Recombinant CD40 Ligand Therapy Has Significant Antitumor Effects on CD40-positive Ovarian Tumor Xenografts Grown in SCID Mice and Demonstrates an Augmented Effect with Cisplatin

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ABSTRACT

CD40 is a member of the tumor necrosis factor receptor family and was first identified with a monoclonal antibody raised against bladder carcinoma. Recombinant human CD40L has been shown previously to have a direct antitumor effect on an ovarian cancer cell line and ovarian carcinoma cells isolated from ascites fluid. We show here that rhuCD40L inhibits the growth of several ovarian adenocarcinomas derived from surgical specimens and grown as xenografts in severe combined immunodeficient mice. Two 14-day treatment cycles were more effective than one. This effect is apparently not mediated by natural killer cells, because blocking natural killer cell activity by antiaisdio GM-1 did not diminish this effect. In addition to suppression of tumor growth, treatment with rhuCD40L resulted in an increased expression of FasL, an increase in apoptosis, and histological changes including increased fibrosis and areas of tumor destruction. Using this model, we examined the efficacy of rhuCD40L in combination with chemotherapy agents. The antitumor effect of rhuCD40L in combination with 4 mg/kg cisplatin (CDDP) was increased over the effect of CDDP alone. Furthermore, rhuCD40L increased the efficacy of a suboptimal dose of CDDP (2mg/kg) such that it matched that of high-dose CDDP alone. These data suggest a role for rhuCD40L therapy in combination with platinum based regimens for primary treatment of epithelial ovarian tumors.

INTRODUCTION

Ovarian cancer is the fifth most common cancer affecting women in the United States. In 1999, there were 25,200 new cases in the United States with 14,500 deaths attributable to ovarian cancer (1). Unfortunately, 70–80% of these patients have advanced stage disease at the time of diagnosis, and optimal cytoreductive surgery followed by platinum-based chemotherapy is the gold standard of therapy. Patients with advanced disease have a response rate of 73–77% after first line therapy with paclitaxel and CDDP with a median progression-free interval of 16–18 months and a median survival of 35–38 months (2, 3). Unfortunately, most patients will recur, and an important prognostic predictor is whether the recurrence is <6 months (platinum resistant) or >6 months (platinum sensitive) from completion of chemotherapy. The patients with platinum-resistant tumors have response rates of <10% when retreated with platinum compounds, and prognosis in these patients remains dismal (4). Overall, the 5-year survival rate for International Federation of Gynecologists and Obstetricians stage III disease is 20–25% and for stage IV disease is 5% (5). These data illustrate the pressing need to develop novel therapeutic immuno-ological approaches concurrently with additional research to define newer effective chemotherapeutic agents.

The CD40 receptor and CD40 ligand belong to the TNF<sup>3</sup> receptor-ligand family (6). Most of these receptors are characterized by the presence of a death domain and interact with a specific ligand. The prototype of this group is the TNF, a naturally occurring cytokine, and there was considerable optimism that this would play a clinically relevant role in the therapy of cancer. However the toxicity of systemically administered TNF was prohibitive because of the interaction with the receptors on endothelial cells and macrophages causing vascular leakage and severe inflammatory responses (7). Similarly the therapeutic application of another member of this family, FasL, has been curtailed because the liver has a high level of Fas expression, and systemically administered FasL is hepatotoxic (8). However, the TNF family of receptor-ligand pairs contains several other members leading to ongoing efforts to identify modulators that will be capable of inducing apoptosis in tumor cells without the toxicity demonstrated by TNF and FasL. The two other molecules with sequence homology to the TNF that have been found to have antitumor properties are CD40L and TNF-related apoptosis-inducing ligand/Apo2L (9–11).

The role of CD40/CD40L interaction in the control of immune cell interaction and function has been intensely studied, especially in context of its expression on B cells (12, 13). The CD40 receptor was first identified with a monoclonal antibody raised against human bladder carcinoma in 1984 (14), yet its role in oncology was not explored until recently. The CD40 receptor is a type I transmembrane protein with a molecular weight of M, 50,000. It belongs to the TNF superfamily of receptors and is expressed on a variety of normal cells including B lymphocytes, macrophages, fibroblasts, dendritic cells, and endothelial cells (15–17). It exists in both membrane-bound and soluble forms. Malignant transformed cells like lymphomas and leukemias, and lung, breast, prostate, ovarian, colon, and renal carcinomas have also been known to express the CD40 receptor (15–17). It has been reported that although CD40 does not contain the full sequence of the originally defined death domain, the cytoplasmic domain does contain a 52aa sequence with a 26% homology to TNFR and 39% homology to Fas (18). These sequences in TNF<sub>R</sub> and Fas, the most closely related members of this family, share a 45% homology.

The CD40L is a type II transmembrane protein expressed by activated CD4 T lymphocytes on activation and interacts with CD40 receptor on B cells to rescue them from apoptosis (12, 13). There is formation of the germinal center, proliferation of B cells, and isotype switching from IgM to IgG and IgE. Deficiency of CD40L has also been known to express the CD40 receptor (15–17). It has been reported that although CD40 does not contain the full sequence of the originally defined death domain, the cytoplasmic domain does contain a 52aa sequence with a 26% homology to TNFR and 39% homology to Fas (18). These sequences in TNF<sub>R</sub> and Fas, the most closely related members of this family, share a 45% homology.

Recombinant CD40L has been shown to have antitumor properties in a number of preclinical models (19–21). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; SCID, severe combined immunodeficiency; NK, natural killer; IHC, immunohistochemistry; CDDP, cisplatin; IL, interleukin.

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have been shown to inhibit tumor growth (9, 19). Similarly CD40L transfected fibroblasts have been shown to induce apoptosis in CD40+ carcinomas in vitro (10).

This differential effect of CD40 ligand on normal and malignant cells suggests that the CD40 receptor expressed on transformed cells may be a novel therapeutic target for significant antitumor effect. The effectiveness of CD40L against an ovarian carcinoma cell line as well as freshly obtained ovarian carcinoma cells isolated from ascites fluid has been tested, and CD40L treatment was found to inhibit tumor cell growth in both cases (17, 20). These authors also found that CD40L has the ability to sensitize these cells to the effects of other apoptosis inducing agents; in particular, they observed that CD40L treatment resulted in enhanced apoptosis in response to CDDP. Because these experiments were carried out in vitro, the possibility of the response being mediated indirectly through activation of immune cells is eliminated; thus, these experiments suggest that the combination of CD40L and CDDP has a direct effect on ovarian carcinoma cells. It has been reported that CD40 ligation can also resensitize CDDP-resistant ovarian carcinoma cell lines (17).

The sensitivity of established ovarian cell lines and ascites single cells is not necessarily predictive of a similar sensitivity of an established solid tumor. We have developed a SCID mouse/ovarian tumor xenograft model that allows for engraftment and passage of surgical specimens (21). These tumors resemble the original lesion more closely in histology, tumor markers, and growth patterns than cell lines that have been in culture for years and have the advantage of exhibiting clinically relevant tumor heterogeneity. Because major elements of an immune response are absent, we have used the model to study the effect of soluble rhuCD40 ligand therapy on CD40+ human ovarian tumors in SCID mice to evaluate direct antitumor activity.

The goal of the current study is to assess the sensitivity of patient-derived human ovarian tumors to rhuCD40L and to determine whether CD40L is able to potentiate the efficacy of chemotherapeutic agents.

MATERIALS AND METHODS

Patient Tumor-SCID Mouse Model. The SCID mouse-patient tumor xenograft model used in these experiments has been described previously (21). Briefly, surgical specimens were received through the Tissue Procurement Facility of Roswell Park Cancer Institute. Specimens were cut into 2 mm × 2 mm pieces in tissue culture medium (DMEM F-12). SCID mice were then anesthetized by 0.4 ml of avertin injected i.p. and, under sterile conditions, these tumor pieces were implanted s.c. in the abdominal wall. Tumors that successfully engrafted were subsequently serially passed into several mice thereby providing sufficient tumor material for experiments with large numbers of mice. For these experiments, a total of six different patient papillary serous adenocarcinomas were used. The mice used in all of the experiments were 7–8-week-old female CB17 SCID mice with an average weight of 20–22 g. They were kept in sterile cages, four to five mice to a cage, and fed with autoclaved chow and water. They were maintained in air-conditioned and light-controlled rooms (12 h cycles). All of the procedures, injections, and tumor measurements were carried out under a laminar flow hood using aseptic precautions.

Experimental Design. The most common histological subtype of ovarian cancer is papillary serous adenocarcinoma and, therefore, this type was chosen from the bank of engrafted tumors for these experiments. Ovarian tumors that successfully engrafted into SCID mice were passaged into experimental mice. In different experiments, tumors were either implanted s.c. or i.p. in the gonadal fat pad (to more closely mimic the natural site of the disease). After the tumors were well established, 4 weeks, SCID mice were divided into groups of similar tumor sizes. When indicated, blockade of NK cell function was accomplished by pretreatment with 20 μl of antiasialo GM-1 24 h before initiation of treatment with rhuCD40L and followed by subsequent treatment every 4 days for the duration of the experiment.

A cycle of treatment with rhuCD40 ligand (Immunex Laboratories, Seattle, WA) consisted of daily i.p. injection for 14 days. In experiments, mice received one of the following: (a) 30 μg of rhuCD40L/dose; (b) 75 μg/dose; or (c) 100 μg/dose. The control used was 100 μl of a Tween tetramethylsilane buffer (25 mM Tris 4% manitol 10% sucrose) used for reconstitution of the rhuCD40 ligand. Tumor growth was assessed daily by measuring the tumors with vernier calipers; the mean of three readings each of the longest and shortest tumor diameters were taken after wetting the fur with sterile PBS. Tumor volume was calculated with the formula V = LD × (SD/2), where V is the tumor volume, LD is the longest tumor diameter, and SD is the shortest tumor diameter. Relative tumor volume growth was calculated for each tumor by dividing the final tumor volume with the initial tumor volume. The Mann-Whitney two-sided test and the Student t test were used to compare the relative tumor volume growths when indicated (Instat Graphic Package, San Diego, CA). In experiments with multiple treatment arms, ANOVA and factorial ANOVA using data that had been transformed using logs were applied where indicated.

At various time points during and at the termination of an experiment, mice were bled and then sacrificed by cervical dislocation. Each of these mice underwent an autopsy, and the tumor and the spleen were weighed and fixed in formalin for pathology. Sections of all of the tumor and spleen samples were processed for light microscopy by standard methods, and sections were stained with H&E.

IHC. Sections (5 μm) of formalin-fixed, paraffin-embedded specimens were mounted on electrostatically charged slides (Superfrost; Fisher), warmed at 60°C for 1 h on a slide warming tray, and stored at 4°C until use. For IHC, slides were deparaffinized in xylene and rehydrated. High-temperature antigen retrieval was carried out by microwaving slides in Antigen Unmasking Solution (Vector Laboratories Burlingame, CA). The solution was brought to a boil, boiled for 10 min, and then allowed a 20 min cool-down period. Slides were transferred to PBS and then used for IHC. Anti-CD40 (a rabbit polyclonal antibody raised against a peptide corresponding to aa258–277; Santa Cruz Biotechnology Inc.) was used at 1 μg/ml; controls included antibodies absorbed with the blocking peptide and/or rabbit IgG. Anti-FasL (mouse monoclonal raised to residues 116–277 of human FasL; Transduction Laboratories) was used at 2.5 μg/ml The isotype control used was anti-CD45 (Zymed Laboratories). After blocking for endogenous peroxidase, sections were blocked with 10% either goat or horse serum. Sections were then labeled with the ABC Vectastain Elite kit (Vector Laboratories).

3,3′-Diaminobenzidine was used as the chromagen.

TUNEL Assay. Apoptosis was evaluated by TUNEL staining (ApopTag; Intergen Corp.) according to the manufacturer’s instructions. ApopTag staining was carried out on either formalin-fixed, paraffin-embedded material or sections of frozen specimens embedded in OCT compound. Labeling was visualized by either fluorescein or 3,3′-diaminobenzidine.

Chemotherapy. For experiments requiring ovarian tumors that are sensitive to paclitaxel and CDDP, in vitro sensitivity testing was performed by Oncotech Corporation, Tustin, CA. Paclitaxel (T) was used at 20 mg/kg and CDDP at 4 mg/kg; both were obtained from Bristol Myers Squibb, Princeton, NJ.

RESULTS

rhuCD40L Inhibits the Growth of Human CD40+ Ovarian Xenografts in SCID Mice. When ovarian tumors were implanted in a s.c. site, one cycle of rhuCD40L treatment resulted in a significant inhibition of tumor growth compared with the control group of mice (two-tailed P = 0.0155; Fig. 1A). This response is not dose-dependent, because there was no difference in the antitumor effect between dosages of 30 μg/ml versus the 100 μg/ml. The potential involvement of murine NK cells in the antitumor effect was assessed by blocking NK cell function with antiasialo GM-1. No significant difference in tumor growth was seen in these mice compared with those that did not receive antiasialo GM-1, indicating that NK cells are not involved in the rhuCD40L effect (Figs. 1B-1, 1B-2). Tumor growth was monitored for 3 weeks after cessation of therapy with a single cycle of CD40L,
and these tumors showed a resurgence of growth in mice in all of the three arms with no significant differences in their growth patterns.

To determine whether this effect was limited to tumors implanted in a s.c. site, the experiment was repeated with a second CD40/H11001 ovarian tumor implanted intra-abdominally in the gonadal fat pad to mimic the natural history of the disease. rhuCD40L (an intermediate dose of 75 μg/ml) was administered i.p. for two 14-day cycles separated by a 2-week rest and recovery interval. In this case, a more pronounced tumor inhibition was seen as assessed by both tumor volume and weight (two-tailed $P = 0.0095$; Fig. 1C).

Treated tumors were evaluated for maintenance of CD40 expression by IHC. The tumor sections showed that the CD40 expression was maintained after CD40L treatment (Fig. 2, A and B). Fas expression was not appreciably different in the treatment and the control groups, but the treated mice had up-regulation of FasL expression on the tumors when compared with the control group (Fig. 2, C and D). After cessation of therapy (3 weeks), the growing tumors of mice in both the treatment and the control group did not demonstrate a similar overexpression of FasL. This suggests a role of Fas/FasL interaction in the observed tumor inhibition.

Histology of the treated tumors demonstrates that rhuCD40L treatment causes a disruption of tissue architecture and increased fibrosis. This fibrosis was more pronounced at the periphery of the lesion than at the center (Fig. 2, E–H). Tumors were evaluated for apoptosis by
both light microscopy of H&E sections and by ApopTag staining. After one cycle of rhuCD40L treatment, an increase in the number of apoptotic cells was observed in the treated compared with the tumors in the control arm. However this increase was not consistently seen after treatment with two cycles of CD40L. It may be that most of the apoptotic changes occur during the initial phases of CD40L treatment and that after two cycles of treatment, most of the apoptotic cells have been replaced by the increased fibrotic tissue. No significant differences in spleen weights or histology were noted between the control and the two treatment groups.

To additionally investigate the induction of apoptosis by CD40L, we designed another experiment in which a CD40+ ovarian tumor (9928–3p) was implanted s.c. in CB17/SCID mice as described previously. CD40L-treated mice received daily i.p. injections of 75 µg of CD40L, and the control mice were treated with sterile Tween tetramethylsilane buffer used for suspending CD40L. Mice from each group (two controls and two CD40L-treated mice) were sacrificed at 8 h, 24 h, 48 h, 5 days, 10 days, and 14 days during treatment and at 1 week after cessation of treatment. The frozen tumors were then stained with ApopTag (TUNEL assay) to assess apoptosis at these different time intervals. Three different representative sections for each tumor slide were selected, and an apoptosis count was performed and expressed as a percentage of cells in a low power confocal field. An increase in apoptosis was observed in the CD40L-treated mice at 8 h; similar differences were not observed at other time points.

We decided to additionally explore the observation that a stronger augmentation of disease control was achieved after two cycles of rhuCD40L compared with a single cycle. A different ovarian papillary serous adenocarcinoma xenograft (9927–2p) was used, and the tumor growth patterns after one versus two cycles of rhuCD40L treatment were evaluated. The median relative growth of tumors in the two weeks after the second cycle of treatment was significantly less than that seen in the 2-week interval after the first cycle of rhuCD40L (P = 0.027; Fig. 3). Subsequent follow up showed that the tumor inhibition achieved after two cycles appeared to be more lasting than that achieved after a single cycle.

**CD40L Therapy in Combination with CDDP Demonstrates an Augmented Effect.** Having thereby established the efficacy of soluble CD40L in the therapy of ovarian xenografts in SCID mice, we decided to test the hypothesis that addition of CD40L to the front line chemotherapy drugs for the treatment of epithelial ovarian cancer, namely CDDP and paclitaxel, can improve response rates and provide longer disease-free survival.

Two different papillary serous adenocarcinomas (the most common epithelial ovarian tumor) were selected from our human ovarian-SCID mice xenograft library. These patient-derived tumors were platinum- and paclitaxel-sensitive as judged by the clinical response of these patients to platinum/paclitaxel combination therapy after primary optimal cytoreductive surgery (residual disease was <5 mm in diameter). Additional confirmation of drug sensitivity was provided by in vitro sensitivity testing that demonstrated a lack of extreme drug resistance to both paclitaxel and CDDP.

Each of these two CD40+ tumors (10332–2p and 10250–2p) was passaged in SCID mice; 2-mm pieces from donor tumors were implanted s.c. in the anterior abdominal wall. Four weeks later, after established tumor growth for each of the two tumors, the mice were placed into eight treatment groups: (a) control; (b) rhuCD40L at 100 µg; (c) CDDP at 4 mg/kg; (d) paclitaxel (T) at 20 mg/kg; (e) CDDP + 30 µg CD40L; (f) CDDP + 100 µg CD40L; (g) T + 30 µg CD40L; and (h) T + 100 µg CD40L. Each cycle consisted of daily i.p. injection of rhuCD40L for 14 days followed by a 7-day washout period. Chemotherapeutic agents were administered i.p. on day 1 and day 8 of each cycle. All of the mice in both the tumor groups were treated with their respective drug/placebo for two cycles. Tumor volumes for each of the mice were calculated every 48 h by tumor measurements. After completion of the two cycles of therapy, response was evaluated by calculating the relative tumor volume as described before.

After the end of the second cycle, survival studies were initiated, and the experiment was terminated 45 days later (80 days − experiment time). The mice were sacrificed by cervical dislocation. Any mouse that had a large tumor burden with a tumor diameter of 20 mm was sacrificed earlier than 45 days to avoid unnecessary suffering.

Tumor 10332–2p (group A) was a “fast-growing tumor,” whereas tumor 10250–2p (group B) had a relatively slower growth curve, thereby providing us with growth pattern heterogeneity. The relative tumor volumes at the end of the two cycles of therapy are summarized in Table 1.

For the mice in the rapidly growing group, all three of the agents caused inhibition of tumor growth, with CDDP being the most effective drug. However, the combination of rhuCD40L at either dose of 30 or 100 µg with CDDP showed an augmented effect in that the therapeutic response achieved was 3 times the expected additive effect of these drugs without any increase in toxicity (Fig. 4A and B). In the CDDP arm alone, 43% of the tumors showed regression with a 57% progression rate. In the combination arm of CDDP and CD40L at either dose, 73% of established tumors evidenced regression, 14.7% were stable, and there was only a 13.3% progression rate.

For the mice with the slow growing tumor, again a significant cytotoxic additive effect was demonstrated in the CDDP combination groups with CD40L. This effect was observed in both the low- and high-dose groups (Fig. 4, C and D). In the CDDP alone arm, 60% of the tumors showed growth inhibition, and there was a 40% progres-
The addition of CD40L to CDDP greatly improved the effect, because all of the tumors (100%) showed growth inhibition with a 60% regression rate.

Furthermore, survival studies reflected the above response rates and demonstrated that these effects were lasting (Table 2). Overall, 23/26 mice (88.46%) of all of the mice treated with CDDP and CD40L were alive with tumor diameters of $\leq 2$ cm 45 days after the cessation of therapy as compared with 10/14 mice (71.4%) treated with CDDP alone, 6/13 (46.2%) treated with CD40L alone, and 3/14 mice (21.4%) treated with placebo.

Paclitaxel alone had marginal activity as compared with the control arms, but there was no significant additive or synergistic effect with CD40L in these tumors.

**rhuCD40L Increases the Efficacy of a Suboptimal Dose of CDDP.** To answer the question of whether the antitumor effect of rhuCD40L could augment suboptimal dosages of CDDP, mice bearing a CDDP sensitive tumor (10332–3p) were treated with either 4 mg/kg (the dosage used previously) or 2 mg/kg of CDDP either alone or in combination with rhuCD40L. Treatment consisted of chemotherapy once a week with or without daily administration of CDDP or placebo, all of which were injected i.p. The treatment regimen consisted of two 2-week cycles of therapy with a 10-day rest interval. Growth of the tumors was assessed during and after cessation of treatment.

Treatment with rhuCD40L alone resulted in a noticeable tumor inhibition as discussed above. Treatment with the optimal dosage of CDDP (4 mg/kg) alone resulted in a significant level of tumor inhibition. However, tumors in animals treated with half of that dosage (2 mg/kg) showed a lesser degree of tumor inhibition approximately equivalent to that seen with rhuCD40L alone. Interestingly, the combination of rhuCD40L and the suboptimal dose of CDDP (2 mg/kg) dramatically improved the efficacy of these treatments, resulting in a significant degree of tumor inhibition that matched that of high dose CDDP alone (Fig. 5). There was no significant difference between these two treatments ($P = 0.61$; ANOVA). These mice were monitored for several weeks after cessation of treatment to ascertain whether this increased tumor inhibition resulted in any long-term benefit. Tumors were measured weekly, and mice were sacrificed when the tumor reached $1500 \text{mm}^3$ ($\sim 1.5$ cm diameter) The addition of rhuCD40L to the CDDP treatment improved the long-term control of tumor growth in these mice. By week 12 (7 weeks after treatment) all of the control mice had reached the terminal stage of tumor growth. By week 14 (9 weeks after treatment), 66.6% of mice in the combin-

![Fig. 4. The effect of CD40L as a single agent and in combination with either Taxol or CDDP chemotherapeutic agents. In two different tumors, a relatively “fast-growing” (A) and a more “slow-growing” tumor (C), CD40L alone was seen to exert an antitumor effect. Although tumor growth was suppressed by both chemotherapy agents, the most effective treatment was CDDP in combination with CD40L ($P = 0.0007$). The degree of tumor inhibition relative to the controls (e.g., median tumor volume of the control group/median tumor volume of the treated groups) is shown in B and D.](image-url)

**Table 2. Survival of SCID mice with human ovarian tumor xenografts 45 days after therapy**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Group A (10332-2p)</th>
<th>Group B (10250-2p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1/8 (12.5%)</td>
<td>2/8 (33%)</td>
</tr>
<tr>
<td>CD40 L</td>
<td>3/8 (37.5%)</td>
<td>3/5 (60%)</td>
</tr>
<tr>
<td>Paclitaxel (T)</td>
<td>1/8 (12.5%)</td>
<td>2/5 (40%)</td>
</tr>
<tr>
<td>Paclitaxel + CD40L 30 µg (T30)</td>
<td>1/7 (14.3%)</td>
<td>1/5 (20%)</td>
</tr>
<tr>
<td>Paclitaxel + CD40L 100 µg (T100)</td>
<td>1/7 (14.3%)</td>
<td>3/5 (60%)</td>
</tr>
<tr>
<td>Cisplatin (P)</td>
<td>6/9 (69%)</td>
<td>4/5 (80%)</td>
</tr>
<tr>
<td>Cisplatin + CD40L 30 µg (P30)</td>
<td>6/8 (75%)</td>
<td>5/5 (100%)</td>
</tr>
<tr>
<td>Cisplatin + CD40L 100 µg (P100)</td>
<td>7/8 (87.5%)</td>
<td>5/5 (100%)</td>
</tr>
</tbody>
</table>
nation arms were alive as compared with 40% and 25% treated with CD40L and low-dose CDDP, respectively.

These preliminary data suggest a possible role for rhuCD40L therapy in combination with platinum-based regimens for primary treatment of epithelial ovarian tumors.

DISCUSSION

Contrary to the original belief that CD40-CD40L function was limited to regulation of humoral immunity, there has been an explosion of data suggesting a much wider role in many other immune functions. T cells can use membrane or soluble CD40L to interact with receptors on adenomatous polyposis colii such as dendritic cells, macrophages, and even stem cells to either induce or up-regulate various cytokines/chemokines such as IL-6, IL-8, IL-12, or TNF-α. Up-regulation of other costimulatory factors like Fas, FasL, and CD54 (ICAM-1) has been reported (16, 17). CD40 ligation also can lead to a rapid cytotoxic T-cell response with expansion of CD8+ T cells independent of the T helper cells (22).

Our efforts have been focused on investigating the direct antitumor effects of soluble rhuCD40L on patient-derived human ovarian tumor xenografts in an immunodeficient system. The rhuCD40L is a soluble recombinant ligand specific for human CD40 receptor with negligible cross-reactivity with murine CD40 receptors (i.e., it is 100 times more reactive with human than mouse CD40). It is homotrimERIC and used in an isoleucine zipper form to increase stability (19).

The efficacy of rhuCD40L against breast carcinoma cell lines has recently been demonstrated both in vitro where it was determined that the observed inhibition of proliferation was attributable to an induction of apoptosis and in vivo in the SCID mouse where treatment with CD40L significantly increased survival (23). CD40 ligation has also been reported to be proapoptotic and to cause an induction of functional Fas ligand, TNF-related apoptosis-inducing ligand, and TNF in apoptosis-susceptible carcinoma cells. This activity depends on the membrane proximal domain but not on the TNFR-associated factor interfacing PXQXT motif in the CD40 cytoplasmic tail and can be blocked by caspase inhibitors (24). Overexpression of FasL and subsequent apoptosis in cultured human hepatocytes has been shown to be induced by CD40 ligation during allograft rejection (25). On the other hand, CD40 ligation has also been shown to inhibit apoptosis in response to fludarabine in B chronic lymphocytic leukemia cells via the NF-κB/BRel transcription factors, and this antiapoptotic response was abolished by a phosphorothioate κB oligodeoxynucleotide (26).

Our experiments have also demonstrated up-regulation of FasL. In this study, we have seen increased apoptosis in the CD40L-treated group, particularly at 8 h after treatment. Although subsequent time points did not reveal an obvious continued increase in apoptosis in treated tumors, it was evident from H&E analysis of tumors after treatment that treated tumors were smaller and consisted of relatively more connective tissue and decreased tumor epithelium. It is quite possible that this increase in fibrotic tissue obscured increases in the numbers of apoptotic cells that might still be present in treated tumors for longer periods of time.

All of our experiments with rhuCD40L have consistently demonstrated a significant antitumor effect in SCID mice with established solid ovarian human xenografts. It appears that this effect is more a function of the duration of therapy (two cycles is more effective than one) than the dose of CD40L. This activity does not appear to be NK cell mediated and is consistent with the observations of others (27). CD40L exerts a direct effect on the tumor as is apparent by the histological disruption of tumor architecture and increased fibrosis in the CD40L-treated groups. Tumor necrosis and direct inhibition appear to play a major role. It is notable that all of the different ovarian tumors tested in the first phase of these experiments were actually resistant to CDDP, and these patients fared poorly and succumbed to their disease. Therefore, it is remarkable that rhuCD40L exerted a significant antigrowth effect on these tumors.

The introduction of paclitaxel in the armamentarium of drugs to treat ovarian cancer has been the most significant advance in this field over the last 2 decades. This success in clinical practice has been duplicated in nude mice and SCID mice ovarian cancer models in the past (28, 29). Yet to our surprise CD40L alone appeared to be more effective in inhibiting tumor growth than paclitaxel in two different paclitaxel-sensitive tumors (groups A and B). Furthermore, this response was lasting as demonstrated by the improved survival rates of mice in the CD40L group; 46.2% (6/13) of mice treated with CD40L were alive at 45 days after therapy compared with 23.1% (3/13) for the paclitaxel group and 26% (6/23) for the paclitaxel + CD40L combination group. It is possible that with higher doses and a different schedule, a better response may be seen with paclitaxel.

The paradox of a better response with CD40L alone as compared with combination therapy with paclitaxel and CD40L is intriguing. Paclitaxel is a mitotic spindle poison and acts by promoting assembly and stabilization of microtubules, preventing depolymerization. This inability to depolymerize microtubules prevents cellular replication (30). Consequently paclitaxel is most effective in the M phase of the cell cycle. CD40 ligation has been recently reported to result in a G2-M phase arrest. So it is quite conceivable that the administration of CD40L prevents all of the cells from entering the M phase and actually diminishes the efficacy of the CD40L-paclitaxel combination.

CDDP has been the cornerstone of all of the chemotherapeutic regimens for ovarian cancer and is not cell cycle specific. The anti-tumor effects are exerted by the ability of the drug to bind with DNA to produce intrastructural cross-linking and DNA adduct formation thereby affecting cell replication (31). Although the mechanism by which CDDP enhances the cytotoxicity of CDDP is not clear, there is evidence that CD40 ligation potentiates the apoptosis induced by CDDP (20). CD40 ligation has also been reported to partially resensitize CDDP-resistant ovarian cancer cell lines (17). Another hypothesis that is currently being explored by us is to determine whether CD40 ligation may increase the formation of CDDP-DNA adducts by measuring the levels of these adducts in the tumors of
mice treated with CDDP with and without CD40L. A G2-M cell cycle arrest by CD40 ligation could potentially allow higher levels of CDDP-DNA adduct formation.

It should be noted that all of the data collected here was derived from using CD40+ tumors, although it would be important to compare CD40+ and CD40− tumors in the future to better predict patient response to CD40L treatment. However, in our studies, we have not yet observed an ovarian tumor from an engrafted patient to be CD40−, and so this study could not yet be done.

This preliminary data showing the enhancement of both response rates and better overall survival rate without any severe additional toxicity for the combination arm of CDDP and CD40L for epithelial ovarian tumors has important therapeutic significance. Incorporation of soluble rhuCD40L in CDDP regimens has the potential to perhaps reduce the relapse rates and enhance the cytotoxicity for epithelial ovarian cancer. Additionally, considering the results demonstrating the effectiveness of CD40L and a suboptimal dose of CDDP, the addition of CD40L to CDDP regimens may also result in reduced exposure to CDDP and reduced development of drug resistance.

In conclusion, soluble rhuCD40L has a significant antitumor activity against ovarian tumor xenografts in SCID mice. Combination with CDDP appears to hold the promise of enhancing the clinical response and progression-free interval without significant toxicity.

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Recombinant CD40 Ligand Therapy Has Significant Antitumor Effects on CD40-positive Ovarian Tumor Xenografts Grown in SCID Mice and Demonstrates an Augmented Effect with Cisplatin

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