of ICAM-1 mRNA was observed at 1 h and 5 h after endotoxin administration (Figure 1). In contrast, no selectin mRNA was detected in hepatocytes either before or after endotoxin. Although no basal expression of E- or P-selectin mRNA was found in hepatic endothelial cells, endotoxin induced a drastic increase of both selectin mRNAs at 1 h (Figure 2). Only P-selectin mRNA was still moderately expressed at 5 h. Endothelial cells showed a substantial basal expression of ICAM-1 mRNA, which further increased 1 h after endotoxin and returned to baseline values at 5 h (Figure 2). Qualitatively similar changes in adhesion molecule mRNA expression as in endothelial cells were found in Kupffer cells before and after endotoxin administration (Figure 2). However, baseline expression of ICAM-1 and

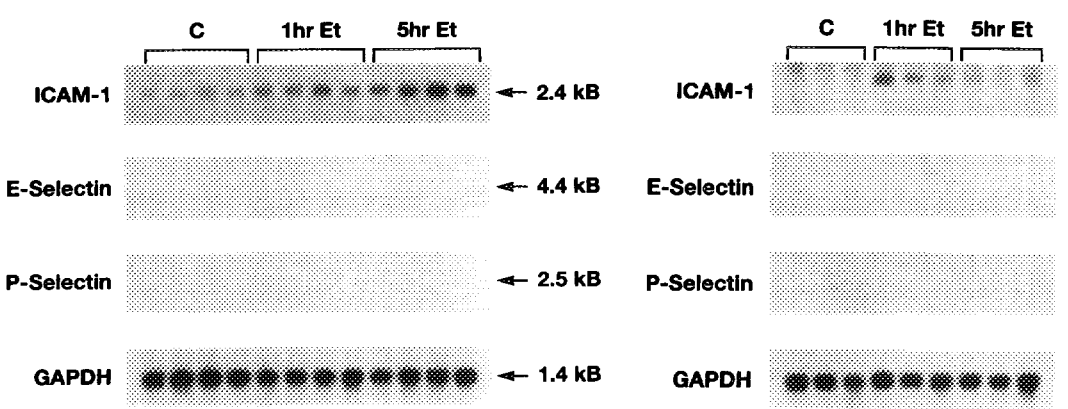

Figure 1. Analysis of mRNA levels for ICAM-1, E- and P-selectin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in whole livers (left graph) and isolated hepatocytes (right graph) of Fischer rats. Untreated controls (C) were compared with animals 1 h and 5 h after injection of 0.5 mg/kg *Salmonella enteritidis* endotoxin. Results from 4 animals are shown for each group and time point.

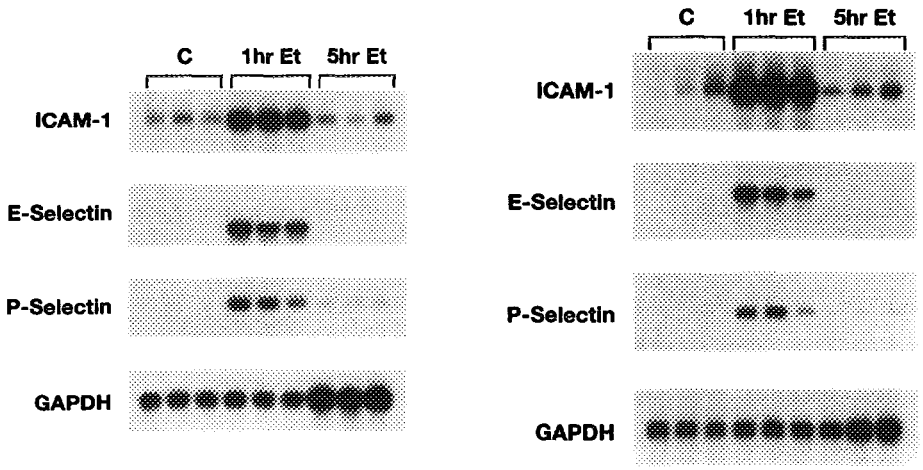

Figure 2. Analysis of mRNA levels for ICAM-1, E- and P-selectin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in hepatic endothelial cells (left graph) and Kupffer cells (right graph) isolated from livers of Fischer rats. Cells from untreated controls (C) were compared with those from animals 1 h and 5 h after injection of 0.5 mg/kg *Salmonella enteritidis* endotoxin. Results from 4 animals are shown for each group and time point.

endotoxin-induced induction of ICAM-1 and selectins in Kupffer cells were quantitatively less than in endothelial cells (Figure 2). The reference mRNA of GAPDH also increased during endotoxemia at 5 h (Figure 2). Ethidium bromide staining was used to verify that there was equal loading of RNA on the gel (data not shown). These data indicate that the increase of GAPDH mRNA is indeed due to increased expression. For more accurate quantitative comparisons of changes in adhesion molecule mRNA levels during endotoxemia, the ratio between CAM mRNA and the reference mRNA of GAPDH of each sample was determined by densitometry for all livers or isolated liver cell fractions (Table 1). The results indicate a high baseline expression of ICAM-1 in endothelial cells relative to other liver cell types. Endothelial cells also respond with the highest increase of ICAM-1 and selectin mRNA during endotoxemia.

DISCUSSION

The objective of this paper was to investigate potential differential induction of CAM mRNAs in individual liver cells during endotoxemia. Regarding ICAM-1 mRNA, our results demonstrated at the whole liver level a low baseline expression, which seems to come mainly from endothelial cells and to a lesser degree from Kupffer cells but not from hepatocytes. Endotoxin was able to induce ICAM-1 mRNA

TABLE 1

DENSITOMETRIC ANALYSIS OF ICAM-1 AND SELECTIN mRNA EXPRESSION DURING ENDOTOXEMIA

	Control	1 h ET	5 h ET
A. ICAM-1			
Whole Liver	0.16 ± 0.01	0.47 ± 0.05*	0.66 ± 0.14*
Hepatocytes	0	0.35 ± 0.01*	0.24 ± 0.01*
Endothelial Cells	2.63 ± 0.27	11.38 ± 0.61*	0.78 ± 0.08*
Kupffer Cells	0.94 ± 0.53	4.29 ± 0.26*	0.54 ± 0.04
B. E-Selectin			
Endothelial Cells	0	4.72 ± 0.37*	0
Kupffer Cells	0	1.58 ± 0.46*	0
C. P-Selectin			
Endothelial Cells	0	2.28 ± 0.41*	0.13 ± 0.02
Kupffer Cells	0	0.74 ± 0.23*	0.06 ± 0.01

The ratio between ICAM-1 mRNA and the reference mRNA of GAPDH was determined for each sample by densitometry for all livers or isolated liver cell fractions. Untreated controls are compared with animals at different time points after injection of 0.5 mg/kg *Salmonella enteritidis* endotoxin (ET). Data represent means ± SE of n = 4 animals. *$P < 0.05$ (compared to control).

in all three cell types *in vivo*, however, the relative increase was most prominent in the endothelial cell fraction followed by Kupffer cells and hepatocytes. The detection of ICAM-1 mRNA is unlikely due to contamination of hepatocytes with other cell types. The parenchymal cell fraction consisted not only of >95% hepatocytes but there was also no evidence for selectin mRNA expression, which was very prominent in the other cell types. Furthermore, each non-parenchymal cell fraction was also highly pure (>95%); a cross-contamination of less than 5% can not explain the substantial increase found in both endothelial cells and Kupffer cells. Our results expand *in vitro* findings, where an upregulation of ICAM-1 expression was shown in isolated hepatocytes (32-34) and isolated sinusoidal endothelial cells (35) after *in vitro* stimulation with cytokines. At the whole organ level, TNF-α and IL-1 were identified as critical mediators of endotoxin-induced ICAM-1 mRNA upregulation (13). Immunohistochemistry also showed increased ICAM-1 expression on endothelial and Kupffer

cells and a new induction of ICAM-1 on hepatocytes during inflammatory liver diseases in humans (21-25) and in experimental models of hepatic ischemia-reperfusion injury (14). The importance of ICAM-1 expression in the liver was demonstrated by the beneficial effects of anti-ICAM-1 antibodies in endotoxin shock (13) and ischemia-reperfusion (14). The mechanism of protection may vary slightly between the models. In murine endotoxin shock, where no relevant endothelial cell injury occurs, the injury to hepatocytes depends on transendothelial migration of neutrophils and attack on the parenchymal cells (11). The anti-ICAM-1 antibody seems to protect because it almost totally prevented the ICAM-1-dependent extravasation of neutrophils (13). On the other hand, during hepatic ischemia-reperfusion injury, the initial injury phase is induced by activated Kupffer cells (36), which are responsible for extensive vascular injury (37). Under conditions where the endothelial cell layer is seriously damaged or even destroyed, an ICAM-1-dependent extravasation step may be less important for the injury than the ICAM-1 and Mac-1-dependent adhesion of neutrophils to the parenchymal cells (14). This suggests that induction of ICAM-1 on sinusoidal lining cells and on hepatocytes may be important under different circumstances.

Much less is known about the role of selectins in the pathogenesis of neutrophil-mediated hepatic injury. Hepatic selectin expression was described during inflammatory liver diseases in humans (21) and during sepsis in baboons (20). In rats, neither E- nor P-selectin mRNA was expressed in any liver cell under control conditions consistent with the reported absence of both selectin molecules on sinusoidal endothelial cells (39). However, endotoxin injection induced a drastic increase of both selectin mRNAs within an hour in endothelial cells and to a lesser degree in Kupffer cells. The capacity to induce E- and P-selectin gene transcription in endothelial cells confirms immunohistochemical detection of the proteins in liver tissue during sepsis (20), endotoxemia (39) and IL-1 injection (40). However, these reports did not distinguish between endothelial cells and Kupffer cells. Our findings clearly demonstrate also in Kupffer cells the capability to induce both selectin mRNAs. The selective response of sinusoidal lining cells to upregulate mRNA for selectins is consistent with their known function of being involved in neutrophil rolling (2-5), which is a prerequisite for firm adhesion of neutrophils and transendothelial migration. Neutrophil rolling has also been described in the hepatic vasculature during endotoxemia (41) and ischemia-reperfusion (42) suggesting a potential role for selectins in the pathogenesis.

In summary, our findings demonstrated the differential expression of CAM mRNA in hepatocytes and sinusoidal lining cells with ICAM-1 and selectin mRNAs induced in vascular cells (endothelial cells and Kupffer cells) and only ICAM-1 mRNA expressed in parenchymal cells during endotoxemia. These results are consistent with the involvement of selectins in neutrophil rolling in the vasculature and ICAM-1 in transendothelial migration and adherence to parenchymal cells. Our approach allows studies of the regulation of CAM gene expression in individual cell types of the liver *in vivo*.

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