Microenvironment and Immunology

Dose-Dense Chemotherapy Improves Mechanisms of Antitumor Immune Response

Chih-Long Chang1,2,3, Yun-Ting Hsu2, Chao-Chih Wu2, Yan-Zen Lai2, Connie Wang4, Yuh-Cheng Yang1, T.-C. Wu1,5,6,7, and Chien-Fu Hung4,5

Abstract

Dose-dense (DD) regimens of combination chemotherapy may produce superior clinical outcomes, but the basis for these effects is not completely clear. In this study, we assessed whether a DD combinatorial regimen of low-dose cisplatin and paclitaxel produces superior immune-mediated efficacy when compared with a maximum tolerated dose (MTD) regimen in treating platinum-resistant ovarian cancer as modeled in mice. Immune responses generated by the DD regimen were identified with regard to the immune cell subset responsible for the antitumor effects observed. The DD regimen was less toxic to the immune system, reduced immunosuppression by the tumor microenvironment, and triggered recruitment of macrophages and tumor-specific CD8+ T-cell responses to tumors [as determined by interleukin (IL)-2 and IFN-γ secretion]. In this model, we found that the DD regimen exerted greater therapeutic effects than the MTD regimen, justifying its further clinical investigation. Fourteen patients with platinum-resistant relapse of ovarian cancer received DD chemotherapy consisting of weekly carboplatin (AUC2) and paclitaxel (60–80 mg/m2) as the third- or fourth-line treatment. Serum was collected over the course of treatment, and serial IFN-γ and IL-2 levels were used to determine CD8+ T-cell activation. Of the four patients with disease control, three had serum levels of IL-2 and IFN-γ associated with cytotoxic CD8+ T-cell activity. The therapeutic effect of the DD chemotherapy relied on the preservation of the immune system and the treatment-mediated promotion of tumor-specific immunity, especially the antitumor CD8+ T-cell response. Because the DD regimen controlled drug-resistant disease through a novel immune mechanism, it may offer a fine strategy for salvage treatment. Cancer Res; 73(1); 119–27. ©2012 AACR.

Introduction

Epithelial ovarian carcinoma (EOC) has the highest mortality rate among gynecologic malignancies, as it is typically asymptomatic and undiagnosed until the disease has progressed to advanced stages. Typical treatment for ovarian cancer is cytoreductive surgery when possible, followed by adjuvant chemotherapy. The introduction of modern platinum-based combination chemotherapy with paclitaxel has improved the 5-year survival rate of patients with advanced EOCs; however, long-term prognosis remains unfavorable. Disease relapse, acquired drug resistance, and short duration of progression-free survival are common and therefore, have motivated the search for better treatments.

Authors' Affiliations: Departments of 1Obstetrics and Gynecology and 2Medical Research, 3Institute of Biomedical Sciences, Mackay Medical College, Sanzhi, New Taipei, Taiwan; Departments of 4Pathology, 5Oncology, 6Obstetrics and Gynecology, and 7Molecular Microbiology and Immunology, The Johns Hopkins University, Baltimore, Maryland

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Corresponding Authors: Chien-Fu Hung, Department of Pathology, The Johns Hopkins University School of Medicine, CRB II Room 307, 1550 Orleans Street, Baltimore, MD 21231. Phone: 410-502-8215; Fax: 443-287-4295; E-mail: chung2@jhmi.edu; and Chih-Long Chang, clchang@mmc.edu.tw
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(5). Notably, evidence suggests that DD platinum-based combination therapy is a worthwhile option that can be safely extended, even for heavily treated patients with notoriously difficult drug-resistant disease (9). In theory, patients with platinum-resistant disease should be unresponsive to any platinum-based treatment; however, this supposition is contradicted by evidence. We speculate that platinum agent and paclitaxel given in a DD schedule at low dosages lead to the use of an immune-mediated tumor-killing pathway that is compromised when the drugs are given in the MTD regimen.

This study compares the efficacy and toxicity of platinum-based combination therapy administered in the DD and MTD regimen. We then determined whether antitumor effects involved the immune system. Because the experimental results from mouse models of platinum-resistant tumors suggested the DD regimen is superior and has immune-mediated efficacy, the treatment was examined in 14 patients with relapse of EOCs.

Materials and Methods

Mouse and cell lines

C57BL/6, (C57BL/6×C3/He) F1 mice, and athymic nude mice were purchased from BioLASCO. All animals were maintained under specific pathogen-free conditions. All procedures were conducted in accordance with approved protocols and recommendations for the proper care and use of laboratory animals. Mouse ovarian cancer cell lines, HM-1 (C57BL/6×C3/He F1 origin) and ID8, were used to establish the tumor model in syngeneic mice. ID8 was derived from the mouse ovarian carcinoma C3H/F1 origin) and ID8, were used to establish the tumor model in syngeneic mice. ID8 was derived from the mouse ovarian carcinoma C3H/F1 origin) and ID8, were used to establish the tumor model in syngeneic mice. ID8 was derived from the mouse ovarian carcinoma C3H/F1 origin) and ID8, were used to establish the tumor model in syngeneic mice. ID8 was derived from the mouse ovarian carcinoma C3H/F1 origin) and ID8, were used to establish the tumor model in syngeneic mice. ID8 was derived from the mouse ovarian carcinoma C3H/F1 origin) and ID8, were used to establish the tumor model in syngeneic mice. ID8 was derived from the mouse ovarian carcinoma C3H/F1 origin) and ID8, were used to establish the tumor model in syngeneic mice.

Intracellular cytokine staining and flow cytometry analysis for immunohistoassay

After chemotherapy, cells were harvested from the peritoneal lavage of tumor-bearing mice. Analysis of tumor-infiltrating lymphocytes (TIL) was conducted on the R HM-1 tumors extracted from mice treated with DD, MTD, or control PBS. R HM-1 tumors were dissociated, weighed, chopped into small pieces, and washed with HBSS. Tissues were incubated with a mixture of enzymes [collagenase type IV (Gibco), hyaluronidase (Sigma), and DNase I (Sigma)] in HBSS at 15 minutes at 37°C. After enzyme digestion, dissociated single cells were harvested and cultured in 24-well plates coated with monoclonal antibody OKT3 for 18 hours.

For antigen stimulation, isolated cells (5×10⁶) from each group were cultured and stimulated in vitro with 2×10⁶ live HM-1 or ID8 cells in culture medium containing IL-2 (100 U/mL) for 1 day. They were then cultured in medium added with 2 μg Golgistop (BD Pharmingen) for another 18 hours. Cells were then washed once in FACScan buffer and stained with allophycocyanin (APC)-conjugated monoclonal anti–mouse CD8a (1:100; eBioscience) for 20 minutes, and fixed using the Cytofix/Cytoperm kit in accordance to manufacturer’s instructions (BD Pharmingen), followed by staining with fluorescein isothiocyanate (FITC)-conjugated rat anti–mouse IFN-γ (1:50; eBioscience) for 20 minutes. Flow cytometric analysis was conducted on a Becton Dickinson FACScan (BD FACScan). Each group was measured in triplicate.

Peritoneal lavage and cytokine assays

The peritoneal cavity of experimental and control R ID8 tumor–bearing mice was lavaged with HBSS to obtain cells. The washout was passed through a 35-μm nylon mesh and collected. After red blood cell (RBC) lysis and repeated washes, cells were processed for in vitro culture and stimulation for further intracellular cytokine staining and flow cytometric analysis.
Macrophage inhibition
Cisplatin-resistant (R HM-1) cells (1 × 10^6) were s.c. injected into female (C57BL/6 C3H/He) F1 mice on day 0 (5 in each group). On day 4, mice began DD regimen [seven 3-day cycles of paclitaxel (5 mg/kg) plus cisplatin (3 mg/kg)]. The control group mice were treated by PBS. Macrophage inhibitor, clodronate liposome (Encapsula NanoScience), and control liposome were i.p. administered since day 1 (1.5 mg/mice at 5-day intervals).

Blood component analysis in mice receiving chemotherapy
Female (C57BL/6 C3H/He) F1 mice were treated with different formats of chemotherapy. Blood from the tail vein was collected in heparinized tube. Component cells were separated by centrifugation (1,000 × g, 5 minutes) and stained with antibodies: FITC-conjugated rat anti–mouse CD3 (AbD serotec, MCA 500F, 1:20), phycoerythrin (PE)-conjugated rat anti–mouse CD4 (BD, 553730, 1:200), APC-conjugated rat anti–mouse CD8a (eBioscience, 17-0081, 1:100), PE-Cy7-conjugated rat anti–mouse NK.1.1 (eBioscience, 25-5941, 1:50), FITC-conjugated rat anti–mouse CD11b (eBioscience, 12-0112, 1:100), PE-conjugated rat anti–mouse CD11c (eBioscience, 12-0114, 1:50), APC-conjugated rat anti–mouse F4/80 (eBioscience, 17-4801, 1:50), and APC-conjugated rat anti–mouse Gr-1 (eBioscience, 17-5931, 1:100). The hematologic components were analyzed by flow cytometry.

Statistical analysis
All data expressed as means ± SE are representative of at least 2 different experiments. Comparisons between individual data points were made using the Student t test or ANOVA.

Results
DD chemotherapy was more effective in controlling platinum-resistant HM-1 tumor
Tumor-bearing mice treated by DD regimen received 7 cycles (3-day intervals) of paclitaxel (5 mg/kg) and cisplatin (3 mg/kg). Mice treated by MTD regimen received 3 courses (10-day interval) of higher dosages of paclitaxel (12 mg/kg) and cisplatin (7 mg/kg) by i.p. injection. The control group received PBS. MTD chemotherapy did not significantly reduce the size of R HM-1 tumor, unlike DD chemotherapy (P = 0.017, control vs. DD; Fig. 1A). The R HM-1 tumor was chemosensitive when low-dose cisplatin and paclitaxel were administered by DD protocol. DD efficacy was also better than MTD, even using treatment with single-agent cisplatin or paclitaxel (P = 0.0003, DD vs. MTD cisplatin; P = 0.002, DD vs. MTD paclitaxel;
Supplementary Fig. S1). This suggests the therapeutic effect is not dose-dependent and might involve another mechanism that we theorize to be immunologic.

**Therapeutic effect of DD chemotherapy against platinum-resistant tumor is immune-dependent**

DD chemotherapy did not produce the same therapeutic effect in immunodeficient tumor-bearing mice (Fig. 1B). The immune profile (CD8⁺, CD4⁺, NK, CD11b⁺, CD11c⁺, and F4/80⁺ cells) of mice following treatment showed that the MTD regimen was toxic to all the immune cells of interest, whereas DD regimen preserved CD8⁺, CD4⁺, and CD11b⁺ cells with minimal reduction of NK, CD11c⁺, and F4/80⁺ cell count (P < 0.0001, P < 0.001, and P < 0.001, respectively; Fig. 2A).

**DD chemotherapy preferentially decreased myeloid-derived suppressor cells**

DD chemotherapy significantly reduced the number of myeloid-derived suppressor cells (MDSC) found in tumor-bearing mice, whereas the same phenomenon was not observed in the control and MTD groups (P < 0.0001, DD vs. control; P < 0.001, DD vs. MTD; Fig. 2B). Selective cytotoxicity toward immunosuppressive MDSCs is important for overcoming cancer immune tolerance, as MDSCs mediate T-cell anergy and promote the development of regulatory T (Treg) cells that inhibit effective antitumor immune response (10, 11). Other treatments found to preferentially reduce MDSCs have also documented a corresponding boost in the response of T cells and number of TILs with associated therapeutic response (12–14).

![Image](cancerres.aacrjournals.org)
Ideally, cancer treatment should also be able to interfere with Treg function or quantity. The primary function of Treg is to maintain peripheral tolerance by suppressing self-reactive T cells that have escaped the primary lymph node; however, the prevention of autoimmunity also contributes to cancer immune tolerance (15). In mouse tumor models, the reduction of Treg cells with low-dose cyclophosphamide was able to recover the antitumor effects induced by immunotherapy (16–18). While the present study did not find DD regimen to be more effective than MTD regimen at reducing the percentage of CD4⁺CD25⁺ Treg cells (P = 0.85; Fig. 2C), both regimens led to the decline of Treg count (P = 0.048, control vs. DD; Fig. 2C). From this, we believe cisplatin and paclitaxel to have an intrinsic, dose-independent drug effect that is selectively cytotoxic to Treg cells.

**Therapeutic effect of DD chemotherapy is associated with tumor macrophage recruitment**

DD chemotherapy increased the recruitment of F4/80⁺ macrophages into the tumor. Representative flow cytometric data of tumor-bearing mice treated with DD regimen showed greater numbers of intratumoral F4/80⁺ cells than mice treated with MTD regimen (P < 0.001, control vs. DD, control vs. MTD; Fig. 3A).

The association of tumor macrophage recruitment with antitumor effect was investigated with the administration of macrophage inhibitor, clodronate liposome. The tumor growth curve showed that clodronate liposome partially abolished the antitumor effect of DD chemotherapy, whereas the same was not observed in the control group that only received the vehicle (liposome: P = 0.01, clodronate liposome vs. liposome only; Fig. 3B). We believe the recruitment of macrophages to the tumor is a component of treatment efficacy.

**DD chemotherapy promotes tumor-specific CD8⁺ T lymphocytes responsible for therapeutic effect against platinum-resistant tumor**

To delineate the effector cell types responsible for the antitumor effect of a treatment, selective depletion of lymphocyte subpopulations was achieved by injecting monoclonal antibodies against CD8, CD4, or NK1.1 in R HM-1 tumor-bearing mice. Tumor growth curve indicated the antitumor effect of DD regimen was most dependent on CD8⁺ T cells.

![Figure 3](image-url)

**Figure 3.** DD chemotherapy resulted in the significant induction of peritoneal macrophage and antitumor effect could be abrogated by macrophage depletion. A, DD chemotherapy induced a large amount of F4/80⁺ cells in the peritoneum of tumor-bearing mice. Cells were obtained through peritoneal lavage with 10 mL of PBS in R HM-1 tumor-bearing mice treated with different formats of chemotherapy. Mononuclear cells were separated by Ficoll-Hypaque gradient. Representative flow cytometric data show that a larger population of F4/80⁺ cells was elicited by DD chemotherapy (12,690 in 20,000 cells analyzed), whereas in mice receiving MTD chemotherapy, the population of F4/80⁺ cells became scant (1,571 in 20,000 cells). **P** < 0.001, control versus DD, control versus MTD. B, tumor growth curve shows diminishing antitumor effect of DD chemotherapy when macrophage was depleted by injection of clodronate liposome (macrophage inhibitor) into mice. R HM-1 cells (1 × 10⁶) were injected s.c. into female (C57BL/6 C3H/He) F1 mice (5 in each group, day 0). On day 4, mice began DD chemotherapy (5 mg paclitaxel plus 3 mg cisplatin every 3 days for 7 doses). Control group mice were treated with PBS. Clodronate liposome and control liposome began on day 1 (1.5 mg/mice) and were intraperitoneally administered at a 5-day interval. Administration of clodronate liposome reduced the antitumor effect by DD chemotherapy in comparison to the vehicle (liposome) control (**P** < 0.001, clodronate liposome vs. liposome only; ***P** < 0.001).
DD regimens results were validated in another intraperitoneal ovarian tumor model

We conducted the same experiments in the ID8 tumor model. ID8 is another aggressive mouse ovarian cancer cell line that is derived from the MOSEC cell line (19). In this platinum-resistant tumor model, DD regimen was again more effective than MTD regimen \( (P = 0.0022, \text{DD vs. MTD}) \). Akin to the R HM-1 tumor model experiments, DD regimen induced the recruitment of CD11b+ F4/80+ macrophages into the peritoneal cavity of ID8 tumor-bearing mice \( (P < 0.001, \text{control vs. DD}; P < 0.01, \text{DD vs. MTD}; \text{Fig. 5A}) \). DD treatment of tumor-naive mice had no macrophage recruitment to the abdominal cavity (Fig. 5B). Flow cytometric analysis found a high proportion of activated IFN-γ-secreting macrophages following DD
tumor peritoneal cavity of tumor-bearing mice. A, in this i.p. tumor model, DD chemotherapy elicited and recruited largest number of CD14+ macrophages inside the peritoneal cavity of R ID8 tumor–bearing mice (**, P < 0.001, control vs. DD; * P < 0.01, DD vs. MTD). B, the proportion of macrophage did not change in tumor-naïve mice regardless of chemotherapy. C, flow cytometric analysis indicated more activated macrophages, as determined by IFN-γ secretion, after DD chemotherapy. Following treatment, cells from the peritoneal lavage of R ID8 tumor-bearing mice were cultured in medium with protein transporter inhibitor BD GolgiPlug. Cells were stained with anti–mouse F4/80 PE, as well as anti–mouse IFN-γ FITC antibodies before analysis by flow cytometry. Representative data show the number of F4/80+ IFN-γ+ cells increased in mice receiving DD chemotherapy but not in the mice receiving MTD and PBS (control; 8.16% in DD vs. 2.11% in MTD and 2.31% in control, both; ***, P < 0.0001). D, in ID8 tumor–bearing mice, DD chemotherapy elicited CD8+ IFN-γ+ TILs. Mice bearing R ID8 tumor were treated with different formats of chemotherapies. After treatment, peritoneal cells were obtained by lavage and prepared for single-cell suspension. Cells were then stained with anti–mouse CD8+ and anti–mouse IFN-γ antibodies before getting analyzed by flow cytometry. Representative flow cytometric data show that peritoneal CD8+ IFN-γ+ cells were found in greater numbers within the tumors of mice receiving DD chemotherapy (***, P < 0.001, control vs. DD and DD vs. MTD). E, CD8+ T cells from peritoneal lavage in mice treated with different formats of chemotherapies were isolated and examined for the expression of IFN-γ and IL-2 by QPCR. A significant enhancement in the expressions of IFN-γ and IL-2 was noted in the peritoneal CD8+ cells in DD group mice (IFN-γ; *, P < 0.01, control vs. DD; DD vs. MTD, IL-2; *, P < 0.01, control vs. DD; #, P = 0.034, DD vs. MTD).

treatment, whereas MTD chemotherapy and PBS (control) did not produce the same results (8.16% vs. 2.11% and 2.31%, both P < 0.0001; Fig. 5C). Higher numbers of activated CD8+ T cells were purified from the peritoneal lavage of mice given DD chemotherapy (P < 0.001, control vs. DD and DD vs. MTD; Fig. 5D). Following DD treatment, CD8+ T cell from the peritoneal lavage of tumor–bearing mice again had higher levels of IFN-γ and IL-2 (IFN-γ; P < 0.01, control vs. DD, DD vs. MTD; IL-2, P < 0.01, control vs. DD, P = 0.034, DD vs. MTD; Fig. 5E). The expression of IFN-γ and IL-2 is known to accompany the activation of cytotoxic T cells. DD chemotherapy is more effective than MTD chemotherapy in the treatment of multiple tumor models.

Change in cytokine profile was correlated with the therapeutic effects of DD chemotherapy in ovarian cancer patients

The DD regimen results were validated in patients with relapse of platinum-resistant ovarian cancer. Following Institutional Review Board (IRB) approval (Protocol number: 09MHHS095), 14 patients were recruited and treated by DD regimen with weekly carboplatin (AUC 2) and paclitaxel.
platinum-resistant disease. We hope the encouraging results would facilitate the examination of DD regimen with low-dose drugs in a larger patient group.

Discussion

Phase I trials of new chemotherapeutic drugs typically focus on the identification of the MTD due to the assumption that it leads to the greatest antitumor cytotoxicity and effectiveness. Consequently, studies have neglected to investigate the possible advantages of low-dose chemotherapy. The data of the present study strongly imply that DD administration of low-dose platinum agent and paclitaxel spares the immune system from major toxicity and modifies the tumor microenvironment in favor of immunogenic tumor cell death, which results in the generation of antitumor immunity. This treatment modality leads to tumor macrophage recruitment, production of tumor-specific CD8+ T cells, and the selective reduction of immunosuppressive MDSCs and Treg cells of the tumor microenvironment. These immunologic changes are associated with the therapeutic response in tumor-bearing mice. It has been theorized that the combined effort of chemotherapy and host immunity results in the best management of persistent malignancies (20). The probability for success may depend on whether or not the drug-induced tumor cell death triggers the mechanisms that promote the development of tumor-specific immune response (21, 22).

The drug-induced immunogenic apoptosis of human ovarian cancer cells produce unique signals that promote dendritic cell maturation, enhance cross-presentation of tumor antigens, and enable T-cell priming that lead to tumor-specific CD8+ activity (23). At Mackay Memorial Hospital, DD chemotherapy with weekly carboplatin and paclitaxel has been a salvage option for platinum-resistant disease. We hope the encouraging results would facilitate the examination of DD regimen with low-dose drugs in a larger patient group.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: C.-L. Chang, C.-F. Hung

Development of methodology: C.-L. Chang, C.-F. Hung

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.-L. Chang, Y.-T. Hou, C.-C. Wu, Y.-Z. Lai, C.-F. Hung

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Analysis and interpretation of data (e.g., statistical analysis, bio-statistics, computational analysis): C.-L. Chang, C. Wang, T.-C. Wu, C.-F. Hung
Writing, review, and/or revision of the manuscript: C.-L. Chang, C. Wang, T.-C. Wu, C.-F. Hung
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Wang, Y.-C. Yang, C.-F. Hung

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