Assessing the Effects of Concurrent versus Sequential Cisplatin/Radiotherapy on Immune Status in Lung Tumor-Bearing C57BL/6 Mice

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Abstract

Concurrent and sequential cisplatin-based chemoradiotherapy regimens are standard therapeutic approaches in cancer treatment. Recent clinical data suggest that these different dosing schedules may adversely affect antigen-specific immunotherapy. The goal of the present preclinical study was to explore the effects of concurrent and sequential cisplatin/radiotherapy on immune status in a lung cancer mouse model. A total of 150 C57BL/6 mice were randomized into six treatment groups: control; 8 Gy thoracic radiotherapy (dose schedules 1 and 2); cisplatin 2.5 mg/kg i.p.; cisplatin + radiotherapy (concurrent); and cisplatin + radiotherapy (sequential; n = 25, all groups). At the end of the study (week 41), serum cytokines were assessed by multiplex immunoassay, surface markers of spleen-derived lymphocytes were assessed by immunostaining and flow cytometry, lung tumor expression of programmed death ligands 1 and 2 (PD-L1/2) was evaluated by immunohistochemistry, and miRNA profiling was performed in serum and lymphocytes by quantitative real-time PCR. Lung whole mounts were prepared to assess treatment effects on lung tumor foci formation. The results showed that sequential chemoradiotherapy (two cycles of cisplatin followed by 8 Gy radiotherapy) had equivalent antitumor activity as concurrent therapy. However, sequential cisplatin/radiotherapy resulted in significant differences in several immune response biomarkers, including regulatory T cells, miR-29c, expression of costimulatory molecule CD28, and serum IFNy. PD-L1 and PD-L2 were strongly expressed in tumor foci, but no trend was seen between groups. These results suggest that monitoring immune status may be necessary when designing treatment regimens combining immunotherapy with chemoradiotherapy. Cancer Immunol Res; 3(7); 741–50. ©2015 AACR.

Introduction

The survival benefits of platinum-based chemotherapy combined with thoracic radiotherapy (RTX) for inoperable stage III non–small cell lung cancer (NSCLC) compared with RTX alone were established in the 1980s and 1990s (1–7). These earlier studies consisted of both sequential (1, 2) and concurrent (3–6) chemoradiotherapy (CRT) regimens, including daily (5, 6) and twice-daily hyperfractionated RTX (3, 4), and sequential delivery of chemotherapy and thoracic RTX treatment groups. An approach in patients with inoperable stage III NSCLC (8). Although concurrent CRT regimens increased the frequency of severe hematologic and nonhematologic toxicities, the survival benefits were considered significant enough to conduct a phase III trial to determine which CRT regimen, concurrent or sequential, was more beneficial (8). This phase III study compared two different concurrent CRT treatment strategies, one with daily and one with twice-daily RTX, with an established sequential CRT regimen (1, 2). The results showed that both concurrent CRT regimens increased median survival compared with that of sequential therapy and that 5-year survival was significantly increased from 10% in patients who received sequential CRT to 16% in patients treated with concurrent chemotherapy and once-daily RTX. Higher rates of acute toxicities were observed with the concurrent CRT regimens (8). Similar results were seen in another phase III trial comparing sequential and concurrent CRT in inoperable stage III NSCLC (9).

It is thought that the radiosensitizing effects of chemotherapy (10) enhance the effectiveness of RTX in concurrent CRT regimens, and it has recently been shown that cisplatin may enhance radiation-induced apoptosis as well as autophagy (11). The enhancement of RTX effects by concurrent chemotherapy, or the dose intensity effect, is reduced when CRT is administered sequentially, which may explain why patients treated with concurrent CRT receive a slight, yet significant, survival advantage compared with those treated with sequential CRT. Administering chemotherapy and RTX concurrently may also result in immunogenic cell death (12–14), which has implications for the use of immunotherapy in combination with CRT. Ionizing radiation (15, 16) and some types of chemotherapy, such as anthracyclines and oxaliplatin (13), are known to induce immunogenic cell death, whereas other therapies may result in tolerogenic cell death (17). Although cisplatin by itself is not known to produce immunogenic cell death (18), it is commonly combined with RTX,
which is a powerful inducer of calreticulin exposure (16), leading to the induction of immunogenic cell death (18). The exposure of calreticulin on the cell surface has been shown to serve as a stimulus for the engulfment of the dying tumor cell by dendritic cells (19).

The antigen-specific cancer immunotherapy, tecemotide, is designed to elicit a cellular immune response against the cell surface glycoprotein mucin 1 (MUC1) that is overexpressed and abnormally glycosylated in NSCLC and several other epithelial cell cancers. Tecemotide was recently evaluated as maintenance therapy in combination with CRT in a phase III clinical trial in unresectable stage III NSCLC (20). Unexpectedly, overall survival, which was the primary endpoint, was not significantly different in the treatment group; however, a survival benefit was observed in the subgroup of patients who received concurrent CRT followed by tecemotide, whereas no such benefit was seen in the patients who received sequential CRT followed by tecemotide (20). The underlying reason(s) for the difference in survival outcome remains unknown, but dose intensity and the induction of immunogenic cell death by concurrent CRT may have played a role. In addition, the status of the immune system at the start of tecemotide treatment could have been a factor (21). The goal of the present preclinical study was to explore the effects of concurrent and sequentially administered cisplatin/radiotherapy on immune status in a lung cancer mouse model.

Materials and Methods

Chemicals

Urethane, cisplatin [cis-diammineplatinum (II) dichloride], tertiary amyl alcohol, and 2,2,2-tribromoethanol were purchased from Sigma-Aldrich.

Mouse lung tumor model

In the transgenic mouse model utilized in the present study, the animals are heterozygous for human MUC1, which is expressed as a self-protein under the control of its own promoter, resulting in a tissue expression pattern consistent with that observed in humans (22, 23). In our experience, expression of the transgene does not appear to have a significant impact on the normal lifespan, as we have kept untreated mice up to 18 months. A total of 150 mixed-sex human MUC1 transgenic (hMUC1.Tg) C57BL/6 mice were supplied by our breeding colony maintained by the UC Davis Mouse Biology Program. The hMUC1.Tg founder mice were purchased from Mayo Clinic. All animal studies were conducted under a protocol approved by the UC Davis Institutional Animal Care and Use Committee. The University of California, Davis is an Association for Assessment and Accreditation of Laboratory Animal Care–accredited institution. For all mouse studies, the week number refers to the age of the mice. To induce the formation of lung bronchioalveolar carcinomas/adenomas, mice received 10 weekly doses of urethane (0.75 mg/g) by i.p. injection (100 μL) using a 25-gauge needle (24, 25) starting at approximately 6 weeks of age. Seven weeks after the last dose of urethane, mice were randomized into six treatment groups (n = 25, all groups) of approximately equal average weight; control; radiotherapy alone in weeks 23 and 27 (RTX 1); radiotherapy alone in weeks 31 and 35 (RTX 2); cisplatin alone; cisplatin + RTX 1 (concurrent cisplatin/radiotherapy); and cisplatin + RTX 2 (sequential cisplatin/radiotherapy). Weekly weight monitoring was performed throughout the study beginning in week 5.

Previously published studies (24, 25) have shown that by 41 weeks, approximately 25 to 27 weeks following the last dose of urethane, multiple lung tumor foci are present in all lobes of the lungs, allowing treatment effects to be assessed. Although previous lung cancer treatment studies in this model were typically ended at 41 weeks, including the present study, tumor-bearing mice can survive up to 56 weeks (DeGregorio MW, unpublished data). With respect to the effects of antigen-specific immunotherapy on the development of lung tumors, we previously showed that two cycles of eight weekly 10-μg doses of tecemotide, a MUC1-specific cancer immunotherapy, are needed to derive an antitumor benefit. Following one cycle of treatment, no difference in the number of lung tumor foci was seen (25).

Cisplatin chemotherapy

Two cycles of cisplatin chemotherapy, each of which consisted of two 2.5-mg/kg doses of cisplatin given by i.p. injection (100 μL) 48 hours apart, were administered to all mice in the cisplatin treatment groups 4 weeks apart in weeks 22 and 26, respectively. Cisplatin was freshly prepared in sterile 0.9% saline before each dose and used within one hour.

Radiation treatment

The radiation treatment protocol was developed by a board-certified medical physicist and a board-certified radiation oncologist to cover the entire thoracic cavity with at least 90% of the prescribed dose. Before treatment, the anteroposterior:posteroanterior diameter of the mice was determined using calipers. All mice assigned to RTX were anesthetized immediately before radiation treatment by i.p. injection with 600 μL of a 1.2% solution of Avertin (tribromoethanol) using a 25-gauge needle. The Avertin solution was prepared by dissolving 2,2,2-tribromoethanol in tertiary amyl alcohol and then sterilized by filtration through a 0.22-μm syringe filter (EMD Millipore). Proper anesthesia was ensured by evaluating the righting and tail pinch reflexes. Anesthetized mice were placed in the prone position on 5-cm solid water at laser isocenter, and the treatment area was covered with 0.5-cm bolus to ensure adequate dose build-up. An 8-Gy dose of local thoracic RTX was delivered using an Elekta linear accelerator (Elekta AB) delivering 9-MeV electrons and incorporating a 3-cm × 3-cm cerrobend electron cutout field to ensure that the entire thoracic cavity was encompassed in the radiation field but sparing as much of the cranial vault and abdominal cavity as possible. All mice were monitored until they had fully recovered from anesthesia following the radiation treatments.

Terminal procedures

At the conclusion of the study in week 41, all mice were euthanized by carbon dioxide asphyxiation. Whole blood was collected by cardiac puncture, allowed to clot for 30 minutes at room temperature, and then centrifuged at 10,000 × g for 10 minutes at 4°C to isolate serum, which was then flash frozen in liquid nitrogen and stored at −80°C until analyzed as described below. Following blood collection, spleens were removed and processed as described below to isolate lymphocytes. To assess the effects of the various treatments on the formation of lung tumor foci, lungs were perfused with 10% neutral-buffered formalin, separated into individual lobes, placed in tissue cassettes, and fixed in formalin for 48 hours followed by 70% ethanol. Lung whole mounts were prepared by standard protocol and examined.
by a pathologist blinded to treatment to determine the numbers of lung tumor foci.

**Multiplex cytokine assays**

A custom 6-plex panel, which consisted of IL6, IL1β, IFNγ, monocyte chemoattractant protein-1 (MCP-1; also known as CCL2), IFNγ-induced protein 10 (IP-10; also known as CXCL10), and macrophage inflammatory protein-1α (MIP-1α; also known as CCL3), was used to analyze the cytokine levels in serum samples. The 6-plex analyses were performed on a Luminex 100/200 system running xPONENT software version 3.1 (Luminex Corporation). Serum cytokine concentrations were determined using Milliplex Analyst version 5.1 software (EMD Millipore). Serum collected from wild-type C57BL/6 mice challenged with 200 µg of lipopolysaccharide from *Escherichia coli* serotype O111: B4 (Sigma-Aldrich) was used as in-house control for the multiplex assays.

**Immunostaining and flow cytometry analysis**

Spleens were processed through sterile cell strainers with 100-µm nylon mesh (BD Biosciences), and lymphocytes were isolated by layering the cell suspension over lymphocyte separation medium (Lonza) and centrifugation at 300 × g for 15 minutes at 4°C. For surface staining, cells were resuspended in staining buffer (2% FBS in PBS) and were incubated for 30 minutes at 4°C in the dark with the following antibodies: PerCP-Cy5.5 rat anti-mouse CD4 (BD Biosciences), PE-Cy5 anti-mouse CD3e, and PE anti-mouse CD4, CD8, CD28, CD160, lymphocyte activation gene 3 (Lag-3), programmed death-1 (PD-1), cytotoxic T-lymphocyte antigen-4 (CTLA-4), and T-cell immunoglobulin domain and mucin domain (TIM3; eBioscience). For intracellular FoxP3 staining, cells were resuspended in freshly prepared Fixation/Permeabilization solution (eBioscience) and incubated at 4°C overnight. After fixation, cells were washed twice with permeabilization buffer and then incubated with PE anti-mouse FoxP3 antibody (eBioscience) for 30 minutes at 4°C in the dark. Data acquisition was performed with a MUSE cell analyzer (EMD Millipore).

**Immunohistochemistry**

Immunohistochemical (IHC) staining was performed by the streptavidin–biotin–peroxidase method. Cross-sections (4-µm thick) of formalin-fixed, paraffin-embedded mouse lung tumor specimens were deparaffinized in xylene for 10 minutes and rehydrated through graded alcohols (100%, 90%, and 70%) to water (5 minutes each). Antigen epitopes were unmasked by boiling in sodium citrate solution (10 mmol/L, pH 6.0) for 10 minutes. Sections were treated with 3% H2O2 for 5 minutes to block endogenous peroxidase activity and then incubated in blocking buffer containing 10% horse serum for 30 minutes. The sections were incubated with rabbit anti-mouse PD-L1 (1:500; ProteinTech Group, Inc.) and rabbit anti-mouse PD-L2 (1:100; ProSci, Inc.) at room temperature for 2 hours. Signals were developed using the Vecta Stain ABC standard Kit with 3,3′-diaminobenzidine peroxidase substrate (Vector Labs) according to the manufacturer’s instructions. Finally, the sections were counterstained with hematoxylin (ThermoFisher Scientific). Slides were scanned using an Aperio AT2 digital scanner (Leica Biosystems). A total of six mice from each of the control, concurrent, and sequential CRT treatment groups were assessed by IHC, and at least two cross-sections per mouse were analyzed.

**Serum miRNA profiling and quantitative real-time PCR**

For the serum miRNA profiling, a Mouse complete SeraMir Exosome RNA amplification and profiling kit (System Biosciences) was used according to the manufacturer’s instructions. Mouse serum (240 µL) was used to isolate the total exosomal RNAs, and 5 µL of exo-RNA was subjected to reverse transcription of exo-cDNA. Each exo-cDNA (10 µL) generated was amplified by quantitative PCR using SYBR Select Master Mix (Life Technologies) with Mouse 384 SeraMir qPCR profiler array (System Biosciences) on a QuantStudio 6 Flex Real Time PCR system (Life Technologies). For lymphocyte miRNA quantitative real-time PCR (qRT-PCR), total cellular RNAs were isolated using Trizol LS reagent (Life Technologies) according to the manufacturer’s instructions. Total RNA concentration was quantified by absorbance at 260 nm using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific). Five nanograms of total RNA were subjected to reverse transcription using a TaqMan miRNA reverse transcription kit (Life Technologies) and then amplified by qRT-PCR using TaqMan Fast Advanced Master Mix (Life Technologies) with sequence-specific primers from TaqMan miRNA assays (Life Technologies) following the manufacturer’s instructions. The miRNA expression levels were quantified using the comparative Ct method and normalized with the expression of U6 snRNA.

**Statistical analyses**

Serum cytokine and lymphocyte miRNA data were analyzed using an unpaired, two-tailed Student t test, and tumor foci data were analyzed using a one-way ANOVA with Bonferroni multiple comparisons post-test. All statistical analyses were performed using GraphPad Prism software version 5 (GraphPad Software, Inc.). All results are expressed as mean ± SEM, and P values <0.05 were considered statistically significant.

**Results**

**Toxicity of cisplatin/radiotherapy in the hMUC1.Tg lung cancer model**

To investigate the effects of concurrent versus sequential cisplatin/radiotherapy on the immune system in a lung cancer mouse model, a total of 150 hMUC1.Tg mice were administered 10 weekly doses of urethane starting at 6 weeks of age to induce lung adenoma and then assigned to six treatment groups as follows (n = 25, all groups): control; radiotherapy in weeks 23 and 27 (RTX 1); radiotherapy in weeks 31 and 35 (RTX 2); cisplatin alone; cisplatin + RTX 1 (concurrent cisplatin/radiotherapy); and cisplatin + RTX 2 (sequential cisplatin/radiotherapy; see Fig. 1A for treatment timeline). Weekly weight monitoring of all mice was performed starting at 5 weeks of age through the end of the study in week 41, based on our previous work (24, 25). As shown in Fig. 1B, distinct weight losses in all treatment groups compared with control were observed that corresponded with current, and sequential CRT treatment groups were assessed by IHC, and at least two cross-sections per mouse were analyzed.
compared with the control mice (Fig. 2). Although no significant differences among treatment groups were observed, the level of IFN-γ, IL-12p70, IL-10, TNF-α, and CXCL10, in serum at week 41 to monitor changes in cytokine profile following chemotherapy and/or radiotherapy, was significantly lower following sequential cisplatin/radiotherapy compared with that of control and concurrent treatment groups, whereas concurrent cisplatin/radiotherapy did not affect CD28 expression (Fig. 3D). This result suggests that sequential cisplatin/radiotherapy induced an immunosuppressive environment that may result in immune tolerance for cancer or may interfere with the response to immunotherapy.

**Effect of concurrent versus sequential cisplatin/radiotherapy on the immune system**

We previously showed that increases in proinflammatory cytokines and chemokines are detectable in serum during lung tumor development in this mouse model, and that two cycles of tecemotide are required to achieve a tumor-suppressive effect (25). To determine the effect of concurrent and sequential cisplatin/radiotherapy on the immune system, we assessed the levels of six different cytokines, which include proinflammatory cytokines (IFN-γ, IL-1β, and IL-6) and chemokines (CCL2, CCL3, and CXCL10), in serum at week 41 to monitor changes in the immune response following chemotherapy and/or radiotherapy (Fig. 2). Although no significant differences among the treated groups were observed, the level of IFNγ was significantly lower following sequential cisplatin/radiotherapy compared with the control mice (P = 0.03; Fig. 2A). Next, we examined the lymphocyte populations by surface marker staining. As shown in Fig. 3A and B, cytotoxic CD8+ T cells were significantly downregulated, whereas CD4+ T cells were upregulated in both concurrent and sequential cisplatin/radiotherapy compared with that of control. It is well known that CD4+ T cells, including helper, memory, and regulatory T cells (Treg), are important for immune regulation. Among the CD4+ subsets, CD4+ FoxP3+ Tregs, also known as suppressor T cells, are crucial for tumor immune escape by suppressing antitumor immune responses. The increase of CD4+ T cells after cisplatin/radiotherapy prompted us to ask whether Tregs were also modulated. Interestingly, only sequential cisplatin/radiotherapy treatment induced significant upregulation of Tregs compared with that of control and concurrent treatment groups (Fig. 3C). We further examined the expression of other inhibitory markers, such as PD-1, CTLA-4, Lag-3, TIM3, and CD160, but no significant difference was observed in any of these markers compared with those of control (Fig. 3E–I). However, CD28, a costimulatory molecule that is essential for T-cell activation, was significantly decreased following sequential cisplatin/radiotherapy compared with that of control and concurrent treatment groups, whereas concurrent cisplatin/radiotherapy did not affect CD28 expression (Fig. 3D). This result suggests that sequential cisplatin/radiotherapy induced an immunosuppressive environment that may result in immune tolerance for cancer or may interfere with the response to immunotherapy.

**Effect of concurrent versus sequential cisplatin/radiotherapy on the tumor microenvironment**

In response to immune surveillance, most tumors develop immune evasion by expressing immune checkpoint ligands to inhibit T-cell activation. The engagement of the PD-1 receptor with PD-L1 or PD-L2, which are expressed by cancer cells or antigen-presenting cells, impairs the activation and effector functions of T cells (26). To determine the effect of concurrent and sequential cisplatin/radiotherapy on the tumor microenvironment, we assessed the expression of PD-L1 and PD-L2 in lung tumor specimens at the end of study in week 41 following the administration of two cycles of concurrent and sequential cisplatin/radiotherapy. As shown in Fig. 4, the overall expression levels of PD-L1 and PD-L2 were similar in the control and treatment groups.

**Serum and lymphocyte miRNA profiling**

miRNAs are small (18–25 nucleotides), noncoding RNA molecules that posttranscriptionally regulate gene expression through direct interaction with specific sequences on target messenger RNAs. It has been found that miRNAs are released into the extracellular space in association with exosomes, which are nanovesicles that have been extensively investigated as intercellular communication modules in the immune system (27). The release of RNA through exosomes is not a random process, but is rather tightly regulated. Recent evidence indicates an exosome-mediated transfer of miRNA from T cells to antigen-presenting cells during antigen recognition in turn to functionally regulate gene expression in recipient cells (28). The changes in circulating miRNA signatures also specifically reflect the microenvironments in different diseases as well as the response to therapy, making it a new class of blood-based, noninvasive biomarkers (29).

Having observed that Tregs were modulated differently in concurrent versus sequential cisplatin/radiotherapy, we sought to evaluate the regulatory mechanism systematically through profiling the changes of circulating miRNA to identify a potential biomarker for treatment efficacy. A total of 18 serum samples collected at the end of the study in week 41 from the control and concurrent and sequential cisplatin/radiotherapy groups (n = 6) were used to profile the exosome-miRNA by qRT-PCR containing 380 miRNAs (System Biosciences). Fourteen of 380 miRNAs were significantly different between concurrent and sequential cisplatin/radiotherapy (Fig. 5A). Among these miRNAs, miR-29c in the sequential cisplatin/radiotherapy group had the most distinct expression pattern apart from concurrent cisplatin/radiotherapy.
To validate the profiling result, the expression levels of miR-29a (Fig. 5B) and miR-29c (Fig. 5C) were assessed in lymphocytes from control, concurrent, and sequential cisplatin/radiotherapy groups. Only miR-29c in the sequential cisplatin/radiotherapy group showed a significant difference compared with that of control and concurrent cisplatin/radiotherapy treatment groups, which correlated with serum miR-29c levels.

Antitumor effects of concurrent and sequential cisplatin/radiotherapy

The numbers of lung tumor foci were assessed in week 41 following the administration of two cycles of cisplatin chemotherapy combined with concurrent and sequential RTX. As shown in Fig. 6, the average numbers of lung tumor foci (+SEM) in all treated groups were significantly reduced compared with that of the control group. The RTX 1 and RTX 2 groups are, respectively, the RTX alone control groups for the concurrent and sequential cisplatin/radiotherapy groups. The average numbers of lung tumor foci observed in the concurrent and sequential cisplatin/radiotherapy treatment groups were similar compared with each other and with the mice treated with radiation and cisplatin alone. Compared with mice treated with cisplatin alone, mice treated with concurrent and sequential cisplatin/radiotherapy showed slightly reduced numbers of lung tumor foci, suggesting a subadditive effect.

Discussion

Recently, unexpected results were seen in a phase III trial of the antigen-specific cancer immunotherapeutic agent tecemotide as maintenance therapy following CRT in unresectable stage III NSCLC (20). Median overall survival among all
Tecemotide-treated patients was not significantly different from that in the placebo-treated patients; however, overall survival was significantly increased by approximately 10 months (30.8 months vs. 20.6 months) in the predefined subset of patients treated with concurrent CRT compared with those treated with sequential CRT, a difference that suggests a benefit of tecemotide immunotherapy in this patient population (20). The underlying reason for the discrepancy in the findings in the phase III study between patients who received prior concurrent and sequential CRT remains unclear, but it suggests that the timing of chemotherapy, radiotherapy, and immunotherapy is important (30). In the treatment of melanoma, which has been the most frequent target of immunotherapeutic intervention in cancer, numerous different vaccine-based approaches have been attempted for over 20 years, but unfortunately, there have been no significant improvements in overall survival to date (31). Speculation about the reasons for the failures of these immunotherapies has included choice of poor adjuvant, HLA restriction, choice of peptide, inadequate antigen presentation, and tumor immune evasion mechanisms. The most promising immunotherapy strategies for melanoma so far have included the combination of the adjuvant GM-CSF with ipilimumab (32), which is an anti–CTLA-4 antibody, and the recombinant viral vector-based vaccine OncoVex GM-CSF, now known as T-VEC (talimogene laherparepvec; refs. 33–36).

One overlooked possibility for previous cancer vaccine failures is the changing immune status of patients with cancer following chemotherapy. The scheduling of antigen-specific immunotherapy with radiotherapy or chemotherapy appears to be critical in setting the baseline immune status that is necessary for a productive immune response. In the present study, concurrent and sequential cisplatin/radiotherapy resulted in equivalent antitumor activity, but the effects of these different treatment schedules on immune status were quite different. Surprisingly, an elevation of Tregs and the downregulation of...
Effects of Cisplatin/Radiotherapy on Immune Status

Figure 4.
Effects of concurrent and sequential cisplatin/radiotherapy on PD-L1/2 expression in lung tumors. Representative IHC staining of PD-L1 (A-D) and PD-L2 (E–H) is shown following treatment with control, concurrent, or sequential cisplatin/radiotherapy. Negative controls (secondary antibody only) are shown in A and E. Scale bars, 100 µm. Magnification factor, ×100.

CD28, which is a costimulatory molecule that interacts with CD80/86 on dendritic cells (37), at the end of the study were seen only in mice that received sequential cisplatin/radiotherapy.

Another potential difference between the effects of concurrent and sequential CRT may relate to immunogenic cell death (ICD). There are three hallmarks of ICD, calreticulin cell surface exposure, the release of high mobility group box 1 (HMGB1) protein, and the liberation of ATP, all of which are critical for the activation of dendritic cells (38, 39). Clinically, it has been shown that concurrent CRT regimens significantly improve 5-year survival compared with sequential CRT (8, 9), and ICD may be responsible for these improved treatment outcomes (40). Ionizing radiation (15, 16) and some types of chemotherapy, such as oxaliplatin and anthracyclines (13), are known to induce ICD. Using an in vitro model, Golden and colleagues (41) recently showed that ionizing radiation dose-dependently induced ICD, and when combined with carboplatin or paclitaxel, radiation treatment enhanced the ICD effect. These researchers found that the combination of radiation and chemotherapy stimulated all three components of ICD. In the present study, we found that concurrent administration of cisplatin and radiotherapy resulted in a proimmunogenic environment, whereas sequential CRT resulted in an immunosuppressive environment. Although cisplatin alone is not known to induce ICD, the combination of cisplatin and radiation results in ICD, which may explain our observations. Thus, concurrent CRT may prime the immune system, via ICD induction, to respond to tumor antigens, which could then augment the response to immunotherapy.

Weight loss was also more pronounced in the concurrent cisplatin/radiotherapy group, suggesting increased toxicity associated with dose intensity, which is consistent with clinical observations (8). Taken alone, this would suggest that concurrent CRT would be less favorable for antigen-specific immunotherapy. However, we believe that a prolonged period of no treatment allowed the mice to repair or recover from any adverse effects of concurrent CRT on the immune system. Mice that received sequential cisplatin/radiotherapy in this study experienced less weight loss; however, because of the prolonged chronic treatment with cisplatin and radiotherapy, an adaptive immune response was noted, i.e., Tregs were elevated. This suggests that there is an antigen-specific immunotherapy treatment window during which the immune system is functioning and able to sufficiently respond to antigen-specific immunotherapies.

Furthermore, we observed differences between the concurrent and sequential treatment groups in a number of miRNAs associated with immune response, specifically, miR-29, which is a critical regulator of innate and adaptive immune responses (42–45). The role of miR-29 in the immune system has been implicated in elevating the threshold for infection-associated thymic involution through inhibiting IFNAR1 expression and signaling (44). miR-29 also suppresses the generation of Th1 cells in response to intracellular bacterial infection by directly targeting the expression of IFNγ, which is known as a positive regulatory cytokine for Th1 but negative regulator for Th2 and Th17 differentiation (43). In addition, a recent study showed that miR-29 could rescue the Th1 polarization and aberrant IFNγ overexpression in miR-29-deficient CD4+ T cells through targeting two transcription factors of IFNγ, T-bet, and eomesodermin (45). In this study, we demonstrated that miR-29c was elevated in the serum and in lymphocytes, whereas serum IFNγ was significantly decreased from mice treated with sequential cisplatin/radiotherapy. Further studies to confirm this association and to identify a potential biomarker of immune system health following chemotherapy/radiotherapy are under way.

In the present study, radiotherapy and cisplatin chemotherapy were administered in two cycles based on previously published research showing that two cycles of antigen-specific immunotherapy are required to achieve any antitumor benefits (25). For this reason, single doses of cisplatin and radiotherapy were not
studied. As shown in the present work, two cycles of radiotherapy and cisplatin alone had equivalent antitumor effects, but the effects of these treatments on survival in this model are currently unknown as survival was not an endpoint of the current study design. Similarly, although titrating down or fractionating the dose of radiotherapy may have enabled a more informative investigation of the effects on survival of treatment sequence, survival was again not an endpoint of the present study. The principal aim of this study was to examine the effects on immune status of two cycles of radiotherapy administered concurrently or sequentially with two cycles of cisplatin. The results of the present study were intended to lay the groundwork for future studies combining concurrent or sequential CRT with immunotherapy to investigate the differences between these treatments with respect to overall survival seen in clinical trials (20).

Inhibition of the interaction between PD-1 on lymphocytes and the ligands PD-L1 and PD-L2 in tumor cells is currently the focus of intense research efforts in cancer immunotherapy (46). There are currently two agents that target the PD-1/PD-L1/2 system, nivolumab and pembrolizumab, both anti–PD-1 antibodies, that have been approved by the FDA, and numerous others are in various stages of clinical development. Interestingly, the lung tumors that develop following urethane induction express PD-L1 and PD-L2, respond to chemotherapy (24) and radiation treatment, and respond to antigen-specific immunotherapy (24, 25). Although in the present study neither sequential nor concurrently administered cisplatin/radiotherapy appeared to alter the expression of PD-L1 or PD-L2, this mouse lung tumor model may be ideal for studying investigational PD-1/PD-L1/2 inhibitors in combination with immunotherapy and CRT.

Finally, a limitation of the current study design is that the immune status was evaluated at only a single time point (week...
Acquisition of data (provided animals, acquired and managed patients, C.-J. Kao, G.T. Wurz, Y.-C. Lin, M.W. DeGregorio, provided facilities, etc.)

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: C.-J. Kao, G.T. Wurz, Y.-C. Lin, M. Wolf, M.W. DeGregorio
Development of methodology: C.-J. Kao, G.T. Wurz, Y.-C. Lin, M.W. DeGregorio
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.-J. Kao, G.T. Wurz, Y.-C. Lin, D.P. Vang, S.M. Griffey, M.W. DeGregorio

References


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