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TUMOR NECROSIS FACTOR α AND INTERLEUKIN-1β BUT NOT INTERFERON γ INDUCE VASCULAR CELL ADHESION MOLECULE-1 EXPRESSION ON PRIMARY CULTURED MURINE HEPATOCYTES

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Summary: Inflammatory cytokines such as tumor necrosis factor α (TNFα), interferon γ (IFN γ) and interleukin-1β (IL-1β) play important roles in the mechanisms of hepatitis. The effects of these cytokines on the expression of vascular cell adhesion molecule-1 (VCAM-1) in hepatocytes were examined. TNFα and IL-1β but not IFNγ or IL-6 induced VCAM-1 expression on primary cultured murine hepatocytes in a dose- and a time-dependent fashion. TNFα is significantly more effective than IL-1β on the induction of VCAM-1 expression. The results of RT-PCR demonstrate that these cytokines regulate VCAM-1 expression at mRNA level. These results suggest that TNFα and IL-1β participate in the pathogenesis of hepatitis via induction of VCAM-1 molecules on hepatocytes.

Hepatitis is a malignant inflammatory disease induced in the liver by various causes (virus infection, germ infection, alcohol, drug injury, etc.). Although there are many unsolved problems relative to the mechanisms, it is the case that hepatocytes are the major target cells damaged in hepatitis. However, it is still unclear what kind of molecules and what kind of regulation are involved in the interaction between hepatocytes and effector cells. There are some reports that T lymphocytes are involved in hepatitis, and histological observations indicate that lymphocytes bind to hepatocytes at the site of inflammation in the liver (1-4). The induction and maintenance of inflammation requires

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the adhesion of leukocytes to target cells. Even though the notion has been established that the interaction via adhesion molecules between lymphocytes and endothelial cells plays an important role in the onset of inflammation (5-7), little is known about the adhesion between hepatocytes and lymphocytes. Some pathological observations suggest that hepatocytes at inflammation sites express intercellular adhesion molecule-1 (ICAM-1) antigen (8-10). In the previous report, we demonstrated that the ICAM-1 expression on hepatocytes is upregulated by inflammatory cytokines (TNFα, IL-1α and IFN-γ) (11), some of which are often detected in the serum of patients with hepatitis (12), in the liver infected with bacteria (13) or in the experimental hepatitis (14, 15). Like ICAM-1, VCAM-1, a ligand for very late antigen-4 (VLA-4), is a member of immunoglobulin superfamily and is thought to participate in the infiltration of lymphocytes to site of inflammation (16). VCAM-1 expresses on activated endothelial cells (17), bone marrow stroma cells (18, 19), synoviocytes (20) and astrocytes (21). In some cases, VCAM-1/VLA4 interaction has been critically implicated in inflammation (22-25), allograft rejection (26, 27) and tumor metastasis (28). The recent report has suggested that VCAM-1 expression is also involved in liver diseases (29, 30). In this report, we demonstrate that TNFα and IL-1β but not IFN-γ induce VCAM-1 expression on primary cultured murine hepatocytes. The VCAM-1 expression is presumed to be a mechanism in the pathogenesis of hepatitis.

Experimental Procedures

Materials. Murine recombinant IFN-γ (mrIFN-γ) and TNFα were gifts from Genentech Inc. (South San Francisco, CA). Human recombinant HGF (hrHGF) was a gift from Snow Brand Milk Products Co. Ltd. (Tokyo, Japan). MrIL-1β and IL-6 were purchased from R & D Systems (Minneapolis, MN). Other reagents were purchased from Sigma (St. Louis, USA). The hybridoma, M/K2.7 (19), producing anti-murine VCAM-1 antibodies was obtained from American Type Culture Collection (ATCC) (Maryland, USA). Collagen type I for coating culture dishes was a gift from Kawasumi Laboratories Inc. (Tokyo, Japan). Female ICR (6-12wks) mice employed in the experiments of this study were purchased from Charles River Japan Inc. (Kanagawa, Japan). All animal experiments were conducted in accordance with local institutional guidelines for the care and use of laboratory animals.

Cell preparation. Parenchymal hepatocytes were isolated from an adult mouse by the modified in situ perfusion method (31, 32). Briefly, after the liver was perfused with

0.0125% collagenase solution, the liver was excised and dispersed in cold Hank's solution. Parenchymal hepatocytes were separated from nonparenchymal cells by differential centrifugation at 50 x g for 90 sec. Then, the dead parenchymal hepatocytes were removed by density gradient centrifugation on Percoll (Pharmacia). The live parenchymal hepatocytes in Williams'E medium containing 10% FCS, 20ng/ml EGF, 10^{-9}M insulin and antibiotics in RPMI1640 with 10% FCS were plated at a density of $2.7x10^5$ cells/well, $1.3x10^5$ cells/well and $3x10^4$ cells/well in flat-bottomed 6 well, 24 well and 96 well (respectively) plates (Sumitomo Bakelite Co.Ltd., Tokyo, Japan) pre-coated with collagen. The purity of the hepatocytes was confirmed by microscopic observation counting and flow cytometry analysis. Only when the purity was more than 98% were the isolated hepatocytes subjected to the following experiments. The hepatocytes were incubated at 37°C for 10 hr in order to adhere them on the collagen-coated plates and were washed before being subjected to the experiments. When the hepatocytes were treated with lymphokines, EGF and insulin were removed from the medium to eliminate the effect of potent interaction between lymphokines and these growth factors on hepatocytes.

Enzyme linked immunosorbent assay (ELISA). Stimulated hepatocytes were fixed with 0.125% glutaraldehyde-PBS for 3 min. After the glutaraldehyde solution in the wells was removed and the cells were washed with PBS containing 0.1% BSA and 30mg/ml glycine, these cells were incubated with 50ul/well of anti-mVCAM-1 antibodies solution (final concentration; 2.2ng/μl) for 1hr. at 4°C. After further washing, peroxidase-conjugated goat anti rat IgG (0.13ng/μl) was added to the wells and they were allowed to stand at room temperature for 1hr. Next the wells were washed 4 times with PBS and then, 100μl/well of 1ng/ml OPD (o-phenylenediamine dihydrochloride; GIBCO BRL) solution was added as a substrate. Fifty μl/well of 3N H_2SO_4 was added to stop the enzyme reaction after incubation at room temperature. Absorbance at 492nm was measured with a MTP-120 microplate reader (Corona Electric Co., Ltd., Ibaragi, Japan).

RT-PCR. Cytoplasmic RNA was extracted from stimulated or non-stimulated cells with RNA zol. (CINNA/BIOTEX Lab.Inc., Texas,USA) by following the manufacturer's instructions and the cDNA was synthesized with 50mM Tris-HCl (pH8.3), 75mM KCl, 3mM $MgCl_2$, 10mM DTT, 10μg/ml oligo $(dT)_{12-18}$ and 0.5mM dNTPs using Moloney murine leukemia virus reverse transcriptase (BRL, Gaithersburg, MD) and incubation at 37°C for 1hr. The polymerase chain reaction (PCR) was conducted by the addition of 80ul of PCR reaction mixture (12.5mM of Tris-HCl, pH 8.3, 62.5mM KCl, 1.88mM $MgCl_2$, and 0.0013% gelatin) containing 50pmol of primer 1 (5'CTCTGTACA TCCCTCCACA), 50pmol of primer 2 (5'GGGACTGTGCAGTTGACAG) and 0.3 unit of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) with 25 thermal cycles, 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min. The primers were designed according to the reported mouse VCAM-1 cDNA sequence (33) Twenty microliters of the PCR products was analyzed using 2% Nusive agarose gel electrophoresis.

Results and Discussion

Primary cultured mouse hepatocytes were stimulated with various cytokines, and the VCAM-1 expression on these hepatocytes was quantified by ELISA as previously described. As shown in Figure 1, the VCAM-1 expression on stimulated hepatocytes was significantly increased when cells were treated with TNFα or IL-1β. On the other hand, either IFN-γ or IL-6 did not increase the expression at the concentration employed in the study although these cytokines induce several metabolic actions in the hepatocytes, for example, the expression of ICAM-1 (11) and C-reactive protein (34), respectively. Other factors such as the growth factors (HGF, EGF and bFGF) did not induce the expression (Fig. 1). It is interesting that the cytokines inducing the VCAM-1 expression on hepatocytes are the same as those inducing the expression on endothelial cells including the incompetence of IFN-γ (23). Taking into consideration the fact that the hepatocytes are developmentally derived from gut epithelial cells (35), hepatocytes appear to keep the ancestral characteristics after terminal differentiation. The VCAM-1 expression began to increase by only 0.1 ng/ml of TNFα and reached a plateau at 10 ng/ml (Fig. 2A) Compared to TNFα, the activity of IL-1β to induce the VCAM-1 expression is relatively weak. A time-course analysis indicates that the VCAM-1 expression on TNFα or IL-1β stimulated hepatocytes reached a peak at about 6-12 hrs after stimulation and lasted for at

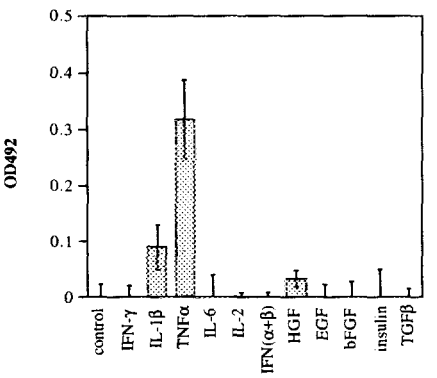

Figure 1. Effect of various cytokines on VCAM-1 expression of primary cultured hepatocytes.

Isolated hepatocytes were cultured with IFN-γ (100U/ml), IL-1β (100ng/ml), TNFα (100ng/ml), IL-6 (100ng/ml), IL-2 (100ng/ml), IFN-α/β (100U/ml), HGF (100ng/ml), EGF (100ng/ml), bFGF (100ng/ml), insulin (10^{-6}M) or TGFβ (10^{-10}M) for 24 hrs. The VCAM-1 expression was then measured by ELISA as described in the Experimental Procedures.

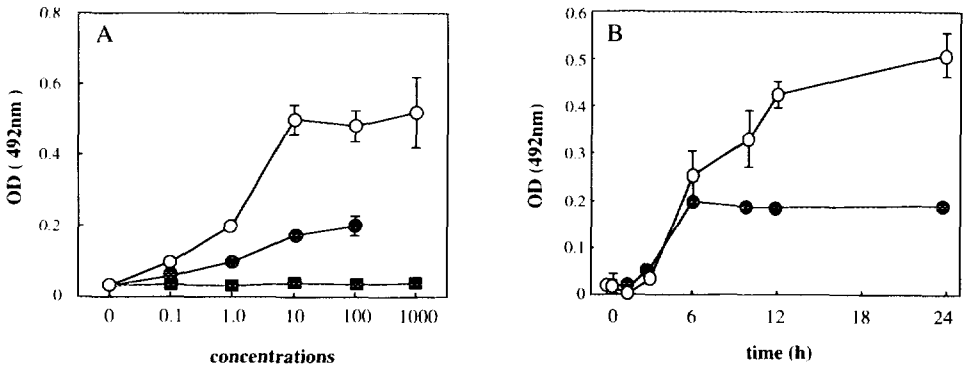

Figure 2. Analysis of VCAM-1 expression on primary cultured hepatocytes.

(A) VCAM-1 expression on hepatocytes is dependent on the concentration of the cytokines. Isolated mouse hepatocytes were cultured with TNFα (O-O), IL-1β (●-●) or IFN-γ (■-■) at various concentrations for 24 hrs. The VCAM-1 expression was then measured by ELISA. (B) Kinetics of the VCAM-1 expression induced by TNFα or IL-1β on hepatocytes. Isolated hepatocytes were cultured with TNFα (100ng/ml) (O-O) or IL-1β (100ng/ml) (●-●) for various periods. The VCAM-1 expression was measured each time by ELISA.

least 24 hrs (Fig. 2B). Furthermore, RT-PCR was performed to examine the mechanism of these cytokines to upregulate the VCAM-1 expression. Figure 3 shows that both TNFα and IL-1β induced mRNA for VCAM-1 in hepatocytes while the mRNA was only slightly detected in the control and IFN-γ treated samples. In this case, TNFα induced VCAM-1 mRNA more than IL-1β. The RNAs for β-actin were almost constant in all samples. These results by RT-PCR are consistent with those by ELISA. Therefore, the protein expression of VCAM-1 is considered to be regulated at the mRNA level. The expression of VCAM-1 is regulated by NF-κB (36, 37) and the cell specificity is

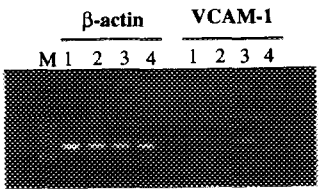

Figure 3. Cytokines induce VCAM-1 mRNA expression.

The RNAs from cytokine-treated hepatocytes were analyzed by RT-PCR with primers for β-actin or VCAM-1 as described in the Experimental Procedures. Lane 1: control, 2: IFN-γ (100U/ml)-treated, 3: TNFα (100ng/ml)-treated, 4: IL-1β (100ng/ml)-treated.

dependent on the specific combination of NF-κB subunits (38). Because hepatocytes are responsive to the same cytokines (TNFα and IL-1β, but not IFN-γ) as endothelial cells are in the VCAM-1 expression, hepatocytes would have the same specific combination of NF-κB subunits as endothelial cells although this needs to be examined.

Both ICAM-1 and VCAM-1 antigens are involved in the lymphocyte invasion into inflammatory tissues although which antigen is more important for the function is still controversial. However, both antigen expressions seem to be the sufficient condition for lymphocytes infiltration (16, 25, 39). In this report and in our previous study (11), we demonstrated that hepatocytes express both ICAM-1 and VCAM-1 antigens by inflammatory cytokines. Therefore, in conclusion, localized inflammatory cytokines would induce ICAM-1 and VCAM-1 antigens, and then these antigens trigger lymphocytes such as T cells and monocytes to infiltrate into the internal parenchymal tissues. This mechanism is considered to play an important role in the pathogenesis of hepatitis.

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