Original Article

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Expression of Intercellular Adhesion Molecule-1 in the Livers of Rats Treated with Diethylnitrosamine

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Summary **It has been reported that levels of soluble intercellular adhesion molecule-1 (ICAM-1) in the blood are elevated in hepatocellular carcinoma patients. In the present study, serial observations of the localization of ICAM-1 in the liver were made by light and electron microscopy in rats with carcinogen-induced cancer. Male Fisher rats were given diethylnitrosamine (DEN) orally in their drinking water. Rats were sacrificed at 6, 8, 12, or 14 weeks after the start of DEN administration and the liver tissue was collected. ICAM-1 expression in liver was assessed using indirect immunoperoxidase staining with anti-rat ICAM-1 antibody. Although ICAM-1 expression by endothelial cells in livers of DEN-treated rats was lower than in the control group at 8 weeks, it was higher in the membrane and cytoplasm of hepatocytes. The expression of ICAM-1 in mesenchymal cells was decreased, paralleling development of cellular atypia, whereas in hepatocyte membranes and cytoplasm it was increased in these atypia. ICAM-1 was localized to the cytoplasm of cancer cells, but to the membrane of hepatocytes in the treated livers at 14 weeks. Furthermore, the levels of ICAM-1 in mesenchymal cells tended to be lower in the cancerous area than in the atypical hyperplastic nodule, and were reduced as the density of cell atypia increased, in comparison to cells in areas without cancerous nodules. We concluded that ICAM-1 may be influenced the development of cancer induced in the rat liver by a chemical carcinogen.**

Key Words**: intracellular adhesion molecule-1 (ICAM-1), rat, Diethylnitrosamine (DEN), cancer, immunoperoxidase staining**

Introduction

Intercellular adhesion molecule-1 (ICAM-1) is member of the immunoglobulin superfamily and a ligand for leukocyte function associated antigen 1 [1–3]. It has been reported already that levels of soluble(s) ICAM-1 in the blood correlate with the intensity of intrahepatic fibrosis in human C-viral chronic hepatitis (CH) or liver cirrhosis (LC) and are elevated in hepatocellular carcinoma (HCC) [4–6]. Recently, enhanced expression of ICAM-1 has been reported in human HCC and ICAM-1 is localized in the cell membrane and endoplasmic reticulum of the tumour cells [7, 8]. In particular, it has been reported that ICAM-1 promotes the growth of residual liver cells, improves delayed hepatic regeneration in disturbed livers, and inhibits the development of hepatic fibrogenesis or cirrhosis [9–11]. We have detected already sICAM-1 in blood and intrahepatic ICAM-1 in patients with C-viral CH, LC, and HCC [12]. In these studies, levels of sICAM-1 in blood were significantly higher in patients with HCC than in chronic hepatitis and LC patients. Furthermore, we reported that measurement of sICAM-1 levels in blood

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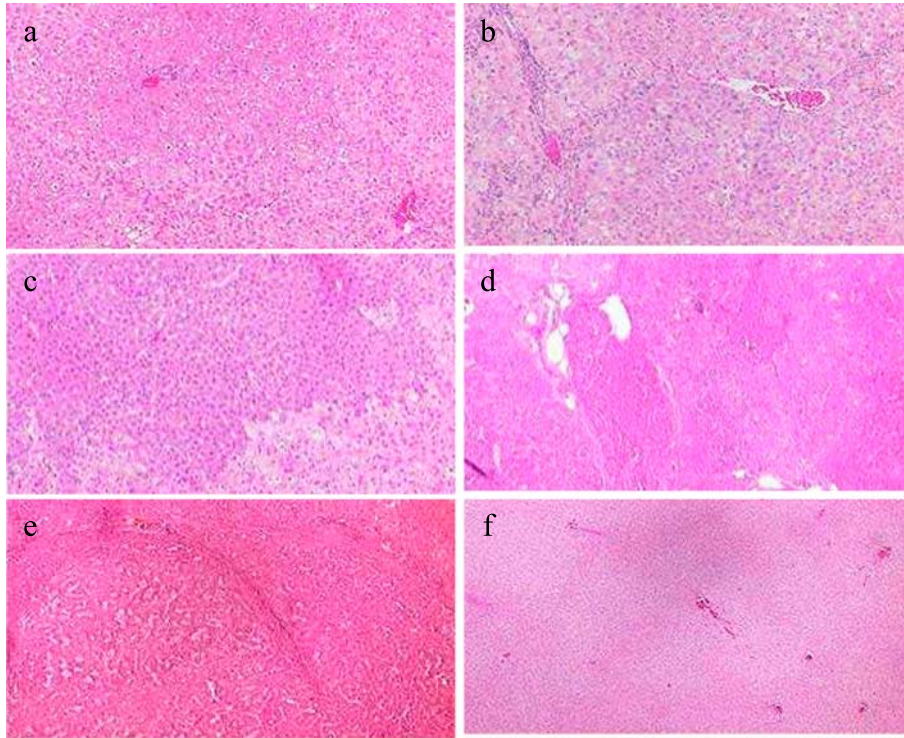E-mail: moriyama@med.nihon-u.ac.jp

Fig. 1.   Histological findings in the livers of DEN-treated and control rats. (a) Hepatocyte nuclei are irregular in size and changes in the staining of the cytoplasm are visible in some animals sacrificed at 6 weeks. (b) Distorted structure of hepatic lobules, and size variation of hepatocyte nuclei as well as a tendency to basophilic staining is increased further in some animals sacrificed at 6 weeks. (c) In some areas, groups of hepatocytes with strong atypia are present. (d) Marked nodulation observed in each animal sacrificed at 12 weeks and parts of the nodules are considered to be AH. (e) Small CA observed in all rats except for No. 13. (f) No histological changes in either control animal sacrificed at 14 weeks (Hematoxylin and eosin staining, original magnification: a, ×10; b, ×10; c, ×10; d, ×4; e, ×4; f, ×4).

was useful clinically for early diagnosis of HCC and decisions of efficacy of therapy in HCC patients, because ICAM-1 was expressed in the cancer cells of these patients. Therefore, in the present study, we made serial observations of the localization of ICAM-1 in liver by light and electron microscopy in rats with carcinogen-induced cancer. We sought to clarify whether ICAM-1 expression also are related to hepatocarcinogenesis in rat livers treated with a chemical carcinogen.

Materials and Methods

Animals

Male Wistar rats (200–225 g) were given Diethylnitrosamine (DEN; 100 mg/litle Sigma Chemical Co., St. Louis, MO), dissolved in their drinking water, and libitum for up to 14 weeks. Of the DEN-treated rats, 5 were sacrificed at 6 weeks (Rats No. 1 to No. 5), 4 at 8 weeks (Rats No. 6 to No. 9), 5 at 12 weeks (Rats No. 10 to No. 14), and 3 at 14 weeks (Rats No. 15 to No. 17). The control rats were given water without DEN, and one was sacrificed at each of weeks 6, 8,

12 and 14. These experimental animals were treated in accordance with the guidelines of Nihon University (1989).

Histological analysis of liver tissue

Livers were obtained following sacrifice and divided into two parts. One part was fixed in 10% buffered formalin and embedded in paraffin and the other used for the detection of ICAM-1. Each paraffin-embedded specimen was cut into 3 to 4 micrometer sections, which were stained with hematoxylin and eosin (HE).

Localization of ICAM-1 in liver

The liver tissue was frozen rapidly with dry ice and acetone, with OCT compound for embedding, and stored at −80°C until use. Four micrometer sections were fixed with acetone and subjected to two sessions of microwave treatment for 30 sec. After 20 min at room temperature, the ICAM-1 in liver was detected using an indirect immunoperoxidase staining method with polyclonal anti-ICAM-1 antibody (1:500 dilution) as the primary antibody and peroxidase-labeled anti-rabbit Immunoglobulin G (MBL,
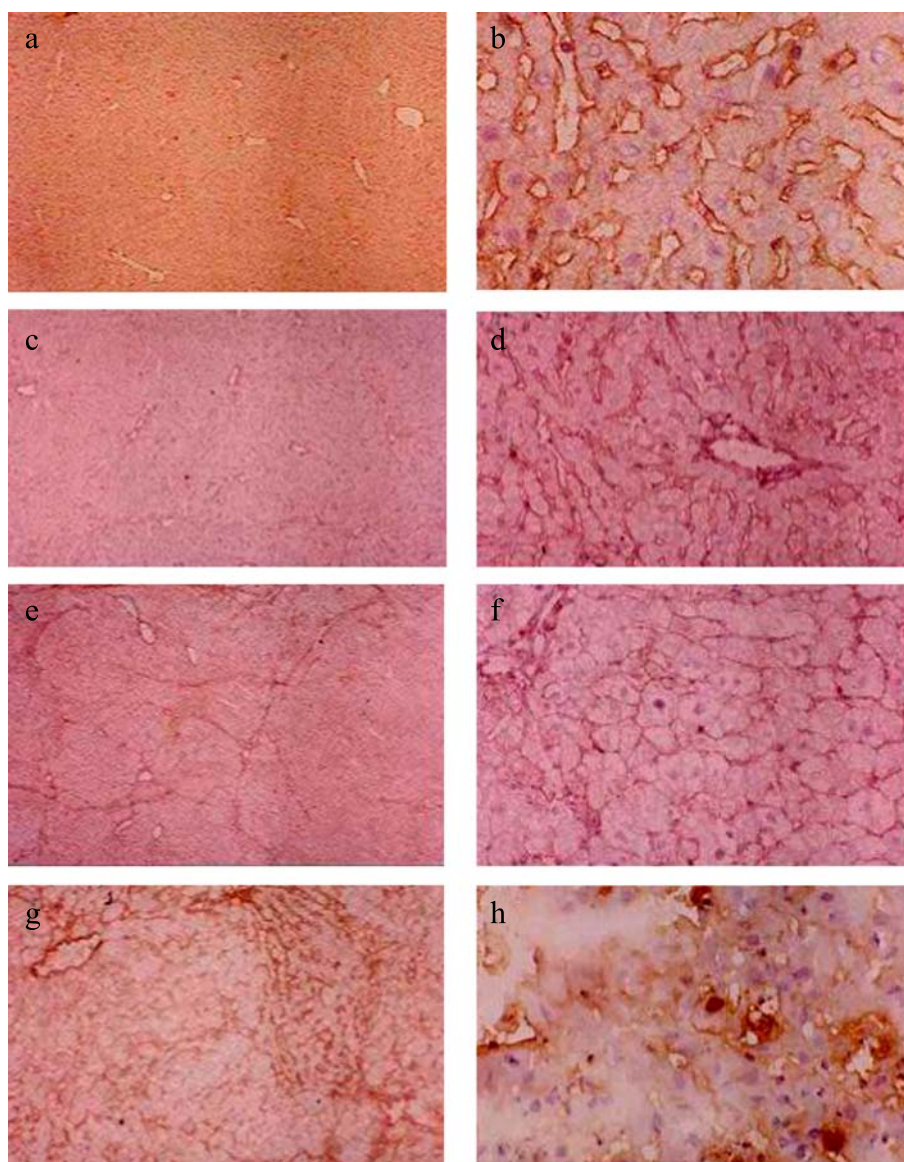
Fig. 2.   Localization of ICAM-1 in the livers of rats sacrificed at each time point. Intrahepatic ICAM-1 staining is diffuse and observed predominantly in the sinusoidal endothelial cells and hepatocyte membranes of the two control animals sacrificed at each time point (a and b). The intrahepatic ICAM-1 in DEN rats sacrificed at 6 weeks is expressed at moderate levels by endothelial cells and hepatocyte membranes without cellular atypia and less strongly in the membranes of hepatocytes with cellular atypia (c and d). The expression of intrahepatic ICAM-1 in DEN rats sacrificed at 8 weeks is moderate in the endothelial cells and hepatocyte membranes without cellular atypia, and slight to moderate with cellular atypia (e and f). (g) ICAM-1 expressed at higher levels in hepatocytes with strong atypia located with a map-like distribution than in areas without cellular atypia. (f) Intrahepatic ICAM-1 in the cancerous region expressed at low levels in the cancer cell membranes of rats sacrificed at 14 weeks (Counterstained by hematoxylin, original magnification: a, ×4; b, ×20; c, ×4; d, ×10; e, ×4; f, ×20; g, ×4; h, ×20).

Tokyo, Japan, 1:300 dilution) as the secondary antibody. This investigation was carried out in animals from the DEN administration and control groups at 6, 8, 12 and 14 weeks. The degree of ICAM-1 expression in liver was semiquantitatively classified into 5 grades (none, absent; slight, presence of only several positive cells in all lobules; mild, scattered positive cells in several lobules; moderate,

scattered positive cells in all lobules; marked, diffuse positive cells in all lobules).

For immunoelectron microscopy (IEM) after the 3,3'-diaminobenzidine (DAB, SIGMA, St. Louis) reaction, the tissues were incubated with 2.5% glutaraldehyde (NISSIN EM Co., Ltd., Tokyo, Japan) and 2% osmic acid (NISSIN EM Co., Ltd.) for 60 min at room temperature, and then

Table 1. Intrahepatic I CAM-1 expression in Irat livers with DEN Chemostimulated Carcinogenesis.

Rat No.		Mesenchymal Cells	Hepatocyte		Atypical hyperplasia		Cancer Cell	
			Membrane	Cytoplasm	Membrane	Cytoplasm	Membrane	Cytoplasm
6w	1	modrate	none	none	n.d	n.d	n.d	n.d
	2	moderate	none	none	n.d	n.d	n.d	n.d
	3	moderate	slight	none	n.d	n.d	n.d	n.d
	4	moderate	slight	none	n.d	n.d	n.d	n.d
	5 (ct)	marked	none	none	n.d	n.d	n.d	n.d
8w	6	moderate	none	none	n.d	n.d	n.d	n.d
	7	mild	mild	none	n.d	n.d	n.d	n.d
	8	mild	mild	none	n.d	n.d	n.d	n.d
	9 (ct)	marked	none	none	n.d	n.d	n.d	n.d
12w	10	moderate	moderate	none	slight	mild	mild	mild
	11	mild	moderate	none	mild	mild	mild	slight
	12	mild	moderate	none	mild	mild	slight	mild
	13	mild	mild	none	none	none	n.d	n.d
	14 (ct)	moderate	none	none	none	none	none	none
14w	15	mild	slight	none	slight	slight	slight	mild
	16	mild	slight	none	none	slight	mild	mild
	17 (ct)	moderate	none	none	none	none	none	none

ct: control, n.d: not detected

embedded in epon 812 (NISSIN EM Co., Ltd.) for 48 h at 60°C. Ultra thin section were obtained using an ULTRATOME III^R (LKB, Bromma, Sweeden) and observed on a JEM-100C electron microscope (JEOL, Tokyo, Japan).

Sequence analysis of ICAM-1 in the cancerous foci

Parts of the liver, apparently including cancerous regions, were taken from two rats (Nos. 15 and 16) sacrificed at 14 weeks. The nucleotide sequences of ICAM-1 DNA in the excised tissues were determined as described below, and compared to that reported previously [13]. DNA was extracted from 100 mg of frozen liver using DNAzol (Sanko Junyaku, Tokyo, Japan) according to the manufacturer's instructions. DNA amplification was carried out using nested PCR, generating a 1769 bp product. The sequences of the outer primer pair were 5'-CTGCTGCCTGCACTTT GCCCT-3' [ICAM–s1; sense, nucleotide position (nt) 1–21], and 5'-GTGTCCAGGTCACGAGTTCAC-3' (ICAM-a1; antisense, nt 1831–1851), and the sequences of the inner primer pair were 5'-ACCCGTGCCAGGCCCATGCTG-3' (ICAM-s2; sense, nt 39–60), and 5'-TTAGGCCTGAGG CTACAA GTA-3' (ICAM-a2; antisense, nt 1788–1808). The first round PCR reaction contained 1 µmol of each outer primer, 1U of Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany), 200 µl M of each of the four deoxy-nucleotides and 1x Taq buffer containing 1.5 mM MgCl$_2$, and was amplified for 35 cycles of 94°C for 40 s, 55°C for 40 s and 72°C for 150 s. The second round PCR amplified 3 µl of the first PCR product using 1 pico mole of each inner primer and the same buffer and cycling conditions as in the first round reaction. The second round PCR products were sequenced as described by Shibahara *et al.* [14] using an ABI model 310 DNA Sequencer.

Results

Histological findings

No cancer lesions were apparent in any animal sacrificed at 6 weeks. However, hepatocyte nuclei were irregular in size and changes in the staining of the cytoplasm were observed in some animals (Fig. 1a). No atypical hyperplastic areas (AH) and cancerous areas (CA) were found in the livers of DEN-treated rats sacrificed at 8 weeks. However, the structure of the hepatic lobules was distorted, and varia-tions in hepatocyte nuclear dimensions and the tendency to basophilic staining were increased further (Fig. 1b). Groups of hepatocytes with strong atypia were observed in some areas (Fig. 1c). A marked nodulation was observed in each animal sacrificed at 12 weeks and parts of the nodules were considered AH (Fig. 1d). In addition, small CA were observed in all rats, except No. 13 (Fig. 1e). There were no histological changes in any animals from the control group, sacrificed at 6, 8, 12 or 14 weeks (Fig. 1f).

Localization of ICAM-1 in the liver

ICAM-1 expression in the liver was diffuse and observed

predominantly in the sinusoidal endothelial cells and membranes of hepatocytes in the one animal of the control group sacrificed at each time point (Fig. 2a and b). ICAM-1 expression in DEN rat livers at 6 weeks was moderate in the endothelial cells and slight in the membranes of hepatocytes with atypia (Fig. 2c and d). However, the levels in both endothelial cells and hepatocyte membranes were lower than in control rats sacrificed at 6 weeks (Table 1). ICAM-1 expression in liver endothelial cells of DEN rats sacrificed at 8 weeks was slight to moderate in areas where the hepatocytes were without atypia (Fig. 2e), and only slight in areas with atypia (Fig. 2f). In particular, ICAM-1 expression was stronger in membranes of hepatocytes with strong atypia located in a map-like distribution area, than in hepatocytes without atypia (Fig. 2g). ICAM-1 expression in CA was observed in the cytoplasm and membranes of the cancer cells in rats sacrificed at 12 or 14 weeks (Fig. 2h). The expression of ICAM-1 by endothelial cells in CA was much lower than in what were considered to be normal areas of the same tissue. Moreover, ICAM-1 levels in endothelial cells tended to be lower in CA than in the AH areas and were further reduced as the density of atypical hepatocytes increased, as compared with hepatocytes in non-cancer areas. On the other hand, the expression of ICAM-1 in cell membranes and cytoplasm of cancer cells or hepatocytes with severe atypia was increased compared to hepatocytes without atypia in non-cancerous areas. In particular, the degree of expression of ICAM-1 in liver correlated with a map-like distribution or anisocytosis in irregular regeneration of hepatocytes [15–17].

Ultrastructural findings

Morphology of the tissue sections was studied by electron microscopy. ICAM-1 was detected in the membranes of mesenchymal cells and those hepatocytes with seemingly severe atypia (Fig. 3a). Furthermore, ICAM-1 was observed in the endoplasmic reticulum (ER) of cancer cells (Fig. 3b).

Sequence analysis

There were no coding changes in 3 clones isolated from rat no. 15 and another 3 from no. 16 compared to the consensus sequence [13].

Discussion

In rats with DEN-induced cancer, the pattern of expression of ICAM-1 in liver differed clearly between hepatocytes and endothelial cells in parenchyma without hepatocyte atypia and the cancerous area and hepatocytes within areas of atypia, even in the same liver. The level of endothelial cell ICAM-1 expression decreased as the degree of cellular atypia increased, whereas, in contrast, it was increased in hepatocyte membranes. In particular, the
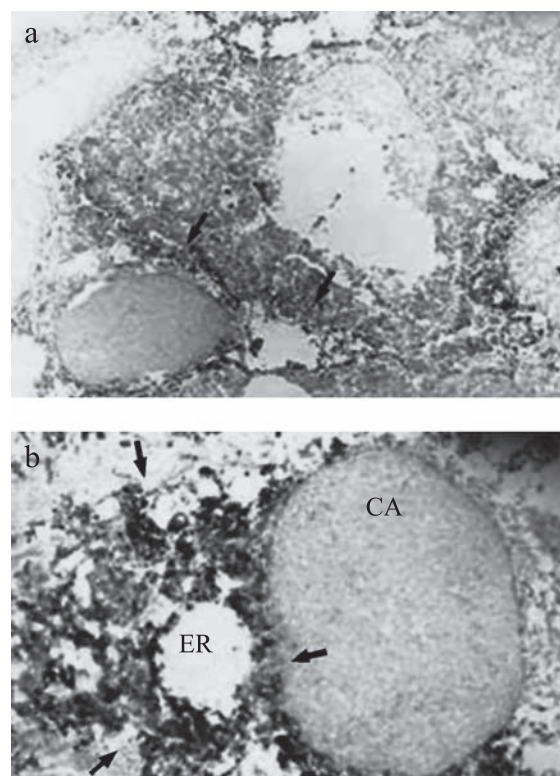

Fig. 3. (a) ICAM-1 detected by electron microscopy in the membranes of infiltrating cells, which seem to be endothelial cells, and in the membranes of hepatocytes (arrows). (b) ICAM-1 observed in the endplasmic reticulum (ER; arrows) of cancer cells (CA) (Ultra thin section EM: a, ×6,000; b, ×12,000).

expression of ICAM-1 with a map-like distribution of irregular regeneration of hepatocytes was higher than in other areas not showing this pattern. Furthermore, the expression of ICAM-1 in cell membranes and cytoplasm of cancer cells in the CA and hepatocytes in the AH areas was clearly greater than in areas lacking CA and AH. Thus, ICAM-1 density in cancer cells and hepatocytes with severe atypia was increased more than in areas without CA or severe hepatocyte atypia. Therefore, there is a clear distinction between areas with or without hepatocyte atypia and CA and AH. Furthermore, the cancer cells themselves produced the ICAM-1, because it was observed by IEM in their ER and in hepatocytes within areas of atypia. The role of ICAM-1 in hepato-carcinogenesis was not clear from the morphological observations above, but it was possible to distinguish areas with marked cellular atypia from normal areas. From these results, we consider that ICAM-1 influenced the development of hepatocyte atypia. Levels of sICAM-1 are increased parallel to development of F stages and HCC in type C liver diseases. In the present study, the expression of ICAM-1 in mesenchymal cells decreased as

cellular atypia increased. Reciprocally, it increased in the membrane and cytoplasm of hepatocytes, paralleling the development of cellular atypia. Furthermore, the ICAM-1 was present in both the cell membrane and cytoplasm of cancer cells. The degree of expression of ICAM-1 in mesenchymal cells in CA was clearly decreased compared to the non cancerous areas of the same rat liver. Therefore, the reason that sICAM-1 concentration in humans is reported to increase as F stages or HCC develop is suggested to be that ICAM-1 secretion by hepatocytes with atypia or by cancer cells is increased.

In addition, we analyzed the gene sequence of ICAM-1 from parts of livers including CA two different rats (Nos. 15 and 16) sacrificed at 14 weeks. The nucleotide sequences of ICAM-1 DNA from the excised tissues were determined and compared with that reported previously [13]. This analysis showed no coding changes in CA from rat liver relative to the consensus sequence. This indicated that ICAM-1 secreted by the cancer cells was homogeneous and the cells did not produce a variant molecule. Therefore, we consider that variant ICAM-1 does not participate in the progression of the liver fibrosis or hepatocarcinogenesis.

What are the factors which promote the expression of atypia of hepatocytes in liver? Some factors have been identified whose serum levels increase in parallel to the development of F stage in chronic hepatitis and liver cirrhosis and some factors are present at high levels in patients with HCC. Such factors include, ICAM-1 [4–6], human hepatocyte growth factor (HGF) [18, 19], vascular endothelial growth factor [20] and others. It is thought that these factors are produced and secreted by hepatoma cells. It has been confirmed that HGF is expressed by hepatocytes with strong atypia in our previous studies [21, 22]. In this study, we also confirm that strong expression of ICAM-1 is found in hepatocytes with strong atypia, decreased expression of ICAM-1 is shown in endothelial cells is shown in AH and small CA. Therefore, it is suggested that ICAM-1 is produced and secreted by these cells. Although it was not clear from our present study, we suggest that ICAM-1 influences the development of atypia of hepatocytes by one of the mechanisms mentioned above.

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