Kupffer cell-mediated cytotoxicity against hepatoma cells occurs through production of nitric oxide and adhesion via ICAM-1/CD18

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Abstract

Rat Kupffer cell (KC)-mediated cytotoxicity against both the syngeneic hepatoma cell line AH70 and hepatocytes was evaluated by changes in mitochondrial function, and the possible role of ICAM-1/CD18 in the interaction between the cells was studied. Rhodamine 123 fluorescence, a marker of the mitochondrial membrane potential, decreased in AH70 cells after co-culture with KC, while that in hepatocytes was unchanged by co-culture. This decrease was blocked by anti-ICAM-1, anti-CD18 and the inhibition of nitric oxide synthesis. Cytometric studies demonstrated that ICAM-1 expression on AH70 cells increased after addition of IFN-γ, IL-1β, tumor necrosis factor (TNF)-α or KC, while in hepatocytes ICAM-1 was not increased. Anti-ICAM-1 pretreatment inhibited the increase in ICAM-1 expression and the decrease in rhodamine 123 fluorescence on AH70 cells after co-culture with KC. CD18 on KC was increased only after co-culture with AH70. TNF-α but not IFN-γ was detected in the supernatant of co-culture between KC and AH70 cells, and this production was partially inhibited by anti-ICAM-1 and anti-CD18. The activity of inducible nitric oxide synthase in Kupffer cells and the levels of nitrites and nitrates in the co-culture supernatant increased over time, and this increase was attenuated either by addition of NO synthesis inhibitors, anti-ICAM-1 or anti-CD18. These results indicate that the rat KC causes mitochondrial dysfunction in cancer cells via the production of NO and cell-to-cell adhesion via ICAM-1/CD18 has an important role in this cytotoxic process.

Introduction

One of the important functions of Kupffer cells (KC) is antitumor activity in the liver (1) after activation by cytokines, biologic response modifiers or endotoxin (2). We have demonstrated that rat KC may damage cancer cells without prior activation (3) and that direct membrane-to-membrane interaction may be the first step in this cytotoxic process by electron microscopy (4). Our previous study indicates that nitric oxide (NO) may damage cancer cells via the inhibition of mitochondrial function in this process (5). However, the factors which define the direct interaction between KC and target cells have not yet been clarified.

In hepatoma, the first defensive barrier is thought to be non-specific cytotoxic cells, such as NK cells (6), neutrophils (7) and monocytes/macrophages. Since KC comprise the largest population of fixed tissue macrophages in the body (8), the elimination of hepatoma cells may largely depend upon KC function.

Adhesion molecules may be involved in cell-mediated cytotoxicity systems, such as ICAM-1 and one of its ligands, LFA-1/CD18. In addition, cytokines such as IFN-γ, IL-1β, and tumor necrosis factor (TNF)-α are produced and released by hepatic sinusoids with bacterial or viral infection or cancer.
invasion. Moreover, these cytokines may be induced by cell-to-cell contact and subsequently modulate the functions of these cells. Roland et al. (9) have demonstrated that IFN-γ is released during co-culture of Th1 lymphocytes and murine KC; IFN-γ then induced NO release by KC, which subsequently blocked Th1 lymphocyte proliferation. The release of NO in this microenvironment may regulate antigen presentation to KC.

In the present study, we investigated the possible involvement of ICAM-1/CD18 and the cytokines, IFN-γ, IL-1β, and TNF-α, in KC-mediated cytotoxicity to cancer cells. We also examined whether NO is involved in this process

**Methods**

**Cell preparation**

Male Donryu rats with a body weight of 150–200 g were used for preparation of KC and hepatocytes. KC were isolated from intact livers by the method of Knook et al. (10) with modifications previously described (3–5). Hepatocytes were isolated according to the method of Drochmans et al. (11). They were cultured in DMEM (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% FCS (Gibco), 100 IU/ml of penicillin and 100 μg/ml of streptomycin (Gibco). The viability of both isolated KC and hepatocytes was >90% judged by Trypan blue exclusion. A rat hepatoma cell line, AH70, was kindly provided by the Japanese Cancer Research Resources Bank. AH70 were cultured in RPMI 1640 (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% FCS (Gibco), 100 IU/ml of penicillin and 100 μg/ml of streptomycin (Gibco). Tumor cells or hepatocytes were seeded onto a culture dish made of non-fluorescent thin glass and cultured at 37°C in a CO2 incubator.

**mAb**

Anti-ICAM-1 (anti-CD54, subtype IgG1) (1A29; Seikagakukogyo, Tokyo Japan), anti-LFA-1 (anti-CD11a, subtype IgG1) (WT1; Seikagakukogyo), anti-CD18 (subtype IgG1, WT-3; Seikagakukogyo) and anti-rat macrophage (subtype IgG1, ED2; Serotec, Kidlington, Oxford, UK) were used in this study.

**Agents studied**

To investigate the involvement of KC-derived NO in the cytotoxic process, L-NMMA (Sigma), an analog of l-arginine, in the range of 5–500 μM, or dexamethasone (DEX) (1 μM) was added to the culture medium just prior to the start of the co-culture. To study the effect of cytokines on expression of adhesion molecules, IFN-γ (100 U/ml), IL-1β (20 U/ml) and TNF-α (10 ng/ml) were separately added to KC or AH70 cells and incubated in 5% CO2 for 2 h at 37°C.

**Mitochondrial function in target cells**

We determined injury in target cells by the decrease in rhodamine 123 (Sigma, St Louis, MO) fluorescence. Rhodamine 123 is known as a mitochondrial energy-sensitive fluorescent probe and the assay was conducted according to the methods described previously (5). Briefly, cultured target cells were incubated with DMEM containing 800 nM of rhodamine 123 for 10 min at 37°C in the dark. The cells were washed three times to remove any extracellular rhodamine 123. Cells were visualized using an inverted microscope (Diaphot, TMD-25; Nikon, Tokyo, Japan). The rhodamine 123 fluorescence in each target cell was 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; Hamamatsu Photonics, Hamamatsu, Japan). The microscopic image of cultured cells was recorded using the transillumination light source and the individual cells in the microscopic field were matched to the image on the television monitor. Temporal alterations in fluorescence intensity in the microscopic field were automatically calculated by a digital imaging processor and expressed as fluorescence intensity (count)/area (pixel).

To confirm any changes in the mitochondrial function of AH70 cells under co-culture conditions, a fluorescence microscope (TMD-25; Nikon) equipped with a laser scanning confocal imaging system (MRC-500, BioRad, UK) was used. Rhodamine 123 fluorescence in AH70 cells before and after co-culture with Kupffer cells was examined and the images were recorded using the laser scanning imaging system.

**Determination of cell injury**

At the end of each experiment, 5 μM propidium iodide was added to the culture medium. Propidium iodide is a red fluorescent DNA stain that is taken up by dead cells. Propidium iodide fluorescence was visualized by fluorescence microscopy using an excitation filter at 535 nm and an emission filter at 590 nm. The images were recorded using the laser imaging system.

**Expression of adhesion molecules**

Changes in ICAM-1 and LFA-1/CD18 expression on cells after co-culture or addition of cytokines were quantitatively analyzed by flow cytometry according to the methods previously described (12). ICAM-1 expression on KC, AH70 cells and hepatocytes, and LFA-1/CD18 expression on KC were analyzed. After co-culture, KC were purified using a cell sorter (EPICS ELITE; Coulter, Hialeah, FL) and a mAb antibody against rat macrophages. For flow cytometric analysis, cells were washed three times with 1% BSA in PBS and pelleted. Twenty microliters of antibody (20 μg/ml) was added to the cell pellet and mixed, followed by incubation at 4°C for 30 min. After washing three times, cells were incubated with FITC-conjugated goat anti-mouse Ig (Cappel, Westchester, PA) at 4°C for 30 min. After three washes, cells were analyzed on the EPICS Profile II (Coulter). Irrelevant mouse Ig was used as a control antibody and as another control.

To visualize ICAM-1/CD-18 expression on AH70 cells, hepatocytes and KC under co-culture conditions, the laser confocal imaging system was used. Co-cultured cells on non-fluorescent glass were fixed in 100% methanol for 15 min and washed three times with PBS. Cells were then incubated with a mouse mAb against rat ICAM-1 or CD18 for 24 h at 4°C. After washing three times, cells were incubated with FITC-conjugated rabbit anti-mouse IgG1 antibody (40 μg/ml; Japan ICN Biomedicals, Tokyo, Japan) for 2 h at room temperature. Cells were washed and examined with the fluorescence microscope.
**TNF-α and IFN-γ production**

To determine whether TNF-α and IFN-γ were produced by the co-culture, the production of TNF-α and IFN-γ in culture supernatants was assayed by ELISA kits (TNF-α, mouse TNF-α ELISA kit, Factor-test-α, Genezyme, Cambridge, MA; IFN-γ, Hbt rat IFN-γ ELISA test kit, HyCult Biotechnology, Uden, The Netherlands). The assays were performed according to the instructions indicated by the manufacturers.

**Nitrite and nitrate production**

To demonstrate the involvement of NO in KC-mediated cytotoxicity, we measured NO, in the co-culture supernatant from KC and target cells (K/T = 10) KC (1×10⁶/ml) were cultured in a 24-well plate (Falcon, Nippon Becton Dickinson, Tokyo, Japan; each 1 ml/well) and 1×10⁵ AH70 cells with or without prior treatment with 10 ng/ml 1A29 or WT-3 were added to the culture. At 4 h after the addition of AH70 cells, the culture supernatant was collected and stored at -80°C until use. NOx concentration was measured according to the method of Bartholomew (13). Briefly, 0.3 M formate and 0.05 U of nitrate reductase (Sigma) were added to 50 μl of supernatant, and the mixture was incubated at 37°C for 4 h. After incubation, 2 vol. of Griess reagent were added and the mixture was incubated at room temperature for 10 min, followed by the measurement of optical density at 543 nm.

**NOS activity**

After co-culture, KC were isolated using the cell sorter and mAb ED2. Inducible-type NOS (iNOS; Ca-independent) and constitutive-type NOS (cNOS; Ca-dependent) activities in KC and AH70 cells were measured in isolated cells as described by Sailer et al. (14) and Knowles et al. (15) with minor modifications. Briefly, cells were homogenized in 20 mM HEPES buffer (pH 7.4) containing 0.1 mM EDTA, 1 mM dithiothreitol, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 2 μg/ml aprotinin and 0.2 mM phenylmethanesulfonyl fluoride. The homogenate was centrifuged at 3000 r.p.m. and the supernatant and pellet were kept on ice. The supernatant was assayed using the L-NMMA-inhibitable conversion of [14C]-labeled L-arginine to [14C]citrulline. Small aliquots (0.05 ml) of supernatant were added to the reaction mixtures containing 50 mM HEPES (pH 7.2), 1.0 mM CaCl₂, 1.0 mM MgCl₂, 10 mM [14C]-l-arginine, 1 mM citrulline (to inhibit catabolism of [14C]-citrulline), 10 mM L-valine (to inhibit non-specific arginase activity), 1 mM flavin adenine dinucleotide and 0.1 mM NADPH in a total volume of 200 μl. The reaction mixtures were incubated for 60 min at 37°C, after which 1 ml of a 1:1 suspension of Dowex (1X8-50; Sigma) and buffer was added to bind any unreacted radiolabeled L-arginine. This assay measures both cNOS and iNOS isoenzymes. The iNOS activity was measured under the Ca²⁺-free conditions with 1 mM ethylene glycol-bis(β-aminoethyl ether) added.

Distributions of iNOS and cNOS in AH70 cells and KC were examined using the same confocal imaging system described above. Co-cultures of AH70 and KC on non-fluorescent glass were fixed with 100% methanol for 15 min, and the cells were reacted with a mouse mAb directed against iNOS (N3020, Transduction Laboratories, Lexington, KY; final concentration 0.5 μg/ml in PBS containing 1% BSA) or cNOS (N30020, Transduction Laboratories; final concentration 0.5 μg/ml in PBS containing 1% BSA) for 24 h at 4°C. After washing three times, cells were reacted with FITC-conjugated rabbit anti-mouse IgG antibody (Southern Biotechnology Associates, Birmingham, AL; final concentration 50 μg/ml in PBS containing 1% BSA) for 2 h at room temperature. Cells were washed and examined with a fluorescence microscope.

**Statistical analysis**

The significance of differences in mean values between groups was evaluated by Student's t-test for paired and unpaired samples. Fluorescent data are expressed as the mean ± SEM for six experiments. The analysis of variance was calculated using the Scheffe's post hoc test.

**Results**

**KC-mediated mitochondrial dysfunction and cell injury in AH70 cells and hepatocytes**

Mitochondrial dysfunction and cell injury in AH70 cells induced by the co-culture with KC are summarized in Fig. 1. Changes in relative fluorescence intensity of rhodamine 123 after treatment are shown in Fig. 1(a). KC decreased rhodamine 123 fluorescence intensity in co-cultured AH70 cells to 70% of the baseline value within 60 min and 50% of baseline at 120 min. L-NMMA and DEX significantly abrogated this decrease in rhodamine 123 fluorescence. Addition of 10 ng/ml of 1A29 and 10 ng/ml of WT-3 significantly attenuated this decrease (Fig. 1b). Propidium iodide-positive AH70 cells appeared 60 min after co-culture with KC and the fluorescence intensity increased thereafter. The percentages of propidium iodide-positive AH70 cells 120 min after the treatment are shown in Fig. 1(c). Treatment of AH70 cells with 100 μM L-NMMA, 1 μM of DEX, 10 ng/ml of 1A29 and 10 ng/ml of WT-3 inhibited these changes. These changes did not occur when the supernatant of co-culture between KC and AH70 cells was given to AH70 cells. Co-culture of the hepatocytes with KC did not affect rhodamine 123 fluorescence and propidium iodide-positive hepatocytes did not appear (data not shown).

**ICAM-1 expression**

ICAM-1 was expressed on some KC in their resting state and the ICAM-1-positive KC number increased after co-culture with AH70 cells (Fig. 2a). ICAM-1 fluorescence on KC was significantly increased by addition of IFN-γ (100 U/ml), IL-1β (20 U/ml) or TNF-α (10 ng/ml). The co-culture with AH70 increased KC ICAM-1 expression more than any cytokine. By contrast, the co-culture with hepatocytes did not increase KC ICAM-1 expression (Fig. 2b).

ICAM-1 expression on AH70 cells was lower than on KC, but AH70 ICAM-1 increased by the co-culture with KC (Fig. 3a) as well as by the addition of IFN-γ (100 U/ml), IL-1β (20 U/ml) or TNF-α (10 ng/ml) (Fig. 3b). When AH70 cells were pretreated with 10 ng/ml of 1A29 for 30 min and then co-cultured with KC after washing, ICAM-1 expression on AH70 cells was significantly attenuated. Treatment with WT-3 also decreased AH70 ICAM-1 expression (Fig. 3c). Co-culture did...
Kupffer cell-mediated mitochondrial dysfunction

Fig. 1. Mitochondrial function and propidium iodide-positive cell number after treatments. (a) Temporal changes in relative rhodamine 123 fluorescence intensity. Data are expressed as mean ± SD from five experiments. *P < 0.05 versus baseline value; **P < 0.05 versus corresponding value of co-cultured group. (b) Relative rhodamine 123 fluorescence intensity in AH70 cells after co-culture with KC for 2 h and effects of anti-ICAM-1 and anti-CD18. (c) Percent propidium iodide-positive cell number 120 min after treatment. AH70, AH70 cells alone; AH70 KUP, AH70 cells were co-cultured with KC; AH70 KUP DEX, 1 μM of DEX was added to the co-culture; AH70 KUP L-NMMA, 100 μM of L-NMMA was added to the co-culture; AH70 KUP 1A29, 10 ng/ml of 1A29 was added to the co-culture; AH70 KUP WT-3, 10 ng/ml of WT-3 was added to the co-culture. Data are expressed as mean ± SD from each 10 flames in five experiments. *P < 0.05 (comparison between co-culture with and without treatment); **P < 0.01 (comparison between AH70 alone and co-culture).

Fig. 2. ICAM-1 expression on KC after treatment (a) Flow cytometric analysis of KC. The negative peak for the histogram is stained only with the FITC-conjugated goat anti-mouse secondary reagent. Cell number is given on the ordinate and log of fluorescence intensity is given on the abscissa. The expression of ICAM-1 on KC 2 h before (open) and after (shadowed) co-culture with AH70 cells is shown. (b) Changes in percent fluorescence-positive cells after cytokines or co-culture are shown. Data are expressed as mean ± SD from each of 10 flames in five experiments. *P < 0.05 compared with baseline value, **P < 0.01 compared with baseline value, NS, not significant; HEP, co-cultured with hepatocytes, AH70, co-cultured with AH70 cells not increase ICAM-1 expression on hepatocytes, nor did IFN-γ, IL-1β or TNF-α (data not shown).

CD18 expression

CD18 expression on KC and AH70 cells increased after co-culture each other (Fig. 4a and b); however, three cytokines did not change CD18 expression. Hepatocytes also did not change its expression (Fig. 4c). Treatment of AH70 cells with 10 ng/ml of 1A29 for 30 min significantly decreased KC CD18 expression.

TNF-α and IFN-γ production

TNF-α and IFN-γ production from cells (2.5×10^5 KC/ml, 2.5×10^6 AH70 cells/ml) in culture supernatants was assayed. TNF-α production was significantly increased by the co-culture but IFN-γ production was not detected at all. Addition of 10 ng/ml of 1A29 or WT-3 significantly decreased the production of TNF-α; however, these antibodies did not completely abrogate its production (Table 1).
NOS activity in KC

KC from co-culture could be purified successfully. Figure 5(a) shows the iNOS and cNOS activities in KC. A marked increase in iNOS activity was observed in KC after co-culture with AH70 cells, although no significant increase in cNOS activity was observed. L-NMMA and DEX significantly attenuated the increase in iNOS activity in KC. In AH70 cells, cNOS and iNOS activities were not increased by co-culture with KC (data not shown). NOS activity was serially measured after co-culture (Fig. 5b), reaching a peak value about 12 h after co-culture.

\[ NO_x \] concentration in co-culture supernatant

[NO\(_x\)] concentrations from co-culture increased gradually over 48 h. [NO\(_x\)] in the supernatant of KC alone (1x10\(^6\) KC/ml) and that of AH70 cells (1x10\(^7\) AH70 cells/ml) was measured after 2 h culture. TNF-\(\alpha\) (50 ng/ml) and IFN-\(\gamma\) (1000 IU/ml) significantly increased [NO\(_x\)] in culture medium of KC (1x10\(^6\) cells/ml for 4 h) (Table 2). [NO\(_x\)] in the co-culture supernatant after 2 h was significantly higher than that of KC alone, and L-NMMA, DEX, 1A29 and WT-3 significantly attenuated this increase.

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**Fig. 3.** ICAM-1 expression on AH70 cells after treatment. (a) Flow cytometric analysis of AH70 cells. The negative peak for the histogram is stained only with the FITC-conjugated goat anti-mouse secondary reagent. Cell number is given on the ordinate and log of fluorescence intensity is given on the abscissa. The expression of ICAM-1 on AH70 cells 2 h before (open) and after (shadowed) co-culture with KC is shown. (b) Percent ICAM-1 fluorescence-positive AH70 cells before and after various treatments. Data are expressed as mean ± SD from each of 10 flumes in five experiments. *P < 0.05, **P < 0.01, NS, not significant; AH70 cells were co-cultured with Kupffer cells (KUP), pretreated with 1A29 and co-cultured with Kupffer cells (KUP 1A29), pretreated with WT-3 and co-cultured with Kupffer cells (KUP WT-3).

**Fig. 4.** CD18 expression on KC after treatment. (a) Flow cytometric analysis of KC. The negative peak for histogram is stained only with the FITC-conjugated rabbit anti-mouse secondary reagent. The expression of CD18 on KC 2 h before (open) and after (shadowed) co-culture with AH70 cells is shown. (b) CD18 expression on AH70 cells after treatment. (c) CD18 expression on Kupffer cells. Changes in percent fluorescence-positive cells after cytokines treatments or co-culture are shown. Data are expressed as mean ± SD from each of 10 flumes in five experiments. *P < 0.05, compared with baseline value; NS, not significant; KC were co-cultured with hepatocytes (HEP); co-cultured with AH70 cells (AH70); treated with 1A29 and co-cultured with AH70 cells (AH70 1A29).
to an enhancement of adhesion between endothelial cells, membrane expression of ICAM-1 increases several-fold. stimulation of endothelial cells with IFN-γ, IL-1β or TNF-α.

Discussion

It has been known that KC are activated by cytokines; in this study, we used IFN-γ, IL-1β and TNF-α to stimulate KC. These cytokines increased ICAM-1 expression on KC and AH70 cells, but CD18 expression on KC was not changed by these cytokines. These observations are compatible with other studies of lymphocytes or endothelial cells (16-21). Upon stimulation of endothelial cells with IFN-γ, IL-1β or TNF-α, membrane expression of ICAM-1 increases several-fold. Increased ICAM-1 expression on endothelial cells may lead to an enhancement of adhesion between endothelial cells and leukocytes. The interaction between KC and AH70 cells induced the production of TNF-α in this study and this cytokine in turn stimulated an increased expression of ICAM-1 but not CD18. TNF-α produced by 2.5x10^5 KC was about 2 ng and a compatible dose was used in the KC stimulation. However, IFN-γ, IL-1β and TNF-α did not sufficiently increase ICAM-1 expression on AH70 cells to the level induced by co-culture, and an increase in CD18 was not seen by the treatment with these cytokines. It also was demonstrated that blocking of the ICAM-1 molecule partially inhibits TNF-α production.

Table 1. TNF-α production in culture supernatants

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<th>Cells and treatments</th>
<th>TNF-α (pg/ml)</th>
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<tr>
<td>AH70</td>
<td>32.0 ± 2.7</td>
</tr>
<tr>
<td>KC</td>
<td>65.3 ± 11.0</td>
</tr>
<tr>
<td>Co-culture</td>
<td>2047.5 ± 110.4</td>
</tr>
<tr>
<td>+1A29</td>
<td>946.0 ± 34.9a</td>
</tr>
<tr>
<td>+WT-3</td>
<td>842.3 ± 36.5a</td>
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*P < 0.05 compared with co-culture level

Table 2. NOx concentrations

<table>
<thead>
<tr>
<th>Cells and treatments</th>
<th>NOx concentration (μM)</th>
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<tbody>
<tr>
<td>AH70</td>
<td>9.6 ± 0.4</td>
</tr>
<tr>
<td>KC</td>
<td>25.3 ± 3.3</td>
</tr>
<tr>
<td>+TNF-α</td>
<td>85.4 ± 11.3a</td>
</tr>
<tr>
<td>+IFN-γ</td>
<td>46.9 ± 5.7a</td>
</tr>
<tr>
<td>Co-culture</td>
<td>160.4 ± 12.8</td>
</tr>
<tr>
<td>+1A29</td>
<td>50.3 ± 5.3b</td>
</tr>
<tr>
<td>+WT-3</td>
<td>44.5 ± 6.2b</td>
</tr>
<tr>
<td>+DEX</td>
<td>27.8 ± 4.8b</td>
</tr>
<tr>
<td>+L-NMMA</td>
<td>25.7 ± 3.2b</td>
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</tbody>
</table>

*P < 0.01 compared with KC level

Fig. 5. NOS activity in KC. (a) The activities of iNOS and cNOS in KC, and the effect of L-NMMA and DEX on this activity. Data are expressed as mean ± SD from five experiments. *P < 0.05 versus NOS activity at 0 h, #P < 0.05 versus corresponding value of the coculture group. (b) Serial alterations in NOS activity in KC after co-culture. KC were co-cultured with AH70 cells for the indicated time. Each experiment was carried out at each time. *P < 0.01 versus NOS activity at 0 h.
NO originates from KC. This is compatible with the results described by Isebo et al. (26) and Aono et al. (27). In leukocytes, cell adhesion by ICAM-1/CD18 induces production of oxidants leading to activation of leukocytes (28). Similarly, KC and cancer cell adhesion induced iNOS activity leading to NO production in KC. Since the cloning of the NOS gene was achieved (29), a direct effect of cytokines on induction of NOS has been reported (30). IFN-γ has been demonstrated to promote NO synthesis (31,32). Likely, TNF-α is demonstrated to generate NOX in this study. However, our finding that the NOX increase was inhibited by treatment with anti-ICAM-1 indicates that ICAM-1/CD18-mediated cell–cell adhesion plays a role in this cytotoxicity. A recent demonstration that NO production by murine macrophages is dependent on LFA-1 expression (33) is compatible with our results. From our results combined with these observations, induction of NO in KC after co-culture with AH70 cells is complicated. After the co-culture, ICAM-1/CD18 adhesion may occur and this adhesion may up-regulate CD18 expression. The increase in CD18 expression may enhance adhesion and induce TNF-α release which then promote NO synthesis. NO induces mitochondrial dysfunction and may subsequently destroy the cell membrane.

NO may have an important role for the cytotoxicity (34) and the induction of apoptosis in target cells (35). Pou et al. (36) and Heinzel et al. (37) have reported that NOS induces not only NO but also other oxygen radicals. It has been considered that the cytotoxicity caused by such radicals is not accompanied by necrosis in target cells, which is always seen in NK cell- or lymphocyte-mediated cell cytotoxicity. Recently, the MTT cleavage assay has been used for evaluating the cytotoxicity of macrophages (38). This test measures formazan produced in effector and target cells. However, as observed here, cell-cell contact between KC and target cells further activates KC, resulting in increased formazan production compared with KC alone. Furthermore, KC can eat labeled materials and include them into their bodies by phagocytosis. Thus, MTT cleavage assay may not be suitable for evaluating the cytotoxicity of KC. The rhodamine 123 assay system used in our study, in which fluorescence changes can be evaluated in an individual cell, is a more suitable method to evaluate KC-mediated cytotoxicity.

Acknowledgements
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Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<tr>
<td>KC</td>
<td>Kupffer cells</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>Nω-monomethyl-L-arginine</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
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<tr>
<td>NOX</td>
<td>nitrite and nitrate (NO_2^- + NO_3^-)</td>
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References

Kupffer cell-mediated mitochondrial dysfunction


