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INCREASED RELEASE OF KC/GRO PROTEIN, INTERCRINE CYTOKINE FAMILY, FROM HEPATOCYTES OF THE CHRONICALLY ETHANOL FED RATS

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SUMMARY: In an attempt to clarify the mechanisms of neutrophil-accumulation in the liver of alcoholics, release of KC/gro protein (intercrine cytokine family) from hepatocytes in the chronically ethanol fed rats was examined. Chemotactic activity for rat neutrophils was demonstrated in the culture supernatant of hepatocytes isolated from the ethanol fed rats, which was then inhibited in the presence of antibody against rat KC/gro protein. Immunoblot analysis revealed that the peptide reacted with antiserum against KC/gro protein was demonstrated at a molecular weight of 20-23kDa, and an amount of KC/gro protein released from the hepatocytes of the chronically ethanol fed rats was increased, as compared with that of the control rats.

Alcoholic liver injury is characterized by hepatocyte necrosis with neutrophil-infiltration (1-5). Neutrophil accumulation at inflammatory sites is known to be mediated by several kinds of chemotactic factors produced at infalmmatory sites, such as C_{5a} and leukotriene B_4 (6,7). Recent studies revealed that hepatocytes, Kupffer cells or monocytes release chemotactic factors for neutrophils (8-12). Furthermore, several kinds of cytokines such as IL-1, IL-6 and TNF-a are increased in sera of patients with alcoholic hepatitis (14-17), and correlated with clinical activities of liver disease (15,16). Recently, IL-8 concentration in the circulation is found to be increased in these patients (17,18). IL-8 is known to be one of the strong chemotactic cytokines (19-21). In rodents, cytokine-induced neutrophil chemoattractant was purified from the rat kidney epithelioid cell line, NRK-52E (22), and it relates to the KC/gro protein (IL-8 related protein), one of intercrine cytokine family (23). These cytokines are characteristically base, heparin-binding polypeptides (20).

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In the previous study, we have demonstrated a 20-25kDa chemotactic factor released from hepatocytes of the chronically ethanol fed rats (24), and neutrophils migrate toward concentration gradients of the chemotactic factors (24). In the present study, we have analyzed the release of KC/gro protein from the hepatocytes of the chronically ethanol fed rats.

MATERIALS AND METHODS

Experimental animals; Sprague-Dawley rats at the age of 4 week-old (Doken, Ibaraki, Japan), were divided into two groups. The animals were housed in individual cages and fed regular liquid diets containing 5% ethanol (Oriental Yeast Ind., Tokyo) or control liquid diets (Oriental Yeast Ind., Tokyo) according to the regime of Lieber and DeCarli (25) for 8 weeks. Although carbohydrate in the ethanol diet was isocalorically replaced by ethanol to the extent of 36% of the total calories, over all composition of the ethanol and control diets was identical.

Isolation and culture of hepatocytes; Hepatocytes were isolated from the rat liver according to the method of Seglen (26). In brief, the liver was perfused with Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY) containing 0.05% collagenase (Collagenase type IV; Sigma, St. Louis, Mo). After washing the cells with HBSS by centrifugation at 50 x g for 5 min three times, the isolated hepatocytes were were suspended in William's medium E (Gibco, Grand Island, NY) supplemented with or without 10% fetal calf serum (FCS; Gibco, Grand Island, NY) at a cell density of $1x10^5$ cells/ml. Ten ml of hepatocytes were placed in the 100mm culture dish (Corning, Iwaki Glass, Tokyo) coated with collagen type I (Sigma, St. Louis, Mo) and incubated at 37°C in 5% CO_2 humidified atmosphere. After 24hr in culture, culture supernatant was then collected and centrifugated at 2000 x g for 10 min to remove debris of the cells.

Chemotactic activity of culture supernatant; After collecting blood from the inferior vena cava, rat neutrophils were prepared by the dextran sedimentation technique (27), followed by centrifugation over ficoll-conray gradient (28). The islolated neutrophils were suspended in William's medium E at a cell density of $2x10^6$ cells /ml.

Chemotactic activity of the conditioned medium was measured using chemotactic chambers (Sanki Co., Kyoto, Japan) with a 5um membrane filter (Nucleopore, Pleasanton, CA) according to the method of Onozaki et al (29). Briefly, conditioned medium was placed into the lower well of chemotactic chamber, and rat neutrophils ($2x10^6$ cells/ml) were placed on the upper compartment. Chemotactic chambers were then incubated at 37°C for 3hr, and the number of neutrophils migrating through a filter and situated on the bottom glass of the lower compartment was counted using an inverted microscope.

Effect of antibody against KC/gro protein on chemotaxis of neutrophils; Chemotactic activity of the conditioned medium was measured in the presence of antibody against rat KC/gro protein. Briefly, conditioned medium was mixed with various concentration of antibody against rat KC/gro protein (Peptide Institute, Osaka, Japan), and placed into the lower compartment of chemotactic chamber. The number of neutrophils migrating into the lower well was counted as described above, and compared with that in the absence of antibody.

Immunoblot analysis; Immunoblot analysis was performed using 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Conditioned medium of hepatocytes (7.5 ul) was applied on SDS- polyacrylamide gels, which were then elctrophoresed according to Laemmli (30). Peptides were transferred from the gels to nitrocellulose membrane sheets (Immunobilon^{TM}-P, Millipore Japan, Yonezawa, Yamagata, Japan). After incubation at 37°C with blocking solution (3% bovine albumin (Sigma, St. Louis, Mo) in PBS), the sheets were incubated with antibody against rat KC/gro protein at a dilution of 1:2,000. After washing, the sheets were incubated with biotin labeled antibody against rabbit immunoglobulin (Amersham Japan, Tokyo, Japan). The protein reacted with the antibody was then stained using a Amersham Blotting detection kit (Amersham Japan, Tokyo, Japan).

Statistics; Each data represents mean±SD. Statistical analysis was performed student's t test.

RESULTS

Chemotactic activity of the hepatocyte-conditioned medium;

When hepatocytes were cultured in the presence of FCS, the number of migrating neutrophils by stimulation with the conditioned medium of the hepatocytes isolated from the chronically ethanol fed rats was markedly enhanced, as compared with that of the control rats (Fig. 1). In contrast, when hepatocytes were cultured in the absence of FCS, chemotaxis of neutrophils was demonstrated by stimulation with the conditioned medium of hepatocytes isolated from the control rats as well as the chronically ethanol fed rats (Fig. 1).

Enhanced chemotactic activities of the conditioned media were almost completely abolished in the presence of antibody against rat KC/gro protein (Fig. 2).

Immunoblot analysis; As indicated in Fig. 3, the protein reacted with antibody against rat KC/gro protein was demonstrated at a molecular weight of 20-23 kDa. When hepatocytes were cultured in the presence of FCS, an amount of KC/gro protein in culture supernatant of the chronically ethanol fed rats was enhanced, as compared with that of control rats (Fig. 3-a). On the other hand, when the cells were cultured in the absence of FCS, moderate amount of KC/gro protein was demonstrated in the culture supernatant of the chronically ethanol fed rats as well as the paired control rats (Fig. 3-b).

DISCUSSION

Histological feature of alcoholic hepatitis is characterized by neutrophil-infiltration surrounding hepatocytes containing alcoholic hyalin (Mallory bodies) (1-5). Although neutrophils are reported to contribute to tissue injury in several experimental tissue damage (31), mechanisms of neutrophil accumulation into the

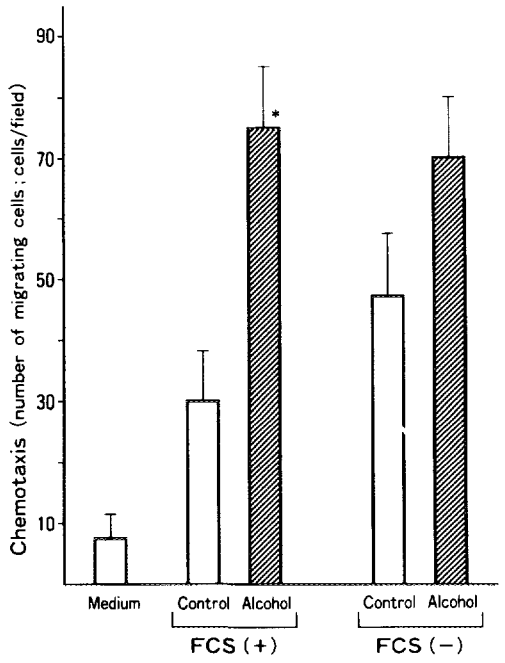

Figure 1. Chemotactic activity of the conditioned medium of hepatocytes cultured in the presence or absence of fetal calf serum

(a) When the cells were cultured in the presence of FCS, an enhanced chemotactic activity was demonstrated in the conditioned medium of chronically ethanol fed rats, as compared with that of control rats (*$p<0.01$). However, when the cells were cultured in the absence of FCS, chemotactic activity of the conditioned medium was increased in control rats as well as in the chronically ethanol fed rats.

liver and role of these infiltrating cells in alcoholic liver injury has not been clearly elucidated yet. Recently, to clarify the role of neutrophil accumulation into the liver, several chemoattractants have been proposed, such as monocyte-derived chemotactic factor when exposed to mallory bodies (13), leukotriene B4 (9), lipid peroxidation products that are metabolized from ethanol (11-12). Recently, IL-8 concentration in the circulation is found to be increased in patients with alcoholic hepatitis (17, 18). IL-8 is one of strong chemotactic cytokines (19-21), and is reported to be produced from hepatocytes (32,33).

In the present study, when the isolated hepatocytes were cultured in the presence of FCS, chemotactic activity of the conditioned medium of hepatocytes isolated from the ethanol fed rats was enhanced as compared with that of control rats. In contrast, when the cells were cultured in the absence of FCS, the chemotactic activity was demonstrated in the conditioned medium of control hepatocytes as well as the hepatocytes isolated from the ethanol fed rats. These enhanced chemotactic activities of the conditioned media were reduced by the antibody against rat KC/gro protein.

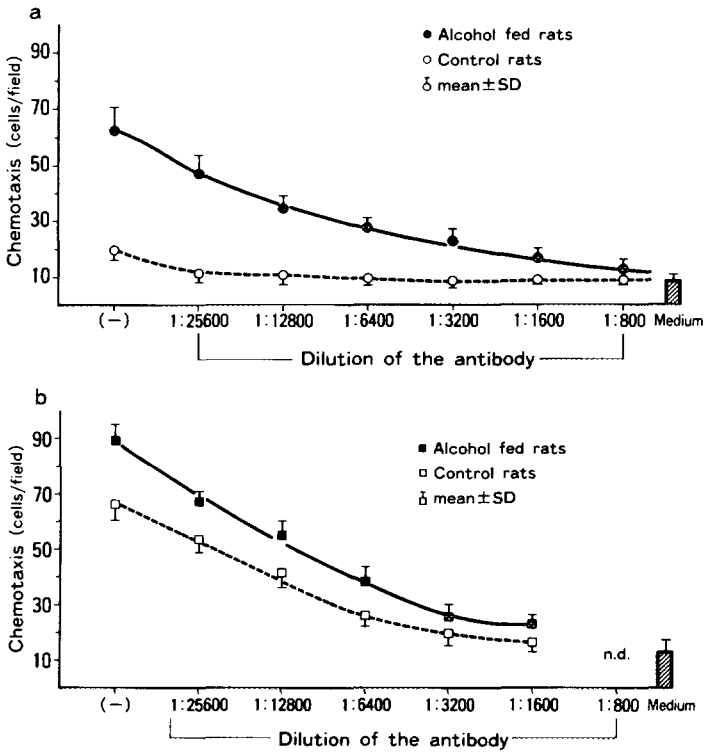

Figure 2. Effect of the antibody against KC/gro protein on the chemotactic activity of conditioned medium

Hepatocytes isolated from the chronically ethanol fed rats or the paired control rats were cultured in the presence of FCS (a) or in the absence of FCS (b). Enhanced chemotactic activities of the conditioned medium were reduced by the antibody against rat KC/gro protein.

Immunoblot analysis revealed that the peptide reacted with the antibody against KC/gro protein was demonstrated at a molecular weight of 20-23 kDa. This data could be in consistence with the fact that dimetric form of IL-8 related cytokines exert their biological activity(19). This peptide could be related to a 20-25kDa chemotactic factor produced by the hepatocytes reported previously (24). When the hepatocytes were cultured in the presence of FCS, an amount of KC/gro protein released from hepatocytes of the chronically ethanol fed rats was enhanced as compared with that of the paired control rats. In contrast, when the cells were cultured in the absence of FCS, moderate amount of KC/gro protein was found to be released from the hepatocytes of control rats as well as the chronically ethanol fed rats. As hepatocytes cultured in the absence of FCS are known to be in critical conditions for maitenance of the cells, this condition could be different from the condition of the cells in vivo. The cells cultured in the presence of FCS could be more resemble to the cells in vivo, as compared with those cultured in the absence of FCS.

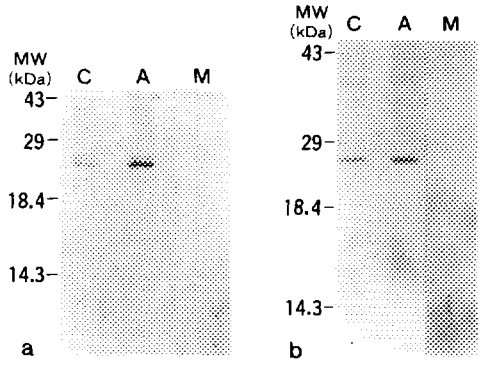

Figure 3. Immunoblot analysis of the hepatocyte-conditioned medium
(a) When hepatocytes were cultured in the presence of FCS, an amount of the protein reacted with the antibody against rat KC/gro protein was increased in the hepatocyte-conditioned medium of the ethanol fed rats, as compared with that of control rats. (b) In contrast, when the cells were cultured in the absence of FCS, an amount of KC/gro protein in the conditioned medium was almost identical in control rats and the chronically ethanol fed rats.
(A; alcohol fed rat, C; control rat, M; medium)

These results suggest that an enhanced release of KC/gro protein from the hepatocytes of the chronically ethanol fed rats could induce chemotaxis of neutrophil in the liver of patients with alcoholics, as KC/gro protein is a strong chemotactic cytokine. Thus, an inhibition of this cytokine released from hepatocytes or neutralization of its activity by selective antibody might be effective to prevent accumulation of neutrophil in the liver, and might prevent against alcoholic hepatitis.

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