Activated Murine Macrophages Induce Apoptosis in Tumor Cells through Nitric Oxide-dependent or -independent Mechanisms

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ABSTRACT

Work reported here investigated aspects of macrophage-mediated tumor cell death, in particular the role of apoptosis as a mechanism for nitric oxide (NO)-mediated macrophage tumor cytotoxicity. Nitric oxide induced apoptosis in P815 cells in macrophage P815 cocultures where fragmentation of tumor cell DNA was characterized to fulfill the criteria for apoptosis (8–10). This occurred in a NO-dependent manner and was prevented by a specific inhibitor of NO synthesis. DNA from P815 cells separated from macrophages in culture by a cell-impermeable membrane or exposed to authentic NO gas showed the pattern of internucleosomal cleavage that is characteristic of apoptosis. Additionally, culture of P815 cells with the NO donor sodium nitroprusside was followed by DNA fragmentation. Macrophages also induced apoptosis in L929 cells but, in this case, apoptosis was NO independent and partially inhibited in cocultures by an anti-tumor necrosis factor (TNF-α) monoclonal antibody. The anti-tumor necrosis factor monoclonal antibody fully prevented apoptosis when macrophages and L929 were separated by a cell-impermeable membrane. Exposure of L929 cells to NO gas or sodium nitroprusside did not result in their apoptotic death. Like other immune cytotoxic cells, macrophages can determine tumor cell death through the induction of apoptosis and do so through more than one effector mechanism.

INTRODUCTION

Activated macrophages have the capacity to recognize and destroy neoplastic cells. To eradicate individual tumor cells or circulating micrometastases in vivo macrophages may rely upon the expression of one or more cytotoxic effector mechanisms, some requiring cell-to-cell contact and others dependent on the elaboration of soluble effector molecules such as peroxide, cytolytic proteases, NO, or TNF-α (1–5). While these killing mechanisms do not appear to be mutually exclusive and may act synergistically, tumor cells have been shown to be differentially sensitive to specific lytic mediators. For example, P815 cells are particularly sensitive to the cytotoxic effects of l-arginine-derived NO and reported resistant to TNF-α, while conversely, L929 fibroblasts are prototypically susceptible to the cytotoxic effects of TNF-α but resistant to NO (6, 7).

Although some details on the biological effects of the above mentioned cytotoxic effectors are known, present understanding of the intimate mechanisms by which they cause tumor cell death remains incomplete. In what appeared to be a pertinent observation, recent work from this laboratory demonstrated that macrophages are not themselves immune from the cytotoxic effects of NO (7). When induced to produce NO in culture by activating signals, macrophages exhibit a pattern of metabolic inhibition, including suppression of oxidative metabolism and protein synthesis that is similar to that observed in tumor cells exposed to NO, and die prematurely (2, 7). The mechanism of macrophage death associated with the production of NO was characterized to fulfill the criteria for apoptosis (8–10).

The apparent mechanistic similarity of the toxicity of NO for macrophages or tumor cells suggested that macrophage-dependent NO-mediated killing of susceptible tumor cells could also proceed through the induction of apoptosis in the targets. Results to be shown from experiments employing the NO-sensitive P815 cell line provided evidence in support of this hypothesis.

Additional experiments were suggested by the reported insensitivity of L929 cells to NO and were directed to investigate whether apoptosis is a generalized phenomenon during macrophage tumor killing or one restricted to NO-mediated cytotoxicity (6). Results from those experiments revealed that macrophages also induce apoptosis in L929 cells, but that this effect is independent from NO and partially dependent on the production of TNF-α by the macrophages. Taken together, these results demonstrate that activated macrophages can kill tumor cells, through the induction of apoptosis and that they do so through NO-dependent or -independent mechanisms.

MATERIALS AND METHODS

Cell Culture. Peritoneal macrophages were obtained from male B6D2F1 mice (Taconic, Germantown, NY). The animals were housed in an isolation environment on their arrival and monitored by Brown University/Rhode Island Hospital Veterinary personnel. Cells were obtained by sterile lavage of the peritoneal cavity with Hanks’ balanced salt solution supplemented with 1% heat-inactivated fetal calf serum (HyClone Laboratories, Logan, UT), 10 mM 3-(N-morpholino)-propanesulfonic acid, and antibiotics. The cells thus obtained were washed, resuspended in culture medium consisting of RPMI-1640 (GIBCO, Grand Island, NY), 10% fetal calf serum, 10 mM 3-(N-morpholino)-propanesulfonic acid, 5 × 10–5 M 2-mercaptoethanol and antibiotics, and plated (2 × 106 cells/well) in 24-well tissue culture plates (Costar, Cambridge, MA). Nonadherent cells were removed by repeated washing after 2 h of incubation at 37°C in 7% CO2 in air. Adherent cells were >95% macrophages by Wright and nonspecific esterase staining. All media and additives contained <3 µg/ml endotoxin as determined by a chromogenic assay (QCL 1000; Whittaker Bioproducts, Walkersville, MD).

The macrophages were activated, when so indicated, through the addition of recombinant murine IFN-γ (10 units/ml; Genzyme, Cambridge, MA) and LPS (1 µg/ml from Escherichia coli 055:BS; Difco Laboratories, Detroit, MI) to the cultures. These concentrations were experimentally determined to result in maximal NO2− production and in vitro cytotoxicity against the tumor cells used in this study. The activating agents were dispensed into the wells immediately prior to the addition of the tumor cells. The inhibitor of NO-synthesis N-MMA (0.5 mM; Calbiochem, La Jolla, CA) or an antimurine TNF-α monoclonal antibody (500 ng/ml; Genzyme) were also included in the cultures when indicated. Supernatants collected for NO2− or TNF-α analysis were stored at –80°C until assayed.

Tumor cells used in these studies were of murine derivation. The P815 mastocytoma cells were obtained from Dr. Carl F. Nathan (Rockefeller University, New York, NY) and the L clone 929 fibroblast-like cells from the American Type Culture Collection (Rockville, MD).

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(DuPont New England Nuclear, Boston, MA) for 2 h at 37°C. The cells were then washed extensively and 10^6 tumor cells were added into wells containing peritoneal macrophages adhered as described above. Final culture volume was 1 ml. The cells were then incubated at 37°C in 7% CO₂ in air. At the times indicated in the text, aliquots of supernatants were collected and counted for 51Cr (1470 Wizard; Pharmacia-LKB, Gaithersburg, MD) or used for NO₂⁻ or TNF-α analysis. Total 51Cr content of the cells was determined by lysis in 1% Triton X-100. Spontaneous release was determined in the absence of macrophages but in the presence of IFN-γ/LPS and was 5–25% of the total depending on the tumor and length of incubation. The percentage of specific 51Cr release was determined as follows:

\[
\% \text{ of } 51\text{Cr release} = \frac{\text{Experimental cpm} - \text{spontaneous cpm}}{\text{Total cpm} - \text{spontaneous cpm}} \times 100
\]

All data reported make reference to the specific 51Cr release thus calculated.

**DNA Fragmentation Assay.** Tumor cells in the logarithmic phase of growth were radiolabeled in culture medium with 0.04 μCi/ml [methyl-3H]thymidine (DuPont New England Nuclear) for 18 h, washed, and cultured with macrophages under the conditions described for the 51Cr release assay. Culture supernatants (M) were collected at the indicated time points and saved following centrifugation (700 × g for 15 min) at 4°C. The cells were treated with ice-cold lysing buffer (25 mM sodium acetate, pH 6.6) for 1 h. DNA from these cells was separated by centrifugation at 13,000 × g for 15 min into fragmented low molecular weight (supernatant; S) and intact high molecular weight (pellet; P) species. Radioactivity was determined by liquid scintillation counting. Specific fragmentation was calculated as:

\[
\% \text{ of specific fragmentation} = \frac{\text{Experimental cpm} (M + S) - \text{spontaneous cpm} (M + S)}{\text{Experimental cpm} (M + S + P) - \text{spontaneous cpm} (M + S + P)} \times 100
\]

In this formula, experimental and spontaneous refer to radiolabeled DNA obtained from tumor cells cultured in the presence or absence of macrophages, respectively. IFN-γ/LPS were present in the tumor cell cultures used to determine spontaneous fragmentation. Spontaneous fragmentation was < 20% at all time points for both tumor cell lines. Data presented on DNA fragmentation make reference to the specific fragmentation values calculated using this formula.

**Transwell Experiments.** For experiments using the Transwell 24-well plate system (Costar), macrophages were seeded in quadruplicate in the lower chamber of the system (2 × 10^6/well), washed, and overlaid with 800-μl culture medium containing additives as indicated. Precultured Transwell inserts were placed in the wells and tumor cells (1 × 10^6/200-μl culture medium) were added within the inserts. Experiments measuring NO₂⁻ accumulation, [3H]thymidine-labeled DNA fragmentation, and 51Cr release were performed as indicated above. In additional experiments, the Transwell inserts containing the tumor cells were carefully removed from the culture at the end of 12 h and DNA from these cells was subjected to extraction and electrophoresis as indicated below.

**Nitrile Determination.** The concentration of NO₂⁻ in culture supernatants was measured with Griess reagent and interpreted as indicative of the flux of l-arginine through NO-synthase (7, 11).

**DNA Isolation and Gel Electrophoresis.** Extraction of DNA was performed as previously described (8). Briefly, total DNA was obtained by Pronase E digestion, phenol:chloroform (1:1) extraction, and precipitation in ½/volumes 3 M sodium acetate (pH 6.6) and 2.5 volumes ethanol at −80°C for 20 h. After centrifugation at 13,000 × g for 10 min, DNA pellets were resuspended in 50 μl of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. Samples were then treated with 10 units/ml DNase-free RNase (Sigma Chemical Co., St. Louis, MO) for 1 h at 37°C and quenched with 0.5 mM EDTA. Electrophoresis was performed on a 1% agarose gel at 50 V for 2 h in the presence of 0.1 μg ethidium bromide. A 123-base pair ladder (Gibco-BRL) was included as a molecular weight marker.

**Cell Treatment with Authentic NO.** Aliquots of both cell lines under study (2 × 10^5 cell/sample) were placed in sterile polystyrene tubes with gas-tight rubber stoppers in a 1.5-ml culture medium. The cultures were deoxygenated by flushing with 100% N₂ for 30 min and 1% NO in 99% N₂ (passed through solid KOH and 10% KOH in water prior to its addition to the cultures) or 100% N₂ bubbled gently into the cultures for 30 min thereafter. At the end of this period the cultures were flushed briefly with 95% O₂/5% CO₂ to ensure the complete oxidation of NO, and the cells were cultured overnight at 37°C in 7% CO₂ in air before DNA extraction and electrophoresis (8).

**Tumor Cell Culture with SNP.** P815 and L929 cells were prelabeled with [methyl-3H]thymidine and then cultured (1.5 × 10^6 cells in a 200-μl culture medium) with or without the addition of SNP (Sigma) at a 0.1, 0.5, or 1 mM concentration for 24 h. The concentration of NO₂⁻ in the culture supernatants and the extent of DNA fragmentation were determined as indicated above.

**TNF-α Bioassay.** For the determination of TNF-α bioactivity in culture supernatants, L929 cells were seeded at 3 × 10^5/well in a 150-μl culture medium in 96-well plates and incubated overnight. Following the addition of recombinant murine TNF-α (Genzyme) standard curves. The specificity of the assay for TNF-α bioactivity was demonstrated by the complete suppression of cytotoxicity by the antimurine TNF-α mAb used in these experiments.

**Data Presentation and Analysis.** Data reported are means ± 1 SD from a representative of at least four identical experiments. Data analysis was by analysis of variance F test.

**RESULTS**

Macrophage-derived NO Induces Early DNA Fragmentation and Delayed Plasma Membrane Failure in Cocultured P815 Cells. Fig. 1 shows the time course of the accumulation of NO₂⁻, a degradation product of macrophage-derived NO, the fragmentation of [3H]thymidine-labeled DNA, and the release of 51Cr from P815 cells during coculture with macrophages. As indicated in “Materials and Methods,” macrophage activation was induced through the addition of IFN-γ (10 units/ml) and LPS (1 μg/ml) at the beginning of culture. Following this activation protocol, the appearance of detectable NO₂⁻ in the supernatants occurred after a 6-h culture and proceeded linearly thereafter through the remainder of the experiment. A similar temporal pattern was detected in measurements of DNA fragmentation, which amounted to 4%, 22%, and 41% of total labeled DNA at 6, 12, and 18 h, respectively. DNA fragmentation preceded plasma membrane damage since the release of 51Cr from the P815 cells was 1.3% at 6 h, 5% at 12 h, and 14% at 18 h.

The production of NO₂⁻, DNA fragmentation, and the release of 51Cr required macrophage activation. None were detected in cultures lacking IFN-γ and LPS (data not shown). Moreover, target cell damage was dependent on the production of NO by the macrophages because suppressing NO production through the addition of N-MMA to the cultures prevented the fragmentation of DNA and the release of 51Cr. Treatment of P815 cells with recombinant murine TNF-α in concentrations up to 50 ng/ml for 24 h did not result in DNA fragmentation or the release of 51Cr (data not shown).

**The Fragmentation of DNA in P815 Cells Associated with the Production of NO by Macrophages Follows the Specific Pattern of Internucleosomal Cleavage Found in Apoptosis.** To determine whether the fragmentation of P815 DNA detected by the use of labeled thymidine conformed to the pattern of oligonucleotides with molecular weight multiples of 180–200 base pairs, characteristic of apoptotic death, macrophages and P815 cells were cultured in differ-
from the production of NO by the macrophages, the addition of Table 3, maximal soluble TNF-α bioactivity was present in the The addition of N-MMA decreased nitrite accumulation, DNA fragmentation, and 51Cr release at 12 and 18 h (P < 0.05; analysis of variance F test).

Macrophages and P815 cells were cultured in different compartments of Transwell plates as indicated in “Materials and Methods.” The target cells were prelabeled with either [methyl-3H]-thymidine or 51Cr as indicated. After a 12-h culture, the NO2 content of the supernatants, specific fragmentation of DNA, or release of 51Cr were measured. Nitrite and P815 cocultured with macrophages for 12 h shows the characteristic “ladder” appearance that is a hallmark of apoptosis (Fig. 2).

Table 1 Activated macrophages induce apoptosis in P815 cells in a NO-dependent fashion even when separated by a cell-impermeable membrane

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NO2 (μM)</th>
<th>DNA fragmentation (%)</th>
<th>51Cr release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.6 ± 0.6</td>
<td>2.7 ± 2.9</td>
<td>2.1 ± 1.7</td>
</tr>
<tr>
<td>IFN-γ/LPS</td>
<td>32.6 ± 0.6</td>
<td>16.9 ± 3.4</td>
<td>6.7 ± 1.1</td>
</tr>
<tr>
<td>IFN-γ/LPS + N-MMA</td>
<td>6.6 ± 0.2</td>
<td>0.8 ± 0.2</td>
<td>2.5 ± 0.5</td>
</tr>
</tbody>
</table>

* P < 0.05 versus cultures containing IFN-γ and LPS.

a P < 0.05 versus cultures without additives.

ent compartments of Transwell microtiter plates as indicated in “Materials and Methods.” Data reported in Table 1 demonstrates that IFN-γ/LPS-activated macrophages induced DNA fragmentation and 51Cr release in P815 cells in a N-MMA-inhibitable fashion even when separated by a cell-impermeable membrane. DNA extracted from P815 cocultured with macrophages for 12 h shows the characteristic “ladder” appearance that is a hallmark of apoptosis (Fig. 2).

NO Induces DNA Fragmentation in P815 Cells. Confirmatory evidence that NO can induce apoptosis in P815 was obtained following the exposure of these cells to authentic NO gas or to the NO donor SNP. Results from these experiments are shown in Fig. 3 in which a typical DNA fragmentation pattern is visible after overnight culture of cells exposed to NO gas for 30 min but not in those exposed to N2, and in Table 2, where it is shown that DNA fragmentation observed a dose-response relationship with the concentration of SNP added to the cultures.

NO-insensitive L929 Cells Also Undergo Apoptosis When Cocultured with Activated Macrophages: an Anti-TNF-α Monoclonal Antibody Is Partially Protective. Fig. 4 shows results obtained in cocultures of macrophages and L929 cells. In contrast to findings in similar experiments using P815 cells, DNA fragmentation and 51Cr release were detectable at 6 h, a time point when NO2 was only beginning to appear in the supernatants but when, as shown in Table 3, maximal soluble TNF-α bioactivity was present in the cultures. Cell membrane disruption and DNA fragmentation approached maximal values after a 12-h culture without appearing to maintain a dose-response relationship with the rate of NO2 accumulation. Confirming the independence of the observed cytotoxic effects from the production of NO by the macrophages, the addition of N-MMA to the cultures did not prevent DNA fragmentation (48 ± 2% at 18 h) or 51Cr release (22 ± 3% at 18 h) from the target cells. The inclusion of an anti-TNF-α mAb in the cocultures reduced DNA fragmentation by 33% and 51Cr release by 63% at 12 h, suggesting the involvement of a TNF-α-dependent mechanism in the cytotoxic activity of macrophages against L929 cells. The protective effects of the mAb were reduced but not abolished by 18 h. The evanescent presence of soluble TNF-α bioactivity in the cultures, as well as the efficacy of the antibody to quench cytokine activity, are demonstrated by data presented in Table 3.

Macrophages Induce TNF-α-dependent Apoptosis in L929 Cells When Separated by a Cell-impermeable Membrane. Table 4 shows results from experiments where macrophages and L929 cells were cultured in the different compartments of Transwell microtiter plates. As demonstrated by these data, significant DNA fragmentation and low grade 51Cr release were detectable after a 12-h culture. In

Fig. 1. Evidence that culture of macrophages in the presence of IFN-γ and LPS is followed by the production of NO2 and associated with the fragmentation of labeled DNA and the release of 51Cr from cocultured P815 cells. Macrophages obtained and plated as described under “Materials and Methods” (2 × 106/well) were activated through the addition of recombinant murine IFN-γ (10 units/ml) and LPS (1 μg/ml) and cocultured with P815 cells (104/well) for the times indicated in the figure. The NO-synthase inhibitor N-MMA (0.5 mM) was added when so indicated. The target cells were labeled with either [methyl-3H]-thymidine or 51Cr. The nitrite concentration in culture supernatants, specific DNA fragmentation, and 51Cr release were measured as indicated in “Materials and Methods.” The addition of anti-TNF-α mAb (500 ng/well) was without effect on any of the measured parameters. The addition of N-MMA decreased nitrite accumulation, DNA fragmentation, and 51Cr release at 12 and 18 h (P < 0.05; analysis of variance F test).

Fig. 2. Evidence that the culture of macrophages and P815 cells in separate compartments of a Transwell plate is associated with the fragmentation of P815 DNA in the pattern of internucleosomal cleavage characteristic of apoptosis. Macrophages were plated in the lower chamber of Transwell plates; P815 cells were added into the upper chamber and harvested 12 h later. DNA from these cells was extracted and subjected to electrophoresis as described in “Materials and Methods.” Lane 1, standard 123-base pair DNA ladder; Lane 2, intact P815 DNA obtained from cultures performed in the absence of IFN-γ/LPS; Lane 3, fragmented DNA extracted from cultures where macrophages were activated with IFN-γ/LPS as described in “Materials and Methods”; and Lane 4, inhibition of fragmentation afforded by the addition of 0.5 mM N-MMA to cultures containing IFN-γ/LPS. Treatment of P815 cells alone with IFN-γ/LPS did not result in DNA fragmentation (data not shown).
Fig. 3. Exposure of P815 cells to authentic NO gas (1% in 99% N₂) but not to 100% N₂ is followed by DNA fragmentation characteristic of apoptosis. The tumor cells were treated with the respective gases as described in "Materials and Methods" for 30 min and then cultured overnight in 7% CO₂ in air. DNA extracted after overnight culture was subjected to electrophoresis. Lane 1, standard 123-base pair DNA ladder; Lane 2, DNA extracted from cells treated with N₂; and Lane 3, cells treated with NO.

Table 2: Culture in the presence of the NO donor SNP results in DNA fragmentation in P815 or L929 cells. In marked contrast with findings with P815 cells, the exposure of L929 cells to NO gas, even when prolonged for 1 h, did not result in the appearance of fragmented DNA on electrophoresis (data not shown). Treatment of the cells with SNP was equally ineffective in inducing DNA fragmentation (Table 2).

**DISCUSSION**

The ability to recognize and destroy non-self constitutes the basis for specific and nonspecific immunity. The immune cells capable of cytotoxicity against neoplastic cells have at their disposal a significant arsenal of effector molecules and mechanisms to carry out their task. Although the final outcome of cytotoxic events is the destruction of the neoplastic cell, the mode of target cell death appears to vary. For cytotoxic T-lymphocyte and natural killer cells, for example, both necrotic and apoptotic forms of cytotoxicity have been described (13-15). Presently reported studies demonstrate that macrophages can eliminate tumor cells by inducing them to undergo apoptotic death and, taking advantage of the specific susceptibility of two tumor cell lines to different macrophage effector molecules, that apoptosis of the tumor targets can be induced through more than one mediator.

Apoptosis is characterized by specific molecular and morphological alterations in the dying cell. At the molecular level, genomic DNA is fragmented by a specific endonuclease. Morphologically, the cell exhibits early nuclear and cytoplasmic condensation and goes on to fragment into well-defined, membrane-bound apoptotic bodies. In contrast with findings in cells undergoing primary necrosis, cell membrane failure and the release of intracellular contents occur in the later stages of apoptosis in a process described as secondary necrosis (9, 10, 16, 17).

Results obtained in macrophage-tumor cell coculture cytotoxicity assays demonstrated extensive fragmentation of tumor cell DNA, an effect that quantitatively exceeded the magnitude of ⁵¹Cr release from the targets. These observations were confirmed in experiments using the Transwell system where the extent of DNA fragmentation was, again, significantly greater than the degree of cell lysis, and where electrophoresis of tumor cell DNA revealed the ladder appearance characteristic of apoptosis. The quantitative discrepancy between DNA fragmentation and cell membrane failure and the pattern of DNA fragmentation mentioned above provided the evidence for the apoptotic nature of macrophage-mediated tumor cell death that is the basis for this communication.

The extent of DNA fragmentation detected by the labeled thymidine method did not exceed 40–50% of total labeled nucleic acids for
Table 3 TNF-α bioactivity in culture supernatants (units/ml). Effects of an anti-TNF-α mAb

<table>
<thead>
<tr>
<th>Time of harvest (h)</th>
<th>Absent</th>
<th>Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>1525 ± 446</td>
<td>26 ± 6</td>
</tr>
<tr>
<td>12</td>
<td>238 ± 59</td>
<td>ND*</td>
</tr>
<tr>
<td>18</td>
<td>66 ± 20</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, none detected.

Table 4 Activated macrophages induce apoptosis in L929 cells in Transwell plates in a NO-independent fashion. Apoptosis is prevented by an anti-TNF-α mAb

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nitrone (μM)</th>
<th>DNA fragmentation (%)</th>
<th>51Cr release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.7 ± 0.3a</td>
<td>2.2 ± 1.1a</td>
<td>7.6 ± 0.9</td>
</tr>
<tr>
<td>IFN-γ/LPS</td>
<td>20.3 ± 1.1b</td>
<td>27.1 ± 2.1b</td>
<td>7.7 ± 0.5</td>
</tr>
<tr>
<td>IFN-γ/LPS + anti-TNF-α mAb</td>
<td>31.3 ± 1.2b</td>
<td>2.0 ± 1.0d</td>
<td>ND</td>
</tr>
</tbody>
</table>

a p < 0.05 versus cultures containing IFN-γ and LPS.
b p < 0.05 versus cultures without additives.
d ND, none detected.

Neither cell type. This extent of fragmentation may represent the maximum achievable during apoptosis since a similar degree of DNA damage was evidenced when the tumor cells were exposed to 3-μM gliotoxin, a well-characterized inducer of apoptosis (18, 19), for 6–24 h (data not shown). It appears, then, that this magnitude of DNA damage goes beyond the capacity for repair in these cells and either represents or is followed by cell death.

The sensitivity of P815 cells to macrophage-derived NO is consistent with the reports of others (1, 6, 7, 20). Present findings additionally demonstrate that killing is accomplished through the induction of apoptosis in the target cells, an effect evident even in the absence of cell-to-cell contact. This last observation confirms that of Klostergaard et al. (6), who reported that activated macrophages can express cytotoxicity toward P815 without cell-to-cell contact (6).

The potential cooperativity between NO and TNF-α in mediating cytotoxicity has been proposed by some (20, 21) and denied by others (22). Current results do not support cooperation between NO and TNF-α in the killing of P815 cells since cytotoxicity could be completely abrogated by inhibiting NO production and was not affected by an anti-TNF-α mAb (data not shown).

The NO-dependent cytotoxic mechanism was not operant in the induction of apoptosis in L929 cells. Results in this communication favor the conclusion that TNF-α played a role in the induction of apoptosis in these cells. This notion is supported mainly by the characteristic internucleosomal fragmentation of L929 DNA harvested after culture with macrophages in Transwell microtiter plates and its suppression by an anti-TNF-α mAb.

The observations related to the role of TNF-α in the apoptotic death of L929 cells were incidental to the study of NO-mediated cell death. Nonetheless, it appears appropriate to point out that previous reports demonstrating that TNF-α can result in the apoptosis and/or necrosis of susceptible targets specifically addressed the effects of the addition of exogenous TNF-α onto tumor cells (23–27). Data reported here indicate that bioactive soluble TNF-α actually produced during macrophage-tumor cell interactions is only partially necessary and clearly not sufficient to explain the apoptotic death of the L929 target cells in cocultures. More specifically, the quantitative differences in the amounts of DNA fragmentation and 51Cr release from L929 between coculture (42 and 21%, respectively, at 12 h) and Transwell experiments (27 and 7.7%, respectively, at 12 h) and the partial protection afforded by the anti-TNF-α mAb in the coculture experiments suggest that mechanisms other than soluble TNF-α release which require cell-to-cell interaction may contribute to the induction of tumor cell apoptosis in cocultures. In a potentially relevant observation, Higuchi et al. (20) reported the amount of anti-TNF-α antibody needed to inhibit macrophage-mediated cytolysis in cocultures to be >100 times higher than that required to abolish the cytotoxicity of culture supernatants (20). These authors invoked the presence of high local concentrations of TNF-α in the immediate vicinity of the macrophages or membrane-bound TNF-α as potential explanations for their findings (20). It is possible, then, that the concentration of anti-TNF-α mAb used in the experiments reported here, while enough to quench all soluble TNF-α bioactivity in the cultures, may not have efficaciously neutralized TNF-α present in sanctuaries created during cell-to-cell contact events or membrane-bound TNF-α associated with the macrophages (28). While soluble TNF-α activity appears, then, to fully explain apoptotic cytotoxicity in Transwell microtiter plates, both this form of TNF-α and other effectors requiring cell-to-cell contact may have contributed to cell death in cocultures.

The resistance of L929 cells to NO deserves further study. To date, the cytotoxic effects of this mediator have been explained through the target-independent inhibition of essential metabolic pathways (2). Present results suggest that this explanation may be unduly simplistic and that either some cell types are capable of detoxifying NO or that other more selective means of cell injury are responsible for its antitumor activity.

In summary, macrophage-derived NO induces apoptosis in NO-sensitive P815 cells but not in NO-resistant L929. Additionally, macrophage-derived soluble TNF-α production partially explains macrophage-dependent apoptosis in L929 cells but not in P815. Taken together these observations demonstrate that like other immune cytotoxic cells, macrophages can determine tumor cell death through the induction of apoptosis and that they can do so through more than one effector mechanism.

![Fig. 5. Evidence that culture of macrophages and L929 cells in separate compartments of a Transwell plate is associated with the fragmentation of L929 DNA, and that fragmentation follows the pattern of internucleosomal cleavage characteristic of apoptosis. Culture conditions and postculture DNA extraction and electrophoresis were identical to that described in the legend to Fig. 2. Lane 1, standard 123-base pair DNA ladder; Lane 2, intact L929 DNA obtained from cultures performed in the absence of IFN-γ/LPS; Lane 3, DNA obtained from L929 cells after a 12-h culture in the presence of IFN-γ/LPS; and Lane 4, preservation of intact DNA resulting from the addition of anti-TNF-α mAb to the cultures.](image-url)
REFERENCES


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