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Activation of murine peritoneal macrophages after cisplatin and taxol combination

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Cisplatin and paclitaxel are potent antineoplastic agents. Their distinctly different mechanisms of action have prompted laboratory and clinical research into their use in combination therapies. Murine peritoneal macrophages treated with cisplatin and paclitaxel in combination elicit an increase in their number of lysosomes. Drug–treated macrophages, when co-incubated with sarcoma 180 cells, establish cytoplasmic contact and transfer lysosomes into tumor cells causing tumor cell lysis. In addition, analysis of tissue culture supernatants show increased levels of interleukin-1α and tumor necrosis factor-α. Our study shows that cisplatin and paclitaxel in combination enhance elements of the immune system with greater efficacy and potency than when used alone.

Key words: Cisplatin, interleukin-1α, lysosomes, *in vitro*, macrophages, taxol, tumor necrosis factor-α.

Introduction

The remarkable clinical efficacy of paclitaxel (taxol) has resulted in numerous observations of partial and complete remission of advanced ovarian cancer in women.[1] Recently, reports of the efficacy of the drug in breast, lung and prostate cancer have aroused great interest in the antitumor compound.[1] Taxol has a unique mechanism of antitumor activity in that it binds to a protein, tubulin, thus inhibiting cell division.[2]

Cisplatin [*cis*-diamminedichloroplatinum (II); CDDP], a heavy metal platinum coordination complex, is proven to be effective in the treatment of testicular, ovarian, prostate, bladder, head and neck, and lung cancers.[3] DNA denaturation is one of the accepted methods of its mechanism of action through its intrastrand and interstrand cross-links interfering with DNA replication and transcription.[4] Another mechanism of action is through the activation of the immune system. Activated macrophages have been found to effectively destroy tumor cells by cytotoxic mechanisms.[5,6] The activation process includes the generation of extracellular products including interleukin (IL)-1α and tumor necrosis factor (TNF)-α.[7]

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Cisplatin plus taxol in combination has become increasingly prevalent in clinical treatment.[8] Combination therapy is capable of producing very high response rates and is seemingly less toxic than either of the two drugs when administered alone.[8] Although, the mechanism of action for the cytotoxic interactions between cisplatin and taxol have not been determined. We have here explored the effects of cisplatin plus taxol on macrophages so as to understand their mechanism of action in the immune system enhancement and its efficacy in fighting cancer.

Materials and methods

Cell cultures

Swiss Webster mice (Charles River, Location, MA) were sacrificed by cervical dislocation and peritoneal macrophages were isolated by injection of 5 ml chilled minimal essential medium (MEM; Gibco, Grand Island, NY) without serum containing 1% antibiotic-antimycotic [penicillin G (10 000 U/ml), streptomycin sulfate (10 000 μg/ml) and amphotericin B (25 μg/ml); Gibco] into the peritoneal cavity. After gently massaging the abdominal wall, cells were aspirated and seeded onto 18 mm^2 glass coverslips, placed in 35 mm Petri dishes, at 2–4 × 10^6 cells/ml and incubated for 2 h at 37°C. Coverslips were washed vigorously to remove non-adherent cells. Cell cultures were incubated in normal medium (minimal essential media and 10% heat-inactivated fetal calf serum) at 37°C in a 5% CO_2 incubator. Sarcoma 180 ascites (S180; CCRFS-180II; American Type Culture Collection, Rockville, MD) were maintained in culture using normal medium. Cells were washed with Hank's balanced salt solution (HBSS; Gibco) and centrifuged at 1000 *g* for 5 min for use in experiments. These cells served as target cells for macrophages and were added to cultures at 3 × 10^5 cells/ml concentration.

Treatments

Cisplatin and taxol were prepared in 0.85% NaCl in 5 and 10 μg/ml concentrations, respectively. Macrophages were treated with the drugs for 2 h. The drug(s)-containing medium was replaced by normal medium, and supernatant (500 μl) was collected at 0.5, 1, 2 and 24 h for cytolytic factor/s analysis. Untreated cells in normal medium served as controls.

IL-1α assay

IL-1α was assayed using an ELISA kit (Genzyme, Cambridge, MA). The method used the multiple antibody sandwich principle,[9] where monoclonal anti-murine IL-1α was used to bind murine IL-1α present in the supernatant. A biotinylated polyclonal antibody binding the IL-1α was added and unbound material was washed out. Peroxidase-conjugated avidin was used to bind these biotin tagged complexes. A substrate solution was then added resulting in a color change. The reaction was stopped by acidification and absorbance was read at 450 nm. Standard curves were generated with IL-1α (15–405 pg/ml) provided in the kits and linear regression analysis was performed.

TNF-α assay

TNF-α released from supernatants of the macrophages was assayed using a specific analysis kit (Genzyme). Again, the multiple antibody sandwich principle[9] was utilized with a murine monoclonal antibody specific for murine TNF-α in the samples. A horseradish peroxidase-conjugated anti-murine TNF-α antibody was used to bind the multiple epitopes on TNF-α. A substrate solution was then added resulting in a color change. The reaction was stopped by acidification and absorbance was read at 450 nm. Standard curves were generated with TNF-α (35–2240 pg/ml) provided in the kits and linear regression analysis was performed.

Lysosomal assay

The quantitation of lysosomes before and after various treatments was achieved by exposing macrophage cultures to fresh medium containing acridine orange (5 μg/ml) for 30 min at 37°C in the dark.[10] After careful washing, macrophages were examined under a Zeiss 10 laser scanning confocal microscope and visual counts made.

Macrophage–tumor cell interaction studies

To study macrophage-tumor cell interaction, macrophage monolayers were treated with either cisplatin (5 μg/ml), taxol (10 μg/ml) or cisplatin (5 μg/ml) plus taxol (10 μg/ml) for 2 h at 37°C in a 5% CO_2 incubator. The medium was then replaced by normal medium and the S180 tumor cells were added. Macrophages and tumor cells were co-incubated for 2 h. Coverslips seeded with macrophages and tumor cells were fixed with 1.5% glutaraldehyde on 0.05 M phosphate buffer (pH 7.2) at room temperature for 10 min. Macrophage-tumor cell interaction was viewed using phase contrast microscopy.

Results

IL-1α release

Compared to normal there was a gradual increase in IL-1α levels in the supernatants of macrophages treated with cisplatin (5 μg/ml) plus taxol (10 μg/ml) (Figure 1). There was a gradual increase in macrophages treated with cisplatin alone. However, taxol-treated macrophages demonstrated a large increase (400 pg/ml) in IL-1α after 30 min post-treatment. These levels gradually decreased until 2 h post-treatment. After 24 h again there was an increase reaching approximately 275 pg/ml.

TNF-α release

Compared to normal, a combination treatment of the two drugs cisplatin (5 μg/ml) plus taxol (10 μg/ml) demonstrated increased levels of TNF-α after only 30 min post-treatment. A cyclical release was observed when TNF-α was viewed at 1, 2 and 24 h post-treatment. This cyclical release pattern was also true for both cisplatin and taxol when used alone, but the levels of TNF-α were not as high as after the combination treatment (Figure 2).

Macrophage activation

Murine peritoneal macrophages demonstrated extension formations after 2 h post-treatment with cisplatin alone (5 μg/ml) (Figure 3A). However, taxol (10 μg/ml) and cisplatin (5 μg/ml) plus taxol (10 μg/ml) treated macrophages did not show any extension formation, for up to 24 h, but instead assumed a discoid shape similar to that of the normal macrophages (Figure 3B–D).

Lysosomal studies

Based on fluorescence measurements after acridine orange labeling, a 100-fold increase in the number of lysosomes in the macrophages was observed only after 2 h of cisplatin (5 μg/ml) plus taxol (10 μg/ml) treatment (Figure 4A) as compared to untreated cells (Figure 4B). Both cisplatin and taxol alone demonstrated only a 50-fold increase in the number of lysosomes (Figure 4C and D).

Macrophage-tumor cell interaction studies

Macrophages co-cultured with tumor cells (S180) establish cell-cell contact within 30 min and demonstrated a transfer of their lysosomes into the tumor cells through cytoplasmic continuity assumed after co-incubation. Cisplatin-treated macrophages have been shown to transfer their lysosomes down the cytoplasmic extensions (Figure 5A). As stated earlier taxol and cisplatin plus taxol treated macrophages assume a discoid shape without cytoplasmic extensions. This, however, did not effect the ability of macrophages to establish contact with the tumor cells and transfer their lysosomes (Figure 5B and C). These tumor cells eventually undergo lysis. Untreated macrophages never established contact with tumor cells (Figure 5D).

Discussion

The antineoplastic activity that taxol has demonstrated in advanced ovarian cancer and other neoplasms in which the platinum analogs are among the most active agents has been the impetus for the development of taxol plus cisplatin combination regimens.[8] Taxol and cisplatin are the two most effective agents discovered to date for treating advanced-stage cancers.[11] Learning how best to combine these agents is the focus of preclinical and clinical studies conducted at a number of institutions. The overt effects of the anticancer drugs cisplatin and taxol appear to be DNA modification and microtubule stabilization, respectively, yet the mechanisms by which these drugs elicit tumor cell

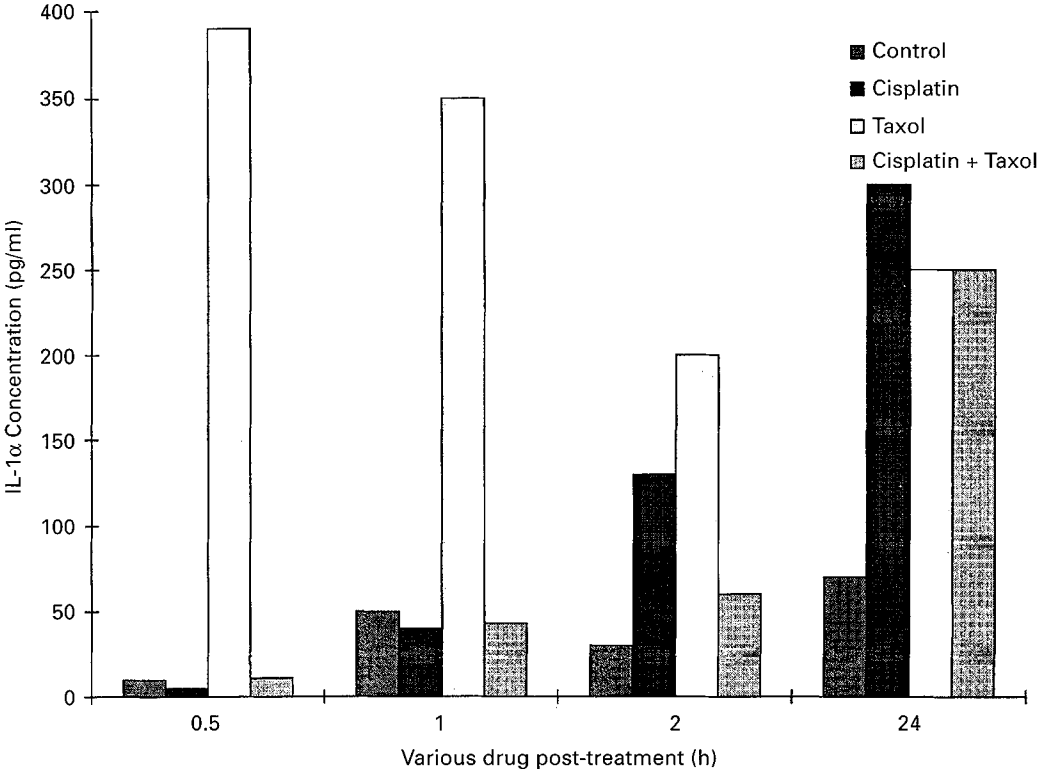

Figure 1. Bar graph showing IL-1α release in the supernatants of murine peritoneal macrophages treated for 2 h with either cisplatin (5 μg/ml), taxol (10 μg/ml) or cisplatin plus taxol after 30 min, 1, 2 and 24 h post-treatment. Note the large increase at 30 min post-treatment with taxol. Both cisplatin and cisplatin plus taxol show a gradual increase in the levels of IL-1α, reaching a maximum level at 24 h post-treatment.

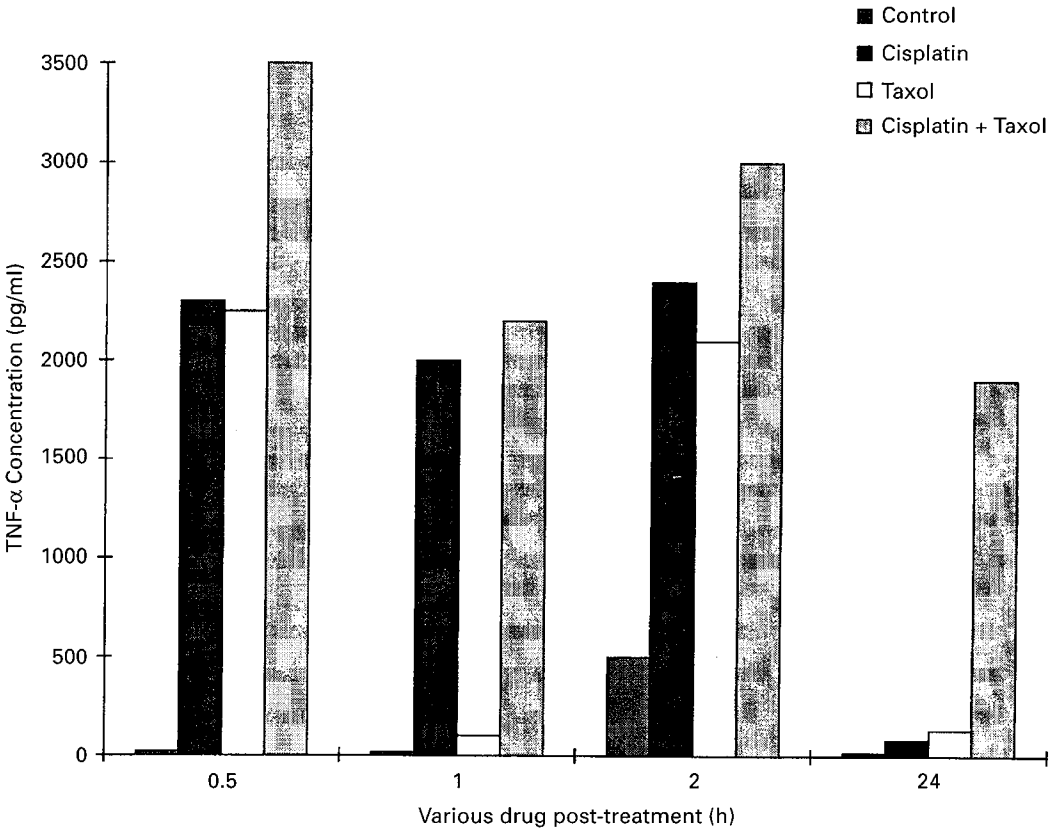

Figure 2. Bar graph showing TNF-α release in the supernatant of murine peritoneal macrophages treated for 2 h with either cisplatin (5 μg/ml), taxol (10 μg/ml) or cisplatin plus taxol after 30 min, 1, 2 and 24 h post-treatment. Note the maximum increase in TNF-α for cisplatin plus taxol at 2 h post-treatment. The release of TNF-α appears to be cyclical in all drug treatments with cisplatin plus taxol showing the highest activity.

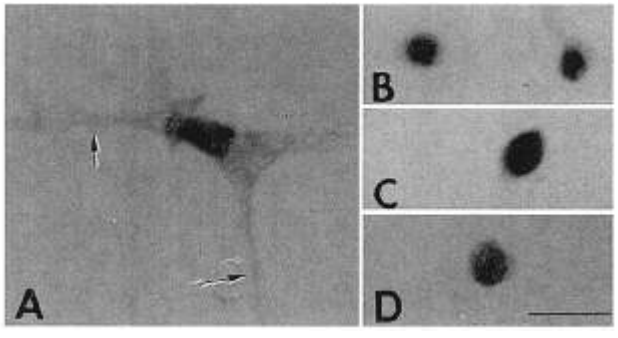

Figure 3. Light micrographs showing macrophages after 24 h of treatment with cisplatin (A), taxol (B), cisplatin plus taxol (C) and normal (D). Note the extension formation after cisplatin treatment (arrows). Taxol and cisplatin plus taxol treated cells show mostly a discoid shape similar to that of normal macrophages. Bar = 0.5 mm.

death are not well understood.[12] Both *in vitro* and *in vivo* studies conclude that taxol interacts synergistically with cisplatin in a manner that is highly schedule dependent.[12]

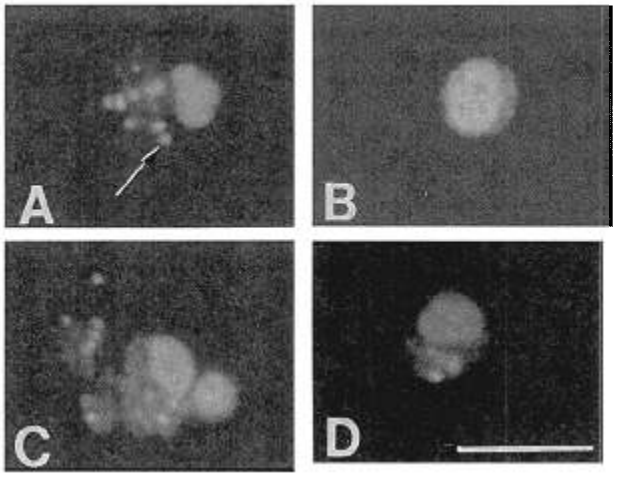

Figure 4. Fluorescent images taken from the Zeiss 10 laser scanning confocal microscope of macrophages labeled with acridine orange (5 μg/ml) showing lysosomal fluorescence in cisplatin plus taxol (A), normal (B), cisplatin (C) and taxol (D). Note the large increase in lysosomal fluorescence after cisplatin plus taxol treatment (arrows). Bar = 10 μm.

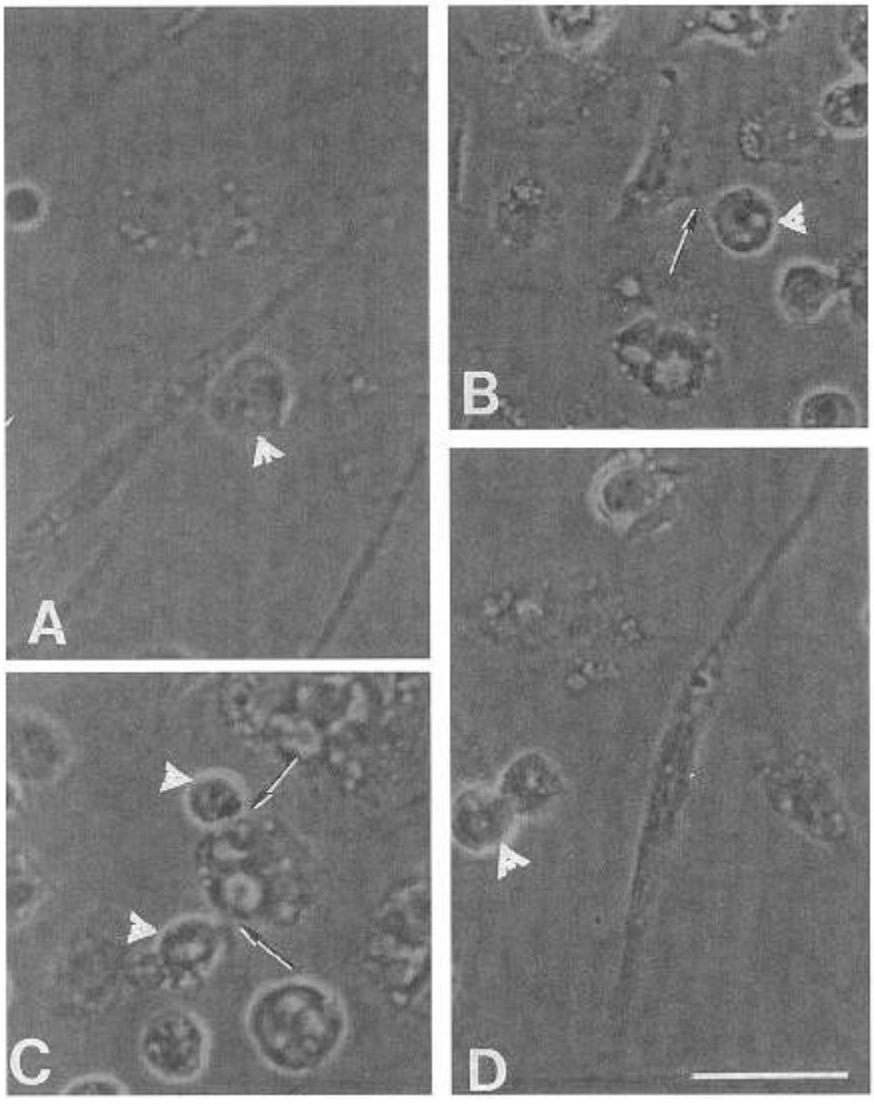

Figure 5. Phase contrast images taken from the Zeiss 10 laser scanning confocal microscope of macrophages co-incubated with S180 tumor cells (arrowheads) after 2 h of treatment with cisplatin (A), taxol (B), cisplatin plus taxol (C) and normal (D). Note that taxol and cisplatin plus taxol treated macrophages do not show long cytoplasmic extensions so very characteristic of cisplatin. However, treated cells are still able to establish contact with tumor cells and destroy them through lysosomal transfer (arrows). Bar = 10 μm.

Cisplatin stimulates immune responses by activating monocyte-macrophages and other cells of the immune system.[13] We have demonstrated here three mechanisms by which the immune system is enhanced by cisplatin and taxol combination compared to cisplatin or taxol treatments: (i) release of cytolytic factors IL-1α and TNF-α, (ii) increase in the macrophage lysosomes, and (iii) cell-cell recognition through contact between activated macrophages and tumor cells.

IL-1α was first described as a lymphocyte activating factor because of its ability to stimulate T cells.[14] Its release by activated macrophages and its cytotoxicity to tumor cells suggests IL-1α as a potent mediator in tumor cell killing by macrophages.[15] Our study demonstrates taxol as having the greatest increase in IL-1α after 30 min, while decreasing by 24 h, but yet stays higher than the untreated macrophages. Both cisplatin and cisplatin plus taxol in combination showed a gradual increase in IL-1α reaching a maximum level after 24 h post-treatment.

Increased levels of TNF-α have also been observed in activated macrophages. TNF-α is known to mediate a variety of functions which include host defense mechanisms and growth.[15] Recent studies have shown increased levels of TNF-α after cisplatin treatment. It is apparent that TNF-α is another important mediator of

tumor cell killing by macrophages. Cisplatin plus taxol combination demonstrated the greatest increase in TNF-α, reaching a maximum level after only 30 min. All three treatments seemed to cause a cyclical release of TNF-α.

Tumor cell death through macrophage activation includes the production of lysosomes.[16,17] Previous studies have shown cisplatin-treated macrophages to increase their number of lysosomes and transfer them via cytoplasmic extensions into tumor cells.[18] This transfer results in eventual lysis of tumor cells.[19] Cisplatin plus taxol combination showed the greatest increase in the level of cytoplasmic lysosomes with a 100-fold increase over that of untreated macrophages. Both cisplatin and taxol independently showed approximately a 50-fold increase in lysosomes.

Lysosomes are only released when cytoplasmic continuity between the macrophage and tumor cell has been established. Past studies have shown that cisplatin activates macrophages to form cytoplasmic extensions which make contact with tumor cells.[18] Through these extensions lysosomes are transferred and cell death occurs. Although taxol and cisplatin plus taxol combination did not activate macrophages to form cytoplasmic extensions they still established contact and formed a cytoplasmic continuity with the tumor cells. Through this cytoplasmic continuity there was an apparent transfer of lysosomes from macrophage to tumor cell and eventual cell lysis.

Conclusion

Cisplatin plus taxol combination stimulates various cytolytic factors of the immune system better than when these drugs are used independently. The macrophage activation includes production of cytoplasmic lysosomes, macrophage-tumor cell contact, and release of cytolytic factors IL-1α and TNF-α. Past *in vitro* and *in vivo* studies have shown that when these two drugs are used in combination they are more effective and less toxic than when they are used separately. Our results support activation of the immune system as an additional mechanism of action of this combination therapy. We also propose, based on our observations, that cisplatin plus taxol combination activates various cytolytic factors of the immune system better than cisplatin or taxol independently.

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