Nitric Oxide Mediates Kupffer Cell-induced Reduction of Mitochondrial Energization in Hepatoma Cells: A Comparison with Oxidative Burst

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ABSTRACT

The metabolic changes in rat hepatoma cell line, AH70 cells, after coculturing with Kupffer cells were visualized using a silicon-intensified target camera and subsequent processing with a computer-assisted digital imaging processor. In cocultured tumor cells, nonactivated Kupffer cells reduced mitochondrial energization as indicated by the decrease in the fluorescence intensity of rhodamine 123 (Rh123) and induced lipid peroxidation as shown by the dichlorofluorescein (DCF) activation. The reduction in Rh123 could be eliminated by addition of an analogue of l-arginine (L-4-monomethyl-l-arginine), suggesting the involvement of nitric oxide (NO) in the decrease in mitochondrial energization. Superoxide dismutase did not inhibit the reduction in Rh123 but significantly inhibited DCF activation. These findings indicate that the latter reaction was mediated by superoxide anion. Two h after the cells were cocultured, propidium iodide-positive, severely injured tumor cells significantly increased in number. This increase was significantly attenuated by addition of L-4-monomethyl-l-arginine but not by superoxide dismutase, suggesting that NO may be greatly involved in Kupffer cell-mediated injury of AH70 cells.

In another set of experiments, the culture medium of Kupffer cells caused no significant alteration of Rh123, DCF, and propidium iodide-associated fluoronances in AH70 cells. In addition, ultrastructural observation revealed that the membrane-to-membrane attachment between Kupffer cells and tumor cells occurred within 30 min after coculturing. These results suggest that Kupffer cell-derived NO release, triggered by the close contact with tumor cells, may induce damage to tumor cells via inhibition of mitochondrial energization.

INTRODUCTION

Kupffer cells comprise the largest population of fixed tissue macrophages in the body (1), and together with the highly specific immune system, they play a role as the defensive machinery against outer invaders. The liver macrophages are in close contact with the sinusoidal endothelium and are usually found in greater number in the periportal area than near the central vein. The topological position within the liver sinusoidal positions the Kupffer cells as the first macrophages to come into contact with exogenously or endogenously derived toxins, microorganisms, and tumor cells entering the liver via the hepatic portal circulation. Recent advances in the techniques of isolation of Kupffer cells have made the evaluation of Kupffer cell-mediated injury of AH70 cells possible.

MATERIALS AND METHODS

Cell Preparation. Male Wistar rats with an average body weight of 250 g were used for the preparation of effector cells. Kupffer cells were isolated from the intact liver tissue by the Pronase method, which was described in detail by Nook et al. (16). In brief, livers of pentobarbital-anesthetized rats were perfused in situ through the portal vein, first with 10 ml of warm Gey's BSS (0.17 g CaCl2-0.37 g KCl-0.03 g KH2PO4-0.21 g MgCl2-6H2O-0.07 g MgSO4-7H2O-7.00 g NaCl-2.27 g NaHCO3-0.226 g Na2HPO4-7H2O-1.00 g/liter o-glucose; pH 7.4) and then with 10 ml of Gey's BSS containing 0.2% Pronase E (Merek, Germany). Livers were excised, minced, and incubated in Gey's BSS containing 0.2% Pronase E for 45 min at 37°C in a CO2 incubator. After selective destruction of parenchymal cells by this enzyme, nonparenchymal cells were washed twice in the serum-free DMEM (Gibco Laboratories) and purified by gradient centrifugation on metrizamide (Sigma Chemical Co.). Kupffer cells were further purified by a centrifugal elutriation at 2500 rpm (750 x g), between countercurrent rates of 23 and 42 ml/min (model J2-21 ME centrifuge; Beckman Co.). Hepatocytes were also isolated from intact livers of male Wistar rats according to the method of Drochmans et al. (17). The viability of isolated cells, both Kupffer cells and hepatocytes, was >90% as determined by the trypan blue dye exclusion test (14). The collected cells were seeded in a flat-bottomed tissue culture flask (Coming; Iwaki Glass Co., Japan) at a final concentration of 1 x 10^6/ml and incubated with DMEM containing 10% fetal calf serum (Gibco) for 2 days at 37°C in 5% CO2. To confirm that

1 The abbreviations used are: NO, nitric oxide; O2·-, superoxide anion; HO2, hydroxyl radical; ONOO-, peroxynitrile anion; BSS, balanced salt solution; DMEM, Dulbecco's modified Eagle medium; DCFH-DA, 2',7'-dichlorofluorescin diacetale; DCF, dichlorofluorescein; SST, silicon-intensified target; Rh123, rhodamine 123; P1, propidium iodide; SOD, superoxide dismutase; SOD, denatured SOD; L-NMMA, N6-monomethyl-l-arginine; LPS, lipopolysaccharide.

2 To whom request for reprints should be addressed.

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these Kupffer cells were nonactivated, the spontaneous respiratory burst of Kupffer cells was measured by luminol-dependent chemiluminescence assay (18). Briefly, luminol (Sigma) was added to the Kupffer cell suspension (10⁶ cells in 1 ml of culture medium) at a final concentration of 1.0 μg/ml. Chemiluminescence activity from the cell suspension was detected by an ultrasensitive photon-counting imaging camera (C-2400-20; Hamamatsu Photonics, Shizuoka, Japan) equipped with a digital imaging processor (ARGUS-100; Hamamatsu). Kupffer cell-derived chemiluminescence activity was <2000 counts/10⁶ cells/min.

AH70 cells, a cell line of rat hepatoma cells, were kindly provided by the Japanese Cancer Research Resources Bank and used as the target cells. AH70 cells were cultured on RPMI-1640 (Flow Labs) supplemented with 10% fetal calf serum, 100 U/ml of penicillin (Gibco), and 100 μg/ml of streptomycin (Gibco). The tumor cells were seeded on a culture dish, the bottom of which was made of nonfluorescent thin glass, and were cultured at 37°C under a humid atmosphere of 5% CO₂ until the experiments were conducted.

**Lipid Peroxidation in Target Cells.** To investigate temporal alterations of oxidative stress in individual target cells, DCFH-DA (Eastman Kodak Co.) was used according to the method of Cathcart et al. (19) with some modifications (14). Cultured tumor cells were incubated with DMEM (pH 7.4) containing 1 μM DCFH-DA for 10 min at 37°C in the dark. The cells were washed three times with DMEM to remove the extracellular DCFH-DA. Cells, adequate for the assay, were selected in the microscopic frame, and fluorescence was measured with a digital imaging microscopic system. Inoculated cells were visualized using an inverted microscope (TMD-2S). To determine the fluorescent DCF produced by lipid peroxides, DCFH-loaded cells were observed under epifluorescence illumination at 420-490 nm with a 100-W Xe lamp, and the fluorescence image of DCF, a fluochrome derived from DCFH oxidation by hydroperoxides (19), was obtained according to our previously reported method (14) using a SIT camera (C-2400-08; Hamamatsu). Thus, obtained images were stored in the digital imaging processor (ARGUS-100). Adherent Kupffer cells were collected by scraping with a cell scraper (Falcon 3086; Becton Dickinson and Co.) and added to AH70 cells in a culture dish to give a final target:effector ratio of 1:10. Kupffer cell-mediated lipid peroxidation in tumor cells was analyzed for 120 min. As a control group, the alteration of fluorescence in DCFH-loaded AH70 cells without Kupffer cells was determined for 120 min.

**Mitochondrial Function in Target Cells.** To investigate the influence of Kupffer cells on the mitochondrial function of cocultured tumor cells, Rh123 (Sigma) was used. Rh123 is known as a mitochondrial energization-sensitive fluorescence probe (20). Cultured AH70 cells were incubated with DMEM (pH 7.4) containing 800 nM of Rh123 for 10 min at 37°C in the dark. The cells were washed three times with DMEM to remove the extracellular Rh123. Cells, adequate for the assay, were selected in the microscope frame, and inoculated cells were visualized using an inverted microscope (Diaphot, TMD-2S, Nikon, Japan). The fluorescence of Rh123 in each tumor cell was visualized and analyzed with a digitized low-light video microscopic system consisting of an inverted phase/fluorescence microscope, a low-light level camera (SIT camera) and video digitizing equipment for averaging, background subtraction, ratio imaging and data storage (ARGUS-100).

**Assay Procedure for Fluorescence Intensities.** The microphotographic image of cocultured cells was recorded using the transillumination light source of a microscope. The area of individual cell in the preslected microscopic field was determined using this image on a television monitor. Temporal alterations of fluorescence intensities (both DCF and Rh123) in the determined area were automatically calculated by the digital imaging processor and expressed as fluorescence intensity (counts/area (pixel)).

**Determination of Injured Cell Number.** At the end of each experiment, 5 μM PI was added to the culture medium. PI is a red fluorescent DNA stain that is only able to pass through the compromised membranes of dead cells. Five min after the addition, the PI-associated fluorescence was visualized by fluorescence microscopy with an excitation filter of 520 nm and an emission filter of 605 nm. The Kupffer cell-mediated cytotoxicity was calculated as the percentage of PI-positive cells per total cell number in the fixed microscopic field.

**Agents Studied.** To determine whether the fluorescence in AH70 cells was altered by Kupffer cell-derived active oxygen species, SOD (Sigma) was added at a final concentration of 3-300 units/ml in the culture medium prior to the addition of Kupffer cells. To confirm the specificity of SOD activity, chemically dSOD was prepared as described below (21). SOD was dissolved in 50 ms sodium carbonate buffer at a concentration of 10 mg/ml (300 μM). H₂O₂ was added to the solution, and the solution was incubated for 2 h at room temperature and subsequently dialyzed overnight at 4°C. The SOD solution was adjusted to pH 7.4 and used for experiments at a concentration of 300 units/ml.

To investigate the possible involvement of Kupffer cell-derived NO in the cytoxic process, l-NMMA (Sigma), an analogue of l-arginine (22, 23), in the range of 5-500 μM, was added to the culture medium just prior to starting the coculture.

**Electron Microscopic Observation.** Ultrastructural observations were made 30 min and 1 h after starting the coculture of AH70 cells with Kupffer cells. Specimens on the culture plate were fixed in a mixture of 2.5% glutaraldehyde (pH 7.4, adjusted with 0.66 M phosphate buffer) at 4°C for 4 h. The samples were washed with the same buffer and further fixed in 2% osmium tetroxide (pH 7.4, with the same buffer) at 4°C for 2 h. They were then dehydrated with serial dilutions of ethanol, embedded in epoxy resin (Epon 812; TAAAB Laboratories Equipment, Ltd., Alsermaston, UK), and polymerized for 72 h at 60°C (24). Ultrathin slices were prepared with an LKB ultramicrotome III (LKB Produkter AB, Stockholm, Sweden). These sections were double stained with uranyl acetate and lead citrate and examined under a transmission electron microscope (CM-30; Philips, Eindhoven, The Netherlands) at an accelerating voltage of 100 kV.

**Statistical Analysis.** The significance of difference in mean values between groups was tested by Student's t test for paired and unpaired values.

**RESULTS**

**Changes in Lipid Peroxidation.** Fig. 1, A and B, shows the temporal alteration of DCF-associated fluorescence in the microscopic field. A remarkable DCF activation was observed in each AH70 cell after the coculture with Kupffer cells. By visualizing the fluorescence, we clearly demonstrated that a heterogeneity between the DCF activation in each tumor cell existed. At 120 min after the addition, Kupffer cells induced a significant elevation of DCF fluorescence in 70-80% of tumor cells in each microscopic field, while other AH70 cells showed no remarkable change during the entire course of observation. Fluorographs of DCF were digitally superimposed on the corresponding microphotographs (Fig. 2). The superimposed images show that the Kupffer cell-mediated alteration of fluorescence actually occurred in individual AH70 cells. Typical changes of DCF intensity in target cells that interacted with Kupffer cells are illustrated in Fig. 3A. There was a lag time for the DCF activation in AH70 cells after the addition of Kupffer cells. Before the application of Kupffer cells, the DCF intensity was 2.9 ± 2.0 counts/pixel (mean ± SD) in AH70 cells. After the addition of Kupffer cells (90 min), the mean value of DCF intensity in AH70 cells was significantly elevated to 5.2 ± 1.5 counts/pixel, 120 min after the addition it was 5.2 ± 0.9 counts/pixel. In a Kupffer cell-free system, tumor cells showed no remarkable activation of DCF during the entire course of observation (0 min, 3.0 ± 1.7; 120 min, 2.5 ± 1.2 counts/pixel).

**Changes in Mitochondrial Function.** Fig. 1, C and D, demonstrates the spatial alteration of Rh123-associated fluorescence in AH70 cells before and 120 min after the addition of Kupffer cells. The fluorescence of Rh123 was clearly visualized by our fluorescence microscopic system and was reduced in an individual AH70 cell by adding the nonactivated Kupffer cells. Time-course changes of Rh123-associated fluorescence intensity is summarized in Fig. 3B. The Rh123 fluorescence intensity in AH70 cells was 3.4 ± 0.7 counts/pixel before the addition of Kupffer cells. The fluorescence intensity gradually decreased, and the decrease became statistically significant at 60 min (2.7 ± 0.7 counts/pixel) after the start of coculture with Kupffer cells. The intensity further decreased until the end of observation (120 min, 1.7 ± 0.8 counts/pixel). Without Kupffer cells, AH70 cells showed no significant alteration of Rh123 fluorescence.

**Effects of Agents.** To determine whether active oxygen species and NO participate in these metabolic changes of AH70 cells, cells
were pretreated with SOD or l-NMMA or with SOD plus l-NMMA in both DCF and Rh123 studies (Table 1). Each value was calculated in each tumor cell at 120 min after the start of the coculture with Kupffer cells. SOD significantly attenuated the DCF activation induced by Kupffer cells at a dose of 300 units/ml, while it showed no remarkable effect on the decrease in Rh123 fluorescence intensity. The chemically dSOD has no effect on fluorescence alterations, suggesting the specificity of SOD. By contrast, l-NMMA at concentrations
The number of PI-positive tumor cells was also determined after 120 min of coculture to investigate the role of active oxygen and NO on the tumor cell viability (Table 1). Nonactivated Kupffer cells remarkably increased the PI-positive tumor cells (P < 0.001). Neither SOD nor dSOD attenuated the Kupffer cell-mediated tumor cell damage, and they appeared even to slightly increase the damage. By contrast, l-NMMA at >50 μM reduced the cytolytic effect of Kupffer cells. Treatment with SOD plus l-NMMA also significantly prevented the Kupffer cell-mediated tumor cell damage.

### Table 1: Effects of SOD and l-NMMA on the Kupffer cell-induced alterations of DCF-, Rh123-, and PI-associated fluorences in AH70 cells

<table>
<thead>
<tr>
<th>Treatments</th>
<th>DCF intensity (counts/pixel)</th>
<th>Rh123 intensity (counts/pixel)</th>
<th>PI-positive cell (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH70</td>
<td>2.53 ± 1.18*</td>
<td>3.10 ± 0.89</td>
<td>2.8 ± 2.0</td>
</tr>
<tr>
<td>+ Kupffer cells</td>
<td>5.24 ± 0.88*</td>
<td>1.73 ± 0.83*</td>
<td>20.8 ± 8.39*</td>
</tr>
<tr>
<td>+ Kupffer cells + SOD, 3 units/ml</td>
<td>4.96 ± 1.78*</td>
<td>1.80 ± 0.54*</td>
<td>21.3 ± 5.52*</td>
</tr>
<tr>
<td>+ Kupffer cells + SOD, 30 units/ml</td>
<td>4.43 ± 1.27*</td>
<td>1.92 ± 0.76*</td>
<td>24.1 ± 5.92*</td>
</tr>
<tr>
<td>+ Kupffer cells + SOD, 300 units/ml</td>
<td>3.77 ± 0.71*</td>
<td>2.30 ± 0.53*</td>
<td>25.6 ± 7.42*</td>
</tr>
<tr>
<td>+ Kupffer cells + dSOD, 300 units/ml</td>
<td>5.08 ± 1.41*</td>
<td>1.78 ± 0.63*</td>
<td>19.8 ± 3.42*</td>
</tr>
<tr>
<td>+ Kupffer cells + l-NMMA, 5 μM</td>
<td>5.08 ± 1.45*</td>
<td>1.86 ± 0.63*</td>
<td>21.3 ± 7.82*</td>
</tr>
<tr>
<td>+ Kupffer cells + l-NMMA, 50 μM</td>
<td>5.24 ± 1.61*</td>
<td>2.67 ± 0.72*</td>
<td>25.7 ± 4.82*</td>
</tr>
<tr>
<td>+ Kupffer cells + l-NMMA, 500 μM</td>
<td>4.56 ± 1.34*</td>
<td>3.06 ± 1.02*</td>
<td>4.1 ± 3.1*</td>
</tr>
<tr>
<td>+ Kupffer cells + SOD, 300 units/ml + l-NMMA, 500 μM</td>
<td>3.67 ± 1.17*</td>
<td>3.09 ± 1.72*</td>
<td>3.4 ± 3.3*</td>
</tr>
</tbody>
</table>

* Mean ± SD from 9 experiments.
+ P < 0.001 compared with AH70 alone.
+ P < 0.01 compared with AH70 alone.
+ P < 0.05 compared with AH70 alone.
+ P < 0.001 compared with AH70 + Kupffer cells.
+ P < 0.05 compared with AH70 + Kupffer cells.
+ P < 0.01 compared with AH70 + Kupffer cells.

Fig. 3. Temporal alterations of DCF and Rh123 fluorescence intensities in the individual AH70 cell. Fluorescence intensities in individual AH70 cells were determined using the digital imaging processor. In A, DCF intensity in the AH70 cells gradually increased and reached a 3-fold higher level of control value 90 min after coculture with Kupffer cells. In B, in contrast, Rh123 decreased after the coculture, and the reduction became significant at 60 min. Point, mean (bar, ±SD) from 9 experiments. *P < 0.05, **P < 0.01, ***P < 0.001 as compared with the value of the individual at 0 min. ***P < 0.05, ****P < 0.01, *****P < 0.001 as compared with the value of the Kupffer-free system.

of >50 μM eliminated the alteration of Rh123 intensity in tumor cells, while it did not inhibit the activation of DCF by Kupffer cells. When SOD plus l-NMMA was added to the culture media, both activation of DCF and a decrease in Rh123 intensity induced by Kupffer cells were significantly attenuated.

Fig. 4. Influence of Kupffer cells and culture medium of Kupffer cells on the hepatocytes and AH70 cells. Kupffer cells induced no remarkable alteration of DCF-, Rh123-, and PI-associated fluorescence in cocultured hepatocytes. The culture medium of Kupffer cells also had no effect on the fluorescence intensities in AH70 cells. Data were obtained 120 min after the coculture. Point, mean (bar, ±SD) from 9 experiments.
Involvement of Cell-to-Cell Attachment. To determine the involvement of close contact in this cytotoxic process, we carried out the following study. The culture medium of Kupffer cells was filtered through a 0.22-μm Millipore filter. Then, the medium was added to a culture dish containing AH70 cells. The culture medium of Kupffer cells alone had no effect on the Rh123 or DCF fluorescences in the cultured hepatoma cells (Fig. 4). When hepatocytes taken from intact rats were used instead of AH70 cells, Kupffer cells without stimulation did not cause any significant alteration of either Rh123 or DCF fluorescence intensity in the cocultured hepatocytes, even 2 h later (Fig. 4).

Purified Kupffer cells were characterized by multiple pseudopodia on the cell surface and frequent lysosomes as evidenced by transmission electron microscopy. After addition of Kupffer cells to the culture dish containing AH70 cells (30 min), morphological alterations occurred on activated Kupffer cells (Fig. 5). Pseudopodia occasionally elongated and reached the plasma membrane of the hepatoma cells. The plasma membrane of Kupffer cells formed membrane-to-membrane attachment to AH70 cells. After 1 h, activated pseudopodia started to engulf tumor cells. Thus, a close contact between Kupffer cells and AH70 cells was observed. At this point, no significant morphological damage was present on the tumor cells.

DISCUSSION

It is generally accepted that the synthetic process of NO and O$_2^-$ is closely related to cell-killing activity of macrophages. In brief, O$_2^-$ is formed from O$_2$ through the oxidation by NADPH oxidase, and NO is proposed to be synthesized through the oxidative metabolic process from L-arginine to L-citrulline using O$_2$ and NADPH (25). In addition,
Thus, the spatial and temporal relationship between $\text{O}_2^-$ and NO-reacts with NO to form HO, a potent cytotoxic oxidant, via the steps described below (26, 27).

$$\text{O}_2^- + \text{NO} = \text{ONOO}^- + \text{H}^+$$

Therefore, the reaction $\text{ONOO}^- + \text{NO} = \text{HO} + \text{NO}_2^- + \text{H}^+$

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Therefore, the reaction $\text{ONOO}^- + \text{NO} = \text{HO} + \text{NO}_2^- + \text{H}^+$

with malignant cells activates Kupffer cells derived from rat liver, and the close attachment to tumor cells through some protein structures on Kupffer cell membranes may be taking place in this process. In addition, in the present study, the membrane-to-membrane attachment between Kupffer cells and AH70 cells was clearly observed. Therefore, there is a possibility that the close contact of some particles on hepatoma cells with a specific receptor or binding site is sufficient to trigger responses for NO production from nonactivated Kupffer cells. There was a heterogeneity in the Rh123 alterations between cocultured AH70 cells after the addition of Kupffer cells. It is conceivable that the heterogeneity depends on whether the cell is attached by Kupffer cells or not. The finding from this study that the culture medium of Kupffer cells did not cause any fluorescence alteration in the tumor cells is in accordance with the importance of Kupffer-to-tumor cell attachment.

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