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## Research paper

# Murine peritoneal macrophages treated with cisplatin and interferon-γ undergo NO-mediated apoptosis via activation of an endonuclease

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**We investigated whether murine peritoneal macrophages treated with cisplatin or interferon (IFN)-γ alone, or in combination, could undergo apoptosis, and whether this results either from the cytotoxic effect of the activating agents or indirectly in an autocrine manner by the cytotoxic molecules released by them upon activation. Our data suggest that cisplatin, which has been shown to induce apoptosis in a number of normal as well as tumor cell types, did not induce apoptosis in murine peritoneal macrophages nor was apoptosis caused by IFN-γ. However, combined treatment with cisplatin and IFN-γ induced apoptosis in macrophages as studied by percent DNA fragmentation assay, qualitative analysis of DNA on agarose gel electrophoresis, and morphological and nuclear alterations studied by phase contrast and fluorescence microscopy. The factor responsible for inducing apoptosis in macrophages was found to be a higher concentration of NO produced by them upon activation with cisplatin and IFN-γ. Macrophages treated with cisplatin or IFN-γ alone produced a low level of NO and did not undergo apoptosis. The inhibitor of NO synthase, L-NMMA, prevented apoptosis in macrophages treated with cisplatin and IFN-γ, suggesting the involvement of NO in the induction of apoptosis in macrophages. The role of NO in inducing apoptosis in macrophages was further confirmed by the observation that direct treatment with sodium nitroprusside, a NO donor, resulted in apoptosis in macrophages. We have also shown that NO-induced apoptosis in macrophages activated with cisplatin and IFN-γ requires activation of an endonuclease, as the endonuclease inhibitor, aurine tricarboxylic acid, prevented apoptosis in them.** [© 1998 Lippincott-Raven Publishers.]

*Key words*: Apoptosis, cisplatin, endonuclease, interferon-γ, macrophage, nitric oxide.

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## Introduction

Activated macrophages are potent effecter cells in host defense against malignant neoplasia. To eradicate individual tumor cells or circulating micrometastases *in vivo* activated macrophages release a plethora of cytotoxic molecules such as NO, tumor necrosis factor-α, interleukin-1, peroxidases, cytolytic proteases, etc.[1-4]

Out of these, NO, a small free radical molecule, is of particular significance and is responsible for cytotoxic and cytolytic activity for a variety of tumor cells both *in vivo* and *in vitro*.[5,6] Many recent works have shown that NO causes several metabolic alterations because of its high reactivity with iron- or thiol-containing biological molecules, and thus inhibits the TCA cycle, mitochondrial respiration, DNA synthesis and antioxidative as well as DNA repair enzymes.[7,8] It has also been shown the NO produced by the activated macrophages can kill tumor cells by inducing apoptosis in them.[9,10] However, recently it has been shown that macrophages are not themselves immune from the cytotoxic effects of NO. When induced to produce NO by activating signals, macrophages exhibit a pattern of metabolic inhibition, inducing suppression of oxidative metabolism and protein synthesis that is similar to that observed in tumor cells exposed to NO, and die prematurely through the induction of apoptosis,[11,12] a process which requires *de novo* protein synthesis and is characterized by dramatic condensation of the cell nucleus associated with extensive internucleosomal cleavage of nuclear DNA.[13] However, it is possible that in addition to NO other reactive intermediates or cytokines produced in the course of macrophage activation may be involved in apoptosis in activated macrophages.[14]

*Cis*-dichlorodiamine platinum II (cisplatin), a broad spectrum anticancer drug,[15] has proven effective in the treatment of bladder, lung, ovarian, head and neck, testicular, and breast cancer, and of certain types of leukemias.[16] Reports from our and other's laboratories have also shown the immunomodulatory role of cisplatin. Cisplatin has been shown to activate monocytes, macrophages, natural killer cells, lymphokine activated killer cells, bone marrow-derived macrophages and tumor associated macrophages to a tumoricidal state.[17-23] Further, it has been demonstrated that treatment of macrophages with a combination of cisplatin and IFN-γ results in a synergistic augmentation of macrophage tumoricidal activity.[24] In spite of the promising immunomodulatory potential of cisplatin, the following points need to be carefully examined before designing clinical chemoimmunotherapeutic trials with cisplatin. (i) Cisplatin has been shown to induce apoptosis in many tumor cell types and also in some normal cells.[25] (ii) Cytotoxic effecter molecules produced by activated macrophages have been implicated in the induction of apoptosis as their killing mechanisms.[11,12,14] In view of the above, we decided to study whether macrophages treated with cisplatin or IFN-γ alone, or in combination, undergo apoptosis, resulting either from the cytotoxic effect of the activating agents or indirectly in an autocrine manner by the cytotoxic molecules released from them upon activation.

## Materials and methods

### Materials

All cell cultures were maintained in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml) and gentamycin (20 μg/ml). RPMI 1640 medium, $N^G$-monomethyl L-arginine (L-NMMA), an inhibitor of NO synthase, propidium iodide (PI), diphenylamine (DPA) and proteinase K were purchased from Sigma (St Louis, MO). FCS was purchased from Biological Industries (Haemek, Israel). Murine recombinant IFN-γ was purchased from Boehringer Mannheim (Mannheim, Germany). Sodium nitroprusside (SNP), a NO donor, was purchased from Hi Media (Bombay, India). The endonuclease inhibitor, aurine tricarboxylic acid (ATA), was purchased from Merck (Bombay, India). All the reagents used were free of endotoxin contamination, as determined by Limulus amoebocyte lysate assay (sensitivity limit 0.1 ng/ml).

### Animals

An inbred strain of healthy BALB/c mice of either sex at 8-10 weeks of age was used for obtaining peritoneal macrophages.

### Preparation of peritoneal macrophages

Macrophage monolayers were prepared as described previously.[21] Briefly, peritoneal exudate cells were harvested by peritoneal lavage using chilled serum-free RPMI 1640 and were added to wells of six-well plastic tissue culture plates (Nunc, Roskilde, Denmark). After 2 h incubation at 37°C in 5% $CO_2$ in air in a $CO_2$ incubator, the non-adherent cells were removed by vigorous washing (three times) with warm serum-free medium. More than 95% of the adherent cell population were macrophages as determined by morphology and non-specific esterase staining.

Macrophage monolayers (approximately $2 \times 10^6$ cells/well) were cultured with or without activating agents as indicated, i.e. cisplatin (5 μg/ml) and/or recombinant murine IFN-γ (50 U/ml) in the wells of six-well tissue culture plates for various time intervals as indicated in the presence or absence of L-NMMA (1 mM) or ATA (50 μM). A combination of LPS (5 μg/ml) and IFN-γ (50 U/ml) was used as standard macrophage activating agent. Macrophages were also treated with SNP in the presence or absence of ATA. Culture supernatants were collected for nitrite assay at different time points as indicated and were stored at −20°C until assayed.

### Percent DNA fragmentation assay

Quantitative measurement of DNA fragmentation was carried out following the method given by Sellins *et al.*[26] Briefly, macrophage monolayers were lysed in 0.5 ml of TTE (TE buffer, pH 7.4 containing 0.2% Triton X-100) and fragmented DNA was separated from intact chromatin by microcentrifuging the tube at 13 000 *g* at 4°C for 10 min. Supernatant (S) containing the fragmented DNA was transferred to another microfuge tube and 0.5 ml of TTE was added to the pellet (P) containing intact high molecular DNA. Cell-free culture supernatants (M) were also collected at indicated time intervals. DNA was precipitated overnight in 0.5 ml of 25% TCA followed by microcentrifugation at 13 000 *g* at 4°C for 10 min. Supernatants were discarded and 80 μl of 5% TCA was added to each pellet. DNA was hydrolyzed by heating at 90°C for 15 min. At this stage a blank was included

containing 80 µl of 5% TCA. Then 160 µl of freshly prepared DPA reagent was added to each tube, vortexed and color was allowed to develop for 4 h at 37°C; 200 µl of the colored solution was transferred to the wells of a 96-well flat-bottomed ELISA plate and optical density was measured at 600 nm in a microtiter plate reader. Percent DNA fragmentation was calculated as:

$$\text{percent DNA fragmentation} = \frac{M + S}{M + S + P} \times 100$$

### Agarose gel electrophoresis of DNA

Extraction of macrophage DNA was performed following the method given by Pringt *et al.*[27] with some modifications. Macrophage monolayers, washed in PBS, were lysed in 0.5 ml of lysis buffer containing 10 mM Tris-HCl (pH 8.0), 75 mM NaCl, 10 mM EDTA, 0.5% SDS and 0.15 mg/ml proteinase K, and incubated for 3 h at 50°C. Lysate was spun down at 10 000 *g* for 20 min at 4°C. The supernatant was collected carefully and a solution of 0.5 M NaCl and 50% absolute ethanol was added to precipitate the DNA. The precipitated DNA was resolublized in 30 µl of TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA, pH 8.0) for 1 h at 65°C and then incubated with 200 µg/ml RNase A for 2 h at 37°C. Thereafter, 10 µl of loading dye (0.025% bromophenol blue, 0.25% xylene cyanol and 30% glycerol in water) was added and 40 µl of sample was loaded in the wells of 1.5% agarose gel and electrophoresed for 2 or 3 h at 75 V in TBE buffer in the presence of 0.5 µg/ml ethidium bromide. DNA was visualized and photographed on a UV transilluminator.

### Phase contrast and fluorescence microscopy for study of apoptotic morphology

As oligonucleosomal DNA fragmentation cannot be considered a confirmatory test for apoptosis, we also simultaneously studied changes in the cellular and nuclear morphology by fluorescence and phase contrast microscopy. Macrophage monolayers grown on cover slips in six-well tissue culture plates were fixed for 10 min in a mixture of ethanol:acetic acid (3:1), washed for 1 min in distilled water, and subsequently air dried and stained with a drop of 50 µg/ml PI for 10 min. The cells on the cover slips were again washed twice with distilled water and mounted with 50% glycerol, and observed by phase contrast and fluorescence microscopy to study cellular and nuclear changes associated with apoptosis.

### Determination of percent cell viability and percent apoptotic cells

Quantitation of percent cell viability was done by the Trypan blue exclusion test.[28] Macrophages grown on the cover slips were stained with Trypan blue (0.3% in PBS) and observed under a light microscope. Cells failing to exclude the dye were considered non-viable. Similarly, percent apoptotic cell population was determined by identifying and counting apoptotic cells on the basis of their characteristic cellular and nuclear morphology. The percentage of viable cells or apoptotic cells was determined by counting more than 200 cells for at least three separate determinations.

### Nitrite determination

Cell-free culture supernatants were collected at different time points as indicated and were measured for nitrite using a spectrophotometric assay method described by Ding *et al.*[29] based on the Griess reaction. Briefly, 100 µl of culture supernatant was incubated with an equal volume of Griess reagent [one part of 1% (w/v) sulfanilamide in 2.5% (v/v) phosphoric acid plus one part of 0.1% (w/v) napthethyldiamine dihydrochloride in distilled water] at room temperature for 15 min. The absorbance was taken at 540 nm in a microtiter plate reader. Nitrite concentration was quantified by using sodium nitrite as standard.

### Statistical analysis

Results are expressed as means ± SD of at least three independent experiments. The statistical significance of difference between test groups was analyzed by two-tailed Student's *t*-test. The level of significance was considered $^{*}p < 0.05$.

## Results

### Macrophages undergo apoptosis when treated with cisplatin and IFN-γ

Treatment of macrophage monolayers with a combination of cisplatin and IFN-γ induced apoptosis as studied by percent DNA fragmentation assay, qualitative analysis of DNA by agarose gel electrophoresis, and by the corresponding cellular and nuclear alterations. DNA fragmentation was detectable after 24 h which reached up to approximately 44% at 36 h. Untreated macrophages or macrophages treated with

cisplatin or IFN-γ alone did not undergo DNA fragmentation (Figure 1 and Table 1). Further, to determine if the fragmentation of macrophage DNA followed the specific pattern of oligonucleosomal cleavage as found in apoptosis, agarose gel electrophoresis was carried out. DNA extracted from macrophages treated with cisplatin and IFN-γ showed oligonucleosomal cleavage giving a ladder pattern (Figure 2, lane 5), whereas DNA extracted from untreated control or from macrophages treated with cisplatin or IFN-γ alone showed no sign of oligonucleosomal DNA cleavage (Figure 2, lanes 1-3). The corresponding morphological changes in the macrophages treated with cisplatin and IFN-γ as studied by phase contrast and fluorescence microscope showed membrane blebbing, cell shrinkage along with chromatin condensation and disintegration (Figure 3b), whereas the morphology of untreated macrophages or macrophages treated with cisplatin or IFN-γ alone did not show such alterations (Figure 3a).

Treated or untreated macrophage monolayers were also counted for percent cell viability and percent apoptotic cells. Untreated macrophages or those treated with cisplatin or IFN-γ alone showed more than 95% viable cells, with less than 2% apoptotic cell population even up to a 36 h of culture period. Macrophages treated with cisplatin plus IFN-γ showed a time-dependent, gradual decline in viability whereas the apoptotic cell population increased up to 36% at 36 h (Table 1).

## Macrophage-derived NO induces apoptosis in cisplatin and IFN-γ treated macrophages

In the next part of the study we investigated the factor(s) responsible for inducing apoptosis in macrophages treated with cisplatin plus IFN-γ. The addition of L-NMMA, a competitive inhibitor of NO synthase, to the macrophage cultures treated with cisplatin and IFN-γ completely inhibited oligonucleosomal DNA fragmentation as determined by percent DNA fragmentation assay (Figure 1 and Table 1) and agarose gel electrophoresis (Figure 2, lane 7). L-NMMA also inhibited the corresponding morphological changes indicating apoptosis, suggesting these alterations to be NO dependent. Time kinetics studies of nitrite accumulation in the

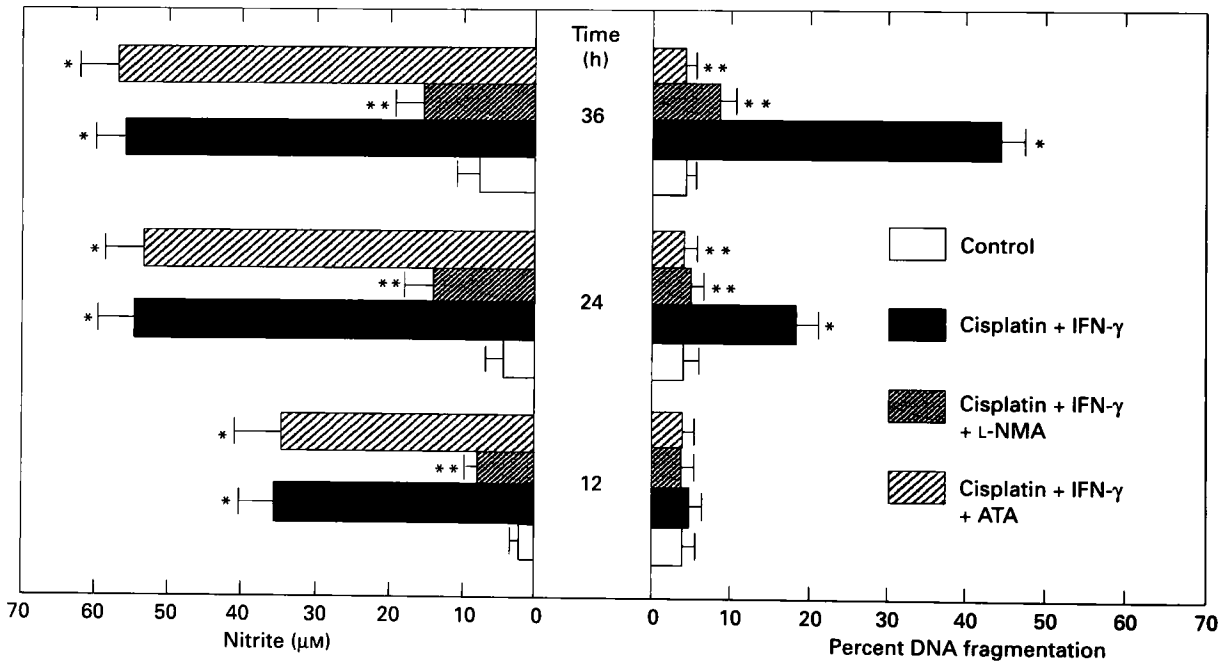


**Figure 1.** Kinetics of NO accumulation and percent DNA fragmentation. Peritoneal macrophages ($2 \times 10^6$ cells/well) treated with cisplatin (5 μg/ml) and/or IFN-γ (50 U/ml) were cultured in the presence or absence of L-NMMA (1 mM) or ATA (50 μM). The nitrite concentration in the culture supernatants and percent DNA fragmentation were assayed at indicated time intervals by the method as described in Materials and methods. Data shown are means ± SD and are representative of three independent experiments done in triplicate. *$p < 0.05$ versus values of control cultures. **$p < 0.05$ versus values of cisplatin and IFN-γ treated cultures.

supernatant of cisplatin plus IFN-γ treated macrophages showed a linear pattern with time, reaching up to 55 μM at 24 h and subsequently becoming stable (Figure 1). Macrophages treated with cisplatin or IFN-γ alone produced a comparatively low concentration of nitrite and did not show DNA fragmentation, a ladder pattern on agarose gel or morphological changes as found in apoptosis (Table 1, and Figures 2 and 3). Production of NO was significantly inhibited by the addition of L-NMMA to the macrophage culture which also prevented apoptosis (Figures 1 and 2, and Table 1). Confirmatory evidence that NO can induce apoptosis in macrophages was obtained following the treatment of macrophage monolayers with different concentrations of the NO donor, SNP, which resulted in dose-dependent DNA fragmentation (Table 2) and showed oligonucleosomal fragmentation, giving a ladder pattern on agarose gel (Figure 4, lanes 2 and 3). However, exposure to low concentrations of SNP, i.e. up to 0.5 mM, did not induce apoptosis in macrophages as less than 3% of the cells underwent apoptosis with 17% DNA fragmentation (Table 2) and did not give a ladder pattern on agarose gel (Figure 4, lane 1).

## NO-mediated apoptosis in cisplatin and IFN-γ treated macrophages involves activation of an endogenous endonuclease

ATA has been shown to inhibit endogenous endonuclease activity and has been utilized to delineate the role of endonuclease in DNA fragmentation.[30] To investigate the role of endonucleases in NO-induced apoptosis in cisplatin and IFN-γ treated macrophages

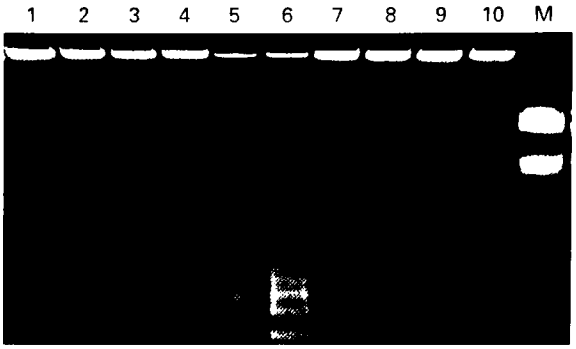


**Figure 2.** Agarose gel analysis of DNA extracted from macrophages. Peritoneal macrophages ($2 \times 10^6$ cells/well) in six-well tissue culture plate, untreated or treated with cisplatin (5 μg/ml) and/or IFN-γ (50 U/ml) or LPS (5 μg/ml) and/or IFN-γ (50 U/ml) were cultured in the presence or absence of L-NMMA (1 mM) or ATA (50 μM). After 36 h culture, DNA was extracted from the macrophages and analyzed by agarose gel electrophoresis. Lane 1, untreated macrophages; lane 2, cisplatin treated macrophages; lane 3, IFN-γ treated macrophages; lane 4, LPS treated macrophages; lane 5, cisplatin plus IFN-γ treated macrophages; lane 6, LPS plus IFN-γ treated macrophages; lane 7, cisplatin plus IFN-γ treated macrophages cultured in the presence of L-NMMA; lane 8, LPS plus IFN-γ treated macrophages cultured in the presence of L-NMMA; lane 9, cisplatin plus IFN-γ treated macrophages cultured in the presence of ATA; lane 10, LPS plus IFN-γ treated macrophages cultured in the presence of ATA. M, marker.

**Table 1.** Effect of NO produced by cisplatin and IFN-γ treated macrophages on percent DNA fragmentation, percent viability and percent apoptotic cell population in the presence or absence of endonuclease inhibitor

| Treatment | Nitrite (μM) | Percent DNA fragmentation | Percent apoptosis | Percent viability |
|---|---|---|---|---|
| Medium | 8.6±2.8[a] | 4.3±2.4 | 1.6±0.6 | 95.1±4.7 |
| Cisplatin | 31.0±4.6* | 4.8±1.3 | 1.5±0.6 | 94.6±4.2 |
| IFN-γ | 28.0±4.2* | 3.9±2.1 | 1.0±0.5 | 96.7±3.6 |
| LPS | 32.3±5.1* | 5.2±2.1 | 1.5±0.2 | 95.3±3.8 |
| Cisplatin + IFN-γ | 56.5±4.1* | 43.8±3.9* | 36.0±8.5* | 83.3±8.1* |
| LPS + IFN-γ | 53.9±4.9* | 45.2±2.8* | 38.2±6.4* | 79.9±5.6 |
| Cisplatin + IFN-γ + L-NMMA | 16.2±3.8** | 8.6±2.3** | 2.0±0.7** | 95.2±3.5* |
| LPS + IFN-γ + L-NMMA | 18.4±4.0*** | 7.9±2.5*** | 1.5±0.5*** | 95.0±4.4*** |
| Cisplatin + IFN-γ + ATA | 57.0±6.4* | 4.2±1.8** | 3.0±1.1** | 94.7±3.4** |
| LPS + IFN-γ + ATA | 54.6±5.2* | 6.3±2.5*** | 1.8±0.8*** | 95.0±3.0*** |

*$P<0.05$ versus values for untreated control cultures.
**$P<0.05$ versus values for cisplatin and IFN-γ treated cultures.
***$P<0.05$ versus values for LPS and IFN-γ treated cultures.
[a]The numbers represent mean±SD of triplicate cultures.
Peritonal macrophages ($2 \times 10^6$ cells/well) in six-well tissue culture plates, treated with cisplatin (5 μg/ml) and/or IFN-γ (50 U/ml) or LPS (5 μg/ml) and/or IFN-γ (50 U/ml) were cultured for 36 h in the presence or absence of L-NMMA (1 mM) or ATA (50 μM) as indicated. The nitrite concentration in the culture supernatants, percent DNA fragmentation, percent apoptotic cell population and percent viability of the macrophages were measured by the method as described in Materials and methods.

we examined the effect of an endonuclease inhibitor. Addition of 50 μM ATA completely inhibited DNA fragmentation (Figure 1 and Table 1), a ladder pattern of DNA on agarose gel (Figure 2, lane 9) and the corresponding morphological changes (Figure 3) in cisplatin and IFN-γ treated macrophages. At this concentration ATA did not interfere with the NO production by the activated macrophages or with the cell viability as tested by the Trypan blue dye exclusion test (Figure 1 and Table 1). The role of endonucleases in NO-mediated apoptosis in macrophages was further confirmed as ATA prevented apoptosis in the macrophages treated with various concentrations of SNP (Figure 4, lanes 5 and 6, and Table 2).

## Discussion

The present investigations were undertaken to determine whether macrophages are induced to undergo apoptosis as a result of their activation *in vitro* with

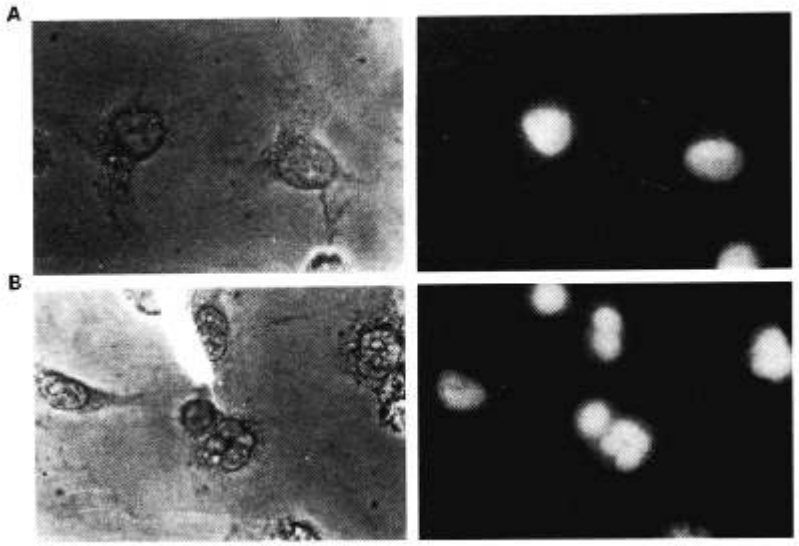


**Figure 3.** Morphology of macrophages stained with PI (5 μg/ml) by the method as described in Materials and methods, and observed under fluorescence microscopy and phase contrast microscopy. The same field of cells was visualized by fluorescence and phase contrast microscopy. Panel (B) represents the typical cellular and nuclear changes of apoptosis, such as membrane blebbing, cell shrinkage and nuclear break down, in the macrophages treated with cisplatin plus IFN-γ. Panel (A) shows that such changes were not observed in the macrophages treated with cisplatin or IFN-γ alone or in the macrophages treated with cisplatin plus IFN-γ in the presence of L-NMMA or ATA.

**Table 2.** Effect of different concentrations of SNP on percent DNA fragmentation, percent apoptotic cell population and percent viable cell population in the presence or absence of the endonuclease inhibitor ATA

| SNP (mM) | Nitrite (μM) | Percent DNA fragmentation | | Percent apoptosis | | Percent viability | |
|---|---|---|---|---|---|---|---|
| | | −ATA | +ATA | −ATA | +ATA | −ATA | +ATA |
| Medium | 8.0±3.2[a] | 4.8±2.3 | 4.2±1.5 | 2.0±0.5 | 2.1±1.1 | 95.8±3.3 | 95.0±3.9 |
| 0.5 | 49.4±4.9* | 16.3±1.6* | 3.9±1.8 | 2.4±0.8 | 2.6±1.0 | 95.0±4.0 | 95.0±4.7 |
| 1.0 | 71.6±5.2* | 25.2±5.2* | 4.1±1.7 | 10.6±4.2* | 2.5±0.6 | 90.2±5.1 | 95.0±4.4 |
| 2.0 | 105.0±5.1* | 36.8±7.2* | 5.1±2.3 | 23.9±6.1* | 3.1±1.3 | 78.4±5.8* | 94.0±4.5 |

*$P<0.05$ versus values of control cultures.
[a]The numbers represent mean±SD of triplicate experiments.
Peritonal macrophages ($2\times10^6$ cells/well), treated with different concentrations of SNP, were cultured in the presence or absence of ATA (50 μM) for 36 h. Nitrate assay, percent DNA fragmentation assay, percent apoptotic cell population and percent viability were assayed by the method as described in Materials and methods. Data shown are means±SD and are representative of three independent experiments done in triplicate.

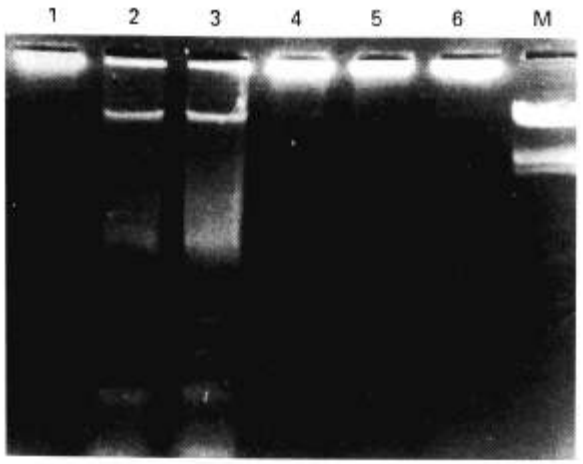


**Figure 4.** Agarose gel analysis of DNA extracted from macrophages treated with different concentrations of SNP for 36 h in the presence or absence of ATA (50 μM). Lanes 1–3, macrophages treated with 0.5, 1 and 2 mM SNP; lanes 3–5, macrophages treated with 0.5, 1 and 2 mM SNP in the presence of ATA. M, marker.

cisplatin or IFN-γ either alone or in combination. We observed that although macrophages could be activated under both conditions, they underwent apoptosis only when activated with cisplatin plus IFN-γ but not when treated with cisplatin or IFN-γ alone. Cisplatin has been shown to induce apoptosis in a number of normal as well as transformed cell types.[25] A likely explanation for the resistance to cisplatin-induced apoptosis of peritoneal macrophages could be the differential sensitivity of the proliferating and quiescent cells to the cisplatin, as reported by others.[31] Many currently used anti-cancer drugs, including cisplatin, are cell-cycle specific, preferentially killing proliferating cells by inducing apoptosis in them.[25] Resident peritoneal macrophages are non-proliferating and this, therefore, may account for their resistance to cisplatin induced apoptosis.

Cisplatin and IFN-γ induced apoptosis was inhibited by the inhibitor of NO synthase L-NMMA, indicating that NO produced by the activated macrophages was involved in the induction of apoptosis in macrophages. NO production was determined to investigate whether there was a correlation between apoptosis and NO produced by the cisplatin and IFN-γ activated macrophages. Interestingly, macrophages treated with cisplatin or IFN-γ alone produced a low level of nitrite and did not undergo apoptosis. However, a synergistic effect was observed by the combined treatment leading to a higher concentration of nitrite production by macrophages and to their apoptosis. These observations suggest the involvement of NO in the induction of apoptosis in cisplatin and IFN-γ treated macrophages. Involvement of NO was further confirmed by the observation that direct treatment with SNP, a NO donor, resulted in apoptosis in macrophages in a dose-dependent manner.

Kinetics of DNA fragmentation indicated onset of irreversible DNA fragmentation upon activation by cisplatin and IFN-γ, which progressed with time. Further, qualitative analysis of DNA on agarose gel electrophoresis confirmed oligonucleosomal cleavage. A comparison of the time kinetics of percent DNA fragmentation with that of the appearance of the DNA ladder shows that although DNA fragmentation was detectable at 24 h, it did not give a ladder pattern on agarose gel. The ladder pattern of DNA appeared only after 36 h when the percent DNA fragmentation was 44%. The reason for this could be that low molecular DNA fragments may not be generated during the early stages of apoptosis, despite the presence of extensive high molecular weight DNA fragments, which are generated prior to internucleosomal DNA fragmentation.[32,33] Further, comparison of results obtained in the percent apoptotic cell assay with those of the percent DNA fragmentation assay suggests the presence of a relatively low number of apoptotic forms even after 36 h in cisplatin and IFN-γ activated macrophage cultures. A possible explanation for this could be the efficient removal of cells undergoing apoptosis by the remaining viable and non-apoptotic macrophages or by progression of the process into a phase of secondary necrosis, as suggested by others.[12]

Results of time kinetics of NO accumulation and the percent DNA fragmentation assay indicated that, although nitrite concentration reached a maximum after 24 h, extensive DNA fragmentation was observed only after 36 h. The quantitative discrepancy between nitrite accumulation and DNA fragmentation suggests NO-induced apoptosis in cisplatin and IFN-γ treated macrophages to also be a time-dependent process.

Although some details of the biological effects of NO are known,[7,8] our present understanding of the intimate mechanisms by which it induces apoptosis remains incomplete. Some reports suggest the role of a protein kinase C inhibitor and antioxidants in the suppression of activation-induced death of macrophages. A protective role of certain cytokines, like granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage CSF and granulocyte CSF, has also been implicated, and these all have been used to promote the recovery of granulocytes and macrophages after systemic chemotherapy for cancer, in the supression of activation-induced macrophage death.[34,35] A complete study regarding the molecular mechanisms involved in death signaling pathways and

the protective mechanisms may help to develop new approaches for the treatment of diseases. Although it might be expected that NO as a free radical can cause direct damage to DNA in the cells, in the present study we have shown that DNA fragmentation is brought about via mechanisms not involving direct attack by oxidative radicals, but via endonuclease activation, as also suggested by others.[36] The endonuclease inhibitor ATA prevented NO-induced DNA oligonucleosomal strand break in macrophages. Since ATA is known to be an endonuclease inhibitor and is capable of blocking the endogenous endonuclease believed to be involved in apoptosis,[30] it is thus likely that ATA interferes in macrophage apoptosis via its action on the endonuclease. We also confirmed that ATA did not interfere with NO production or with cell viability. However, not all forms of apoptosis necessarily depend on DNA fragmentation and the activation of an endonuclease may not be a critical factor.[37] Therefore, we also observed simultaneous changes in the cellular and nuclear morphology by fluorescence and phase contrast microscopy, which suggested the occurrence of apoptosis. Addition of ATA inhibited these morphological changes, associated with apoptosis, suggesting that activation of endonuclease underlies the NO-induced apoptosis in cisplatin plus IFN-γ treated macrophages. Biological significance of activation-induced death of the cisplatin and IFN-γ treated macrophages could be a regulatory mechanism to control the destructive potential of activated macrophages which may, otherwise, cause extensive local damage to the host tissues through the release of free radicals, lytic enzymes and inflammatory cytokines, which are responsible for many systemic symptoms associated with acute and chronic inflammation.[38,39] A similar mechanism has been implicated in the regulation of the destructive potential of other cells in the immune system. The phenomenon has been most extensively studied in the lymphoid lineage, where autoreactive thymocytes undergo activation-induced apoptosis upon encountering 'self' antigens in the thymus, but apoptosis also appears to play an important role in limiting the persistence of activated T cells, B cells and granulocytes.[34]

An additional benefit of activation-induced apoptosis in macrophages could be in the destruction of the intracellular pathogens of the macrophages which, otherwise, will not be accessible for killing by the conventional immunodefense effector mechanism.

Although optimum activation of cisplatin and IFN-γ treated macrophages leads to their death, it does not restrict the chemoimmunotherapeutic use of cisplatin and IFN-γ against cancer. The form of apoptosis we describe allows for normal activation in the short term, thus permitting the macrophages to perform their antitumor or antimicrobial functions; however, it strictly limits the subsequent persistence of the activated cells.

The unique observation of this study is the occurrence of apoptosis in the activation-induced death of cisplatin and IFN-γ activated macrophages via activation of an endonuclease. One of the most important implications of the involvent of apoptosis in activation-induced death in cisplatin and IFN-γ treated macrophages is that, since this is an active gene-dependent process, there are possibilities of evolving newer therapeutic strategies to treat cancer, using cisplatin and IFN-γ in which sustained macrophage activation could be retained while intervening in the specific death signaling pathways and by taking simultaneous measures to prevent inflammatory damages to the surrounding tissues.

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