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Cisplatin and interferon-γ treated murine macrophages induce apoptosis in tumor cell lines

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Macrophages, treated *in vitro* with a combination of cisplatin and interferon (IFN)-γ, have been shown to develop enhanced tumoricidal activity against a number of tumor cell types, through mechanisms which remain largely unknown. In the present study, we have investigated the mechanism involved in the tumor cell cytotoxicity mediated by cisplatin and IFN-γ treated macrophages, and the effector molecules involved therein. Peritoneal macrophages treated with cisplatin and IFN-γ, when co-cultured with different tumor cell types, caused tumor cell death by induction of apoptosis. Evidence for this was provided by percent specific DNA fragmentation assay, by specific pattern of internucleosomal DNA fragmentation detected by agarose gel electrophoresis and by microscopic examination of the cells, which revealed nuclear alterations, characteristic of apoptosis. The time kinetics studies of DNA fragmentation, loss in cell viability and apoptotic cell population showed linearity with time; most of the cells that underwent apoptosis were found to be viable even after 24 h co-culture. Macrophages induced apoptosis in tumor targets even in the absence of cell-to-cell contact, i.e. via diffusible effector molecules. In P815 cells, NO produced by cisplatin and IFN-γ treated macrophages was found to induce apoptosis as addition of N^G-monomethyl L-arginine (L-NMMA), a specific inhibitor of NO synthase to the co-culture, prevented apoptosis in P815 cells. Further, direct treatment of P815 cells with the NO donor, sodium nitroprusside (SNP), resulted in apoptosis. In L929 cells, the effector molecule was found to be tumor necrosis factor (TNF)-α as apoptosis was blocked by the addition of anti-TNF-α antibodies to the co-culture but the addition of L-NMMA or SNP had no effect. The study thus shows that cisplatin and IFN-γ treated macrophages can kill tumor cells by extracellular release of effector molecules which act by inducing apoptosis in a target cell-specific manner.

Key words: Apoptosis, cisplatin, interferon-γ, macrophages, tumor cells.

Introduction

Cisplatin [*cis*-dichlorodiamine platinum(II)], a widely used potent anticancer drug,[1] has also been shown to activate murine macrophages,[2,3] natural killer cells,[4] human lymphokine activated killer cells[5] and human monocytes[6,7] to a tumoricidal state both *in vivo* and *in vitro*. The tumoricidal effect of macrophages has been shown to be further enhanced when these cells are treated with cisplatin in combination with interferon (IFN)-γ.[8] Combination of cisplatin-based therapy with an immunostimulant has shown a synergistic effect in the antitumor activity of tumor-bearing mice[9,10] and in the *in vitro* cytotoxic action of immune cells.[11] The cytotoxic effects of activated macrophages may involve either cell-to-cell contact[12] or they may trigger cytotoxic action to kill a wide variety of tumors by extracellular release of soluble effector molecules such as reactive oxygen intermediates,[8] reactive nitrogen intermediates,[13] tumor necrosis factor (TNF) or interleukin-1.[14]

However, despite knowledge of the existence of a number of effector molecules, the mechanism(s) by which they trigger target cell death remains unclear. Further, very few reports are available which explain the mode of cell death that the target cells follow. Effector cells have been suggested to kill infected or tumor cells either by necrosis or by apoptosis. These two processes differ both morphologically and biochemically. Necrosis is associated with cell swelling, membrane rupture and dissolution of organized cellular structure. In contrast, apoptosis has little effect on organelle integrity, but is characterized by cell shrinkage, condensation of chromatin and cleavage of DNA into oligonucleosomal fragments of 180–200 bp length or its multiples, giving a characteristic ladder pattern when run on agarose gels.[15,16]

The present investigation reports that killing of tumor cells by macrophages treated with cisplatin and IFN-γ involves induction of apoptosis in target cells via the extracellular release of effector molecules which act in a target cell-specific manner.

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Materials and methods

Materials

All cell cultures were maintained in RPMI 1640, 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 was purchased from Sigma (St Louis, MO). FCS was purchased from Biological Industries (Hamemek, Israel). [methyl-^{3}H]Thymidine ([^{3}H]Tdr) was purchased from Bhabha Atomic Research Centre (Bombay, India). Murine recombinant IFN-γ, murine recombinant TNF-α and anti-TNF-α monoclonal antibody were purchased from Boehringer Mannheim (Mannheim, Germany). Lipopolysaccharide (LPS; *Escherichia coli* 055:B5), propidium iodide (PI) and proteinase K were purchased from Sigma. Sodium nitroprusside (SNP), a NO donor, was purchased from Hi Media (Bombay, India). N^G-monomethyl L-arginine (L-NMMA), the inhibitor of NO synthase, was a kind gift from Dr H Hodson (Department of Medical Chemistry, Welcome Research, UK). All the reagents used were free of endotoxin contamination, as determined by the Limulus amebocyte lysate assay (sensitivity limit 0.1 ng/ml).

Animals

Inbred strains of healthy BALB/c mice of either sex at 8–10 weeks of age were used to obtain peritoneal macrophages.

Tumor cells

Tumor cells used in this study were L929 (a murine fibroblast cell line) and P815 (a murine mastocytoma cell line), obtained from National Tissue Culture Facility (Pune, India).

In vitro activation of macrophages and co-culture with tumor cells

Macrophage monolayers were prepared as described previously.[3] Peritoneal exudate cells were harvested from peritoneal lavage using chilled serum-free RPMI 1640 and were added to wells of 24-well plastic tissue culture plates (Nunc, 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 (3 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 (2×10^6 cells/well) were cultured with or without activating agents as indicated i.e. cisplatin (5 μg/ml) and recombinant murine IFN-γ (50 U/ml). A combination of LPS (5 μg/ml) and IFN-γ (50 U/ml) was used as standard macrophage activating agents. After 12 h incubation at 37°C in a CO_2 incubator, these activating agents were removed from the culture by thorough washing. Tumor target cells (2×10^5) were then added in the prewetted transwell inserts and placed over the macrophage monolayers in the wells of 24-well tissue culture plates. Prior to the addition of tumor cells, the inhibitor of NO synthase L-NMMA (1 mM) or anti-TNF-α monoclonal antibody (0.5 μg/ml) or both were added as indicated in Results. Culture supernatants were collected for nitrite and TNF-α assay at different time-points as indicated, and were stored at − 20°C until assayed.

Quantitative estimation of DNA fragmentation

Percent specific DNA fragmentation was measured following a method given by Cui *et al.*[17] Tumor target cells in logarithmic phase of growth were radiolabeled in culture medium with 0.5 μCi/ml [^{3}H]Tdr for 18 h, washed thoroughly in RPMI 1640, layered over the transwell inserts, and co-cultured with macrophages in 24-well tissue culture plates for various time periods and culture conditions as described. Cell-free culture supernatants (M) were collected at indicated time-points and preserved at 4°C. Tumor cells in the transwell inserts were carefully removed after 3, 6, 12 and 24 h. These cells were lyzed with 50 μl of ice-cold lysis buffer (25 mM sodium acetate, pH 6.6) for 1 h, and DNA from the cells was separated by centrifugation at 1300 *g* for 20 min into fragmented low molecular weight (supernatant; S) and intact high molecular weight (pellet; P) fractions. Radioactivity was determined by liquid scintillation counter (LKB, Turku, Finland).

Percent specific fragmentation was calculated as

$$\text{Percent specific fragmentation} = \left[\frac{\text{experimental c.p.m. (M + S)}}{\text{experimental c.p.m. (M + S + P)}} - \frac{\text{spontaneous c.p.m. (M + S)}}{\text{spontaneous c.p.m. (M + S + P)}}\right] \times 100$$

In the formula, experimental and spontaneous fragmentation refer to radiolabeled DNA obtained from

tumor cells co-cultured with cisplatin and IFN-γ treated macrophages and their respective untreated controls.

Qualitative analysis of DNA fragmentation on agarose gel electrophoresis

Extraction of target cell DNA was performed following the method given by Prigent *et al.*[18] with some modification. Tumor target cells, 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 resolubilized in 30 μl of Tris-EDTA 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 EtBr. DNA was visualized and photographed on a UV transilluminator.

Changes in the nuclear morphology

At various time-points as indicated, changes in nuclear morphology associated with apoptosis were studied by using the DNA fluorescent dye propidium iodide. Cells were fixed in 80% ice-cold ethanol, washed twice with cold phosphate-buffered saline (PBS) and stained with propidium iodide (50 μg/ml in PBS). Observations were made under fluorescence microscopy to observe nuclear changes.

Percent viability and percent apoptotic cell population assay

Percent viability assay of the target cells was done by Trypan blue exclusion test.[19] Cell count was performed on a hemocytometer with an equal volume of cell suspension and Trypan blue (0.3% in PBS). Trypan blue-stained cells were considered non-viable. Similarly, percent apoptotic cells was determined by identifying and counting apoptotic cells, on the basis of their characteristic apoptotic and cellular nuclear morphology.

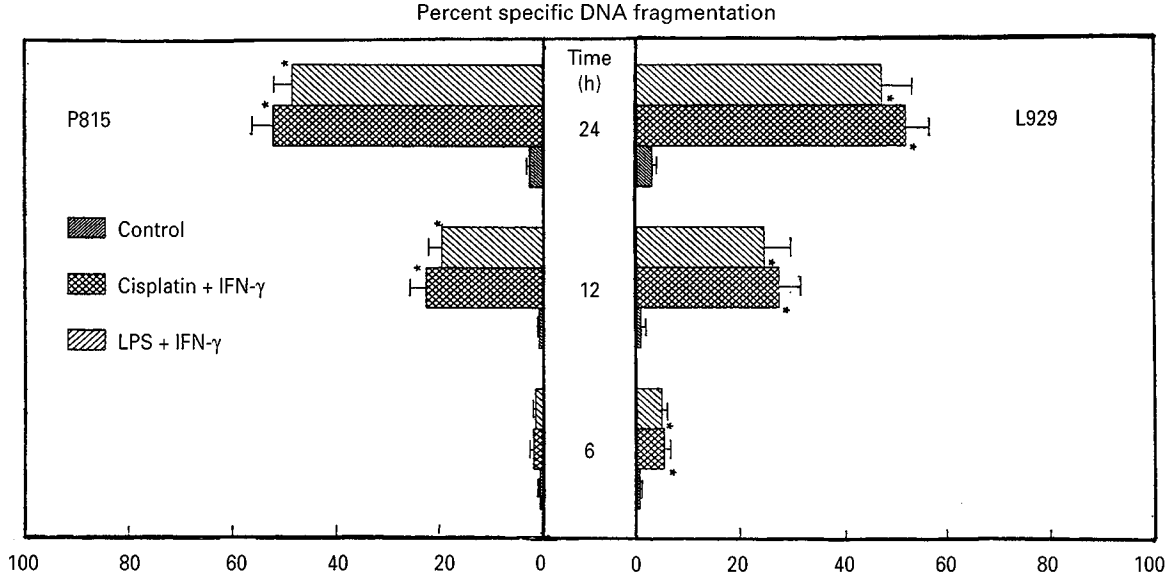


Figure 1. Kinetics of percent specific DNA fragmentation in P815 and L929 cells. Peritoneal macrophages (2×10^6 cells/well) in 24-well tissue culture plates, treated with cisplatin (5 μg/ml) and IFN-γ (50 U/ml) or LPS (5 μg/ml) and IFN-γ (50 U/ml), were co-cultured with [^{3}H]Tdr-labeled tumor target cells (P815 or L929; 2×10^5 cells/well), the co-cultures being separated by prewetted transwell inserts. Percent specific DNA fragmentation in tumor targets was measured 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 co-cultures.

Nitrite determination

Cell-free culture supernatants, collected at different time-points as indicated, were assayed for nitrite by a microplate assay method according to Ding *et al.*[20] Briefly, 100 μl of culture supernatant was incubated with an equal volume of Griess reagent (one part 1% sulfanilamide in 2.5% H_3PO_4 plus one part 0.1% naphthyl-ethylene-diamine dihydrochloride in distilled water) at room temperature for 10 min. The absorbance was taken at 540 nm in a microtiter plate reader. Nitrite concentration was quantified by using sodium nitrite as standard.

TNF-α bioassay

Cell-free culture supernatants, collected at different time-points as indicated, were assayed for TNF-α bioactivity by the method given by Mathews *et al.*[21] Briefly, 3×10^4 L929 cells were plated in complete medium containing actinomycin D (2 μg/ml) in each well of 96-well flat-bottom culture plates and culture supernatants were added in a 1:1 ratio. After 18 h incubation at 37°C in a CO_2 incubator the supernatant was flicked off and cells were fixed in 5% formaldehyde in PBS, followed by staining with 0.5% aqueous crystal violet. Cells were then lysed in 33% acetic acid and optical density was measured in a microtiter plate reader at 540 nm. The TNF activity was expressed in terms of percent cytotoxicity, which was calculated using the formula:

$$\text{percent cytotoxicity} = \frac{C - E}{C} \times 100$$

where C represents the absorbance of control cells and E represents the absorbance of experimental cells.

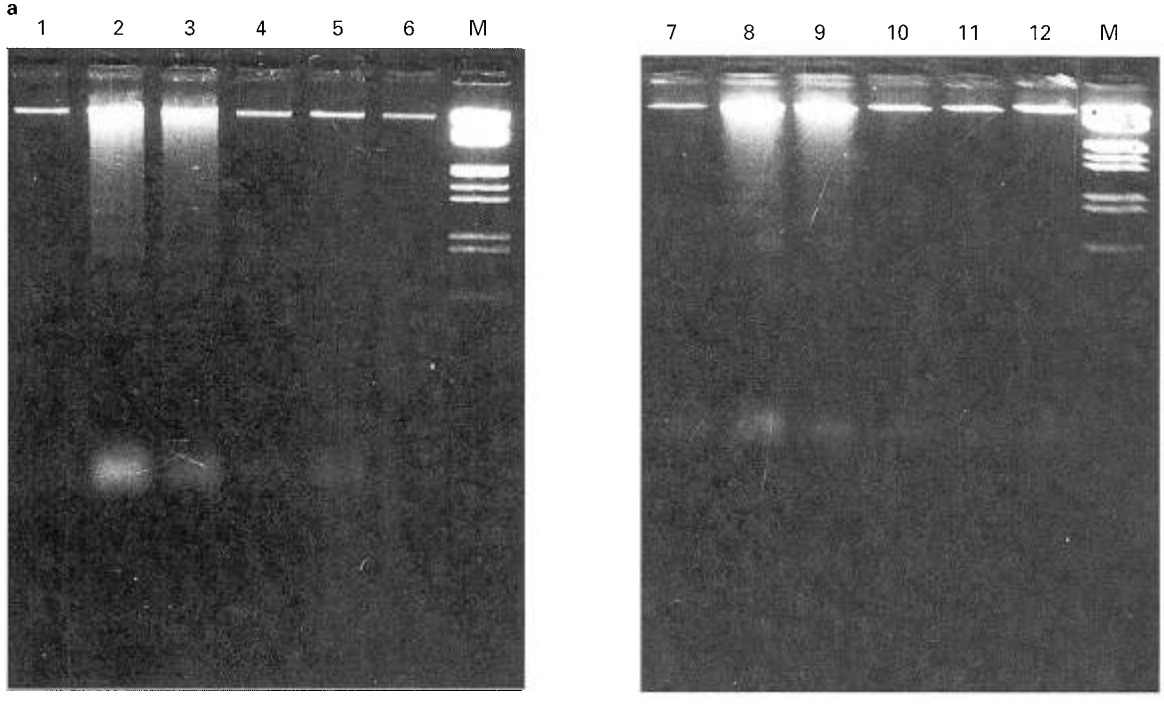
Data presentation

Results are expressed as the means $\pm$ 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

Quantitative estimation of percent specific DNA fragmentation in tumor targets

Macrophages treated with cisplatin and IFN-γ induced DNA fragmentation in P815 and L929 cells after 6 h co-culture. No significant DNA fragmentation was observed in untreated controls. As shown in Figure 1, time



kinetics studies of percent specific DNA fragmentation showed a linear pattern with time in both the tumor targets. A similar pattern of percent specific DNA fragmentation was also observed for LPS and IFN-γ treated macrophages.

Qualitative analysis of DNA fragmentation agarose gel electrophoresis

Although results of the percentage DNA fragmentation assay suggested that cisplatin and IFN-γ treated macrophages induced DNA fragmentation in tumor targets, they did not provide any qualitative data. To investigate whether DNA fragmentation is random or oligonucleosomal, DNA isolated from the target cells was analyzed by agarose gel electrophoresis. DNA extracted from target cells (P815 or L929), co-cultured with cisplatin and IFN-γ or LPS and IFN-γ treated macrophages, showed oligonucleosomal cleavage giving a ladder pattern on agarose gel electrophoresis. The DNA ladder appeared only after 12 h co-culture in both the tumor targets. DNA extracted from target cells, co-cultured with untreated macrophages,

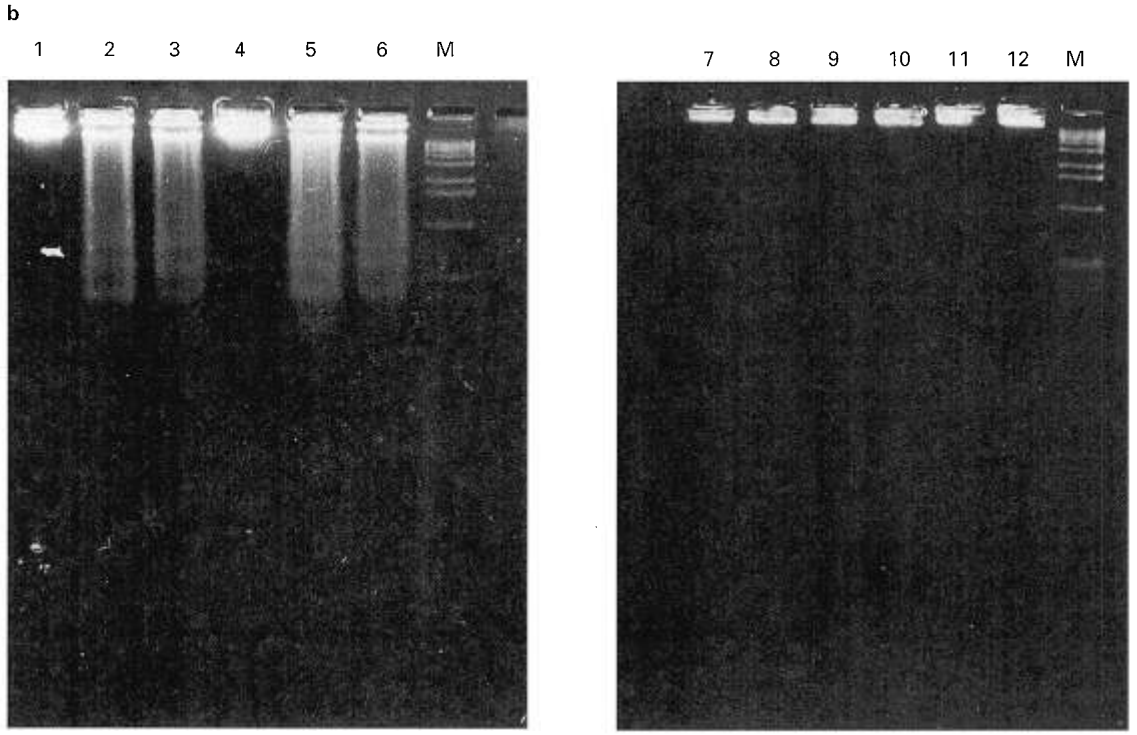


Figure 2. Agarose gel electrophoresis of DNA extracted from tumor targets (P815 or L929). Peritoneal macrophages (2×10^6 cells/well) in 24-well tissue culture plates, treated with cisplatin (5 μg/ml) and IFN-γ (50 U/ml) or LPS (5 μg/ml) and IFN-γ (50 U/ml), were co-cultured with tumor target cells (P815 or L929; 2×10^5 cells/well) in the presence or absence of L-NMMA (1 mM) or anti-TNF-α mAb (0.5 μg/ml) as indicated. The co-cultures were separated by prewetted transwell inserts. After 24 h co-culture, DNA was extracted from the tumor target cells and analyzed by agarose gel electrophoresis as described in Materials and methods. (a) Lane 1, no DNA fragmentation in P815 cells co-cultured with untreated macrophages; lanes 2 and 3, DNA from P815 cells, showing ladder pattern when co-cultured with cisplatin and IFN-γ or LPS and IFN-γ treated macrophages; lanes 5 and 6, addition of L-NMMA prevented oligonucleosomal fragmentation of DNA in P815 cells co-cultured with cisplatin and IFN-γ or LPS and IFN-γ treated macrophages; lanes 8 and 9, addition of anti-TNF-α mAb did not block DNA fragmentation in P815 cells co-cultured with cisplatin and IFN-γ or LPS and IFN-γ treated mcrophages; lanes 4, 7 and 10, addition of L-NMMA or anti-TNF-α or both did not induce DNA fragmentation in P815 cells co-cultured with untreated macrophages; lanes 11 and 12, addition of L-NMMA and anti-TNF-α mAb prevented DNA fragmentation in P815 cells co-cultured with cisplatin and IFN-γ or LPS and IFN-γ treated macrophages. (b) Lane 1, no DNA fragmentation in L929 cells co-cultured with untreated macrophages; lanes 2 and 3, DNA from L929 cells, showing ladder pattern when co-cultured with cisplatin and IFN-γ or LPS and IFN-γ treated macrophages; lanes 5 and 6, addition of L-NMMA did not prevent oligonucleosomal fragmentation of DNA in L929 cells co-cultured with cisplatin and IFN-γ or LPS and IFN-γ treated macrophages; lanes 8 and 9, addition of anti-TNF-α mAb blocked DNA fragmentation in L929 cells co-cultured with cisplatin and IFN-γ or LPS and IFN-γ treated macrophages; lanes 4, 7 and 10, addition of L-NMMA or anti-TNF-α or both did not induce DNA fragmentation in L929 cells co-cultured with untreated macrophages; lanes 11 and 12, addition of L-NMMA and anti-TNF-α mAb prevented DNA fragmentation in L929 cells co-cultured with cisplatin and IFN-γ or LPS and IFN-γ treated macrophages. M, marker.

showed no sign of DNA fragmentation even up to 24 h of co-culture [Figure 2a (lanes 1, 2 and 3) and b (lanes 1, 2 and 3)].

Changes in the nuclear morphology of tumor targets studied by fluorescence microscopy

Figure 3 shows the nuclear morphology of a representative tumor target (P815) co-cultured with cisplatin and IFN-γ treated macrophages (Figure 3b) or untreated controls (Figure 3a). Upon co-culture with treated macrophages, the target cell nuclei showed the characteristic condensed and fragmented appearance (Figure 3b). Nuclear condensation and fragmentation were detectable after 12 h co-culture, which further enhanced with time. Target cells co-cultured with untreated macrophages or the target cells from the co-cultures (treated or untreated) up to 6 h did not show any changes in the nuclear morphology. Similar results were obtained with L929 cells investigated (not shown).

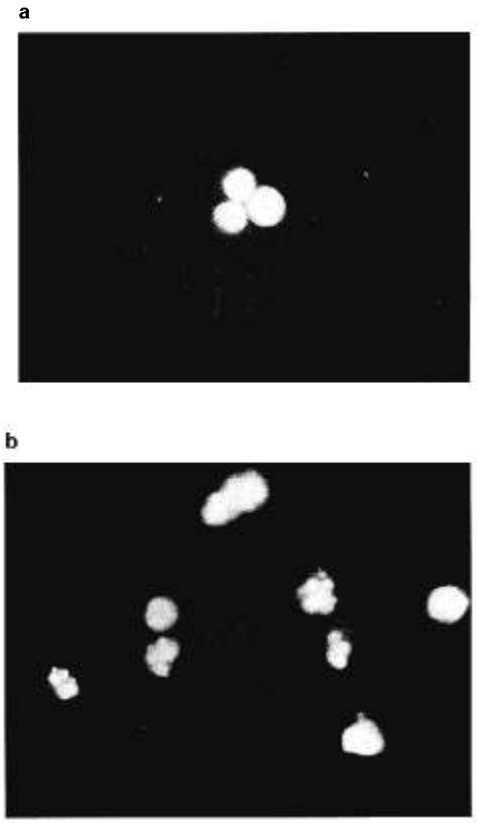


Figure 3. Morphology of target cell nuclei (P815) stained with propidium iodide. P815 cells (2×10^5 cells/well) were co-cultured with cisplatin and IFN-γ (a) or untreated (b) macrophages (2×10^6 cells/well). After 24 h co-culture, P815 cells were stained with propidium iodide (5 μg/ml) by the method described in Materials and methods, and observed under fluorescence microscopy.

Time kinetics studies of percent viability and percent apoptotic cell population in tumor targets

The percent cell viability and percent apoptotic cell population in tumor targets co-cultured with macrophages were also investigated. In both the tumor target cells, percent viability was found to be always greater than 93% with apoptotic cell populations less than 5% after 6 h co-culture or in controls up to 24 h (Figure 4a and b). In the co-cultures of tumor cells with cisplatin and IFN-γ treated macrophages, significant loss of cell viability was observed at 24 h, whereas apoptotic cell population appeared after 12 h.

Effector molecules involved in the apoptosis of tumor targets

In order to characterize the effector molecules involved, DNA fragmentation, nuclear changes, percent viability and percent apoptotic cell population in tumor targets were studied in the presence of various inhibitors. As shown in Table 1, addition of L-NMMA, a specific inhibitor or NO synthase, to the co-culture completely inhibited DNA fragmentation in P815 cells, suggesting this fragmentation to be NO dependent. Nitrite accumulation in the co-culture supernatants with cisplatin and IFN-γ treated macrophages was found to reach approximately 55 μM at 24 h, following a linear pattern with time (Figure 5). The presence or the absence or the type of tumor target cells did not interfere with the amount of NO produced by macrophages (data not shown). A comparison of time kinetics of NO production and percent specific DNA fragmentation (Figure 5 and Figure 1) indicates that DNA fragmentation is paralleled by NO accumulation, suggesting a dose-dependent relationship between the two. Exposure to SNP, a NO donor, resulted in DNA fragmentation in P815 cells (Table 1). Addition of anti-TNF-α mAb (0.5 μg/ml) did not prevent DNA fragmentation in P815 cells or treatment with TNF-α (1 nM) up to 24 h also did not cause DNA fragmentation in P815 cells (Table 1), which rules out the possibility of the involvement of TNF-α in the DNA strand breaks of P815 cells.

However, in contrast to the results obtained for P815 cells, addition of L-NMMA to the co-culture did

not protect DNA fragmentation in L929. Exposure to the NO donor, SNP, did not cause DNA fragmentation in L929 cells (Table 1), suggesting that induction of apoptosis in L929 cells is NO independent. However, blocking TNF-α in the co-culture with anti-TNF-α mAb resulted in a complete inhibition of DNA fragmentation in L929 cells. Further evidence for TNF-α-dependent DNA fragmentation comes from the results of the experiments in which treatment of L929 directly with TNF-α resulted in DNA break (Table 1).

Similar to the findings of quantitative assay experiments, blocking iNOS with L-NMMA completely prevented DNA fragmentation in P815 cells [Figure 2a (lanes 5 and 6)] or blocking TNF-α with anti-TNF-α

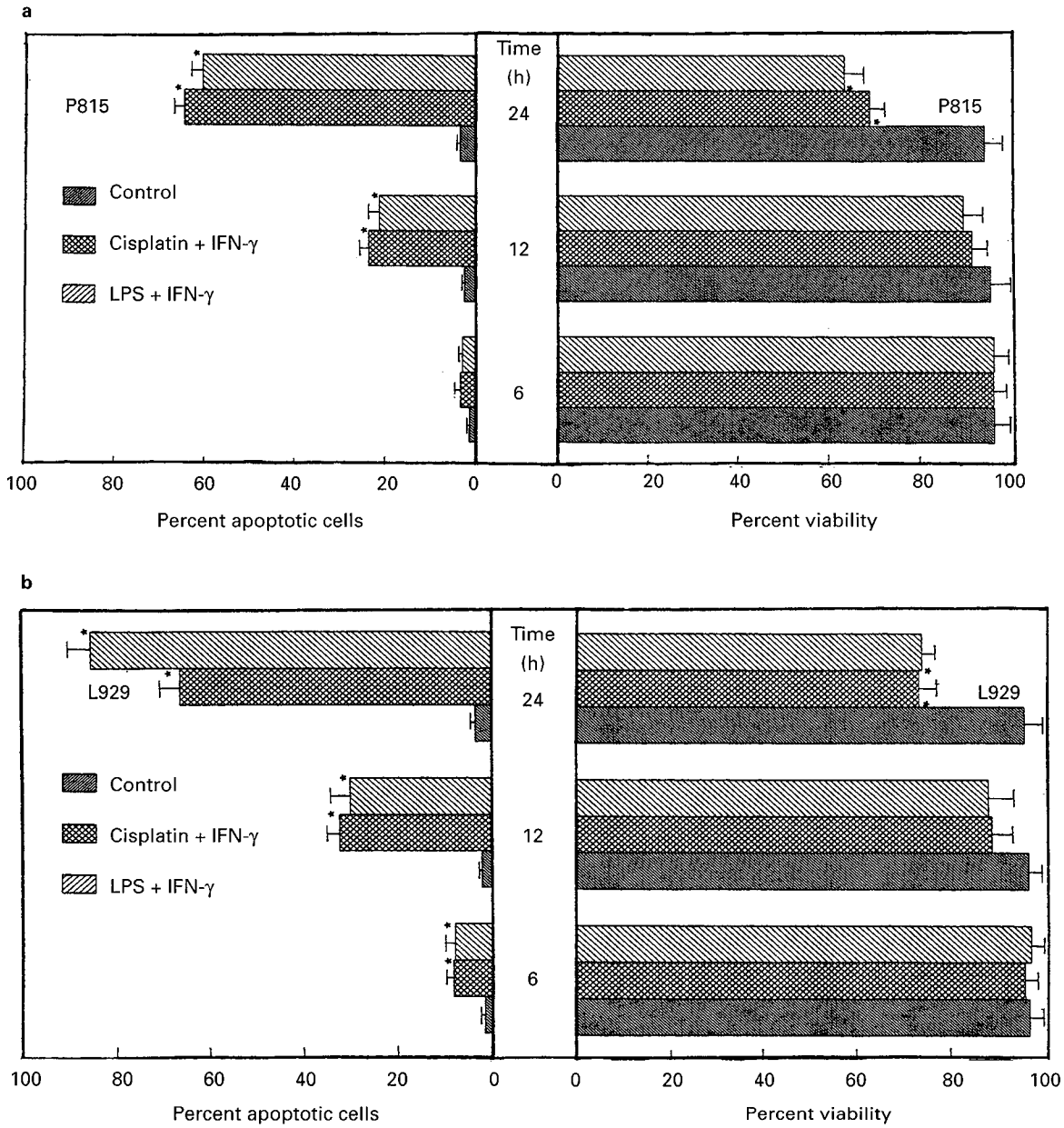


Figure 4. Kinetics of percent apoptotic cell population versus percent viability of tumor target cells: (a) P815 and (b) L929. Tumor target cells (2×10^5 cells/well) were co-cultured with cisplatin (5 μg/ml) and IFN-γ (50 U/ml) or LPS (5 μg/ml) and IFN-γ (50 U/ml) treated macrophages (2×10^6 cells/well), the co-cultures separated by prewetted transwell inserts. Percent apoptotic cell population and percent viability were assayed by the method 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 co-cultures.

Table 1. Percent specific DNA fragmentation, percent apoptotic cell population and percent viability assay in tumor targets in response to effector molecules

Treatment	TNF-α (cytotoxicity)	Nitrite (μM)	Percent specific DNA fragmentation		Percent apoptosis		Percent viability	
			P815	L929	P815	L929	P815	L929
Medium	12.7±2.3[a]	4.2±0.9	2.6±0.4	3.1±0.7	3.3±1.0	3.4±1.1	94.0±2.1	95.0±3.6
Cisplatin+IFN-γ	65.5±4.8*	55.0±3.1*	52.0±4.1*	52.1±4.3*	64.0±2.0*	65.9±4.3*	69.0±3.1*	72.6±3.6*
Cisplatin+IFN-γ+ L-NMMA	66.2±2.9**	12.1±2.0*	29.0±0.5**	51.0±3.2	3.9±0.9**	63.5±2.0	93.1±4.1	74.1±4.6
Cisplatin+IFN-γ+ abTNF-α	14.1±2.1**	53.9±4.1	51.0±3.4	2.8±0.2**	62.0±3.1	4.9±2.2**	66.3±3.7	94.0±3.3
SNP (0.5 mM)	–	48.6±3.2	42.3±4.1	3.5±1.6	55.0±4.9	3.9±1.1	71.3±3.2	94.1±3.3
TNF-α (1 nM)	74.0±5.9	–	2.9±0.6	45.9±3.2	3.9±0.6	51.6±4.1	94.3±2.6	65.1±4.4

*$p<0.05$ versus values for untreated control co-cultures.
**$p<0.05$ versus values for cisplatin and IFN-γ treated co-cultures.
[a]The numbers represent mean±SD of triplicate co-cultures.
Peritoneal macrophages (2×10^6 cells/well) in 24-well tissue culture plates, treated with cisplatin (5 μg/ml) and IFN-γ (50 U/ml), were co-cultured with labeled ([^{3}H]thymidine) or unlabeled tumor target cells (P815 or L929; 2×10^5 cells/well) for 24 h in the presence or absence of L-NMMA (1 mM) or anti-TNF-α mAb (0.5 μg/ml) as indicated. The co-cultures were separated by transwell prewetted inserts. Target cells were also directly treated with SNP (0.5 mM) or TNF-α (1 nM) as indicated for 24 h. The nitrite concentration and TNF-α bioactivity in the culture supernatants, and percent specific DNA fragmentation, percent apoptotic cell population and percent viability of tumor targets were measured as described in Materials and methods.

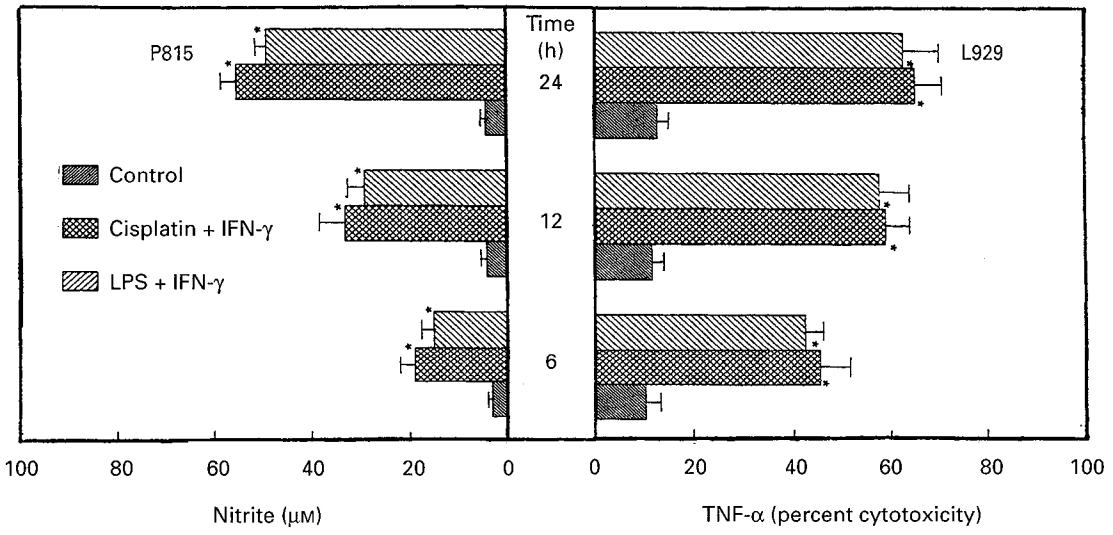


Figure 5. Kinetics of NO accumulation and TNF-α bioactivity (percent cytotoxicity). Peritoneal macrophages (2×10^6 cells/well) treated with cisplatin (5 μg/ml) and IFN-γ (50 U/ml) or LPS (5 μg/ml) and IFN-γ (50 U/ml) were co-cultured with tumor target cells (P815 or L929; 2×10^5 cells/well), the co-cultures separated by prewetted transwell inserts. The nitrite concentration and TNF-α bioactivity in the culture supernatants were assayed at indicated time intervals by the method described in Materials and methods. The presence or the absence or the type of tumor targets did not show significant difference in the amount of nitrite or TNF-α produced (not shown). Data shown are means ± SD and are representative of three independent experiments done in triplicate. *$p<0.05$ versus values of control co-cultures.

mAb prevented oligonucleosomal cleavage in L929 [Figure 2b (lanes 8 and 9)] as observed on agarose gel electrophoresis. Morphological alterations in target cell nuclei were also prevented in the presence of specific inhibitors (not shown). Study of percent cell viability and percent apoptotic cell population of tumor targets, in the presence of specific inhibitors, showed that inhibition of responsible effector molecules resulted in inhibition in the loss of cell viability. More than 93% cells were viable with apoptotic cell populations less than 5% (Table 1). Exposure to SNP (0.5 mM) up to 24 h caused a decline in cell viability

with 55 ± 4.9% apoptotic cell populations in P815 cells but failed to induce apoptosis or loss in cell viability in L929 cells. Conversely, treatment with murine recombinant TNF-α (1 nM) up to 24 h resulted in 51.6 ± 4.1% apoptotic cell population with a corresponding viability of 65.1 ± 4.4% in L929 cells, but caused no effect on P815 cells (Table 1).

Discussion

The widely used anti-cancer drug cisplatin has not only been shown to have cytotoxic effect but has also been shown to have immunomodulatory effect.[2-7] In combination with other immunostimulants, such as IFN-γ, interleukins and LPS, cisplatin has shown a synergistic enhancement in tumoricidal activity of macrophages, both *in vivo* and *in vitro*.[8-10] In the present study, we investigated the mode of target cell destruction by macrophages, treated with cisplatin in combination with IFN-γ, and the effector molecules involved therein. The results of the present study show that death of tumor targets by cisplatin and IFN-γ activated macrophages may involve apoptosis as one of the killing mechanisms for which cell-to-cell contact is not essential. These findings are consistent with others that macrophages may exert their cytotoxic effects via diffusible mediators.[3] Further, there has been much discussion about the additive or independent action of effector molecules. Some reports suggest that these effector molecules act synergistically,[22] while others propose that effector molecules show target cell specificity.[23] Our findings suggest that effector molecules show target cell specificity for inducing apoptosis in L929 and P815 cells. The effector molecule responsible for inducing apoptosis in P815 cells was found to be NO. This finding was confirmed by the observation that addition of L-NMMA, the NO synthase inhibitor, showed a protective effect against apoptosis. Moreover, treatment with SNP, a NO donor, resulted in apoptosis in P815 cells. Addition of anti-TNF-α mAb or direct treatment with TNF-α did not cause apoptosis in P815 cells. Conversely, for L929, the apoptotic factor was found to be TNF-α but not NO. These findings suggest that NO and TNF-α are mutually independent for their action with respect to their cytotoxic action against L929 or P815 cells. Our results are consistent with reports suggesting that L929 cells (TNF-α-sensitive tumor cells) are resistant to NO-mediated cytotoxicity, whereas P815 cells (NO-sensitive tumor cells) are resistant to TNF-α-mediated cytotoxicity.[23] However, the possibility that effector molecules may act synergistically or additively in the case of other target cells is not ruled out. As reported by others, the killing mechanism against HL-60 and Raji was largely blocked by L-arginine depletion, slightly blocked by the addition of anti-TNF-α mAb and completely blocked by the combined effect of the two.[22] Further, apoptosis cannot be the sole mechanism for target cell killing, and the same effector molecule may induce apoptosis in one target cell and necrosis in the other. TNF-α induces apoptosis in F17 cells and necrosis in L-M cells.[24] Therefore, it is obviously not sufficient to rely on one or two targets to answer the general question on the mechanism of cisplatin and IFN-γ treated macrophage-mediated cytotoxicity and the mode of target cell death.

Several approaches were used to study apoptotic mode of cell death. First, DNA fragmentation in target cells by [^{3}H]Tdr assay was investigated. Time kinetics studies showed a linear relationship between percent specific DNA fragmentation and time, indicating onset of irreversible DNA fragmentation upon induction by effector molecules, which progressed with time. Further, qualitative analysis of DNA on agarose gel electrophoresis confirmed oligonucleosomal cleavage. Since oligonucleosomal fragmentation of DNA by itself cannot be considered as a sufficient test for apoptosis,[25] corresponding nuclear changes were simultaneously observed by fluorescence microscopy which also favored occurrence of apoptosis.

A comparison of the time kinetics of percent specific DNA fragmentation with that of the appearance of a DNA ladder as detected by agarose gel electrophoresis shows that although DNA fragmentation was detectable at 6 h, it did not give a ladder patternon agarose gel. The ladder pattern of DNA appeared only after 12 h, when percent specific fragmentation was greater than 20%. The reason for this could be that low molecular weight DNA may not be generated during early stages of apoptosis, despite the presence of extensive high molecular weight DNA fragments, which are generated prior to internucleosomal DNA fragmentation.[26,27] Time kinetics studies of percent viability and percent apoptosis and its comparison with that of percent specific DNA fragmentation indicate DNA fragmentation to be a very early event, whereas loss of viability occurred at a later stage of apoptosis. We could not detect any necrotic form of cell death as studied by Trypan blue uptake and propidium iodide staining. More than 88% cells were viable at 12 h with approximately 20% apoptotic cells. These data rule out the possibility of cell death, at least by primary necrosis. A comparison of the time kinetics of quantitative and qualitative DNA fragmentation study with that of nuclear changes indicates that all these events are inter-related as they followed same time

kinetics and responded to specific co-culture conditions in a similar manner. NO, TNF-α, IL-1, reactive oxygen intermediates, etc., are the major cytotoxic molecules among more than 100 produced by activated macrophages.[3] Although various cellular responses of these effector molecules are known, how and in what sequence these events result in apoptosis by breaking the protective mechanism of target cells are not known. Furthermore, whether molecular mechanisms involved in NO induced apoptosis and TNF-α induced apoptosis are common or not remains to be investigated.

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