Enhancement of Hepatic Macrophages in Septic Rats and Their Inhibitory Effect on Hepatocyte Function

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In the present study, the function of hepatic macrophages and the modulation of hepatocytes by sepsis-elicited hepatic macrophages were investigated in rats with induced sepsis. The functional state of hepatic macrophages was determined by the following indicators: phagocytic index, protein-synthesizing capacity, and superoxide (O2) producing capacity. These indices of changes in hepatic macrophages were much higher in rats with sepsis than in healthy controls. Moreover, the activated hepatic macrophages had some biological properties which were different from those of the resident Kupffer cells. It was found that protein synthesis by cultured hepatocytes was inhibited in the co-culture system of hepatocytes and sepsis-elicited hepatic macrophages, and that the supernatant of hepatic macrophages from rats with sepsis also reduced the protein-synthesizing capacity of cultured hepatocytes. Thus, activated hepatic macrophages may play a role in inducing hepatic dysfunction in sepsis.

INTRODUCTION

It is assumed that hepatic macrophages are responsible for the prevention of septic complications, because they occupy the main portion of tissue macrophages and they play an important role in clearing and inactivating bacteria, endotoxin, immune complexes, and various blood-borne particles [1, 2]. Recently it has been reported that hepatic macrophages also produce various mediators, such as reactive oxygen intermediates [3], prostaglandins [4], interleukin-1 [5, 6], and collagenase [7]. It has also been suggested that the soluble factors mentioned above which are released by phagocytes may damage host tissues [8].

In sepsis, circulating levels of endotoxin and inflammatory stimulants are increased, and consequently hepatic macrophages may be activated. Finally, hepatic macrophages can injure nearby hepatocytes [9].

According to the above working hypothesis, we examined the properties of hepatic macrophages isolated from rats with induced sepsis, and studied the effect of those macrophages on the ability of cultured hepatocytes to synthesize protein. We will present evidence that hepatic macrophages in sepsis are activated and can inhibit the protein-synthesizing capacity of hepatocytes.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 200 to 300 g were purchased from Shizuoka Agricultural Co-operative Experimental Animals (Hamamatsu, Japan). Throughout the experimental period, the rats were maintained in an air-conditioned animal room.

Sepsis was induced by the method of Wichterman et al. [10]. Briefly, under ether anesthesia a midline incision was made and the cecum was ligated just below the ileocecal valve. Then the cecum was punctured twice with a 22-gauge needle. The abdomen was closed in two layers. On the third day after treatment, the rats were used for the experiments.

Preparation of Hepatic Macrophages and Hepatocytes [11, 12]

Rats were anesthetized with ether and pentobarbital, and after laparotomy the liver was perfused through the portal vein with Ca2+-Mg2+-free (CMF) Hank’s solution containing 5 mM EGTA. Subsequently, the liver was perfused with CMF-Hank’s solution supplemented with 0.05% collagenase. Then the liver was removed en bloc and cut into small pieces. These pieces were incubated at 37°C for 10 min with CMF-Hank’s solution supplemented with 0.05% collagenase and passed through a stainless steel mesh. They were separated into parenchymal cells and non-parenchymal cells (NPC) by differential centrifugation. The NPC pellet was suspended in Eagle’s MEM with 10% FCS (Gibco Laboratories, Grand Island, NY) and placed in a 35-mm culture dish. After 60 min, the dish was washed with MEM to detach the nonadherent cells, leaving the adherent cells, which
were almost all hepatic macrophages. The viability of the cells, as measured by the trypan blue exclusion test, was more than 95%. The morphological characteristics of the cells were checked by light microscopy and phagocytosis of colloidal carbon and latex particles.

**Measurement of Superoxide Production**

Superoxide generation by hepatic macrophages was measured by the reduction of ferricytochrome C that was inhibited by superoxide dismutase (SOD) [13]. Hepatic macrophages were washed twice with Hank's balanced solution without phenol red. Then the reaction mixture containing 60 μM cytochrome C with or without SOD (30 μg/ml) was added to a culture dish. The reaction was initiated by the addition of 1 mg/ml of opsonized zymosan (OZ) as a stimulant. After incubation for 60 min, the reaction was stopped by transfer of the incubation mixture to the tube. Then the tube was centrifuged at 1500 rpm for 10 min at 4°C. The optical density of the supernatant was measured by a spectrophotometer at 550 nm. The amount of superoxide released was calculated as the amount of cytochrome C reduced. The molar extinction coefficient of cytochrome C at 550 nm was used as 2.1 × 10⁴ M. Data were expressed as nmole/60 min/mg protein. The protein content of the digest was determined by the method of Lowry et al. [14].

**Phagocytic Index**

The phagocytic index was measured by the ⁵¹Cr disappearance rate determined as the slope of the logarithm of plasma radioactivity vs time after injection of ⁵¹Cr-labeled endotoxin [15–17]. This is summarized in the equation

\[
\text{Phagocytic index} = \log C_1 - \log C_2 / T_2 - T_1
\]

where C₁ and C₂ represent the radioactivity in the peripheral blood at time T₁ and T₂, respectively. Na₂S₁₃CrO₄ (⁵¹Cr)-endotoxin at a dose of 4 mg/kg body weight was injected via the tail vein, and blood was taken from the femoral vein at 1, 3, 5, 10, and 20 min.

**Co-culture System**

Hepatocytes (5 × 10⁴/0.2 ml) from normal rats were inoculated into a 24-well plastic plate (Corning 25820) in MEM supplemented with 10% FCS, 10⁻⁴M insulin, and 10⁻³M dexamethasone. The plastic plate was placed in a humidified incubator at 37°C under 5% CO₂ in air. After 24 hr of incubation the medium was discarded, and hepatic macrophages isolated from normal or septic rats in MEM supplemented with 10% FCS were added to each well for co-culture. The co-culture continued for 24 hr, then 2 μCi of ³H-leucine was added. Eighteen hours later, protein synthesis assay was performed as described below. Radioactivity (cpm) in the hepatocytes was calculated by subtracting that of hepatic macrophages from the total radioactivity.

**Supernatant Transfer System**

The supernatant obtained from the culture of hepatic macrophages (1 × 10⁶/ml) from normal or septic rats and 2 μCi of ³H-leucine were added to the cultured hepatocytes 24 hr after the initial plating, and 18 hr later, the assay of protein synthesis was performed.

**Assay of Protein Synthesis**

Protein synthesis was determined by the incorporation of ³H-leucine into trichloroacetic acid (TCA)-precipitable material in cells. In brief, 2 μCi of ³H-leucine were added to the culture dish, and incubation proceeded for 18 more hours. The culture medium was discarded, and 10% cold TCA was added to the dish, which was then scraped with a rubber policeman; the pellet was placed on a glass fiber filter (Whatman GF/C). The filter was washed twice with 5% TCA and dried. The filter was then put into a scintillation vial containing scintillation cocktail (POPOP 0.5 g, PPO 4.0 g in 1 liter of toluene). Radioactivity was measured by a Packard scintillation counter.

Statistical analysis was performed with Student’s t test.

**RESULTS**

Figure 1 demonstrates superoxide production by macrophages from different anatomical sites. Hepatic macrophages produced superoxide when triggered with OZ, but did not produce a significant level of superoxide when
TABLE 1

Function of Hepatic Macrophage in Septic Rat and Normal Rat

<table>
<thead>
<tr>
<th>Hepatic macrophage</th>
<th>Control (n = 10)</th>
<th>Sepsis (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phagocytic index</td>
<td>0.103 ± 0.015</td>
<td>0.127 ± 0.025*</td>
</tr>
<tr>
<td>( O_2 ) producing activity (n mole/60 min/mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( O_2 ) stimulation</td>
<td>35.3 ± 6.9</td>
<td>108.2 ± 17.6*</td>
</tr>
<tr>
<td>PMA stimulation</td>
<td>5.6 ± 2.7</td>
<td>27.9 ± 2.1*</td>
</tr>
<tr>
<td>Protein synthetic capacity (cpm)</td>
<td>4410 ± 520</td>
<td>8240 ± 1110*</td>
</tr>
</tbody>
</table>

* \( P < 0.01 \) vs control.

Figures 2 and 3 show the effects of LPS in vivo and in vitro on superoxide production of hepatic macrophages. As is shown in Fig. 2, the intravenous injection of LPS enhanced superoxide production on Days 1 and 3 after administration. The addition of LPS to the culture medium for 24 hr also enhanced superoxide production at concentrations of 0.05 and 0.5 ng/ml. On the other hand, superoxide-producing activity was suppressed at concentrations of LPS higher than 1 \( \mu \)g/ml (Fig. 3).

The inhibitory effects of hepatic macrophages on protein synthesis by cultured hepatocytes are shown in Figs. 4 and 5. Figure 4 shows that in the co-culture system of both hepatocytes and hepatic macrophages from rats with sepsis protein synthesis by hepatocytes was significantly decreased to 80, 55, and 28% of the control value at hepatic macrophage/hepatocyte ratios of 5:1, 10:1, and 20:1, respectively. Figure 5 shows that protein synthesis by cultured hepatocytes was reduced to 90, 66, and 64% of the control values by supplementation with 25, 50, and 100%, respectively, of the supernatant of the cultured hepatic macrophages from rats with sepsis.

DISCUSSION

Sepsis, which frequently occurs in patients with severe infections, usually has a poor prognosis [18]. Many investigations have been performed in a search for ways to combat sepsis.
In this study of the pathophysiology of sepsis, we focused our attention on hepatic macrophages, which play an important role in host defense. The model of sepsis which we used was the rats with cecal ligation and puncture, since we speculated that infection of the portal area would strongly affect the function of hepatic macrophages. The indicators used in evaluating the function of the macrophage were its phagocytic activity, protein-synthesizing capacity, and superoxide-producing activity. Phagocytic activity is responsible for clearing bacteria, endotoxin, and cell debris, etc. Protein-synthesizing capacity is closely related to the synthesis of various enzymes (i.e., lysosomal enzyme), and superoxide-producing activity is necessary for oxygen-dependent killing [19]. The above indicators of the function of hepatic macrophages isolated from septic rats were greatly increased, showing an activated state and good response of the host defense system. The factors which induce activation of the macrophage in sepsis have not been fully identified. One possible factor is endotoxin released from the infectious focus, as suggested by Keller et al. [20]. This theory is supported by our findings that the superoxide-producing activity of hepatic macrophages was enhanced by LPS and that superoxide production was increased in hepatic macrophages from LPS-injected rats.

It is also interesting that in the present study the biological properties of the hepatic macrophages varied with the pathological condition. There are many reports that the number of Kupffer cells increases in the inflammatory state [21-23]. Pilaro and Laskin demonstrated a sixfold increase in the number of mononuclear phagocytes recovered from the livers of rats injected with endotoxin [22]. In previous studies in our laboratory resident Kupffer cells were found to produce superoxide when stimulated by opsonized zymosan but not by phorbol myristate acetate, in contrast with peritoneal macrophages and peripheral monocytes [24]. Such differences in the superoxide-producing activity of the monocyte/macrophage lineage may occur during differentiation. In general, monocytes gradually lose their oxidative capacity as they differentiate. Arthur also noted that a clear relationship could not be demonstrated between PMA concentration and superoxide generation by normal rat Kupffer cells [25]. However, the present study demonstrated that hepatic macrophages obtained from rats with sepsis produced superoxide in response to PMA stimulation. Therefore, it appears that hepatic macrophages in sepsis may include a heterogeneous population, probably composed of locally proliferating Kupffer cells [23], cells recruited to the liver from the bone marrow and peripheral blood monocytes [21]. An alternative hypothesis is that such activated hepatic macrophages acquire responsiveness to PMA, possibly due to the induction of PMA receptors.

Another interesting problem to be discussed is the possible effect of the activated macrophages on the modulation of hepatocytes.

It is well known that hepatic dysfunction frequently develops in sepsis [26-29], although the mechanism of this change remains to be clarified. One hypothesis is that in sepsis hepatic macrophages would be activated and would influence the parenchymal cells and the other sinusoidal cells located in their vicinity. In the present study hepatic macrophages from rats with sepsis impaired the protein synthesis of cultured hepatocytes in the following two experiments: (1) co-culture system of normal hepatocytes and activated macrophages and (2) transfer of the culture medium of hepatic macrophages to hepatocytes. This evidence suggests two kinds of mechanism for hepatocytotoxicity of hepatic macrophages: (1) cell to cell interaction and (2) possible role of soluble mediators released from activated macrophages, such as reactive oxygen intermediates, prostaglandins, interleukin-1, protease, collagenase, etc.

Keller et al. have carried out many detailed investigations using the co-culture system with LPS [30-33]. Although the experimental system presented here is somewhat similar to that of Keller et al., there are substantial differences. In the present study, the authors employed hepatic macrophages (Kupffer cells) from a septic model, because the purpose of this investigation was to clarify the functional changes of hepatic macrophages and to analyze the mechanism of the metabolic abnormalities and immunological disorder, etc., in sepsis.

It is well known that reactive oxygen intermediates probably have a harmful effect on hepatocytes by damaging proteins, DNA, biomembranes, etc. Nathan also reported that reactive oxygen intermediates play an important role in cell-mediated cytotoxicity in vitro [34, 35]. We have previously reported that protein synthesis by cultured hepatocytes was decreased when they were exposed in vitro to superoxide generated by the hypoxanthine-xanthine oxidase system [36].

Therefore, excessive superoxide from hepatic macrophages in sepsis might be one of the factors leading to hepatocytotoxicity in the co-culture system. However, in the transfer system, reactive oxygen intermediates do not play a primary role, because they are unstable and
disappear rapidly from the culture medium. With regard to soluble factors secreted by activated hepatic macrophages which inhibit protein synthesis by hepatocytes, no definite mediators have yet been discovered, although one of the candidates is an interleukin-1-like substance, as pointed out by Keller et al.

In an attempt to elucidate hepatocytotoxicity in the transfer system, a biochemical analysis of the culture medium of activated hepatic macrophages is now in progress.

REFERENCES