Cisplatin-treated murine peritoneal macrophages induce apoptosis in L929 cells: role of Fas–Fas ligand and tumor necrosis factor–tumor necrosis factor receptor 1

Puja Chauhan, Ajit Sodhi and Shikha Tarang

Cisplatin [*cis*-diamminedichloroplatinum (II)]-treated murine peritoneal macrophages interact with L929 cells *in vitro* in a sequential manner, resulting in the formation of contact between the two cells. This interaction leads to the death of L929 cells by the process of apoptosis. The detailed investigations have suggested the involvement of two different pathways in macrophage-mediated L929 cell apoptosis. It is observed that the induction of apoptosis in L929 cells by cisplatin-treated macrophages is contact dependent and is mediated through Fas–Fas ligand and tumor necrosis factor–tumor necrosis factor receptor 1 pathways. This conclusion was based on the Western blot and immunoprecipitation analysis of Fas–Fas ligand, tumor necrosis factor–tumor necrosis factor receptor 1, Fas-associated death domain and tumor necrosis factor receptor-associated death domain. The Fas–Fas ligand interaction between macrophages and L929 cells increased the expression of Fas-associated death domain, and tumor necrosis factor–tumor necrosis factor receptor 1 interaction between macrophages and L929 cells increased the expression of tumor necrosis factor receptor-associated death domain in L929 cells. The induction of apoptosis in L929 cells was investigated by DNA fragmentation, Annexin V staining and Western blot analysis of Bax, Bcl-2, Bid, cytochrome *c*, poly(ADP ribose) polymerase, CAD, caspase-8 and caspase-3. *Anti-Cancer Drugs* 18:187–196 © 2007 Lippincott Williams & Wilkins.

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Introduction

Macrophages are a part of the mononuclear phagocyte system and are professional antigen-presenting cells for adaptive immunity [1–3]. Macrophages effectively kill tumor cells through both antibody-dependent and antibody-independent mechanisms [4,5]. Macrophages on treatment with the anticancer drug cisplatin [*cis*-diamminedichloroplatinum (II)] are activated and mediate cytotoxicity against tumor cells [6]. Although the mechanism responsible for the tumoricidal activity of activated macrophages remains poorly defined, it appears to involve both cell contact and secretion of a number of distinct cytotoxic molecules [7,8]. Nitric oxide and tumor necrosis factor (TNF)-α produced by activated macrophages are potent mediators of tumor cell death [9–11]. The Fas (APO-1/CD95) and TNF receptor (TNFR) are members of the TNF/nerve growth factor receptor superfamily involved in various forms of physiological and pathological cell death [12]. Treatment with anisomycin, ultraviolet radiation or cytotoxic drugs induces the expression of Fas ligand (FasL) and TNF in macrophages and T cells [13–15]. According to Ichinose *et al.* [16], tumor cell killing by activated macrophages might be in part due to the release of interleukin-1, TNF-α and partly due to the membrane associated forms of these molecules. It has been reported that when activated macrophages were treated with cytochalasin B, it inhibits binding of macrophages with tumor cells and the macrophage-mediated cytotoxicity was significantly inhibited [17]. These findings prompted us to determine whether membrane-associated TNF-α and FasL are responsible for the induction of apoptosis of L929 cells by cisplatin-treated macrophages, subsequent to establishment of a contact.

Fas–FasL and TNF–TNFR1 interactions are important cellular pathways regulating the induction of apoptosis in a wide variety of tissues [18–20], through Fas–FasL- and TNF–TNFR1-independent, as well as Fas–FasL-dependent cell apoptosis, have been reported in cisplatin-treated cells [21,22]. Apoptosis triggered by Fas ligation is crucial within the immune system, as the ligand for Fas is FasL, and is chiefly expressed on cytotoxic T cells and the phagocyte cells [23]. TNF-α is a potent cytokine produced by many cell types, including activated macrophages, monocytes and lymphoid cells in response to inflammation, infection or in contact with tumor cells [24]. These responses are elicited by TNF-induced

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trimerization of two distinct cell surface receptors, TNFR1 and TNFR2 [25,26]. TNFR1 induces apoptosis when it is ligated with TNF, and TNFR2, a poor inducer of apoptosis, is involved in activation of nuclear factor-κB and cell survival [27,28]. The molecular mechanisms involved in Fas and TNFR1-induced apoptosis in tumor cells involve death domains and activation of caspases [12]. Activation of TNFR1 results in the recruitment of the TNFR1-associated death domain (TRADD) protein, whereas Fas interacts with another adaptor protein, i.e. (FADD) Fas-associated death domain. Procaspase-8 binding to FADD results in its cleavage and activation [29]. This entire cluster is sometimes called the death-inducing signaling complex [30]. Thus, FADD binds directly to Fas, whereas recruitment of FADD to TNFR1 is via TRADD [31]. Activated caspase-8 acts upon the downstream caspases that participate in the execution of the apoptotic process. In most cells, TNFR1 and Fas-induced apoptosis is associated with DNA fragmentation, which is induced by the activation of DNase. In the cells, the DNase is kept in its inactive state by association with ICAD. When ICAD is degraded by caspase-3, DNase is activated, causing DNA fragmentation [32].

Apoptotic cells only become sensitive to the Fas-mediated cell death once antiapoptotic Bcl-2 levels decrease. Caspase-8 cleaves the cytosolic proapoptotic protein Bid. Upon cleavage, Bid translocates to mitochondrial membranes and binds to Bad, and induces the release of cytochrome *c* from mitochondria [33,34]. The results presented here suggest a contact-mediated mechanism by which cisplatin-treated macrophages induce L929 cell apoptosis via Fas–FasL and TNF-TNFR1 pathways.

Materials and methods

Mice

Inbred strains of BALB/c mice of either sex at 8–10 weeks (20–22 g) of age were used for obtaining peritoneal macrophages.

Cell cultures and reagents

L929 cells were obtained from the National Tissue Culture Facility (Pune, India). The L929 cells were maintained by subculturing in complete RPMI-1640 medium.

Macrophage monolayers were cultured in RPMI-1640 medium supplemented with heat-inactivated fetal calf serum (10%), penicillin (100 U/ml), streptomycin (100 U/ml) and gentamicin (20 μg/ml) at 37°C in humidified air containing 5% CO_2. Fetal calf serum was from Biological Industries (Haemek, Israel) and cisplatin was obtained from Cadila Pharmaceuticals (Mumbai, India). Polyclonal antibodies against poly (ADP ribose) polymerase (PARP), caspase-3, caspase-8, Fas, FasL, FADD, TNF, TNFR1, TRADD, cytochrome *c*, Bid, actin and horseraddish peroxidase-conjugated rabbit, mouse and goat IgGs were obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA). RPMI-1640, caspase-3 inhibitor Ac-DEVD-CHO and caspase-8 inhibitor Ac-IETD-CHO were purchased from Sigma-Aldrich (St Louis, Missouri, USA). All the reagents were endotoxin-free as determined by the Limulus amoebocyte lysate assay (sensitivity limit, 0.1 ng/ml).

Isolation of macrophages and coincubation with L929 cells

Macrophage monolayers were prepared as described previously [7]. Peritoneal exudate cells were harvested from peritoneal lavage using chilled serum-free RPMI-1640 medium and added to wells of 24-well tissue culture plates (Nunc, Roskilde, Denmark). After 2 h of incubation at 37°C, in an atmosphere of 5% CO_2, the nonadherent cells were removed by washing (three times) with warm serum-free medium and the adherent cells were further incubated in complete medium overnight to form macrophage monolayers. More than 95% of the adherent cell population was macrophages as determined by morphology and nonspecific esterase staining. Macrophage monolayers were treated with cisplatin (10 μg/ml) for 2 h. Thereafter, macrophages (2×10^6/well) were washed with phosphate-buffered saline (PBS) and further coincubated with L929 cells (1 : 1 ratio) in complete medium for various time intervals as indicated in the Results. In another set of experiments, the L929 cells were plated (2×10^6/ml), and were allowed to attach and spread for 2 h. A wet (RPMI-1640 medium) nitrocellulose disk of 1.2 μm pore size was placed on L929 cells and then cisplatin-treated or cisplatin-untreated macrophages (2×10^6 cells) were layered on the nitrocellulose disk. In the third set of experiments, the macrophage monolayers (treated or untreated) were fixed with paraformaldehyde (4%) for 15 min at room temperature. Fixed cells were washed with PBS (three times) and then coincubated with L929 cells in fresh complete medium for different time intervals.

Treatment of macrophages with cisplatin for 2 h, washing and further incubation for 36 h in complete medium did not show any cell death.

Preparation of cell lysates and immunoblotting

After specific time periods of coincubation the nitrocellulose disks were taken out with macrophages. The L929 cells in the wells were washed with ice-cold PBS containing 1 mmol/l Na_3VO_4, then lysed in 50 μl of lysis buffer [20 mmol/l Tris–HCl, pH 8, 137 mmol/l NaCl, 10% glycerol (v/v), 1% Triton X-100 (v/v), 1 mmol/l Na_3VO_4, 2 mmol/l ethylenediaminetetraacetic acid, 1 mmol/l phenylmethylsulfonyl fluoride, 20 μmol/l leupeptin and 0.15 units/ml aprotonin] for 20 min at 4°C. The lysates were centrifuged at 10 000 *g* for 10 min and the

supernatants (containing Triton X-100 soluble proteins) were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis were (SDS–PAGE) at 20 mA. Forty micrograms of protein was loaded in each well of SDS–PAGE. The separated proteins were transferred to nitrocellulose membrane (1 h at 125 V using Bio-Rad Mini Trans Blotter; Bio-Rad, Hercules, California, USA). The transferred proteins on the membrane were then immunoblotted with primary antibody, incubated with horseradish peroxidase-conjugated secondary antibody and visualized by the Chemiluminescence Western Blotting Kit (Santa Cruz Biotechnology). To monitor equal loading of protein, Western blot analysis using antibody directed against actin was performed for each experiment, as shown in the lower panels.

Immunoprecipitation

The macrophage–L929 cocultures after specific time periods of incubation were lysed using the lysis buffer. In another set, L929 cells after specific time periods of coincubation with cisplatin-treated macrophages separated by a 1.2-μm nitrocellulose disk were lysed. The cell lysates (100 μl) were incubated with 2 μg Protein A at 4°C for 1 h. The cell lysates were centrifuged at 10 000 *g* for 10 min. The supernatants were incubated with 5 μl of antibody overnight at 4°C with constant shaking. Five micrograms of Protein A was added, and the samples were incubated for 4 h at 4°C and centrifuged at 10 000 *g*. The pellet was washed five times with 1 ml lysis buffer by centrifugation, and suspended in 50 μl of lysis buffer and an equal volume of sample buffer. The samples were boiled for 3 min at 95°C, centrifuged at 10 000 *g* and the supernatants were run on 10% SDS–PAGE. Western blotting of samples was performed as described.

Fluorescence microscopy

Macrophages grown on cover slips in single-well plates were treated with cisplatin, washed and cocultured with L929 cells for different time intervals. Cocultures were washed with PBS (three times), fixed in 4% paraformaldehyde for 10 min at room temperature, stained with Annexin V–fluorescein isothiocyanate (FITC) or FasL–FITC or TNFR1–FITC, and observed and photographed under an Olympus (BX51) fluorescence microscope (Olympus Optical, Tokyo, Japan). The white bar on the micrographs is 10 μm.

Assay of caspase-8 activity

The caspase-8 activity was determined using a calorimetric assay kit bought from Chemicon International (Temecula, California, USA). Briefly, L929 cells were coincubated with cisplatin-treated macrophages separated by a 1.2-μm nitrocellulose disk for different time intervals. The nitrocellulose disks were removed at stipulated time periods and the L929 cells were lysed as per the protocol provided. Caspase-8 colorimetric substrate IETD-pNA was added to the cell lysate (20 μl) and assay was performed in a 100-μl volume in 96-well flat-bottomed plates. The absorbance was measured on a microplate reader at a wavelength of 405 nm after 1 h of incubation at 37°C and the results are expressed as nanomoles of pNA per milligram of protein per 60 min.

Assay of caspase-3 activity

The caspase-3 activity was determined using a fluorimetric assay kit bought from Sigma-Aldrich. Briefly, L929 cells were coincubated with cisplatin-treated macrophages separated by a 1.2-μm nitrocellulose disk for different time intervals. The disks were then removed and the L929 cells were lysed as per the instructions provided. Five microliters of cell lysates were added to the wells of a 96-well flat-bottomed plate. For blank, 5 μl of 1 × assay buffer was used. To some wells, 200 μmol/l of caspase-3 inhibitor was added. Two microliters of reaction mixture were then added gently to each well. The plate was covered for 1 h in the dark, and the reading was taken at excitation 360 nm and emission 460 nm. The results are expressed as nanomoles of 7-amino-4-methyl coumarin per milligram of protein per 60 min.

Quantitative estimation of DNA fragmentation

Quantitative measurement of DNA fragmentation was measured by the method given by Sellins and Cohen [35]. Briefly, L929 cells after specific time periods of coincubation with cisplatin-treated macrophages separated by a 1.2-μm nitrocellulose disk were lysed in 0.5 ml of TTE (Tris ethylenediaminetetraacetic acid buffer, pH 7.4 containing 0.2% Triton X-100). Fragmented DNA was separated from intact chromatin by centrifuging the microcentrifuge tubes at 13 000 *g* at 4°C for 10 min. Supernatant (S) containing the fragmented DNA was transferred to another microcentrifuge tube. Then, 0.5 ml TTE buffer was added to the pellet (P) containing intact high-molecular-weight DNA. Cell-free culture supernatants (M) were also collected at indicated time intervals. DNA was precipitated overnight in 0.5 ml of 25% trichloroacetic acid (TCA) followed by centrifugation at 13 000 *g* at 4°C for 10 min. Supernatants were discarded, and 80 μl of 5% TCA was added to each pellet and heated at 90°C for 15 min to hydrolyze DNA. At this stage, a blank was included containing 80 μl of 5% TCA. Then, 160 μl of freshly prepared diphenylamine reagent containing 240 mmol/l diphenylamine, 1.5% sulfuric acid and 0.01% acetaldehyde in acetic acid was added to each tube and vortexed, and the color was allowed to develop for 4 h at 37°C. A total of 200 μl of the colored solution was transferred to the wells of 96-well flat-bottomed enzyme-linked immunosorbent assay plates and the optical density was measured at 600 nm in a microtiter plate reader (Molecular Devices, California, USA).

% DNA fragmentation was calculated as

$$\%\,\text{DNA fragmentation} = \frac{M + S}{M + S + P} \times 100.$$

Results

Coculturing of L929 cells with cisplatin-treated macrophages results in the formation of a contact between the two cells by 2 h of incubation [7]. This contact formation is due to the fusion of the membranes of L929 cells with cisplatin-treated macrophages, as investigated by staining the two cells differentially with green or red membrane-labeling fluorescent dyes (Fluorescent Cell Linker kit; Sigma-Aldrich) (data not shown). Untreated macrophages do not make any such contact with L929 cells.

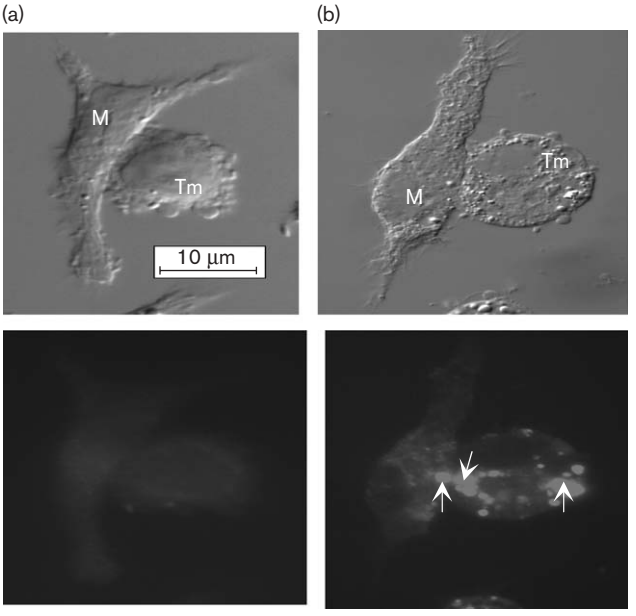
Fluorescence microscopical studies of Annexin V–fluorescein isothiocyanate staining of cocultures of L929 cells with cisplatin-treated macrophages

Bright Annexin V–FITC staining is observed in L929 cells after 3 h of coincubation with cisplatin-treated macrophages (Fig. 1b). L929 cells cocultured with untreated macrophages did not stain for Annexin V–FITC (Fig. 1a).

Fluorescence microscopical studies on the expression of Fas ligand on the cisplatin-treated macrophages cocultured with L929 cells

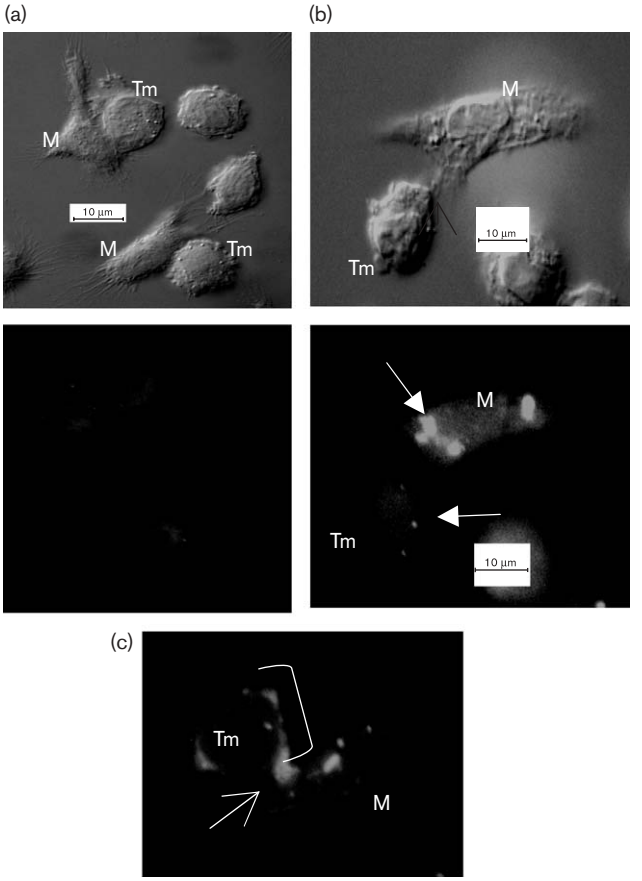
At 2 h of coculture, the expression of FasL is seen at the base of contact between macrophages and L929 cells

Fig. 1



Annexin V–fluorescein isothiocyanate staining of L929 cells cocultured with macrophages for 3 h: (a) untreated macrophages + L929 cells and (b) cisplatin-treated macrophages + L929 cells. Upper panel shows the corresponding cells observed under differential interference contrast. M, macrophage; Tm, L929 cell. The figure is representative of three independent experiments with similar results.

Fig. 2



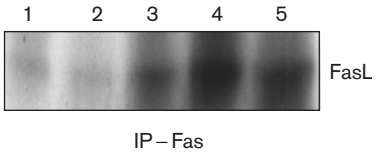
Immunofluorescence staining of cocultures of L929 cells and macrophages with anti-Fas ligand (FasL)–fluorescein isothiocyanate antibody: (a) untreated macrophages + L929 cells, (b) cisplatin-treated macrophages + L929 cells cocultured for 2 h; upper panel shows the corresponding cells observed under differential interference contrast. M, macrophage; Tm, L929 cell and (c) cisplatin-treated macrophages + L929 cells cocultured for 8 h. The formation of cytoplasmic contact between cisplatin-treated macrophages and L929 cells is indicated by black arrow in (b) and by white arrows in (c). In (b), the presence of FasL at the base of contact between macrophages and L929 cells is clearly seen (white arrows). The presence of FasL is prominent at the base of contact between macrophages and L929 cells (c) (white arrow). The spreading of macrophage over the L929 cell with bright expression of FasL is evident (c) (white bracket). The figure is representative of three independent experiments with similar results.

(Fig. 2b). At 8 h of coculture, the FasL is clearly observed at the area of contact between L929 cells and cisplatin-treated macrophages (Fig. 2c). The spreading of macrophage cup-like extension with FasL over the L929 cells is evident. No expression of FasL is seen on the untreated macrophages cocultured with L929 cells (Fig. 2a).

Expression of Fas–Fas ligand in L929 cells cocultured with cisplatin-treated macrophages

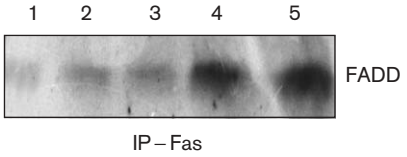
Cisplatin-treated or untreated macrophages were cocultured with L929 cells. After stipulated time intervals,

Fig. 3



Fas–Fas ligand (FasL) interaction as observed by immunoprecipitation (IP) with anti-Fas antibody, followed by immunoblotting with anti-FasL antibody in cocultures of L929 cells with macrophages. Lane 1, untreated macrophages + L929 cells; lane 2, cisplatin-treated macrophages + L929 cells cocultured for 2 h; lane 3, cisplatin-treated macrophages + L929 cells cocultured for 4 h; lane 4, cisplatin-treated macrophages + L929 cells cocultured for 8 h; lane 5, cisplatin-treated macrophages + L929 cells cocultured for 12 h. The figure is representative of three independent experiments with similar results.

Fig. 4



Fas–Fas-associated death domain (FADD) interaction as observed by immunoprecipitation (IP) with anti-Fas antibody followed by immunoblotting with anti-FADD antibody in L929 cells coincubated with macrophages separated by 1.2-μm nitrocellulose disks. Lane 1, untreated macrophages + L929 cells; lane 2, cisplatin-treated macrophages + L929 cells coincubated for 4 h; lane 3, cisplatin-treated macrophages + L929 cells coincubated for 8 h; lane 4, cisplatin-treated macrophages + L929 cells coincubated for 12 h; lane 5, cisplatin-treated macrophages + L929 cells coincubated for 16 h. The figure is representative of three independent experiments with similar results.

cells were lysed and immunoprecipitated with anti-Fas antibody and immunoblotted with anti-FasL antibody. Maximum expression of FasL was observed at 8 h of coculture of L929 cells with cisplatin-treated macrophages (Fig. 3). No or little expression of FasL is observed in cocultures of L929 cells with untreated macrophages.

Expression of Fas–Fas-associated death domain by immunoprecipitation in L929 cells coincubated with cisplatin-treated macrophages

L929 monolayers were coincubated with cisplatin-treated macrophages separated by a 1.2-μm nitrocellulose disk. After stipulated time intervals, the nitrocellulose disks were removed with macrophages. The L929 cells were then lysed, immunoprecipitated with anti-Fas antibody and immunoblotted with anti-FADD antibody. Maximum expression of FADD was observed at 12–16 h of coincubation of L929 cells with cisplatin-treated macrophages (Fig. 4). Only minimum expression of FADD is seen in L929 cells coincubated with untreated macrophages.

Fluorescence microscopical studies on the expression of tumor necrosis factor receptor 1 on L929 cells cocultured with cisplatin-treated macrophages

A diffused staining of TNFR1 is seen on L929 cells and untreated macrophage cocultures (Fig. 5a). At 2 h of coculturing of L929 cells with cisplatin-treated macrophages, enhanced TNFR1 staining is, however, observed at the base of the contact formed between L929 cell and macrophage (Fig. 5b). At 4 h of coculturing, bright TNFR1 expression is seen at the cytoplasmic contact area formed between the cisplatin-treated macrophages and L929 cells (Fig. 5c). At 12 h of coculturing, the TNFR1 staining is seen spread over the L929 cells (Fig. 5d).

Expression of tumor necrosis factor–tumor necrosis factor receptor 1 in L929 cells cocultured with cisplatin-activated macrophages

Paraformaldehyde-fixed cisplatin-treated and untreated macrophages were cocultured with L929 cells. After stipulated time intervals, cells were lysed and immunoprecipitated with anti-TNF antibody and immunoblotted with anti-TNFR1 antibody. Maximum expression of TNFR1 was observed between 8 and 12 h of coculture of L929 cells with cisplatin-treated macrophages (Fig. 6).

Expression of tumor necrosis factor receptor 1–tumor necrosis factor receptor 1-associated death domain by immunoprecipitation in L929 cells coincubated with cisplatin-treated macrophages

L929 monolayers were coincubated with cisplatin-treated macrophages separated by a 1.2-μm nitrocellulose disk. After stipulated time intervals, the disks were removed with macrophages. The L929 cells were then lysed, immunoprecipitated with anti-TNFR1 antibody and immunoblotted with anti-TRADD antibody. Maximum expression of TRADD was observed at 8 h of coincubation of L929 cells with cisplatin-treated macrophages (Fig. 7).

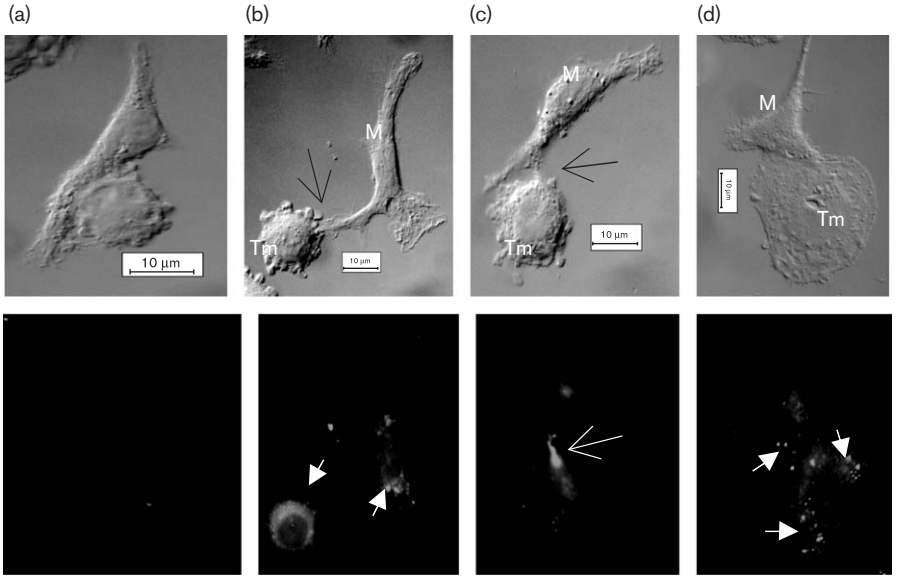
Expression of tumor necrosis factor receptor-associated death domain–Fas-associated death domain by immunoprecipitation in L929 cells coincubated with cisplatin-treated macrophages

L929 monolayers were coincubated with cisplatin-treated macrophages separated by a 1.2-μm nitrocellulose disk. After stipulated time intervals, the disks were removed with macrophages. The L929 cells were then lysed, immunoprecipitated with anti-TRADD antibody and immunoblotted with anti-FADD antibody. Maximum expression of FADD was observed at 16 h of coincubation with cisplatin-treated macrophages (Fig. 8).

Expression of Bax, Bcl-2, caspase-8, caspase-3, Bid, CAD, poly (ADP ribose) polymerase and cytochrome *c* in L929 cells coincubated with cisplatin-treated macrophages

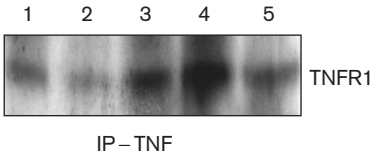
L929 monolayers were coincubated with cisplatin-treated macrophages separated by 1.2-μm nitrocellulose disks.

Fig. 5



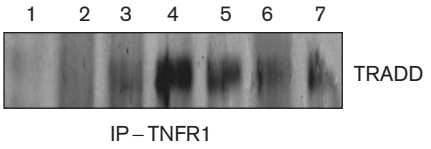
Immunofluorescence staining of cocultures of L929 cells and macrophages with anti-tumor necrosis factor receptor 1 (TNFR1)–fluorescein isothiocyanate antibody: (a) untreated macrophages + L929 cells, (b) cisplatin-treated macrophages + L929 cells cocultured for 2 h, (c) cisplatin-treated macrophages + L929 cells cocultured for 4 h and (d) cisplatin-treated macrophages + L929 cells cocultured for 12 h. Upper panel shows the corresponding cells observed under differential interference contrast. M, macrophage; Tm, L929 cell. The formation of the contact between cisplatin-treated macrophages and L929 cells is clearly seen from 2 to 4 h of coculture (b and c) (black and white arrows). Panels (b–d) also clearly show the sequential accumulation of TNFR1 at the base of the contact formed between two cells, its high concentration at the contact and finally spreading over the surface of L929 cells (white arrows). The figure is representative of three independent experiments with similar results.

Fig. 6



Tumor necrosis factor (TNF)–tumor necrosis factor receptor 1 (TNFR1) interaction, as observed by immunoprecipitation (IP) with anti-TNF antibody followed by immunoblotting with anti-TNFR1 antibody in cocultures of L929 cells with macrophages. Lane 1, untreated macrophages + L929 cells; lane 2, cisplatin-treated macrophages + L929 cells cocultured for 2 h; lane 3, cisplatin-treated macrophages + L929 cells cocultured for 4 h; lane 4, cisplatin-treated macrophages + L929 cells cocultured for 8 h; lane 5, cisplatin-treated macrophages + L929 cells cocultured for 12 h. The figure is representative of three independent experiments with similar results.

Fig. 7



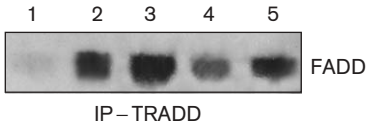
Tumor necrosis factor receptor 1 (TNFR1)–tumor necrosis factor receptor-associated death domain (TRADD) interaction as observed by immunoprecipitation (IP) with anti-TNFR1 antibody followed by immunoblotting with anti-TRADD antibody in L929 cells coincubated with macrophages separated by 1.2-μm nitrocellulose disks. Lane 1, untreated macrophages + L929 cells; lane 2, cisplatin-treated macrophages + L929 cells coincubated for 2 h; lane 3, cisplatin-treated macrophages + L929 cells coincubated for 4 h; lane 4, cisplatin-treated macrophages + L929 cells coincubated for 8 h; lane 5, cisplatin-treated macrophages + L929 cells coincubated for 12 h; lane 6, cisplatin-treated macrophages + L929 cells coincubated for 16 h; lane 7, cisplatin-treated macrophages + L929 cells coincubated for 18 h. The figure is representative of three independent experiments with similar results.

After appropriate time intervals, the disks were removed with macrophages and the L929 cells were lysed. The cell lysates were immunoblotted to observe the expression of caspase-8, caspase-3, Bax, Bcl-2, Bid, cytochrome *c*, CAD and PARP. The DNA fragmentation was also assessed quantitatively.

The expression of activated (cleaved) caspase-8 in L929 cells on coincubation with cisplatin-treated macrophages was seen at 8 h, but was maximum at 16 h (Fig. 9a and b). The expression of activated (cleaved) caspase-3 was observed only at 24 h (Fig. 10a and b).

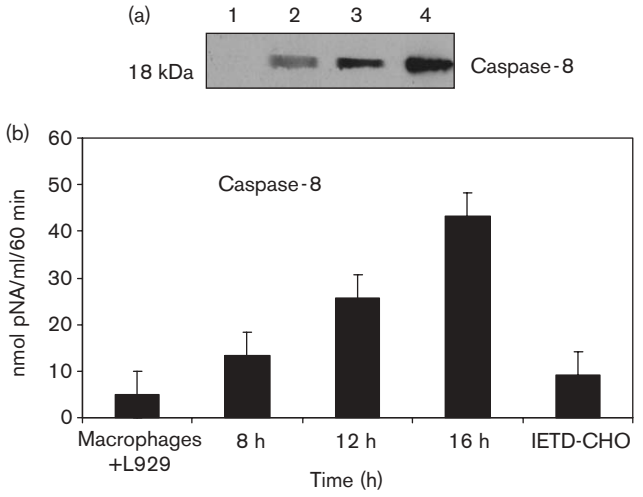
The expression of Bax in L929 cells was seen at 6 h of coincubation with cisplatin-treated macrophages,

Fig. 8



Tumor necrosis factor receptor-associated death domain (TRADD)–Fas-associated death domain (FADD) interaction, as observed by immunoprecipitation with anti-TRADD antibody followed by immunoblotting with anti-FADD antibody in L929 cells coincubated with macrophages separated by 1.2-μm nitrocellulose disks. Lane 1, untreated macrophages + L929 cells; lane,2, cisplatin-treated macrophages + L929 cells coincubated for 4 h; lane 3, cisplatin-treated macrophages + L929 cells coincubated for 8 h; lane 4, cisplatin-treated macrophages + L929 cells coincubated for 12 h; lane 5, cisplatin-treated macrophages + L929 cells coincubated for 16 h. The figure is representative of three independent experiments with similar results.

Fig. 9



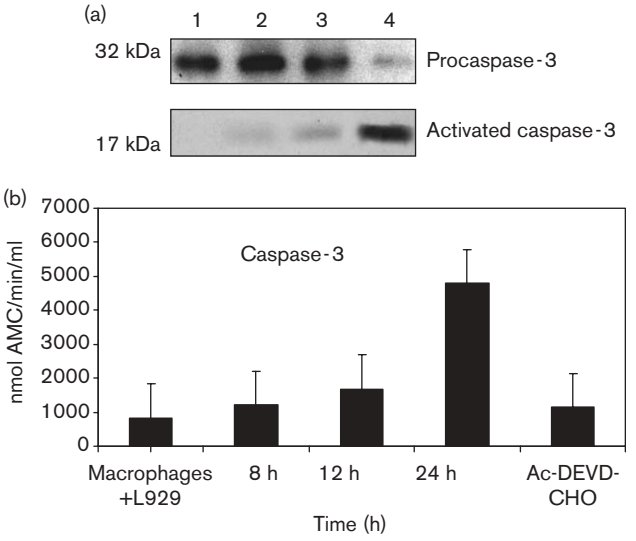
(a) The expression of cleaved caspase-8 in L929 cells, as observed by immunoblotting with anticleaved caspase-8 antibody. The L929 cells were coincubated with macrophages separated by 1.2-μm nitrocellulose disks. Lane 1, untreated macrophages + L929 cells; lane 2, cisplatin-treated macrophages + L929 cells coincubated for 8 h; lane 3, cisplatin-treated macrophages + L929 cells coincubated for 12 h; lane 4, cisplatin-treated macrophages + L929 cells coincubated for 16 h. The figure is representative of three independent experiments with similar results. (b) Caspase-8 activity in L929 cells as measured by colorimetric analysis. Each bar represents the standard error of three independent experiments.

whereas Bcl-2 expression was seen at 3 h. Bcl2 expression was significantly decreased at 6 h (Fig. 11).

The maximum expression of truncated Bid was at 16 h (Fig. 12), and cytochrome *c* expression was observed at 18 h (Fig. 13).

The activation (cleavage) of PARP was seen at 24 h (Fig. 14), and the enhanced expression of CAD is observed between 18 and 36 h of coincubation (Fig. 15).

Fig. 10



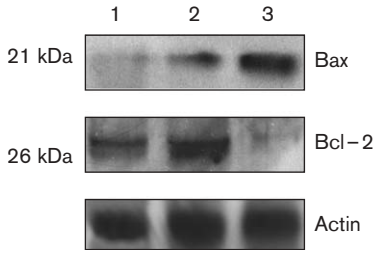
(a) The expression of procaspase-3 and cleaved caspase-3 in L929 cells, as observed by immunoblotting with specific antibodies. The L929 cells were coincubated with macrophages separated by 1.2-μm nitrocellulose disks. Lane 1, untreated macrophages + L929 cells; lane 2, cisplatin-treated macrophages + L929 cells coincubated for 12 h; lane 3, cisplatin-treated macrophages + L929 cells coincubated for 16 h; lane 4, cisplatin-treated macrophages + L929 cells coincubated for 24 h. The figure is representative of three independent experiments with similar results (b) Caspase-3 activity in L929 cells as measured by fluorimetric assay kit. Each bar represents the standard error of three independent experiments.

The DNA fragmentation in L929 cells on coincubation with cisplatin-treated macrophages gradually increased from 18 to 36 h (Fig. 16).

Discussion

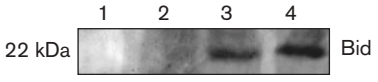
Apoptosis, the genetically programmed mode of physiologically relevant cell death, has been identified as an important determinant of tumor growth. Apoptotic molecules have emerged as appropriate therapeutic targets for the effective control and elimination of tumor cells [36]. Activated macrophages are able to affect diverse aspects of the biology of neoplastic tissues, including vascularization, growth rate and apoptosis [37]. The antitumor mechanism of macrophages is not completely understood, although induction of apoptosis in tumor cells by activated cytotoxic T lymphocytes, activated macrophages and natural killer cells is the aim of most immunotherapies of cancer [38]. The treatment of murine peritoneal macrophages with cisplatin activates them to tumoricidal state [7]. Cisplatin-activated macrophages can kill tumor cells by two mechanisms, one in which direct contact of macrophage and tumor cell is required, and a second in which the activated macrophages release tumor cell-specific cytotoxic factors [7,8].

Fig. 11



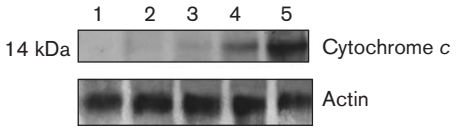
The expression of Bax and Bcl-2 in L929 cells, as observed by immunoblotting with anti-Bax and anti-Bcl-2 antibodies. The L929 cells were coincubated with macrophages separated by 1.2-μm nitrocellulose disks. Lane 1, untreated macrophages + L929 cells; lane 2, cisplatin-treated macrophages + L929 cells coincubated for 3 h; lane 3, cisplatin-treated macrophages + L929 cells coincubated for 6 h. The figure is representative of three independent experiments with similar results.

Fig. 12



The expression of t-Bid in L929 cells, as observed by immunoblotting with anti-t-Bid antibody. The L929 cells were coincubated with macrophages separated by 1.2-μm nitrocellulose disks. Lane 1, untreated macrophages + L929 cells; lane 2, cisplatin-treated macrophages + L929 cells coincubated for 8 h; lane 3, cisplatin-treated macrophages + L929 cells coincubated for 12 h; lane 4, cisplatin-treated macrophages + L929 cells coincubated for 16 h. The figure is representative of three independent experiments with similar results.

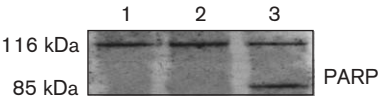
Fig. 13



The expression of cytochrome *c* in L929 cells, as observed by immunoblotting with anti-cytochrome *c* antibody. The L929 cells were coincubated with macrophages separated by 1.2-μm nitrocellulose disks. Lane 1, untreated macrophages + L929 cells; lane 2, cisplatin-treated macrophages + L929 cells coincubated for 8 h; lane 3, cisplatin-treated macrophages + L929 cells coincubated for 12 h; lane 4, cisplatin-treated macrophages + L929 cells coincubated for 16 h; lane 5, cisplatin-treated macrophages + L929 cells coincubated for 18 h. The figure is representative of three independent experiments with similar results.

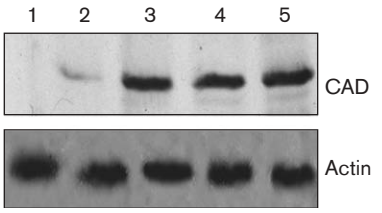
Activated macrophages show enhanced expression of FasL and TNF on their cell surface [39,40]. Macrophages have been reported to mediate apoptosis of a broad range of tumor cell types via FasL and TNF [40]. Engagement

Fig. 14



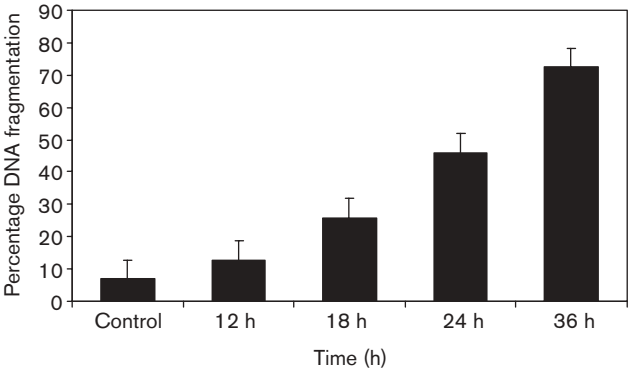
The expression of cleaved poly (ADP ribose) polymerase (PARP) in L929 cells, as observed by immunoblotting with anti-PARP antibody. The L929 cells were coincubated with macrophages separated by 1.2-μm nitrocellulose disks. Lane 1, untreated macrophages + L929 cells; lane 2, cisplatin-treated macrophages + L929 cells coincubated for 12 h; lane 3, cisplatin-treated macrophages + L929 cells coincubated for 24 h. The figure is representative of three independent experiments with similar results.

Fig. 15



The expression of CAD in L929 cells, as observed by immunoblotting with anti-CAD antibody. The L929 cells were coincubated with macrophages separated by 1.2-μm nitrocellulose disks. Lane 1, untreated macrophages + L929 cells; lane 2, cisplatin-treated macrophages + L929 cells coincubated for 12 h; ane 3, cisplatin-treated macrophages + L929 cells coincubated for 18 h, lane 4, cisplatin-treated macrophages + L929 cells coincubated for 24 h; lane 5, cisplatin-treated macrophages + L929 cells coincubated for 36 h. The figure is representative of three independent experiments with similar results.

Fig. 16



The percentage DNA fragmentation in L929 cells, as observed by the diphenyl amine method. The L929 cells were coincubated with macrophages separated by 1.2-μm nitrocellulose disks. Each bar represents the standard error of three independent experiments.

of Fas or the p55 component of the receptor for TNF (TNFR1) results in cell death through the controlled process of apoptosis. Such a response has a clear adaptive benefit to the host in facilitating the elimination of transformed cells or terminating an activated inflammatory response [41,42]. The Fas–FasL pathway, i.e. the ligation of Fas, which is expressed on the surface of target tumor cells and FasL on the surface of cytotoxic T lymphocyte, is the best-known mechanism that induces apoptosis of tumor cells [43]. Activated macrophages have been shown to induce apoptosis in glioma cells via the Fas–FasL pathway [37]. Fas is ubiquitously expressed in lymphoid and nonlymphoid tissues, and in many primary tumors and tumor cell lines [44].

TNF-α is a pleiotropic cytokine that is primarily produced by activated macrophages, and is a mediator of inflammatory, immunological responses and tumor cell lysis [24]. The molecular mechanisms of TNF-induced activation of survival pathways (nuclear factor-κB, Jun N-terminal kinase) have been reasonably well elucidated [45]. The principle and mechanism of membrane TNF-mediated cell death, however, remains largely unknown. TNFR1 are mostly present on the cells that undergo apoptosis, whereas its ligand, i.e. TNF, is present as a cell surface-anchored molecule on activated macrophages [26]. The data presented here provide evidence that the plasma membrane-associated TNF and FasL on cisplatin-treated macrophage binds to TNFR1 and Fas expressed in L929 cells, which is a contact-mediated process, resulting in the activation of FADD and finally apoptosis of L929 cells.

It has been reported that tumor cells are lysed by membrane-associated TNF as expressed by paraformaldehyde-fixed activated macrophages [46]. Perez *et al.* [47] has generated nonsecreted and uncleavable TNF cell surface mutants that express cytotolytic activity. On the basis of their observations, they have suggested that the essential requirement for the cytotoxicity activities of the membrane-associated TNF and FasL is cell-to-cell contact. Blood monocytes treated with lipopolysaccharide and phorbol 12-myristate 13-acetate were found to express an integral transmembrane component of TNF [48].

In the present study, it is reported that cisplatin-treated macrophages when coincubated with L929 cells, separated with a 1.2-μm nitrocellulose disk, resulted in L929 cell death by apoptosis, mediated through the formation of Fas–FasL–FADD complexes. Furthermore, when cisplatin-treated macrophages were fixed with paraformaldehyde and incubated with L929 cells, the expression of TNF–TNFR1–TRADD–FADD complexes were observed by immunoprecipitation. Expression of Fas–FasL and TNF–TNFR1 complexes by immunoprecipitation suggests the interaction and direct contact formation between cisplatin-treated macrophages and L929 cells. The formation and expression of the complexes of TNFR1–TRADD, TRADD–FADD and Fas–FADD suggests the induction of apoptosis in L929 cells, through the death signals triggered after contact formation between cisplatin-treated macrophage and L929 cells. If the cisplatin-treated macrophages were, however, coincubated with L929 cells, separated by 0.22-μm nitrocellulose disks, expression of TNF–TNFR1–TRADD and Fas–FasL–FADD complexes were not observed (data not shown). Cisplatin-treated macrophage-induced L929 cell apoptosis was also investigated by Annexin V–FITC staining. It was observed that the L929 cells within 3–4 h of coincubation with cisplatin-treated macrophages stained positively with Annexin V–FITC. The induction of cell death of L929 cells by apoptosis on coculturing with cisplatin-treated macrophages (separated by 1.2 μm nitrocellulose disk) was further confirmed by observing the enhanced activation/expression of downstream apoptosis mediating molecules like caspase-8, caspase-3, CAD, PARP, Bid, Bax, cytochrome *c* and DNA fragmentation, and the downregulation of Bcl-2.

The data presented clearly show that cell death of L929 cells with cisplatin-treated macrophages is contact mediated, and Fas–FasL and TNF–TNFR1 dependent. This may not be, however, the only pathway. Our other observations have shown that L929 cell death may also be mediated via the soluble factor nitric oxide (unpublished data).

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