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Cisplatin and carboplatin mediated release of cytolytic factors in murine peritoneal macrophages *in vitro*

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The anticancer drugs cisplatin and carboplatin have been shown to activate murine peritoneal macrophages *in vivo* and *in vitro*. These activated macrophages have enhanced tumoricidal activity mediated by extension and contact formation with the tumor cells leading to an increase and transfer of lysosomes with eventual lysis of the tumor cells. Cisplatin (10 μg/ml) or carboplatin (50 μg/ml) for 2 and 24 h treatment of macrophages *in vitro*, in addition, show a significant increase in the release of various cytolytic factors, like hydrogen peroxide, superoxide anion, interleukin-1α, lysozyme and β-*N*-hexoseaminidase, that are also responsible for the destruction of tumor cells.

Key words: Anticancer drugs, carboplatin, cisplatin, cytolytic factors, interleukin-1, macrophage activation.

Introduction

Cisplatin [*cis*-diamminedichloroplatinum (II); CDDP], a broad spectrum anticancer drug, proven to be effective in the treatment of testicular, ovarian, prostate, bladder, head and neck, and lung cancers,[1] has been proposed to act through an inter- and intrastrand cross-linking of DNA as its major mechanism of action.[2] This interaction with DNA is assumed to be the crucial event leading to the inhibition of DNA synthesis. However, it has been shown that repair enzymes are able to delete these cisplatin–DNA cross-links and the cells are able to continue to divide.[3] Other possible mechanisms of action of this drug not directly explained by DNA–drug interactions do exist.[4–6]

Activated macrophages have been found to effectively destroy target cells by cytotoxic mechanisms.[7,8] This activation process includes the generation of extracellular products such as reactive oxygen metabolites,[9,10] proteases,[11] arginases,[12] interleukin (IL) tumor necrosis factor (TNF),[13,14] and nitric oxide.[15,16] Previous investigations have demonstrated that cisplatin[17] and its analog, carboplatin, that has been shown to have less toxic side effects, also act through the enhancement of the immune system.[18] These drugs have been shown to induce extension formations in the murine peritoneal macrophages which seek out tumor cells developing cell–cell contacts, an increase in the lysosomes and subsequent transfer of these lysosomes to the tumor cells leading to their lysis. In our present study, we have tried to establish the activation of the immune system through the release of biologically active cytolytic factors like hydrogen peroxide, superoxide anion, lysozyme, β-*N*-hexoseaminidase (βNH) and IL-1α from the macrophages as another possible mechanism of action of cisplatin and carboplatin.

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Materials and methods

Cell cultures

Murine macrophages were isolated from the peritoneal cavity of Swiss Webster mice 6–8 weeks old (Charles Rivers Laboratories, MA).[18] Mice were sacrificed by cervical dislocation and peritoneal macrophages were isolated by injection of 5 ml chilled minimal essential medium (Gibco BRL, NY), seeded in 35 mm petri dishes at 2–4 × 10^6 cells/ml and incubated for 2 h at 37°C after which cells were washed vigorously to remove non-adherent cells. Cell cultures were incubated in normal medium (minimal essential media with 10% fetal calf serum and 1% antibiotic antimycotic, 10 mM HEPES; Gibco BRL) and incubated at 37°C overnight
before use for experiments.

Treatments

Cisplatin (10 μg/ml) and carboplatin (50 μg/ml) (gifts from Johnson Matthey Research Laboratories and the Chemistry Branch of the National Cancer

Table 1. Effect of cisplatin and carboplatin on the release of cytolytic and tumoricidal factors

Treatment and duration (h)	H_2O_2 (nM)	Superoxide anion (nM)	IL-1α (pg/ml)	Lysozyme (units/ml)	βNH (munits)
Normal medium					
2	143.75 ± 1.28	28.9 ± 0.199	4.0 ± 0.0	0.35 ± 0.09	0.084 ± 0.002
24	73.14 ± 0.23	38.9 ± 0.26	9.5 ± 0.7	0.77 ± 0.07	0.142 ± 0.003
Cisplatin					
2	172.92 ± 2.65	31.77 ± 0.431	55.5 ± 0.7	0.75 ± 0.05	0.109 ± 0.007
24	74.07 ± 0.29	42.93 ± 0.27	10.5 ± 0.7[a]	1.01 ± 0.01	0.115 ± 0.003
Carboplatin					
2	161.0 ± 3.91	31.24 ± 0.06	42.0 ± 2.83	0.53 ± 0.01	0.11 ± 0.006
24	76.39 ± 0.00	44.27 ± 0.409	7.0 ± 1.41[a]	0.9 ± 0.00	0.131 ± 0.008
LPS					
2	—	—	482.06 ± 14.5	0.57 ± 0.20	0.109 ± 0.007
24	—	—	535.35 ± 29.9	0.9 ± 0.00	0.086 ± 0.005
PMA					
2	217.36 ± 6.97	32.7 ± 0.89	15.0 ± 1.41	0.48 ± 0.00	0.121 ± 0.001
24	79.17 ± 1.06	43.37 ± 0.93	25.5 ± 4.9	0.95 ± 0.01	0.072 ± 0.004

Values are expressed as means ± standard deviation from three independent experiments. Results represent experiments done in triplicate.
[a] Values not found to be significantly different from controls after using Student's *t*-test ($p < 0.05$).

Institute) were issolved in normal medium containing di-methylsulfoxide (DMSO) (3 µl/ml medium; Sigma, St Louis, MO). Cells were treated with the drugs at 37°C for various times as specified (Table 1). Cells treated with phorbol myristate acetate (PMA; 10 ng/ml DMSO), lipopolysaccarhide (LPS, 10 µg/ml) or normal medium plus DMSO (3 µl/ml) were used as controls (all from Sigma).

Cytolytic factors

The data presented in Table 1 was collected in triplicate from three independent experiments and analyzed using the Student's *t*-test (two tailed, $p < 0.05$).[19]

H_2O_2. H_2O_2 was assayed according to the modified method previously outlined.[10] Horseradish peroxidase (165 U/ml; Sigma) dissolved in phenol red solution (0.1 g/ml; Sigma) as the reaction mixture was added to macrophage cultures and incubated at 37°C for 30 and 60 min. The supernatant was collected and 0.1 N NaOH was added (30 µl/ml reaction mixture), and absorbance was read with a spectrophotometer at 615 nm. Standards were tested using the same batch of reaction mixture with 10–1000 nM H_2O_2 (Sigma). Reaction mixture without cells were used as blanks.

O_2^-. Superoxide anion release was assayed according to the method described by Johnston *et al.*[20] Ferricytochrome *c*, type III from horse heart (80 µM) in Hank's balanced salt solution (both from Sigma) was added to macrophage cultures (1.5 ml) and incubated at 37°C for 90 min. This reaction mixture was then transferred to test tubes and placed in ice to stop the reaction. Absorbance was then read with a spectrophotometer at 550 nm. Reaction mixtures without cells were used as blanks. Superoxide anion detection was inhibited by treatment with superoxide dismutase (SOD; 40 µg/ml; Sigma) as a control. Values of superoxide anion release were calculated as described by Adams and Hamilton.[7]

βNH. βNH enzyme was assayed according to the method described by von Figura.[21] Fetal calf serum hexoseaminidase was first inactivated by adjusting the pH to 10.4 with NaOH and incubated from 30–60 min at 37°C after which the pH was neutralized back to 7.5 using HCl. An assay mixture was prepared by mixing 10 mM *p*-nitrophenyl-2-acetamido-2-deoxy-D-glucopyranoside in 0.1 M sodium citrate buffer containing 0.04% $NaNO_3$ and 0.2% bovine serum albumin (Sigma). Substrate solution (0.1 ml) was added in each tube containing an equal volume of culture supernatant and incubated for 24 h at 37°C. Stop reaction mixture (2.8 ml of 0.4 M glycine–NaOH, pH 10.4; Sigma) was then added and optical density read at 405 nm. The substrate solution alone was read as blank control.

Lysozyme. Macrophage culture supernatants were assayed for lysozyme according to the method outlined of Barret.[22] Macrophage culture supernatants (0.1 ml) were added to a substrate suspension of *Micrococcus lysodiekticus* (1 mg/ml, Sigma) in 2.5 ml substrate buffer and incubated at 37°C for 1 h. Substrate suspension alone was used as blanks. Absorbance readings were taken at time 0 and 1 h after incubation at 600 nm.

IL-1α. IL-1α was assayed using ELISA test kits purchased from Genzyme (Cambridge, MA). Supernatants collected from macrophage cultures were assayed using a solid-phase enzyme immunoassay employing the multiple antibody sandwich principle.[23] A 96-well microtiter assay pre-coated with monoclonal anti-murine IL-1α was used to capture murine IL-1α present in standards and test samples. After the unbound material was removed, a biotinylated polyclonal antibody binding the captured murine IL-1α was added and, again, unbound material was washed out. Peroxidase-conjugated avidin, binding biotin tagged complexes, was added. The addition of a substrate solution resulting in a color change was stopped by acidification and subsequently read at 450 nm. Standard curves were generated using 15–405 pg/ml of murine IL-1α and linear regression analysis was performed.

Cytotoxicity assay

Macrophage mediated cytotoxicity was assessed by measuring cell lysis of sarcoma-180 solid tumor cells (American Type Cell Culture, Rockville, MD) using crystal violet[24] after incubation with the cytolytic factors and supernatants collected from macrophage cultures at different times. Cells were seeded in 96-well plates at 4×10^5 cells/200 µl in normal medium and incubated for 3 h at 37°C after which they were incubated with hydrogen peroxide (2, 50, 150 and 300 nM), egg white lysozyme (1, 5, 10 and 20 U/ml, 0.067 M phosphate buffer, pH 6.25), IL-1α (5, 25, 50 and 100 pg/ml), PMA (10 ng/ml DMSO), LPS (10 µg/ml), SOD (40 µg/ml), DMSO (3 µl/ml normal medium), cisplatin (10 µg/ml), carboplatin (50 µg/ml) or PMA and SOD together for 24 h. Each treatment was done in triplicate. Controls were incubated in normal medium only. After incubation, the medium was removed and cells were fixed with methanol for 30 s and stained with 100 µl of crystal violet (0.5%; Sigma) for 20 min. Wells were then washed with distilled water and left to dry after which optical density was measured using a Titertek Multiscan MCC/340 plate reader at 620 nm.

Results

Macrophage activation

Normal murine peritoneal macrophages (Figure 1A) after treatment with cisplatin (10 µg/ml) or carboplatin (50 µg/ml) for 2 h at 37°C in culture show

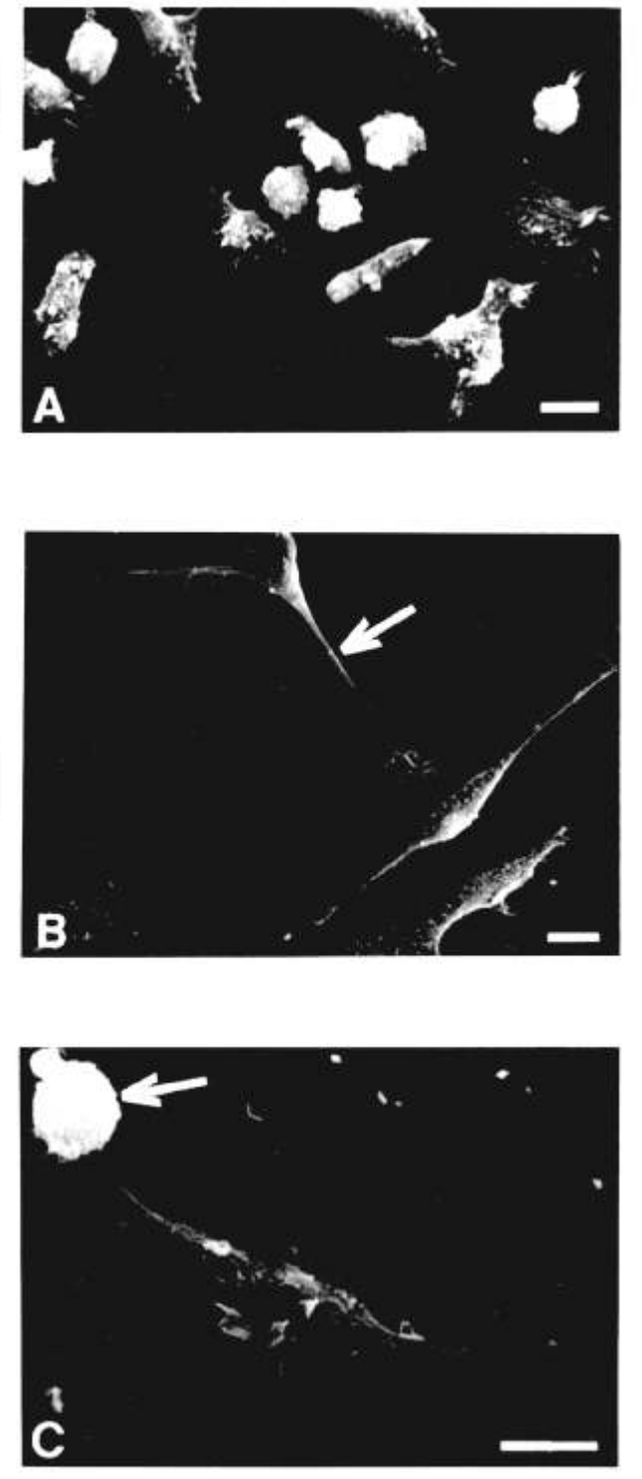

Figure 1. Scanning electron micrographs of murine peritoneal macrophages. (A) Normal cells showing no extension formations. (B) Cisplatin-treated (10 µg/ml) macrophages for 2 h showing the extension formations (arrow). (C) Cisplatin-treated (10 µg/ml) macrophages for 2 h and co-incubated with sarcoma-180 cells for 1 h. Note the close association of the macrophage and sarcoma-180 cell (arrow). Bar = 10 µm.

extension formations within 10 min (Figure 1B). When cultured with tumor cells (sarcoma-180), such macrophages assume cell–cell contact within 30 min and show shortening of these extensions pulling the tumor cells towards them (Figure 1C). Previously we have shown that the treated macrophages demonstrate an increase and transfer of their lysosomes to the tumor cells through the extensions. These tumor cells eventually undergo lysis. In addition macrophages are able to secrete cytolytic factors that help in the lysis of the tumor cells.

Cytolytic factors

Treatment of murine peritoneal macrophages with cisplatin (10 μg/ml) or carboplatin (50 μg/ml) *in vitro* shows a significant increase in the release of various cytolytic factors like H_2O_2, superoxide anion, IL-1α, lysozyme and βNH (Table 1). This increase was seen after 2 and 24 h of treatment with both drugs. There was a greater increase in the release of H_2O_2 after 2 h compared with 24 h of treatment. This increase was more so in the case of cisplatin treatment compared with carboplatin. Positive controls treated with PMA demonstrated a similar pattern of increase. Although there was an increase in the release of superoxide anions after cisplatin and carboplatin treatment, more so after carboplatin treatment, it was still not so significant as with other cytolytic factors (Table 1). IL-1α did not show significant increases after 24 h of cisplatin or carboplatin treatment compared to 2 h of treatment. In addition, LPS seemed to induce a greater release of IL-1α than PMA. Lysozyme increased after 2 and 24 h treatments with both of the drugs; however, carboplatin comparatively induced a greater increase. βNH was increased after 2 h treatments only and no significant increases were seen after 24 h of the treatment with either of the drugs. Cells incubated in normal medium with DMSO as the vehicle for dissolution of the drugs did not show any increase in the release of the various cytolytic factors.

Cytotoxicity assay

When sarcoma-180 cells were incubated in lysozyme, H_2O_2 and IL-1α at various concentrations, measurements of cell density demonstrated a decrease in cell number in a dose-dependent manner (Figure 2). Similarly both cisplatin and carboplatin show significant lysis of the tumor cells (Figure 3). LPS and PMA treatment also demonstrate a decrease in tumor cell number. PMA in combination with SOD demonstrated a protective action against cell lysis compared with PMA alone. Interestingly, when tumor cells were incubated in supernatants from cisplatin or carboplatin treated macrophages, a similar decrease in cell number was observed (Figure 4). DMSO incubated tumor cells did not show any decrease in cell number.

Discussion

Macrophages lyse tumor cells in three ways, i.e. lysis, rapid antibody dependent cell cytotoxicity and slow antibody dependent cell cytotoxicity. These are very distinct processes while all require the activation of the macrophages by biological response modifiers or activators.[7,25] Calcium ionophore A23187, *Corynebacterium parvum* and lymphokine mediators have been demonstrated to be some such agents.[26] Cisplatin and carboplatin have been shown to enhance macrophage activity by increased cell–cell recognition through the formation of extensions, the transfer of lysosomes and eventual lysis of the tumor cell.[17,18]

Lysozyme is involved in host defense mechanisms in the body. It plays a role in localization of microorganisms and have also been shown to have a modulating effect on neutrophils as well as mediating tumorcidal functions of the macrophages. Cisplatin or carboplatin treatment has been shown to increase lysozyme levels in the macrophages,[27,28] thus representing an activated state.

βNH release by cisplatin or carboplatin treated macrophages was seen only after 2 h of treatment and not after 24 h of treatment. This enzyme is a lysosomal enzyme[29] and deficiency of this enzyme has been shown to cause genetically determined lysosomal storage diseases. Macrophage activation is related to an increase in the number of lysosomes after cisplatin or carboplatin treatment.[17,18]

In the four electron reduction of dioxygen to water in oxidative phosphorylation, partial reduction can occur producing superoxide, H_2O_2 and hydroxyl radical depending on whether one, two or three electrons are added, respectively. Though only a small fraction of these are produced (3–5% of mitochondrial electron flow), these reactive oxygen species can be damaging to the cell.[30,31] Reactive oxygen species are known to interact with DNA as well as lipids. The interaction with DNA results in damage as characterized by OH^8Gua and the inter-

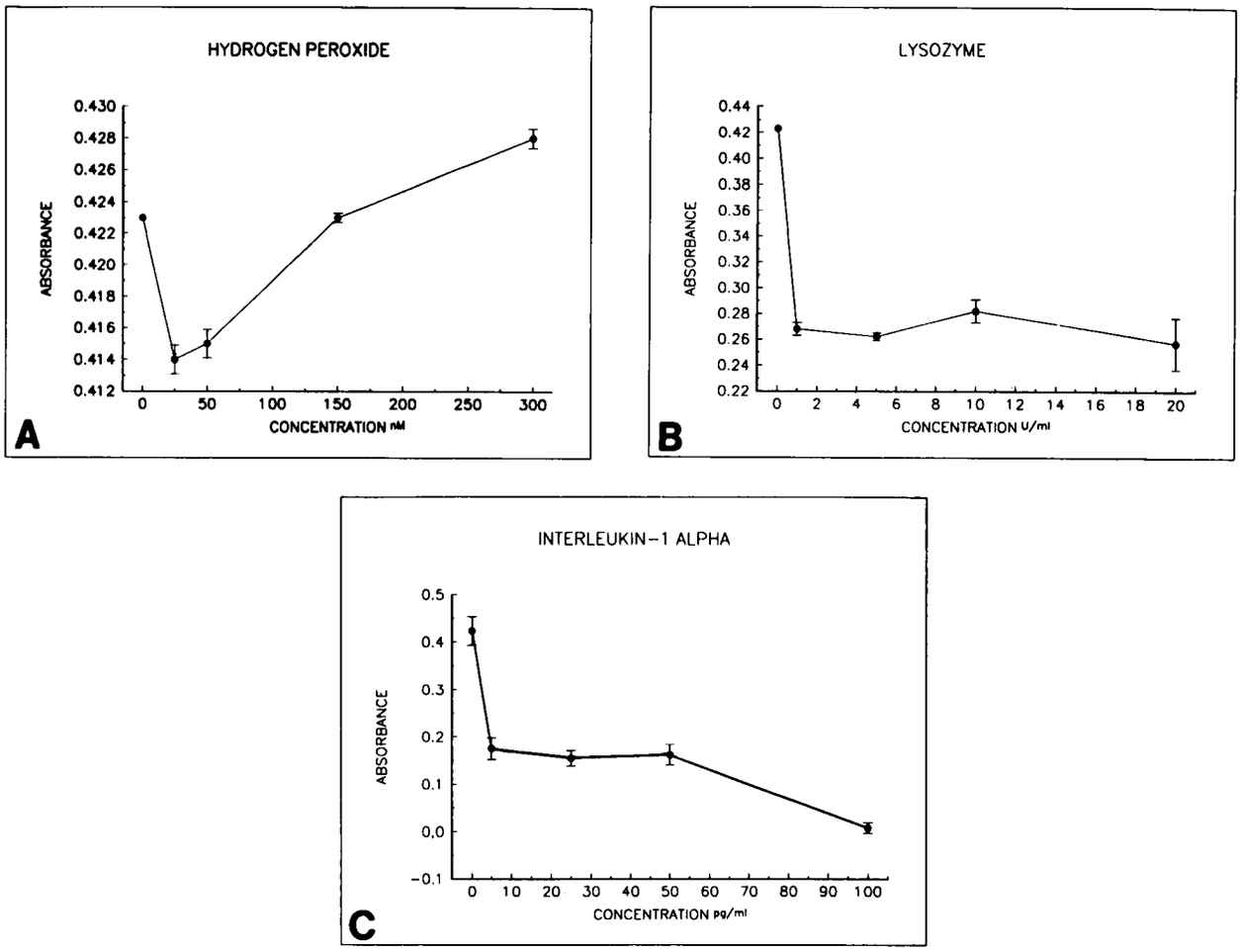

Figure 2. Graphs showing sarcoma-180 cell densities after treatment with H_2O_2, lysozyme and IL-1α at different doses. Cells were seeded at 4×10^5 cells/200 µl in 96-well plates, treated for 24 h and stained with crystal violet. Cell densities are represented by absorbance measurements. Note that cell numbers decrease with incubation in lysozyme (B) and IL-1α (C), while cell densities first decrease and gradually increase after incubation in 150 nM H_2O_2 (A). Standard deviations were found to be less than 10% of the mean.

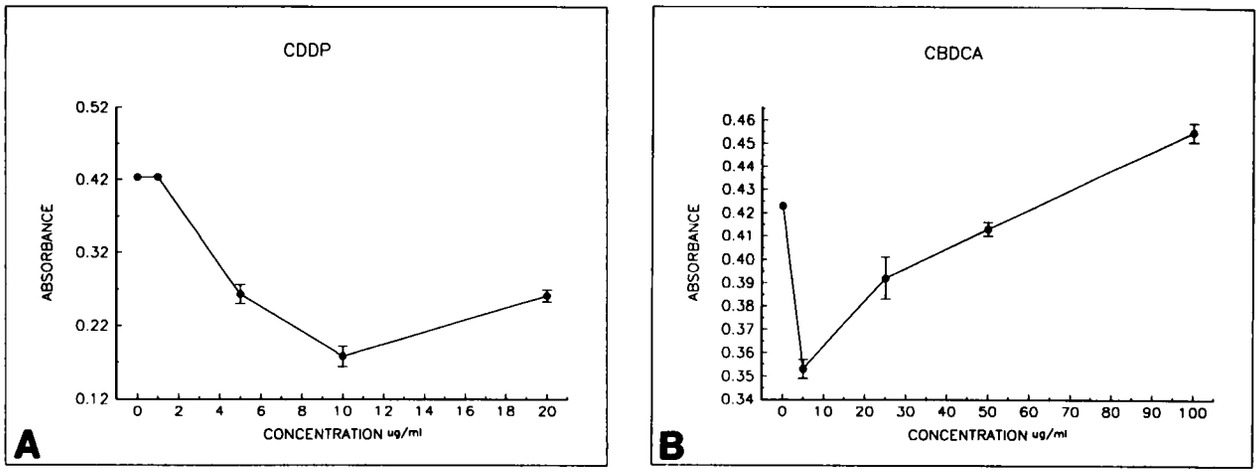

Figure 3. Graphs showing sarcoma-180 cell densities after treatment with cisplatin (10 µg/ml; CDDP) or carboplatin (50 µg/ml; CBDCA) at different concentrations. Cells were seeded and stained as specified in Figure 2. Note that cisplatin induced a greater effect than did carboplatin which is less toxic. Standard deviations were found to be less than 10% of the mean.

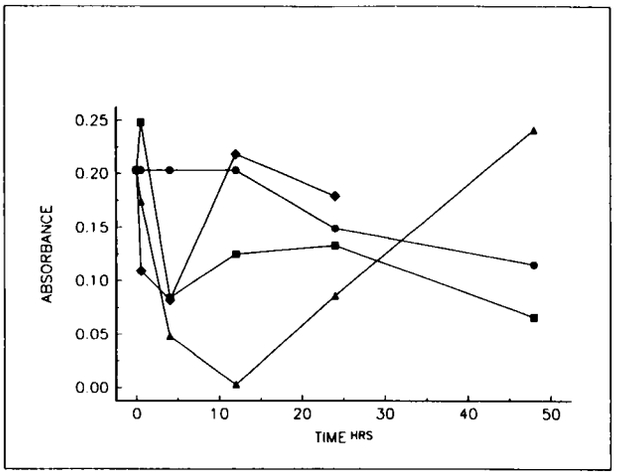

Figure 4. Graphs showing sarcoma-180 cell densities after incubation in supernatants collected from macrophages after treatment with cisplatin (10 μg/ml; CDDP) or carboplatin (50 μg/ml; CBDCA) at different times as specified. Cells were seeded and stained as specified in Figure 2. Note the decrease in cell number after incubation in supernatants from cisplatin and carboplatin-treated macrophages. Standard deviations were found to be less than 10% of the mean. ●, Control; ▲, CDDP; ■, CBDCA; ◆, LPS.

action with lipids results in lipid peroxidation leading to cell death.[31,32] In terms of macrophage mediated cytotoxicity, reactive oxygen species have been implicated, more particularly H_2O_2 and superoxide anions.[20,33] The increased production of reactive oxygen species after cisplatin and carboplatin treatment seems to suggest that damage to DNA and or the stimulation of lipid peroxidation in the target cells could be one of the mechanisms by which these activated macrophages kill. It seems that 2 h drug treatment produces a higher yield of H_2O_2 as compared with 24 h drug treatment.

Activated macrophages are known to release a number of cytotoxic and cytostatic mediators, among which IL-1α is one.[13,34] It was first described as a lymphocyte activating factor because of its ability to stimulate T cells.[35] Its release by activated macrophages and its cytotoxicity to tumor target cells suggests IL-1α as a potent mediator in tumor cell killing by macrophages. Our study has shown not only an increase in the release of this factor, suggesting its role in macrophage mediated cytotoxicity after cisplatin or carboplatin treatment *in vitro*, but also its ability to lyse tumor target cells in a dose-dependent manner. The significant increase in the release of IL-1α after 2 h cisplatin or carboplatin treatment is not seen after 24 h treatments. Similar results were obtained using bone marrow derived macrophages, where insignificant activity of IL-1α was found after 4 h of the treatment with cisplatin.[14] It is interesting to note that βNH was found to have no significant increases after 24 h of drug treatment.

Increased levels of TNF has been observed in activated macrophages as well, especially those stimulated with LPS.[14,36,37] TNF is known to mediate a variety of functions which include host defense mechanisms and growth, and recent studies have shown the increased release of TNF after both cisplatin and carboplatin treatment in the macrophages. It is evident that TNF is another important mediator of tumor cell killing by macrophages primed by various agents like cisplatin and carboplatin.

Currently, the production of nitric oxide, another cytotoxic agent produced by macrophages,[38–40] is also being studied as another possible mediator of cisplatin, carboplatin induced tumor cytotoxicity. The role of calcium in macrophage activation has yet to be elucidated,[18] but we have found that cisplatin induces an increase in the intracellular calcium levels in tumor cells. This probably is involved in activating inducible endonucleases causing DNA fragmentation, a hallmark of apoptosis,[41–44] which cisplatin has been shown to induce as well.[45,46]

Conclusion

The results of this study support the hypothesis that in addition to inducing tumor cell lysis by direct cell–cell contact, cisplatin and carboplatin primed macrophages can also mediate lysis of the tumor cells via the secretion of a variety of cytolytic and cytostatic factors. It is proposed that cisplatin and carboplatin can both inhibit tumor cell proliferation in several ways. One mechanism is through the interaction with DNA, resulting in inter- and intrastrand cross-linking leading to inhibition of DNA replication[2] or through their effect on cytoskeletal elements thus inhibiting cell division.[4–6,47] Now we demonstrate another possible mechanism via the enhancement of the immune system through the activation of macrophages. This priming of macrophages results in an increased binding to tumor cells (direct cell–cell contact)[17,18] and a release of cytolytic and cytostatic factors important in mediating tumor cell cytotoxicity. The activation of macrophages by cisplatin and carboplatin, thus, seems to be a multi-step process involving various mediators as cytolytic factors eventually inducing cell cytotoxic processes as lysis or apoptosis.

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