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—Original Article—

LIPID PEROXIDE FORMATION IN ISOLATED HEPATOCYTES BY CYTOTOXIC FACTORS PRODUCED FROM LYMPHOKINE-ACTIVATED MACROPHAGES

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Summary

When peripheral blood lymphocytes from patients with chronic active hepatitis were stimulated with liver specific lipoprotein (LSP), considerably higher frequencies of lymphocyte transformation and MIF production were induced. Peritoneal macrophages from guinea pigs were activated by lymphokine-containing lymphocyte culture supernatant and produced a cytotoxic (or cytostatic) factor acting on isolated hepatocytes in culture. The cytotoxic (or cytostatic) factor, which was fractionated by Sephadex G-75 column gel filtration followed by DEAE-cellulose column chromatography, had cytotoxic effect on isolated liver cells and produced a significant amount of lipid peroxide. These results suggested the possibility that the cytotoxic effects may be caused at least partially by the lipid peroxid formation.

Key Words: *macrophage-mediated cytotoxicity, cytotoxic factor, lipid peroxide, liver specific lipoprotein, monokine.*

Introduction

It is becoming an accepted idea that autoimmune reactions induced by liver specific lipoprotein (LSP) may play an important role in the pathogenesis of chronic active hepatitis. Although diverse possibilities such as antibody-dependent cell-mediated cytotoxicity, killer T cell-mediated cytotoxicity, antigen-antibody complexes and participation of lymphokines have been proposed in attempts to interpret the immunopathogenesis of liver injury in chronic active hepatitis, none of them permits a clear interpretation of how such immunological mechanisms result in the liver injuries. We have previously reported that activated macrophages may have cytotoxic or cytostatic effects on isolated hepatocytes via effector cell-to-target cell contact[1] or through the production of monokines acting on the hepatocytes[2]. Since lipid peroxidation as a mechanism for liver injury has been reported, particularly in the case of carbon tetrachloride poisoning[3], we investigated whether lipid peroxide is formed in isolated hepatocytes due to cytotoxic factor produced by lymphokine-activated macrophages.

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Materials and Methods

1. *Lymphocytes.* Lymphocytes were obtained from patients with chronic active hepatitis. All of them showed positive lymphocyte transformation by stimulation with LSP, providing evidence of sensitization to LSP.

2. *Preparation of LSP.* Subcellular components of rat liver cells were fractionated according to the method of Meyer zum Büschenfelde[4]. The purification of LSP consisted essentially of a series of gel chromatography columns through which the supernatant of rat liver homogenate was passed. The membrane lipoprotein appearing in the void volume on chromatography over Sepharose 6B was shown to contain LSP.

3. *Preparation of peritoneal exudate cell suspension.* To obtain peritoneal exudate cells (PEC) which mainly consist of macrophages, 20 ml of sterilized mineral oil (Marcol 52, Esso Oil Co.) was injected into the peritoneal cavity of normal guinea pigs. PEC was collected 4 days after oil injection by perfusing the peritoneal cavity with 200 ml of Hanks solution. After the oil phase was decanted, the aqueous phase was centrifuged at 800×g for 10 min at 4°C. The pellets were then washed three times with fresh Hanks solution and suspended in Eagle MEM solution containing 10% fetal calf serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin to make a cell suspension of 5×10^7 cells/ml. The cells mostly consisted of macrophages with a few contaminating lymphocytes and granulocytes.

4. *Assay of macrophage activation.* The peripheral blood lymphocytes were cultured in the presence of LSP at 37°C for 48 hours and the culture supernatant containing the macrophage activating factor (MAF) was added to the PEC prepared from normal guinea pigs as described above. The macrophage activating factor contained in the culture supernatant of activated lymphocytes was estimated by measuring the uptake of [^{3}H]-glucosamine into PEC according to the method of Hammond et al.[5]; the culture supernatant of activated macrophages was added to the PEC and [^{3}H]-glucosamine (1 μCi, specific activity: 20 Ci/mol) was added to the culture after incubation at 37°C for 72 hours. After incubation at 37°C for another 6 hours, 3 ml of phosphate buffered saline was added to each culture and cells were collected on a millipore filter membrane by suction after gentle shaking. The membrane was then washed by 10 ml of ice-cold 5% trichloroacetic acid solution and radioactivity retained on the filter membrane was counted by liquid scintillation spectrometry as described previously[1,2]. As a control the same experiments were performed without adding LSP to determine the stimulation index. Based on analyses of many normal subjects, we judged that positive activation of macrophages occurred when the stimulation index was greater than 180%.

5. *Assay of macrophage-mediated cytotoxicity.* To demonstrate that MAF-activating macrophages are capable of exerting cytotoxic or cytostatic action on liver cells, we chose guinea pig hepatocytes as the target cells because these were readily available. Isolated liver cells were prepared from a normal guinea pig according to the method of Bellemann et al.[6]. Culture supernatants containing MAF were prepared by stimulating lymphocytes from patients with chronic active hepatitis as described above. These were added to PEC taken from a normal guinea pig, followed by incubation at 37°C for 6 hours. After incubation, cells were separated, washed with fresh Eagle MEM solution and resuspended in the same medium to make a cell suspension of 5×10^6 cells/ml. After PEC were incubated for another 24 hours, the culture supernatant was separated and 0.5 ml of the culture superna-

tant or its fractionated sample prepared from activated macrophages (5×10^6) was added to the same volume of the isolated hepatocyte suspension (5×10^6 cells/ml). After incubation at 37°C for 24 hours, 1 μCi of [^{3}H]-L-ornithine (specific activity: 9 Ci/mmol) was added to the cell suspension and incubation was continued for another 24 hours. After culture supernatant was separated by centrifugation, anti-guinea pig serum albumin prepared from a sensitized rabbit was added with carrier albumin to make a coprecipitate with the nascent labeled albumin. The radioactivity contained in the immunoprecipitate was then assayed by liquid scintilation sepectrometry. Exactly the same experiments were carried out using the lymphocyte culture supernatant prepared from normal individuals as a control. The inhibition of albumin synthesis by activated macrophages in patients was calculated by a comparison with the control value of albumin synthesis in normal individuals. Based on control experiments in many normal individuals, we judged that the macrophage-mediated cytotoxicity is positive when albumin synthesis is less than 80% of the average value in normal controls.

6. *Fractionation of the culture supernatant of activated macrophages.* The culture supernatant of MAF-activated macrophages was fractionated by gel filtration using a Sephadex G-75 column (5 × 150 cm) preequilibrated with phosphate buffer (0.02 M, pH 7.2) containing 0.1 M NaCl. Elution was carried out with the same buffer solution and absorbancy at 280 nm of each 10 ml eluate was estimated. After illustrating the protein elution profile as shown in **Fig. 1**, eluates were separated into six portions as indicated, and these were condensed separately to the starting volume by ultrafiltration using a diafilter membrane (G-10T, Bioengineering Co., Tokyo) for assay of cytotoxic activity. Since the activity was found predominantly in the fourth fraction as shown in **Fig. 1**[12], this was further fractionated by DEAE-cellulose column; the condensed material was applied to a column (1 × 5 cm) of DEAE-cellulose (Whatman DE 23 cellulose) equilibrated with Tris-HCl buffer (0.02 M, pH 7.2) containing 0.1 M NaCl. After rinsing out the column with the same buffer, elution was performed by changing the solution pH to 8.0 and 8.5 in a stepwise manner and finally to the same buffer (pH 8.5) containing 0.5 M NaCl. Each 3 ml of eluate was collected and elution pattern was illustrated by estimating the absorbancy at 280 nm. These fractionated materials were divided into 5 portions as indicated in **Fig. 2a**.

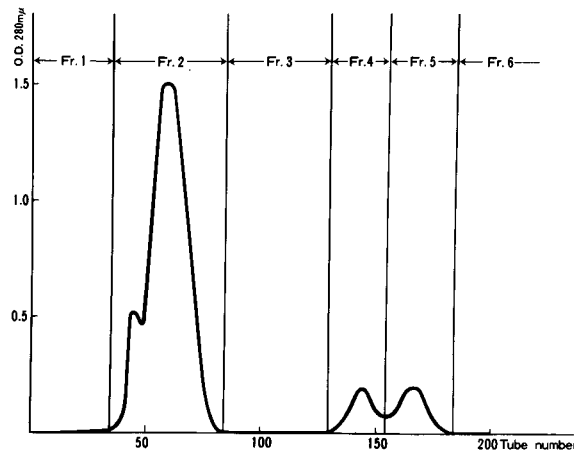

Fig. 1. Sephadex G-75 filtration of the supernatant of activated macrophages.

7. *Assay of lipid peroxide formation in isolated hepatocytes.* Lipid peroxide formation in isolated hepatocytes was assayed according to the method of Yagi et al.[7]. The fractionated materials of MAF-activated macrophage culture supernatant were condensed and each 0.1 ml was added to 0.5 ml of the isolated hepatocyte suspension (2×10^6 cells/ml). These were diluted to a total volume of 1.0 ml by adding Eagle MEM solution containing 10% fetal calf serum. After incubation at 37°C for 2 hours, cells were recovered by centrifugation, washed with Eagle MEM solution and resus-

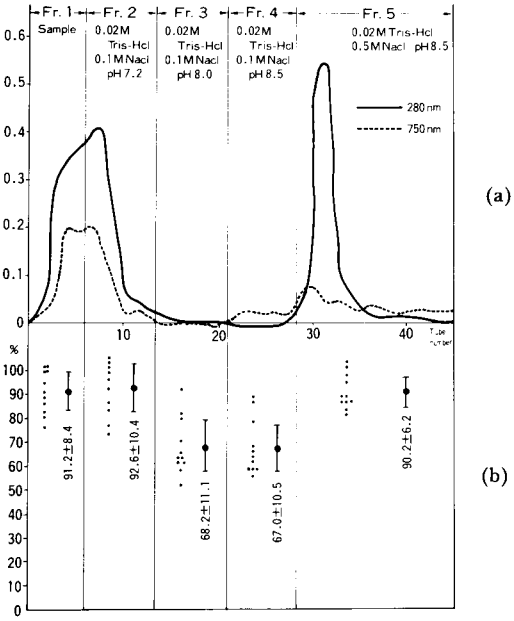

Fig. 2. a) (above) DEAE-cellulose column filtration of the supernatant of activated macrophages.
b) (below) Cytotoxicity of fractionation of the culture supernatant of activated macrophages on isolated hepatocytes.

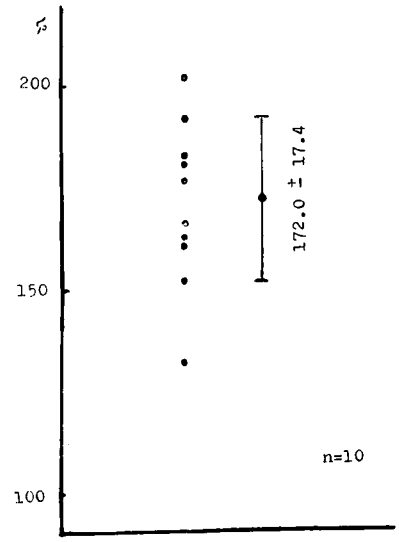

Fig. 3. Lipid peroxides accumulate in the isolated hepatocytes treated with the fourth fractionated culture fluid by gel filtration using a Sephadex G-75 column chromatography. As a control, the same experiments were carried out using the lymphocyte culture supernatant prepared from normal individuals.

pended in 1.0 ml of phosphate buffer (pH 7.4). The cells were then lysed by freezing-thawing and treated with 0.2 ml of 7% sodium dodecyl sulfate solution at room temperature for 10 min to solubilize the cell debris. 2.0 ml of 1/12 N sulfuric acid and 1.0 ml of 10% phosphotungstic acid were added to the lysate and kept standing at room temperature for 5 min. Finally, 1.0 ml of 5% thiobarbituric acid was added and heated at 95°C for 45 min. After cooling, the resulting colored substance was extracted with 4 ml of n-butanol and its absorbancy was estimated at 532 nm.

Results

1. *Macrophage-mediated hepatocyte dysfunction.* We previously reported that the culture supernatant of activated macrophages caused hepatocyte damage[2]; the capacity of protein biosynthesis of the isolated hepatocytes was significantly inhibited by addition of the culture supernatant prepared from MAF-activated macrophages. This activity was found in the fourth fraction fractionated by Sephadex G-75 gel filtration. When this fourth fraction was further fractionated by DEAE-cellulose column chromatography, activity causing a significant reduction of albumin synthesis was detected both in the third and fourth fractions; albumin synthesis in isolated hepatocytes was decreased to 68.2 ± 11.1% and 67.0 ± 10.5% that of controls by adding the third and fourth fractions respectively. On the other hand no such activity was found in any other fractions, as shown in **Fig. 2b**.

2. *Lipid peroxide formation in isolated hepatocytes by activated macrophages.* As indicated above, the fourth fraction separated from MAF-activated macrophage culture supernatant by Sephadex G-75 gel filtration was shown to cause a reduction of albumin synthesis in hepatocytes. This was further investi-

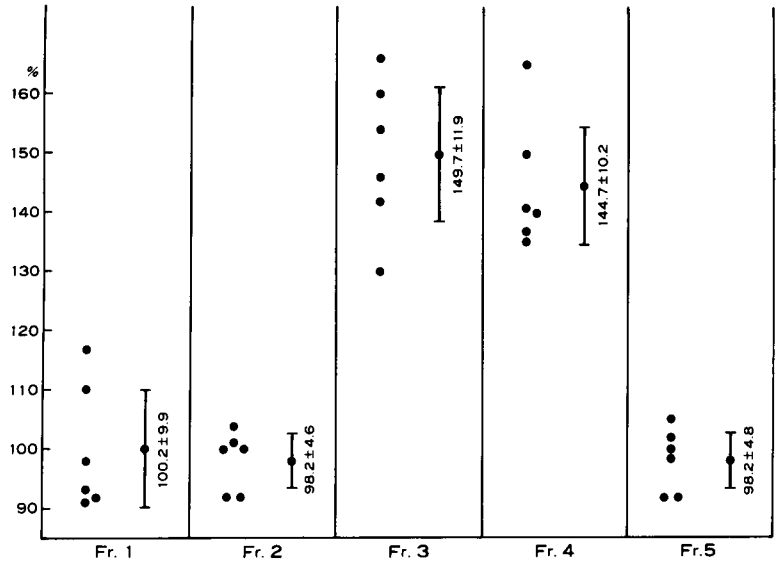

Fig. 4. Lipid peroxide accumulate in isolated hepatocytes treated with the fractionated materials by DEAE-cellulose column chromatography. As a control, the same experiments were carried out using the lymphocyte culture supernatant prepared from normal individuals.

gated with respect to the lipid peroxide formation, because lipid peroxidation as a mechanism for liver damage has been studied in detail in recent years. As shown in **Fig. 3**, the addition of fraction 4 to the hepatocyte culture induced significant lipid peroxide formation, suggesting that the mechanism which caused a reduction of albumin biosynthesis may produce the lipid peroxide in hepatocytes. This is also the case in materials fractionated by DEAE-cellulose column chromatography: lipid peroxide was formed in hepatocytes by fraction 3 and fraction 4 separated by DEAE-cellulose column as shown in **Fig. 4**. On the contrary, no such activity was found in any other fractions.

Discussion

Accumulating evidence suggest that immune reactions to LSP may be involved in the pathogenesis of chronic active hepatitis. Although it is assumed that diverse immune reactions are possibly related to different stages of tissue damage, the concept of liver cell disturbance via ADCC reaction is most attractive because anti-LSP antibody is detectable at a considerably high frequency in serum of patients with chronic active hepatitis. On the other hand, there is considerable information on the crucial role of the macrophages both in the induction of the immune response and as a powerful effector cell. The latter capacity is presumed by evidence that macrophages obtained from immune animals have altered morphology, metabolism and function. Chronic inflammatory lesions may arise by direct interaction of the inflammatory agents with macrophages, or more often, after an immunological response involving either or both T and B cells. The products of lymphocytes may stimulate macrophages to release mediators relevant to the pathogenesis of chronic inflammation. However, if we are to implicate immune mechanisms in liver disease, we should consider how these mechanisms can damage liver tissue and learn how they can be detected by in vitro testing. In this connection, we have previously re-

ported the possibility that the MAF-activated macrophages exert inhibitory action on isolated hepatocytes in a cell-to-cell contact manner or through the production of cytotoxic or cytostatic factors[1,2]. These factors were fractionated into at least two active materials by Sephadex G-75 gel filtration followed by DEAE-cellulose column chromatography. In the present studies, we have provided evidence that the factors produced from the activated macrophages cause a significant formation of lipid peroxide in the isolated hepatocytes. Since lipid peroxidation has been well documented as a mechanism for liver necrosis, particularly in the case of carbon tetrachloride poisoning[3], it is reasonable to assume that liver damage by the activated macrophages may be induced through the formation of lipid peroxide, by which the membranous system of liver cells may be destructed. In the present experiments, we have studied liver injury from the standpoint of the inhibition of protein biosynthesis. It is well known that the first response of the hepatocyte to any injury is an alteration in the profiles of the rough endoplasmic reticulum. The rough endoplasmic reticulum becomes dilated and disrupted, and membrane-found ribosomes become detached. These phenomena may reflect the reduction of albumin biosynthesis. We have also used [^{3}H]-L-ornithine for the assay of protein synthesis. This is because L-ornithine may change to arginine via the urea cycle and be incorporated in the protein. Therefore, we can estimate not only capacity protein synthesis but also the urea cycle. Although we are not sure at the present stage how much lipid peroxide formation will contribute to the induction of liver damage, we assume that lipid peroxide formation may be involved at least partially in the pathogenesis of liver injury.

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