Selective Induction of Interleukin-1 Production and Tumor Killing Activity of Macrophages Through Apoptosis by the Inhibition of Oxidative Respiration

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Suppression of mitochondrial respiration and increased glycolysis are characteristic features of activated macrophages. We show here that antimycin A, a respiratory inhibitor, induced interleukin-1 synthesis and tumoricidal activity without inducing tumor necrosis factor or nitric oxide. The induction of tumoricidal activity was resistant to inhibitors of tyrosine-specific protein kinases and intracellular glycprotein transport. The cognate interaction between macrophages and target cells was not a prerequisite for the tumoricidal activity. In contrast, lipopolysaccharide induced the production of interleukin-1, tumor necrosis factor and nitric oxide, the induction of tumoricidal activity being sensitive to genistein and brefeldin A. Antimycin A, like lipopolysaccharide, induced the release of a cytoplasmic enzyme and apoptosis of macrophages. Antimycin A showed anti-metastatic activity in vivo. These results suggest that the inhibition of oxidative respiration would induce apoptosis and the resultant release of soluble effector molecules of macrophages which inhibit tumor metastasis in vivo.

Key words: interleukin-1; antimycin A; activated macrophage; apoptosis; anti-tumor activity

Macrophage infiltration into tumor tissue, and subsequent lysis of the tumor cells by macrophages is believed to be an example of the host defense system against tumor growth. Cultured macrophages kill tumor cells in response to various stimuli including lipopolysaccharide (LPS), lymphpokiné and muramyl dipeptide. Macrophage-mediated tumoricidal activity is dependent on cognate interaction with tumor cells, does not require a phagocytic process, and takes several days for killing. Soluble factors such as an oxygen intermediate, tumor necrosis factor (TNF), and nitric oxide (NO) have been reported to be mediators of macrophage-tumoricidal activity, and the mechanism for tumor killing differs depending on the mechanism for macrophage activation and the nature of the target cells. We previously reported that the anti-fungal antibiotic, ascosfuranone, activated macrophages resulting in increased glycolysis, tumor-killing activity, and interleukin-1 (IL-1) production, while suppressing the lymphocyte function. Ascosfuranone also increased the tumor killing activity of peritoneal cells and spleen cells in vivo, and had prophylactic antitumor and anti-metastatic activities. We have recently found that inhibitors of respiration also increased the glycolysis and killing activity of macrophages, and that ascosfuranone inhibited succinate and NADPH-dependent O2 uptake of mitochondria. These results suggest that inhibition of respiration would activate macrophages to kill tumor cells and protect the host from tumor invasion. Activated macrophages induce characteristic metabolic changes to target cells, including inhibition of the complex I and complex II activity of mitochondrial electron transport, inhibition of acanitase in the citric acid cycle, and inhibition of [1H] thymidine incorporation into DNA. NO produced by macrophages in response to such stimulators as LPS and interferon (IFN)-γ is assumed to be a causative agent which suppresses mitochondrial respiration since NO inhibits iron sulfur enzymes in electron transport. In addition, macrophage differentiation and expression of tumoricidal activity result in the inhibition of mitochondrial iron-sulfur enzymes in macrophages by the L-arginine-dependent pathway. These observations are consistent with the characteristic metabolic changes of activated macrophages including increased glycolysis and decreased oxidative respiration.

In this paper, we further characterize the property of the activated macrophages induced by a typical respiratory inhibitor, antimycin A. Our results demonstrate that inhibiting the macrophage respiration induced IL-1 and tumoricidal activity, which is distinct from the tumoricidal activity induced by LPS, and provides anti-metastatic activity to the host animal.

Materials and Methods

Macrophages. Peritoneal exudate cells (PEC) were induced by injecting 2.5 ml of a 3.8% sterile Brewer's thiglycollate medium into the peritoneal cavity of
C3H/He mice (female, 6 wk-old, Japan Charles River Inc., Yokohama, Japan). Cells were collected from the peritoneal cavity 4 days after the injection, washed with fetal calf serum (FCS)-free Eagle's MEM and used for experiments without further purification.9 Greater than 90% of cells expressed the Ia antigen and Fc receptor as analyzed by flow cytometry.25

**Tumorcidal Activity.** PEC (4 x 10^6 cells) and ^3^Cr-labeled FM3A target cells (2 x 10^4 cells) were cultured in a flat-bottom microtiter well containing 0.2 ml of RPMI 1640 medium supplemented with 50 μg/ml 2-mercaptoethanol, 50 μg/ml of kanamycin, 8 μg/ml of tylosin tartrate, 10% FCS. To block cognate interaction, macrophages and target cells were separated with a membrane filter (Millicell-HA, Millipore). Macrophages (1.2 x 10^6 cells) were cultured in a flat-bottom 24-well plate, and target cells (3 x 10^4 cells) were cultured in the upper chamber. After 16 h, the radioactivity of the supernatant was measured.9 The percent specific lysis was calculated from the formula: maximum chromium release and spontaneous chromium release were defined as cpm in the presence of 1% sodium dodecyl sulfate (SDS) and as cpm in the absence of macrophages, respectively.

\[
\text{% lysis} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}} \times 100
\]

**TNF Activity.** PEC (1 x 10^6 cells) collected from C3H/He mice were cultured in 0.5 ml of RPMI 1640 medium in a flat bottomed 24-well plate. After the culture, the supernatant was collected, and the TNF activity was measured by a cytotoxic assay using L929 fibroblasts.26 L929 cells (8 x 10^4 cells) were cultured with serially diluted test samples in the presence of actinomycin D (2.5 μg/ml) for 18 h. Following this culture, the cells were washed with phosphate-buffered saline (PBS, 0.8% NaCl, pH 7.4) and stained with 0.2% crystal violet for 15 min. The dye was extracted with 0.1 ml of methanol, and the absorbance at 540 nm was measured. One unit/ml of TNF was determined as the concentration resulting in 50% survival of the cells.

**IL-1 Production.** PEC (1 x 10^6 cells/well, 0.5 ml) were cultured in a flat-bottomed 24-well plate. After removal of supernatant, the cells were detached from the bottom by vigorous pipetting and homogenized in 0.5 ml of the medium. The IL-1 activity of the supernatant and the cell lysate was measured by a thymocyte proliferating assay.25 In brief, thymocytes (5 x 10^5 cells) from C3H/HeJ mice (female, 4-6 wk-old, Sankyo Labo Service Co., Tokyo, Japan) were cultured in 0.1 ml of a medium containing 25% test sample and 1 μg/ml of phytohemagglutinin (PHA). After culturing for 48 h, the cells were pulse-labeled with [3H] thymidine (18.5 kBq/well) for 4 h, and the radioactivity of the harvested cells was measured.

**Immunoprecipitation.** PEC (1 x 10^6 cells/well) were cultured for 4 h in a flat-bottomed 24-well plate in methionine and FCS-deficient RPMI 1640 medium in the presence of 3700 kBq/ml of [35S] methionine. The cells were detached by pipetting, washed with PBS, and resuspended in a mixture of 20 mm Tris-HCl (pH 7.2), 1% NaCl, 1% Nonidet P-40, 2 mm phenylmethane-sulfonyl fluoride, 10 μg/ml of leupeptin and 10 μg/ml of antipain. The lysate was used for immunoprecipitation after centrifugation (10,000 g, 10 min). Anti-mouse IL-1β antibodies were incubated with protein A-Sepharose for 1 h at 4°C, and washed twice with PBS. The cell lysate and antibody-coated Sepharose were mixed gently by rotating overnight at 4°C. The Sepharose beads were washed 5 times with 10 mm Tris-HCl (pH 7.2), 1% NaCl, 1% Nonidet P-40, and 0.1% sodium deoxycholate, and then boiled in 2% SDS, 10% glycerol, 60 mm Tris-Hcl (pH 6.8), 0.002% bromophenol blue, and 5% 2-mercaptoethanol. The samples were resolved by 12.5% SDS-PAGE and detected by autoradiography.

**NO production.** PECs (1 x 10^6 cells) were cultured for 48 h, and the supernatant (0.1 ml) was diluted with 10 ml of distilled water and 60 mg of Griess reagent as described in the previous report.27 The absorbance at 520 nm was measured, and the NO amount was calculated from a standard curve produced with NaN3O.

**LDH assay.** β-NADH (30 mm, 12.5 μl) and 7-hydroxy-3-H-phenoxazin-3-one-10-oxide (0.2 mm, 12.5 μl) were incubated with 250 μl of a substrate (10 mm sodium lactate and 50 mm Tris at pH 9.0) for 10 min at room temperature. The reaction was initiated by adding 12.5 μl of the sample and 125 μl of diaphorase (1 unit/ml) to the reaction mixture, and the increase in fluorescence at 582 nm was monitored. LDH activity was calculated by comparing with that of the purified enzyme.

**DNA Fragmentation.** PECs (1 x 10^6 cells) were lysed in 0.5 ml of 50 mm Tris-HCl (pH 8.0), 10 mm EDTA, and 0.5% (v/v) sarcosine. After an overnight incubation with 360 ng/ml of proteinase K at 37°C, 0.5 ml of a TE buffer (100 mm Tris-HCl at pH 8.0 and 5 mm EDTA) was added, and the lysate was centrifuged for 20 min at 15,000 g. The supernatant was extracted with 0.8 ml of phenol, and DNA in the aqueous layer was precipitated with 70% ethanol and 0.3 M sodium acetate. The precipitate was resuspended in 40 μl of TE buffer containing 100 μg/ml of RNaseA and incubated overnight at 37°C. Ten microliters of the sample were loaded on 1% agarose gel and the gel, was stained with ethidium bromide.

**Anti-metastatic Activity.** Metastatic B16 cells isolated from the lung were expanded in vitro. The cells were collected by a trypsin treatment, washed three times with PBS, resuspended in PBS, and injected via the orbital vein into C57BL/6 mice. Sixteen days after implantation, the lung weight was measured.25

**Chemicals.** Antimycin A and LPS were purchased from Sigma Chemicals Co. (St. Louis, MO, U.S.A). PHA was purchased from Welcome Diagnostics (Dartford, UK), FCS from Bioserum (Victoria, Australia),
and the thiglycolate medium from Becton Dickinson (Cockeysville, MO, U.S.A.). The anti-IL-1β antibody (from hybridoma B122) was purchased from Genzyme (Cambridge, MA, U.S.A.), and radioactive materials were purchased from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Ascofur'anone was kindly provided by Dr. T. Hosokawa, all the other reagents being of analytical grade from commercial suppliers.

**Results**

The induction of tumoricidal activity was studied by using an inhibitor of respiration, antimycin A, which inhibits electron transport between complex III and cytochrome c. Macrophages were cultured with 51Cr-labeled target cells for 16 h in the presence of the respiratory inhibitor, and the radioactivity released into the supernatant was measured (Fig. 1). Antimycin A significantly enhanced the tumoricidal activity of macrophages at concentrations above 10 ng/ml in a dose-dependent manner. The effect was comparable to the cytotoxic activity induced by LPS. The tumoricidal activity might have been necrotic because macrophages activated with antimycin A failed to increase the release of radioactivity from [3H] thymidine-labeled target cells. Since the reagent did not affect the spontaneous release of radioactivity from the target cells, the effect was not due to any direct cytotoxicity of antimycin A to the target cells. Similar tumoricidal activity of macrophages has been observed in experiments with different target tumors including a mouse thymoma and a mastocytoma (ref. 12 and data not shown).

TNF and NO are known to be major effector molecules for the tumoricidal activity of macrophages. As shown in Table I, LPS significantly induced the generation of both molecules. In contrast, we failed to detect any significant activity of either molecule in the supernatant from antimycin A-treated macrophages. Instead, the compound reduced the spontaneous release of NO from unstimulated macrophages. These results suggest that distinct molecules, other than NO and TNF, are involved in respiratory inhibitor-mediated tumoricidal activity.

The induction of tumoricidal activity by LPS has been found to be sensitive to inhibitors of tyrosine-specific protein kinases. Consistent with the previous report, genistein inhibited the induction of tumoricidal activity in macrophages by LPS, while the inhibitor had only a negligible effect on that by antimycin A (Fig. 1). In addition, the induction of tumoricidal activity by LPS was significantly inhibited by brefeldin A, an inhibitor of glycoprotein transport between the endoplasmic reticulum and Golgi apparatus. However, the induction of tumoricidal activity by antimycin A was resistant to brefeldin A. The supernatant of the macrophage culture in the presence of antimycin A showed no cytoxicity to the target tumors (data not shown).

The foregoing results indicate that antimycin A-induced tumoricidal activity was independent of any soluble factors released from macrophages, like Fas-

**Table I. Effect of Respiratory Inhibitors on Inducing TNF and NO**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>TNF (unit/ml)</th>
<th>NO (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>&lt;4</td>
<td>20</td>
</tr>
<tr>
<td>antimycin A</td>
<td>&lt;4</td>
<td>&lt;3</td>
</tr>
<tr>
<td>LPS</td>
<td>59.7</td>
<td>204</td>
</tr>
</tbody>
</table>

Macrophages were cultured with antimycin A (30 ng/ml) or LPS (10 µg/ml) for 16 h and the concentration of TNF and NO in the culture supernatant was measured. Typical results of more than three experiments each are shown.

![Fig. 1. Effect of Brefeldin A and Genistein on the Macrophage Tumoricidal Activity Induced by LPS and Antimycin A.](image1)

**Fig. 2. Independence of the Tumoricidal Activity Induced by Antimycin A from Cognate Interaction.**

PECs and 51Cr-labeled FM3A cells were cultured in the presence of antimycin A (30 ng/ml) or LPS (10 µg/ml) in 24-well flat bottomed plates. A small chamber with membrane filter bottom was placed in each well of the plate. Cognate interaction + FM3A and PEC were cocultured in the lower well; Cognate interaction − FM3A cells were cultured separately in the upper chamber, and PEC in the lower well. Sixteen hours after initiation of the culture, the radioactivity released into the supernatant was measured. A typical result of three independent experiments is shown.
mediated cytolysis by cytotoxic T cells. To verify this, macrophages and target cells were separated by a membrane filter and incubated in the presence of activators (Fig. 2). Unexpectedly, the inhibition of cell-to-cell contact did not affect the antimycin A-induced tumoricidal activity, while the LPS-induced tumoricidal activity was significantly suppressed by inhibiting the cognate interaction. This result suggests that the antimycin A-induced tumoricidal activity of macrophages would be dependent on soluble factors which are released via a mechanism distinct from the Golgi-mediated transport system.

We have previously reported that ascofuranone induced the IL-1 production of macrophages. Therefore, PECs were cultured with antimycin A, and the IL-1 activity was measured by a thymocyte proliferation assay (Fig. 3). In the antimycin A-treated cells, IL-1 activity in the culture supernatant was detected 16 h after initiating culture, although the activity was less than that induced by LPS. In contrast, IL-1 activity in a cell lysate of the antimycin A-treated cells (30 ng/ml) was detected as early as 8 h after the initiation, the amount being comparable to that induced by 10 μg/ml of LPS. The thymocyte-proliferating activity was almost completely neutralized by the anti-IL-1β antibody from hybridoma B122 (data not shown).

To test the requirement of de novo synthesis for the IL-1 production induced by antimycin A, PECs were cultured with antimycin A in the presence of cycloheximide, an inhibitor of protein synthesis. When PECs were incubated with [3H] leucine for 4 h in the presence of cycloheximide, incorporation of the radioactive precursor was inhibited by 71% and 36% at 1 μg/ml and 100 ng/ml of cycloheximide, respectively, without any significant effect on the incorporation of [3H] uridine (data not shown). These concentrations of cycloheximide significantly inhibited IL-1 production induced by antimycin A as well as that induced by LPS (Fig. 4A). This result was further confirmed by immunoprecipitation. PECs were incubated with antimycin A or LPS in the presence of [35S] methionine, and the lysate was immunoprecipitated with the anti-IL-1β antibody. Consistent with the result of Fig. 3, the lysate from antimycin A-treated PEC contained a significant amount of IL-1β, this being comparable to that induced by LPS (Fig. 4B).

It has been reported that IL-1 was secreted via self in-
Fig. 6. Induction of Apoptosis of Macrophages by LPS and Antimycin A.

PECs were cultured with the drugs indicated in the figure for 4 h. After washing, the cells were further incubated in the absence of the drugs for 16 h, and the DNA fragmentation was analyzed by agarose gel electrophoresis.

Table II. Suppression of the Pulmonary Metastasis of B16 by Respiratory Inhibitors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Lung weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>4</td>
<td>420.3 ± 156.4</td>
</tr>
<tr>
<td>antimycin A (0.25 mg/kg)</td>
<td>4</td>
<td>187.0 ± 33.2</td>
</tr>
</tbody>
</table>

BDF1 mice were i.p. treated with antimycin A (0.25 mg/kg), and B16 cells were injected via an orbital vein 24 h after treatment. The lungs were dissected and weighed 16 days after the implantation.

* Mean ± SD.

† Statistically significant compared to the untreated group (p<0.05, t-test).

We finally tested the anti-metastatic activity of antimycin A in vivo against B16 melanoma. Antimycin A was i.p. injected once 1 day prior to the implantation of B16. Antimycin A significantly suppressed the pulmonary metastasis of B16 cells and reduced the weight of lungs resulting from tumor metastasis (Table II). The lung weight of antimycin A-treated mice was not significantly different from that of normal mice (data not shown). The applied dose of antimycin A (0.25 mg/kg) did not reduce the body weight of mice, but treatment with more than 1 mg/kg sometimes killed the mice.

Discussion

We have reported that ascofurane activated macrophages to enhance glycolysis, tumoricidal activity and IL-1 production.\(^9\) We have recently demonstrated that ascofurane inhibited mitochondrial respiration\(^{12-13}\) and antimycin A enhanced glycolysis and tumoricidal activity of macrophages. We have investigated here the property of tumoricidal activity of macrophages induced by antimycin A. The induction of macrophage tumoricidal activity by antimycin A was resistant to genistein and brefeldin A, although no cognate interaction was necessary for this activity. Moreover, antimycin A, like LPS, induced de novo IL-1 synthesis and apoptosis of macrophages. All the other kinds of respiratory inhibitors we tested, including oligomycin and rotenone, also induced tumoricidal activity, IL-1 production, and apoptosis of macrophages (data not shown). We also found that antimycin A showed anti-metastatic activities in vivo. These results suggest that the inhibition of macrophage respiration induced soluble anti-tumor factors through apoptosis which resulted in the anti-metastatic effect in vivo.

In contrast to LPS, antimycin A did not induce TNF and NO production, and the induction of tumoricidal activity by antimycin A was resistant to brefeldin A. However, tumoricidal activity was not inhibited by separating the target cells and macrophages, suggesting that soluble factors involved in the tumoricidal activity were released by a mechanism different from classical Golgi-mediated transport. IL-1 is secreted through a non-vesicle transport system, presumably via self-injury induction by activators of macrophages.\(^{31-36}\) Consistent with these reports, antimycin A, like LPS, induced IL-1 production and apoptosis of macrophages. Thus, it is possible that some cytotoxic factors in the cytoplasm were released by a mechanism similar to that for IL-1 production.

Although IL-1 promotes human monocyte-mediated cytotoxicity,\(^{37}\) it is less possible that IL-1 would mediate antimycin A-induced tumoricidal activity, because IL-1 was stably present in the culture supernatant of antimycin A-treated macrophages while the supernatant had no cytotoxicity toward tumor cells. It might also be possible that labile lysosomal enzymes contributed to the tumoricidal activity. We failed to find any tumor killing activity of antimycin A-treated macrophages by preincubating the macrophages, tumor cells or both. Thus, antimycin A must be present during the tumor killing assay with macrophages and tumor cells. It is possible that
antimycin A directly killed the tumor cells in cooperation with factors from the macrophages. Elucidation of the mechanism for this tumor killing activity is a subject for our future study.

Mitochondria have been suggested to be involved in apoptosis. Hypoxia-induced apoptotic cells release cytochrome c from mitochondria, and the anti-apoptosis protein, bcl-2, blocks the release of cytochrome c. It has also been shown that cytochrome c was necessary to induce apoptosis in a cell-free system. Albina et al. have reported that LPS and IFN-γ induced apoptosis of macrophages via an NO-dependent mechanism. They also demonstrated that inhibitors of the tricarboxylic cycle induced apoptosis. NO suppressed mitochondrial respiration by inhibiting the activity of iron-sulfur enzymes in the citric acid cycle such as aconitase. Decreased oxidative phosphorylation and increased glycolysis have been observed in LPS-activated macrophages. These observations suggest that LPS would also inhibit respiration of macrophages through an NO-mediated mechanism and the respiratory inhibition-dependent mechanism is presumably involved in LPS-mediated apoptosis of macrophages. Alternatively, it is possible that the inhibition of mitochondrial respiration opens a mitochondrial megachannel in an NO-independent manner, as Zanzami et al. have recently suggested.

The induction of IL-1 was selective in the respect that antimycin A did not induce TNF. This suggests that the regulation of TNF and IL-1 expression was differentially controlled, and that the inhibition of respiration activated the signal pathway toward IL-1, but not TNF, expression. While the induction of tumoricidal activity by LPS was inhibited by inhibitors of tyrosine-specific protein kinases including genistein, herbimycin A and tyrphostin, the induction of tumoricidal activity by antimycin A was not inhibited by genistein. Since genistein did not inhibit IFN-γ-induced tumoricidal activity, tyrosine-specific protein kinases would be specifically involved in signal transduction from macrophage LPS-receptors. In contrast, LPS-induced enhancement of the glycolysis of macrophages was inhibited by the calmodulin inhibitors, trifluoperazine and No. 233. Antimycin A-induced enhancement of glycolysis was also inhibited by calmodulin inhibitors (data not shown). It is known that IL-1 production but not TNF and NO production, is regulated by a calmodulin-dependent mechanism. Thus, calmodulin is likely to participate in downstream signal transduction for metabolic changes, IL-1 production, and possibly apoptosis of macrophages activated by LPS and antimycin A.

Antimycin A inhibited the pulmonary metastasis of melanoma, which has also been observed with other respiratory inhibitors such as rotenone (unpublished result) and ascorfuranone. In vivo treatment with ascorfuranone induced cytotoxic inflammatory cells in the spleen and peritoneal cavity, increased phagocytic activity as shown by the results of a carbon clearance test, and increased prophylactic antitumor activity which was suppressed by i.p. treatment with silica. These observations suggest that the antitumor activity of respiratory inhibitors is mediated by the activation of macrophages. However, it is less plausible to assume that the antitumor activity and the anti-metastatic activity were due to the direct cytotoxic effect of macrophages observed in this present work because the antitumor activity was obtained by administering the inhibitors prior to the tumor inoculation. Rather, the activation of peritoneal macrophages by respiratory inhibitors might trigger an activation cascade for inducing effector inflammatory cells which would prevent tumor propagation and metastasis.

In conclusion, our present study demonstrates that the inhibition of mitochondrial respiration activated macrophages to kill tumor cells by the induction of apoptosis. The oxygen concentration at an inflammatory site is assumed to be suppressed. Macrophages secreted angiogenesis factors into the culture medium when cultured in a hypoxic environment. Exposure of macrophages to hypoxia with subsequent reoxygenation resulted in significant production of IL-1, but not TNF. Our results also suggest that the hypoxic environment at an inflammatory sites would locally activate macrophages. These results implicate the inhibition of respiration under the hypoxic conditions to be one of the mechanisms of activation of macrophages infiltrating the tumor site in vivo.

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