Published in final edited form as:

Cancer Res. 2013 January 1; 73(1): 119–127. doi:10.1158/0008-5472.CAN-12-2225.

Dose-dense chemotherapy improves mechanisms of antitumor immune response

Chih-Long Chang^{1,2,3}, Yun-Ting Hsu², Chao-Chih Wu², Yan-Zen Lai², Connie Wang⁴, Yuh-Cheng Yang¹, T.-C. Wu^{4,5,6,7}, and Chien-Fu Hung^{4,5}

¹ Department of Obstetrics and Gynecology, Mackay Memorial Hospital, Taipei, Taiwan.

² Department of Medical Research, Mackay Memorial Hospital, Tamshui, New Taipei, Taiwan.

³ Institute of Biomedical Sciences, Mackay Medical College, Sanzhi, New Taipei, Taiwan.

⁴ Department of Pathology, the Johns Hopkins Hospital, Baltimore, Maryland, USA

⁵ Department of Oncology, Johns Hopkins Hospital, Baltimore, Maryland, USA

⁶ Department of Obstetrics and Gynecology, The Johns Hopkins Hospital, Baltimore, Maryland, USA

⁷ Department of Molecular Microbiology and Immunology, The Johns Hopkins University Baltimore, Maryland, USA

Abstract

Dose-dense (DD) regimens of combination chemotherapy may produce superior clinical outcomes, but the basis for these effects are 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 to 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 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 4 patients with disease control, 3 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. Since the DD regimen controlled drug-resistant disease through a novel immune mechanism, it may offer a fine strategy for salvage treatment.

Address correspondence to Dr. Chien-Fu Hung, Department of Pathology, The Johns Hopkins University School of Medicine, CRB II Room 307, 1550 Orleans Street, Baltimore, MD 21231, USA. Phone: 001-1-(410) 502-8215; Fax: 001-1-(443) 287-4295; chung2@jhmi.edu and co-correspondence to Dr. Chih-Long Chang^{1,2,3}, (clchang@mmc.edu.tw).

Conflict of interest: The authors declare no conflicts of interest.

Keywords

chemotherapy; dose dense; antitumor immunity; drug-resistance; ovarian cancer

Introduction

Epithelial ovarian carcinoma (EOC) has the highest mortality rate among gynecological malignancies since 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 EOC; 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.

Few phase III trials that investigated modifications to standard chemotherapy have produced encouraging results(1-3). Thus far, the condensed treatment interval method first advocated by van der Burg and colleagues, now known as dose dense (DD) chemotherapy, is considered to be one of the most promising treatment strategy(4). Theoretically, DD regimen deprives cancer cells the opportunity to proliferate and impedes tumor progression via the antiangiogenic effects of paclitaxel (5). However, DD regimen is not without complications since it may be overly hematologically toxic and consequently force the premature cessation of treatment (5). To offset toxicity and enhance treatment tolerability, DD regimen may be modified to use low drug dosages (6, 7).

The clinical trials of dose dense platinum-based combination chemotherapy have reported promising outcomes for patients with advanced EOC. One randomized phase I/II trial investigating the efficacy of cisplatin and paclitaxel found response rates of 94% for chemotherapy-naïve patients and 84% for patients with recurrent disease(8). Another phase II study of weekly cisplatin with an etoposide reported similar results(4).

The Japanese Gynecologic Oncology Group (JGOG 3016) conducted a randomized phase III trial comparison of MTD treatment versus DD treatment of advanced ovarian cancer. The “partial” dose dense protocol, consisting of six 21-day cycles of intravenous carboplatin (AUC 6) on day 1 in addition to paclitaxel (80mg/m²) on days 1, 8, and 15, significantly lengthened the median progression-free survival of patients(5). Notably, evidence suggests that dose dense 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 dose dense schedule at low dosages lead to the utilization 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 if antitumor effects involved the immune system. **Since** 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 EOC.

Materials and Methods

Mouse and cell lines

C57BL/6, (C57BL/6×C3/He) F1 mice and athymic nude mice were purchased from BioLASCO, Taiwan. All animals were maintained under specific pathogen-free conditions. All procedures were performed 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 cancer cell line, MOSEC (C57BL/6 origin) and carries the luciferase gene to enable *in vivo* imaging. The cells were cultured in MEM medium (Gibco-BRL, Gaithersburg, MD, USA) with 10% FBS (Hyclone, Logan, UT, USA), 100 U/ml penicillin (Gibco), and 100 pg/ml streptomycin (Sigma, St Louis, MO, USA) under the condition of humidified atmosphere with 5% CO₂/95% air at 37°C. Intraperitoneal tumor growth was monitored by *in vivo* imaging of tumor luminescence (indicating tumor load) by Xenogen IVIS 200 Imaging System.

Development of cisplatin-resistant cell line and cytotoxicity assay

Cisplatin-resistant cell lines (R HM-1) and (R ID8) were produced by culturing the cells with escalating doses of cisplatin (0.1 µg/ml to 2 µg/ml of cisplatin within 2 months, maintained at 2 µg/ml). *In vitro* cytotoxicity was evaluated by MTT assay in accordance with protocol of the manufacturer (Sigma–Aldrich, St. Louis, MO, USA). The absorbance of each well was measured at 570/630 nm within a microplate reader. Each measurement was taken in triplicates and presented as mean ± SD.

Tumor treatment model and depletion of lymphocyte subpopulations

One million R HM-1 cells were inoculated subcutaneously (s.c.) in the left thigh of (C57BL/6×C3/He) F1 mice and athymic nude mice (day 0, 5 in each group). On day 4, mice began receiving paclitaxel and cisplatin intraperitoneally in either DD format [seven 3-day cycles of paclitaxel (5 mg/kg) plus cisplatin (3 mg/kg)] or MTD format [three 10-day cycles of paclitaxel (12 mg/kg) plus cisplatin (7 mg/kg)]. Other sets of mice were given the single-agent treatment either with paclitaxel (16 mg/kg in DD, 40 mg/kg in MTD) or cisplatin (10 mg/kg in DD, 25 mg/kg in MTD) at the same intervals. Chemotherapeutic drugs, paclitaxel (Taxol) and cisplatin (Platinex) were purchased from Bristol-Myers Squibb Company (Princeton, NJ) under the approval of the pharmaceutical committee in Mackay Memorial Hospital. Control group mice were treated with PBS at 3-day intervals. R ID8 cells (5·10⁵) were i.p injected into C5BL/6 mice on days 0 and 5. On day 7, mice began treatment. Depletion of lymphocyte subpopulation was achieved through i.p. injection with 100 µg of rat monoclonal antibody GK1.5 (anti-CD4), 2.43 (anti-CD8), or PK136 (anti-NK1.1). Depletion was initiated 1 week before tumor challenge and continued every other day for the first week, followed by once per week after. Depletion was assessed 1 day after the fourth administration of antibodies by flow cytometry analysis of spleen cells stained with 2.43, GK1.5, or PK136 to confirm the depletion of CD8, CD4, or NK cells. Depletion was maintained by weekly antibody injections for the duration of the tumor growth follow-up.

Intracellular cytokine staining and flow cytometry analysis for immunoassay

After chemotherapy, cells were harvested from the peritoneal lavage of tumor-bearing mice. Analysis of tumor-infiltrating lymphocytes (TILs) was conducted on the R HM-1 tumors extracted from mice treated with DD, MTD, or control PBS. R HM-1 tumors were dissected, weighed, chopped into small pieces, and wash with HBSS. Tissues were incubated with a mixture of enzymes [collagenase type I (Gibco, Grand Island, NY, USA), collagenase type IV (Gibco), hyaluronidase (Sigma), and DNase I (Sigma)] in HBSS for 15 min 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 (5×10^6) from each group were harvested and cultured *in vitro* with 2×10^5 live HM-1 or ID8 cells in culture medium containing IL-2 (100U/mL) for one day. They were then cultured in medium added with 2 μ g Golgistop (BD Pharmingen, San Diego, CA, USA) for another 18 hours. Cells were then washed once in FACScan buffer and stained with APC- conjugated monoclonal rat anti-mouse CD8a (1:100) (eBioscience, San Diego, CA, USA) for 20 minutes, and fixed using the Cytofix/Cytoperm kit in accordance to manufacturer's instructions (BD Pharmingen), followed by staining with FITC-conjugated rat anti-mouse IFN- γ (1:50) (eBioscience) for 20 minutes. Flow analysis was performed on a Becton Dickinson FACScan (BD FACSCalibur). 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- μ m 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 analysis as previously mentioned. In addition, CD8⁺ cells were isolated from pelleted cells using beads coated with CD8 antibody (MACs, MiltenyiBiotec, Auburn, CA). Isolated CD8⁺ cells were subjected to RNA extraction then quantitative real-time polymerase chain reaction (qRT-PCR) for the expression profiling of IFN- γ and IL-2. Human serum IFN- γ and IL-2 were quantified using multiplex bead-based assays (BD Cytometric Bead Array) then analyzed by flow cytometry in accordance with manufacturer's protocol. Blood samples from patients were obtained before treatment, 1 month after the initiation of treatment, and 2 months after the initiation of treatment). Patient enrollment was limited to those with platinum- resistant ovarian cancer recurrence who will receive weekly carboplatin (AUC 2) and paclitaxel (80 mg/m²) as the third or fourth line treatment.

Macrophage inhibition

Cisplatin-resistant (R HM-1) cells ($1 \cdot 10^6$) 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, Nashville, TN) 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 (1000 ·g, 5 minutes) and stained with antibodies: FITC-conjugated rat anti-mouse CD3 (AbD serotec, MCA 500F, 1:20), 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 CD 11c (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 hematological components were analyzed by flow cytometry.

Statistical Analysis

All data expressed as means $\pm$ standard error (SE) are representative of at least two different experiments. Comparisons between individual data points were made using a Student's *t*-test or ANOVA (analysis of variance).

Results

DD chemotherapy was more effective in controlling platinum-resistant HM-1 tumor

Tumor-bearing mice treated by DD regimen received seven cycles (3-day intervals) of paclitaxel (5 mg/kg) and cisplatin (3 mg/kg). Mice treated by MTD regimen received three 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 versus 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 versus MTD cisplatin) ($p=0.002$, DD versus MTD paclitaxel) (Supplemental Fig. 1). This suggests the therapeutic effect is not dose-dependent and might involve another mechanism that we theorize to be immunological.

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 (MDSCs)

DD chemotherapy significantly reduced the number of myeloid-derived suppressor cells (MDSCs) found in tumor-bearing mice while the same phenomenon was not observed in the control and MTD groups ($p<0.0001$, DD versus control; $P<0.001$, DD versus MTD) (Fig. 2B). Selective cytotoxicity towards immunosuppressive MDSCs is important for overcoming cancer immune tolerance since MDSCs mediate T cell anergy and promote the development of regulatory T (T_{reg}) 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 tumor infiltrating lymphocytes (TILs) with associated therapeutic response(12-14).

Ideally, cancer treatment should also be able to interfere with T_{reg} function or quantity. The primary function of T_{reg} 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 T_{reg} 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 $CD4^+CD25^+T_{reg}$ cells ($P=0.85$) (Fig. 2C), both regimens led to the decline of T_{reg} count ($p=0.048$, control versus DD) (Fig. 2C). From this, we believe cisplatin and paclitaxel to have an intrinsic, dose-independent drug effect that is selectively cytotoxic to T_{reg} 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 dose dense regimen showed greater numbers of intratumoral $F4/80^+$ cells than mice treated with MTD regimen ($p<0.001$, control versus DD, control versus 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 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 versus 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 ($p<0.001$, anti-CD8 versus rat IgG)(Fig. 4A), whereas the antitumor effect of MTD regimen did not seem to be associated with any of the monitored immune cells (Fig. 4B). Subsequently, we found DD chemotherapy induced tumor-specific immune responses. Flow cytometry analysis of the splenocytes identified CD8⁺IFN- γ ⁺ T cells in mice that received DD treatment ($p<0.001$, DD versus Control and MTD) (Fig. 4C). Moreover, the percentage of tumor-infiltrating CD8⁺IFN- γ ⁺ T cells was highest in mice treated with DD chemotherapy, compared to MTD or control ($p<0.001$, DD versus control and MTD). The R HM-1 tumor model experiments have shown DD regimen with cisplatin and paclitaxel promotes tumor macrophage recruitment, reduces immunosuppression of the tumor microenvironment, and elicits tumor-specific antitumor CD8⁺ T cell responses.

DD regimen 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 ($p=0.022$, DD versus MTD) (Supplemental Fig.2). Akin to the R HM-1 tumor model experiments, DD regimen induced the recruitment of CD14⁺F4/80⁺ macrophages into the peritoneal cavity of ID8 tumor-bearing mice ($p<0.001$ control versus DD, $p<0.01$, DD versus MTD) (Fig. 5A). DD treatment of tumor-naïve 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 treatment, whereas MTD chemotherapy and PBS (control) did not produce the same results (8.16 % versus 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 versus DD and DD versus MTD, Fig. 5D). Following DD treatment, CD8⁺ T cell from the peritoneal lavage of tumor-bearing mice again had higher levels of interferon- γ (IFN- γ) and interleukin-2 (IL-2) (IFN- γ , $p<0.01$, control versus DD, DD versus MTD; IL-2, $p<0.01$, control versus DD, $p=0.034$, DD versus 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 IRB approval (Protocol number: 09MMHIS095), 14 patients were recruited and treated by DD regimen with weekly carboplatin (AUC 2) and paclitaxel (80 mg/m²) as their third or fourth-line treatment. Disease control was defined as: 1. Stable or lowered CA-125 levels (GCIC criteria) over the period of two or more consecutive months, or, 2. No change/shrinkage of tumor size over the period of two or more months (RECIST

1.1 CT scan criteria). Patient serum levels of IFN- γ and IL-2 were determined by blood samples obtained before treatment, 1 month, and 2 months into treatment. DD chemotherapy controlled the disease in 4 (28.6%) out of 14 patients. Of these four patients, three patients had higher levels of serum IFN- γ and IL-2 two months after the start of chemotherapy (DD2 for IFN- γ , $p < 0.001$, responsive versus unresponsive; DD1 and DD2 for IL-2, $p < 0.001$ responsive versus unresponsive) (Fig. 6). Although the sample size was small, the patient results corroborate what we saw in the mouse tumor models and suggest that this treatment warrants further investigation in a larger study. Based on the response of these 14 patients, platinum-based combination therapy with low dose cisplatin and paclitaxel administered in a dose dense interval can lead to the development of antitumor cytotoxic T cell immune response in patients with 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 maximum tolerated dose 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 dose dense 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 T_{reg} cells of the tumor microenvironment. These immunological 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 relapse of ovarian cancer with platinum-resistance. Since we would expect the activation of CD8⁺ T cells to accompany IFN- γ and IL-2 elevation, we tested and confirmed higher serum levels of these cytokines in 3 of 4 patients whose disease was controlled. While the statistical power of the patient study is challenged by the small sample size, the results are promising enough that the regimen should be investigated in a larger clinical study. We believe that disease control in patients is related to the antitumor immunity that is produced as a consequence of DD treatment. The results of the present study suggest that antitumor effect of DD regimen is potentiated by tumor-specific immune response, and ergo, is more effective against drug-resistant disease. Furthermore, the gentler nature of low-dose dose dense regimen makes it ideal for maintenance therapy following the initial marrow-depriving MTD treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge Dr. Richard Roden for helpful discussions.

Financial support: This work was supported by the National Science Council, Taiwan, No. 97-2314-B-195-007-MY3; and P50CA098252 National Cancer Institute, USA

References

1. Sorbe B. Consolidation treatment of advanced ovarian carcinoma with radiotherapy after induction chemotherapy. *Int J Gynecol Cancer*. 2003; 13(Suppl 2):192–5. [PubMed: 14656279]
2. Hakes TB, Chalas E, Hoskins WJ, Jones WB, Markman M, Rubin SC, et al. Randomized prospective trial of 5 versus 10 cycles of cyclophosphamide, doxorubicin, and cisplatin in advanced ovarian carcinoma. *Gynecol Oncol*. 1992; 45:284–9. [PubMed: 1612505]
3. Bookman MA, Brady MF, McGuire WP, Harper PG, Alberts DS, Friedlander M, et al. Evaluation of new platinum-based treatment regimens in advanced-stage ovarian cancer: a Phase III Trial of the Gynecologic Cancer Intergroup. *J Clin Oncol*. 2009; 27:1419–25. [PubMed: 19224846]
4. van der Burg ME, de Wit R, van Putten WL, Logmans A, Kruit WH, Stoter G, et al. Weekly cisplatin and daily oral etoposide is highly effective in platinum pretreated ovarian cancer. *Br J Cancer*. 2002; 86:19–25. [PubMed: 11857006]
5. Katsumata N, Yasuda M, Takahashi F, Isonishi S, Jobo T, Aoki D, et al. Dose-dense paclitaxel once a week in combination with carboplatin every 3 weeks for advanced ovarian cancer: a phase 3, open-label, randomised controlled trial. *Lancet*. 2009; 374:1331–8. [PubMed: 19767092]
6. Leiser AL, Maluf FC, Chi DS, Sabbatini P, Hensley ML, Schwartz L, et al. A phase I study evaluating the safety and pharmacokinetics of weekly paclitaxel and carboplatin in relapsed ovarian cancer. *Int J Gynecol Cancer*. 2007; 17:379–86. [PubMed: 17362316]
7. Watanabe Y, Nakai H, Ueda H, Hoshiai H. Evaluation of weekly low-dose paclitaxel and carboplatin treatment for patients with platinum-sensitive relapsed ovarian cancer. *Gynecol Oncol*. 2005; 96:323–9. [PubMed: 15661216]
8. de Jongh FE, de Wit R, Verweij J, Sparreboom A, van den Bent MJ, Stoter G, et al. Dose-dense cisplatin/paclitaxel. a well-tolerated and highly effective chemotherapeutic regimen in patients with advanced ovarian cancer. *Eur J Cancer*. 2002; 38:2005–13. [PubMed: 12376205]
9. Sharma R, Graham J, Mitchell H, Brooks A, Blagden S, Gabra H. Extended weekly dose-dense paclitaxel/carboplatin is feasible and active in heavily pre-treated platinum-resistant recurrent ovarian cancer. *Br J Cancer*. 2009; 100:707–12. [PubMed: 19223898]
10. Dolcetti L, Peranzoni E, Ugel S, Marigo I, Fernandez Gomez A, Mesa C, et al. Hierarchy of immunosuppressive strength among myeloid-derived suppressor cell subsets is determined by GM-CSF. *European journal of immunology*. 2010; 40:22–35. [PubMed: 19941314]
11. Kao J, Ko EC, Eisenstein S, Sikora AG, Fu S, Chen SH. Targeting immune suppressing myeloid-derived suppressor cells in oncology. *Critical reviews in oncology/hematology*. 2011; 77:12–9. [PubMed: 20304669]
12. Suzuki E, Kapoor V, Jassar AS, Kaiser LR, Albelda SM. Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. *Clin Cancer Res*. 2005; 11:6713–21. [PubMed: 16166452]
13. Vincent J, Mignot G, Chalmin F, Ladoire S, Bruchard M, Chevriaux A, et al. 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. *Cancer research*. 2010; 70:3052–61. [PubMed: 20388795]
14. Liu P, Jaffar J, Hellstrom I, Hellstrom KE. Administration of cyclophosphamide changes the immune profile of tumor-bearing mice. *J Immunother*. 2010; 33:53–9. [PubMed: 19952956]
15. Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M, et al. Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunological reviews*. 2001; 182:18–32. [PubMed: 11722621]

16. Ghiringhelli F, Menard C, Terme M, Flament C, Taieb J, Chaput N, et al. CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. *The Journal of experimental medicine*. 2005; 202:1075–85. [PubMed: 16230475]
17. Lutsiak ME, Semnani RT, De Pascalis R, Kashmiri SV, Schlom J, Sabzevari H. Inhibition of CD4(+)25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. *Blood*. 2005; 105:2862–8. [PubMed: 15591121]
18. Taieb J, Chaput N, Scharz N, Roux S, Novault S, Menard C, et al. Chemoimmunotherapy of tumors: cyclophosphamide synergizes with exosome based vaccines. *J Immunol*. 2006; 176:2722–9. [PubMed: 16493027]
19. Roby KF, Taylor CC, Sweetwood JP, Cheng Y, Pace JL, Tawfik O, et al. Development of a syngeneic mouse model for events related to ovarian cancer. *Carcinogenesis*. 2000; 21:585–91. [PubMed: 10753190]
20. Emens LA, Jaffee EM. Leveraging the activity of tumor vaccines with cytotoxic chemotherapy. *Cancer research*. 2005; 65:8059–64. [PubMed: 16166275]
21. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat Med*. 2007; 13:54–61. [PubMed: 17187072]
22. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat Med*. 2007; 13:1050–9. [PubMed: 17704786]
23. Wu X, Feng QM, Wang Y, Shi J, Ge HL, Di W. The immunologic aspects in advanced ovarian cancer patients treated with paclitaxel and carboplatin chemotherapy. *Cancer Immunol Immunother*. 2010; 59:279–91. [PubMed: 19727719]

Figure 1. Dose dense (DD) chemotherapy exhibited better therapeutic efficacy against cisplatin-resistant tumor, R HM-1

In order to mimic the treatment of ovarian cancer in a clinical setting, we designed two treatment protocols for a mouse ovarian cancer model to which DD or maximum-tolerated dose (MTD) chemotherapies were administered. (A) DD chemotherapy exhibited better anti-tumor effect in mice bearing R HM-1 cell tumor. R HM-1 cells (1×10^6) were injected subcutaneously (s.c.) into the female (C57BL/6, C3H/He) F1 mice (5 in each group, day 0). On day 4, mice started chemotherapy with paclitaxel and cisplatin delivered intraperitoneally (i.p.) in either DD (paclitaxel 5 mg/kg plus cisplatin 3 mg/kg in a 3-day interval for 7 courses) or MTD (paclitaxel 12 mg/kg plus cisplatin 7 mg/kg in a 10-day interval for 3 courses) regimen. Control group mice were treated with PBS in 3-day interval. Significant therapeutic efficacy was noted in mice treated by DD chemotherapy (# $p=0.017$, control versus DD), which was better than that of MTD ($p=0.039$, MTD versus DD). (B) The specific anti-tumor effect of DD chemotherapy on R HM-1 cells was abolished in nude mice, suggesting host immunity might be involved in the tumor elimination. Cisplatin-resistant HM-1 cells (1×10^6) were injected s.c. into female athymic nude mice (5 in each group, day 0). On day 4, mice began intra-peritoneal administration of paclitaxel and cisplatin chemotherapy given in either DD (paclitaxel 5 mg/kg plus cisplatin 3 mg/kg in a 3-day interval for 7 courses) or MTD (paclitaxel 12 mg/kg plus cisplatin 7 mg/kg in a 10-day interval for 3 courses) format. Control group mice were treated by PBS in a 3-day interval. There is no difference in the tumor size between the different groups of immune-deficient mice.

Figure 2. MTD chemotherapy caused significant myelosuppression while DD chemotherapy preserved the major immune cells and decreased myeloid-derived suppressor cells (MDSC) (A) Female (C57BL/6 C3H/He) F1 mice (without tumor) were treated with different formats of chemotherapy. MTD chemotherapy-treated mice were severely depleted of CD8⁺, CD4⁺, NK, CD11b⁺, CD11c⁺ and F4/80 cells. Conversely, DD chemotherapy preserved the numbers of CD8⁺, CD4⁺ and CD11b⁺ cells. The suppression of NK, CD11C⁺ and F4/80 cells by DD chemotherapy is much less than that by MTD chemotherapy (**p<0.0001, **p<0.001 and **p<0.001, respectively). (B) Female (C57BL/6 C3H/He) F1 mice (with or without R HM-1 tumor) were treated with different formats of chemotherapy. In tumor-free mice, the different treatments did not significantly change the numbers of MDSCs. In R HM-1 tumor-bearing mice, DD chemotherapy drastically reduced the number of MDSCs in comparison to the control and MTD groups (**p<0.0001, control versus DD; **P<0.001, DD versus MTD). (C) DD and MTD regimen similarly reduced the number of T_{reg} cells in tumor-bearing mice (percentage of CD4⁺CD25⁺ cells, p=0.048, control versus DD).

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- Paque gradient. Representative flow cytometry data demonstrated a larger population of F4/80⁺ cells was elicited by DD chemotherapy (12690 in 20000 cells analyzed), whereas in mice receiving MTD chemotherapy, the population of F4/80⁺ cells became scant (1571 in 20000 cells) (**p<0.001, control versus DD, control versus MTD).

(B) Tumor growth curve showed diminishing anti-tumor effect of DD chemotherapy when macrophage was depleted by injection of clodronate liposome (macrophage inhibitor) into mice. R HM-1 cells ($1 \cdot 10^6$) 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 (paclitaxel 5 mg plus cisplatin 3 mg every 3 days for 7 doses). Control group mice were treated by PBS. Clodronate liposome and control liposome began on day 1 (1.5 mg/mice) and was 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=0.01, clodronate liposome versus liposome only).

Figure 4. DD therapeutic effect on drug-resistant tumor is immune-dependent and mediated by CD8⁺ effector cells

(A) The use of neutralizing antibodies for depletion of lymphocyte sub-populations revealed that CD8⁺ T cells are essential for tumor eradication. R HM-1 cells (1×10^6) were injected s.c. into female (C57BL/6, C3H/He) F1 mice (5 in each group, day 0). Neutralizing antibodies were given i.p. (100 μ g/mice/day initiated on D1, given every other day for 2 weeks, then 200 μ g/mice/day every week). On day 4, Mice began DD format chemotherapy. Control group mice were treated by PBS. Tumor growth curve demonstrated that anti-tumor effect was abolished when CD8⁺ cells were depleted by anti-CD8 antibody (** $p < 0.001$, anti-CD8 versus rat IgG), while the anti-CD4 and anti-NK antibodies did not affect tumor growth. (B) In the absence of CD8⁺ T lymphocytes, MTD demonstrates moderately more therapeutic effect than DD. R HM-1 cells (1×10^6) were injected s.c. into female (C57BL/6, C3H/He) F1 mice (5 in each group, day 0). Neutralizing antibodies were given i.p. (100 μ g/mice/day initiated on D1, given every other day for 2 weeks, then 200 μ g/mice/day every week). Lymphocyte depletion assay failed to identify a specific population of effectors cells that is associated with anti-tumor effects of MTD chemotherapy for R HM-1 tumor. (C) In tumor-bearing mice, DD chemotherapy elicited anti-tumor CD8⁺IFN- γ ⁺ T lymphocytic reaction. Mice bearing R HM-1 tumor were treated with different formats of chemotherapies. After treatment, spleens were obtained and the single-cell splenocyte suspension was prepared by tissue dissociation and enzyme digestion. Cells were then stained with anti-mouse CD8 and anti-mouse IFN- γ antibodies before getting analyzed by flow cytometry. Representative flow data showed that CD8⁺IFN- γ ⁺ cells were found in greater numbers in the mice receiving DD chemotherapy (** $p < 0.001$, DD versus control and MTD). (D) Representative flow data demonstrated more tumor-infiltrating CD8⁺IFN- γ ⁺ T lymphocytes were also induced in mice treated by DD (** $p < 0.001$, DD versus control and MTD).

Figure 5. DD chemotherapy elicited the greatest number of IFN- γ -secreting CD14⁺F4/80⁺ macrophage and subsequent CD8⁺IFN- γ ⁺ tumor-infiltrating lymphocytes (TILs) inside the peritoneal cavity of tumor-bearing mice

(A) In this i.p. tumor model, DD chemotherapy elicited and recruited largest number of CD14⁺F4/80⁺ macrophages inside the peritoneal cavity of R ID8 tumor-bearing mice (**p<0.001, control versus DD, *p<0.01, DD versus MTD). (B) The proportion of macrophage did not change in tumor-naïve mice regardless of chemotherapy. (C) Flow cytometry 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 showed 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 versus 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 ⁺ tumor-infiltrating lymphocytes (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 data showed that peritoneal CD8⁺IFN- γ ⁺ cells were found in greater numbers within the tumors of mice receiving DD chemotherapy (**p<0.001, control versus DD and DD versus 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 interleukin-2 (IL-2) by Q-PCR. 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 versus DD, DD versus MTD; IL-2, *p<0.01, control versus DD, #p=0.034, DD versus MTD).

Figure 6. Patients who responded to DD chemotherapy had higher serum levels of IFN- γ and IL-2

Fourteen patients with platinum-resistant recurrence of ovarian cancer received DD chemotherapy with weekly carboplatin (AUC 2) plus paclitaxel (80 mg/m²) as their third or fourth line of treatment. Their serum levels of IFN- γ and IL-2 were measured before treatment (PreDD), one month after (DD1), and two months after (DD2) the start of DD chemotherapy. (A) Of the four patients whose disease was controlled, three had higher serum levels of IFN- γ and IL-2 (red), whereas patients with no response had low or undetectable levels of IFN- γ or IL-2 (black) (DD2 for IFN- γ , ** p <0.001, responsive versus unresponsive; DD1 and DD2 for IL-2, ** p <0.001 responsive versus unresponsive).