Use of a Novel Polymer in an Animal Model of Head and Neck Squamous Cell Carcinoma

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Abstract

Objective. To evaluate the adverse effects and therapeutic efficacy of our biocompatible polymer platform delivering targeted local therapy of cytokine CCL21 and cisplatin in a partially resected xenograft animal model of head and neck squamous cell carcinoma. In addition, to evaluate the efficacy of cotreatment with radiotherapy and assess the biocompatibility of the cisplatin-eluting polymer in the murine neck.

Study Design. Experimental animal study.

Setting. Academic research laboratory.

Subjects and Methods. SCCVII/SF cell injection established head and neck squamous cell carcinoma tumors in C3H/HeJ mice. Subjects underwent surgery, and a chemokine-eluting polymer was implanted into the resected site. Subjects treated with cisplatin received radiation or no radiation, and tissue was harvested after 8 weeks to assess polymer biocompatibility.

Results. Our results with the polymer platform significantly (P < .05) reduced SCCVII/SF tumor size in C3H/HeJ mice with cisplatin (49% ± 8.7%, Δ3.4 ± 0.6 cm³ [95% CI]), CCL21 (42% ± 4.8%, Δ3.5 ± 0.4 cm³), and cisplatin/CCL21 dual-agent polymer (82% ± 4.4%, Δ8.0 ± 0.4 cm³) as compared with controls. Cisplatin polymer with high-dose (16 Gy) and low-dose (4 Gy) radiation reduced tumor mass (respectively, 92% ± 7.2%, Δ6.1 ± 0.5 cm³; 85% ± 7.4%, Δ5.7 ± 0.5 cm³) as compared with the reduction from high-dose radiotherapy alone (70% ± 7.9%, Δ4.7 ± 0.5 cm³). No significant toxicity or inflammation was noted on histopathology after radiotherapy and cisplatin-eluting polymer treatment.

Conclusion. Cisplatin, CCL21, and cisplatin/CCL21 dual-agent polymer all exhibit significant antitumor effects and decrease tumor burden. Moreover, combination cisplatin polymer with radiotherapy may permit a decrease in intensity of radiation therapy in patients having received the cisplatin polymer. Histopathologic analysis suggests that the polymer is free from significant adverse effects in this model and warrants clinical trial.

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The primary obstacle to the long-term survival of patients with head and neck squamous cell carcinoma (HNSCC) continues to be disease recurrence. Recurrence rates among patients with advanced HNSCC (stage III or IV) range from 25% to 50%, resulting in an estimated 13,800 HNSCC recurrences per year in the United States alone. Conventional treatment—typically, surgery with or without radiation therapy—is frequently unsuccessful for patients with recurrent or advanced HNSCC: the diseased area is often unresectable, and radiation therapy uses tumoricidal mechanisms to which recurrent tumor cells may be resistant, rendering the method less effective and more toxic to the patient. Adjuvant chemotherapy is an alternative salvage option. Although chemotherapy has been proven to be effective in clinical trials, it is limited by the lack of selective delivery of anticancer drugs to the tumor site and significant systemic toxicity due to the indiscriminate destruction of normal cells. Furthermore, intravenous delivery of cancer drugs is constrained by the disruption of the microvasculature caused by previous treatments, necessitating higher doses of the drug, thereby exacerbating adverse side effects (eg, nausea, vomiting, ototoxicity, neuropathy).

Improvements in the method of drug delivery, particularly approaches that focus therapy at the tumor site, are of utmost importance to overcome the problems with current adjuvant drug delivery and to significantly extend the quality of life of HNSCC patients. Locoregional chemotherapy via intra-arterial infusion has demonstrated effectiveness and low toxicity for the treatment of HNSCC. The use of intra-arterial chemotherapy, however, has not achieved universal acceptance, largely due to the challenges associated with establishing and maintaining arterial access. Nanoparticle-based drug delivery strategies purportedly offer many advantages over the free drug release observed in intra-arterial chemotherapy, as they prevent the premature degradation of the drug, reduce the renal clearance of the drug, and control its release kinetics. However, no nanocarriers have been approved by the Food and Drug Administration, and only a few clinical trials are currently underway. This failure to clinically translate nanocarriers as a drug delivery vehicle can be attributed to the different physiologic barriers that nanocarriers must traverse to reach and enter cancer cells. Furthermore, when targeting moieties are attached to the nanocarriers, immunogenicity and plasma protein adsorption both increase, reducing the circulation time of nanocarriers in the bloodstream and their ability to target the tumor.

Polymeric drug delivery systems have emerged as an attractive approach for localized cancer chemotherapy: drugs are loaded in a polymer platform, which is injected or implanted within or adjacent to the tumor site and which functions as a reservoir to deliver the drug in a sustained and predictable manner. We have engineered a novel biocompatible modular polymer platform that is designed for patients with advanced or recurrent HNSCC. The polymer is designed for intraoperative application to the surgical bed after tumor removal or debulkment, facilitating enhanced postoperative radiation treatment with localized and sustained delivery of immunomodulators. Prior versions of our polymer platform demonstrated efficacy in discrete delivery of cisplatin or CCL21 to decrease tumor burden. These data suggested that sensitization with cisplatin might further improve antitumor efficacy of CCL21 treatment. In the present study, we seek to reaffirm these findings and integrate both drugs into a new dual-agent polymer in anticipation of synergistic or additive effects.

We also demonstrated that our cisplatin-eluting polymer may sensitize to combined radiation therapy. Our present study aims to verify this beneficial effect of our polymer with a corresponding evaluation of polymer-dependent adverse effects and biocompatibility following radiation. The polymer is already used in current Food and Drug Administration–approved devices, and both the intact polymer and its degradation products are considered well tolerated in humans. The exact polymer-tissue interactions in the vicinity of a dynamic tumor environment, however, are unknown. Herein, we simulate our proposed clinical implantation in the neck of mice to evaluate potential polymer-tissue interactions.

Methods

Mice

The mice used in this study were 8-week-old C3H/HeJ mice (Jackson Laboratory, Bar Harbor, Maine). The mice were maintained in the University of California, Los Angeles, vivarium under specific pathogen-free conditions, and sterilized food and water were available ad libitum. The institutional animal studies committee approved all of the experiments.

ELISA

CCL21 concentration in the 1-mL culture medium was determined by ELISA as described by the manufacturer (R&D Systems, Minneapolis, Minnesota). Briefly, 96-well plates (Costar, Cambridge, Massachusetts) were coated overnight with 4 μg/mL of the appropriate anti-mouse CCL21 monoclonal antibody. The wells of the plate were blocked with 10% fetal bovine serum (Gemini Bio Products, West Sacramento, California) in phosphate-buffered saline (PBS) for 1 hour. The plate was then incubated with 100 μL of medium or standard buffer for 2 hours. The plate was incubated with 2 μg/mL of biotinylated monoclonal antibody to the appropriate cytokine (PharMingen, San Diego, California) for 2 hours, and excess antibody was washed off with PBS-Tween. The plates were incubated with avidin peroxidase,
and after incubation in OPD substrate to the desired extinction, the subsequent change in color was read at 450 nm with a Microplate Reader (Molecular Dynamics, Sunnyvale, California). The recombinant mouse CCL21 used as a standard in the assay was obtained from the same manufacturer.

**Mass Spectrometry**

Release of cisplatin from single-layer polymer was measured by mass spectrometry at 7 time points (0, 1, 6 hours; 1 day; 1, 2, 3 weeks). Films were placed in 5 mL of PBS and incubated at 37°C. Solutions were collected and replaced with fresh PBS every 3 days. At each time checkpoint, respective films were removed and the solutions collected. Each solution was diluted with 20 mL of 5% nitric acid (HNO₃). All the diluted solutions were analyzed with the Thermo Jarrell Inductively Coupled Plasma Spectrometer (Franklin, Massachusetts) at a wavelength of 214 μm according to manufacturer’s instruction.

**Polymers Fabrication**

Polymers were made as previously described in our laboratory. The cisplatin-releasing polymer was designed to be adequately flexible to adapt to irregular tissue contours without tearing. A 70:30 ratio of poly(ε-caprolactone):poly(lactide-co-caprolactone) (PLCL:PCL) was found to offer the optimal flexibility. PCL and PLCL were obtained from Boehringer Ingelheim (Ingelheim am Rhein, Germany) and are manufactured under GMP- and ISO-certified facilities, qualifying these materials’ availability for future clinical testing. Cisplatin (4 mg/kg) and/or recombinant murine CCL21 (10 ng; R&D Systems) was added to the polymer solution.

**Tumorigenesis and Treatment**

Mouse syngeneic SCCA cell SCCVII/SF (n = 5 × 10⁵) was injected subcutaneously into the right flank of 8-week-old C3H mice. When tumors reached an average size of 0.5 to 1 cm³, all animals underwent surgery to debulk their tumors to 0.5 × 0.5 × 0.5 cm³ and were randomly assigned to different groups as indicated. Skin flaps were elevated and the wound closed with sutures over the polymer sheet. Tumor size was monitored daily after surgery until day 14. Tumor volume was calculated by the formula: tumor size = length × width × height. Animals were then randomly assigned to the different treatment groups: (1) plain polymer control, (2) polymer with cisplatin, (3) polymer with mouse recombinant CCL21, (4) polymer with 2 layers (1 each of CCL21 and cisplatin), (5) polymer with mixed CCL21 and cisplatin, (6) plain polymer with radiation, and (7) cisplatin polymer with radiation. Tumors were harvested at the end of the trial for histologic analysis.

**Radiation Therapy**

Three days after surgery, mice were anaesthetized and positioned in a Lucite jig with lead shielding the body, except for the tumor site, which was irradiated with a Gamma Cell 40 Irradiator (Cs-137 source; Atomic Energy of Canada Ltd, Ottawa, Canada) at a dose rate of 0.6 Gy/min. Tumors were irradiated with a total dose of 16 Gy, 8 Gy, or 4 Gy on 4 consecutive days on postoperative days 3 to 6.

**Assessment of Biocompatibility**

After midline cervical exposure, cisplatin-eluting polymer was implanted in direct proximity to the trachea and great vessels of 8-week-old C3H/HeJ mice. The skin was then reaproximated with absorbable sutures. Three days after surgery, the mice were subjected to radiation treatment as previously described or mock treatment. Eight weeks later, the neck was harvested and assessed with histopathologic analysis.

**Chemotaxis Assay**

CCL21-induced cytotoxic T lymphocyte response was assessed by chemotaxis array in T2 cells, which is a T/B hybridoma cell with HLA.A2 and without HLA class II antigen as previously described. Briefly, 10⁴ T2 cells (courtesy of Dr Peter Cresswell’s laboratory, Yale University, New Haven, Connecticut) were loaded into the upper chamber of a standard 24-well plate fitted with 3-μm polycarbonate membrane inserts (Corning Life Science, Corning, New York). Recombinant CCL21 (10 μg) was loaded in polymer, and 500 μL of 5 mL of supernatant containing CCL21 released from polymer over 5 days was added to the lower chamber of each well. Recombinant CCL21 (500 ng/mL) and serum-free RPMI medium were used as positive and negative control. After incubation for 2 hours, migrated cells were recovered from the lower chamber and quantified by flow cytometry based on the number of events per minute collected in a preset T2 cell gate.

**Statistics**

The primary end point was the day on which the tumor burden of any animal in the study reached 10% of normal body weight. A priori power analysis based on Cohen’s d of 1.6 (to estimate treatment effects at the 95th percentile of control) and 80% power suggested 4 animal subjects per arm, for a total of 32 mice. Tumor volume among treatment arms was compared with repeated measures analysis of variance (ANOVA) models that contained terms for time, treatment effects, and the interaction between time and treatment terms. For tumor growth experiments in which there was a significant treatment × time interaction effect (demonstrating differences in tumor growth rates among groups), we compared the mean tumor sizes among treatment arms of interest at specific time points per Bonferroni-corrected 2-sample t tests. P < .05 was considered significant. Statistical values and power analyses were calculated with RStudio (version 1.0.136).

**Results**

**Cisplatin-Secreting Polymer Reduced Tumor Burden**

We previously reported that the cisplatin-eluting polymer reduces tumor burden in the animal model and is facile for surgical use. To test and replicate the feasibility of our modular polymer platform in delivering cisplatin, we first
measured the cisplatin release kinetics from polymer in vitro by mass spectrometry. Time-dependent experiments demonstrated release of cisplatin from polymer in a constitutive fashion over the course of 14 days (data not shown). We then evaluated the antitumor efficacy of the chemotherapeutic layer of the polymer platform in murine models of head and neck cancer. The cisplatin-secreting polymer effectively reduced SCCVII/SF tumors in the C3H/HeJ mice (49% \( \pm \) 8.7%, \( \Delta3.4 \pm 0.6 \) cm\(^3\) [95% CI]) on day 14 (\( P < .01 \)) as compared with the plain polymer control group (Figure 1). We were able to confirm this decrease in tumor burden by excising the tumors that had been treated with the cisplatin polymer. Thus, our current work reported that the cisplatin-secreting polymer was more effective than polymer without cisplatin in decreasing head and neck cancer tumor size. In addition, our previous data indicated the cisplatin-secreting polymer was more effective than the plain polymer plus cisplatin given as an intratumoral bolus injection.\(^{27}\)

**Recombinant CCL21 Released from Polymer Reduced Tumor Burden**

We previously demonstrated the antitumor efficacy of dendritic cell–based CCL21 immunotherapy.\(^{28}\) Therefore, we sought to combine polymer-based cisplatin with CCL21 treatment. To avoid toxicity of cisplatin to cultured dendritic cells, 10 ng of mouse recombinant CCL21 cytokine was mixed into monolayer PCL-PLCL polymer. CCL21 polymer was immersed in PBS solution, and CCL21 release kinetics were assessed by ELISA. Time-dependent experiments revealed that CCL21 was released continuously from polymer in vitro at an average rate of 290 pg/d in the 10-day course (data not shown). ELISA data showed that CCL21 was released continuously from polymer in vitro at an average rate of 450 pg/d in the 10-day course (data not shown). We then evaluated the antitumor efficacy of the CCL21 polymer platform in murine models of head and neck cancer. The animal experiment showed that tumor volume in the polymer-released recombinant CCL21 group was significantly reduced (42% \( \pm \) 4.8%, \( \Delta3.5 \pm 0.4 \) cm\(^3\) [95% CI]) as compared with the control group (\( P < .05 \)), suggesting the antitumor efficacy of polymer-released recombinant CCL21 (Figure 2).

**Concomitant CCL21 and Cisplatin-Secreting Polymer Further Reduced Tumor Burden**

Two versions of our new dual-agent polymer were manufactured. The first version had a combination of cisplatin and CCL21 mixed into a monolayer polymer, while the second comprised the same 2 agents stratified into a 2-layer polymer (Figure 3). CCL21 release kinetics were monitored in each of the polymer settings in vitro. ELISA data showed that CCL21 was released continuously from polymer in vitro at an average rate of 290 pg/d in the 10-day course (Figure 4). Next, we evaluated the antitumor efficacy of the 2 polymer platforms in vivo. In the xenograft model, tumor progression in the 2-layer polymer treatment was similar to the mixed-layer polymer treatment (Figure 5). We then tested whether different orientations of 2-layer polymer would affect therapeutic efficacy. Intriguingly, the 2-layer polymer treatment with the cisplatin side facing the tumor led to a significant decrease in tumor size (82% \( \pm \) 4.4%, \( \Delta8.0 \pm 0.4 \) cm\(^3\) [95% CI]) when compared with the 2-layer polymer treatment with the CCL21 side facing the tumor (46% \( \pm \) 4.6%, \( \Delta4.5 \pm 0.5 \) cm\(^3\)) as well as the cisplatin polymer treatment (65% \( \pm \) 5.1%, \( \Delta6.3 \pm 0.5 \) cm\(^3\)) as compared with the plain polymer control treatment (\( P < .05 \); Figure 6). Subsequent hematoxylin and eosin staining of harvested tumors revealed increased tumor necrosis sites in both the cisplatin polymer treatment group and the 2-layer polymer treatment group, as compared with the plain polymer control. The subsequent hematoxylin and eosin staining indicated that cisplatin released from polymer leads to tumor cell necrosis and accounts for the antitumor efficacy (Figure 7).
Radiation Improved Antitumor Efficacy of Cisplatin Polymer

We investigated whether our cisplatin polymer platform could combine with radiation and decrease the minimum dosage of radiation. Three days after surgical implantation of cisplatin-eluting polymer, animals were randomly assigned to different groups and received a range of radiation on 4 consecutive days. High-dose radiation at 16 Gy (4 Gy/d × 4 days) resulted in significant decreases (70% ± 7.9%, Δ4.7 ± 0.5 cm³ [95% CI]) in tumor volume (P < 0.005); 16 Gy of radiation combined with cisplatin polymer treatment further decreased (92% ± 7.2%, Δ6.1 ± 0.5 cm³) tumor volume versus control (P < 0.05; Figure 8). Low-dosage radiation at 4 Gy (1 Gy/d × 4 days) did not exhibit effective antitumor efficacy (P > 0.05). Radiation of 4 Gy combined with cisplatin polymer, however, led to a significant decrease (85% ± 7.4%, Δ5.7 ± 0.5 cm³) in tumor volume when compared with radiation treatment alone (P < 0.05; Figure 9) or the control polymer group (P < 0.005), indicating that 4-Gy radiation treatment has a synergistic effect with cisplatin polymer. Moreover, 4 Gy of radiation with cisplatin polymer treatment resulted in similar antitumor effect to the high-dosage radiation at 16 Gy or 8 Gy with cisplatin treatment (P > 0.05; Figure 9). In the overall ANOVA model, the interaction effect was statistically significant (P < .0001), indicating that the treatment groups exhibited different rates of tumor growth over time. The observed power (1 − β) through ANOVA was 97% (effect size, f = 1.04).

Cisplatin Polymer Does Not Induce Significant Acute Local Tissue Injury

To determine if our cisplatin polymer causes local tissue injury, we implanted the polymer in direct contact with the trachea and great vessels. We then closed the skin over the polymer and stratified the mice into a cohort that received radiation and one that did not. We observed the mice throughout treatment and analyzed the necks of these mice using routine histopathology after 8 weeks. Regardless of radiation exposure, our results did not indicate significant

Figure 3. Synthesis of the 2 platforms: mixed polymer and 2-layer polymer. Green dot, CCL21; blue dot, cisplatin.

Figure 4. Drug release kinetics. The release kinetics of recombinant CCL21 from the mixed polymer were measured each day by ELISA. Values are presented as mean ± SD.

Figure 5. Polymer treatment and tumor volume. Concomitant CCL21 and cisplatin-secreting polymer further reduced tumor burden. Two-layer polymer resulted in similar and nonsignificant mean tumor volume in vivo as compared with mixed polymer. Error bar indicates SD.

Figure 6. Polymer orientation and tumor size. The 2-layer polymer with the cisplatin facing the tumor significantly decreased the mean tumor size as compared with the 2-layer polymer with CCL21 facing the tumor, the cisplatin polymer, and the control polymer. Error bar indicates SD.
local toxicity from the experiment (Figure 10). The polymer did incite a minor local inflammatory response, but no damage to the trachea or great vessels was observed.

**Discussion**

We developed and evaluated our modular drug delivery device using cisplatin, CCL21, and various combinations of both drugs integrated into a new dual-agent polymer. All 3 types of polymers significantly reduced tumor burden in vivo following partial tumor resection with the cisplatin-CCL21 dual-agent polymer having the most significant effect. Cisplatin is hypothesized to sensitize targeted tissue to CCL21 chemotherapy and thus potentiate the antitumor effects of the chemotactic cytokine CCL21. The 2-layer polymer exerted a greater antitumor effect than a mixed monolayer with the same therapeutic agents. Furthermore, a reverse orientation of the 2-layer polymer, with the CCL21 facing the tumor (cisplatin facing away), was not more effective than a polymer that was solely secreting cisplatin. The superb efficacy of our dual-agent polymer warrants future study regarding safety and combination with radiotherapy. Furthermore, the continuous local delivery of dual chemotherapy by our polymer proposes a viable approach for patient-specific cancer therapy.

In our study, our cisplatin-eluting polymer sensitized cancerous tissue to radiotherapy in a safe and biocompatible fashion. Treatment with cisplatin and low-dose radiation was at least as effective as treatment with high-dose...
radiation alone. Significantly, these results suggest that the postsurgical application of the cisplatin-eluting polymer may permit a reduction in intensity of radiation when treating patients with HNSCC. Thus, a reduction in radiation may beneficially decrease the dose-dependent negative side effects and increase the patient’s quality of life. Sensitization to radiotherapy is a significant finding considering the severity of radiation-associated side effects and their contribution to patient mortality.\textsuperscript{30}

An additional benefit of this polymer system includes prophylaxis against tumor recurrence following resection. Viable squamous cell carcinoma cells have been recovered from the surgical wound following neck dissection and were shown to be capable of growing as colonies in vitro; theoretically, these may implant and cause cancer recurrence.\textsuperscript{31}

Therefore, exposing such cells to this polymer system in combination with radiation therapy may also decrease the chance of recurrence.

In conclusion, our polymer wrap is slowly degradable and biocompatible, and it can deliver immunomodulators and chemotherapeutic agents to maximize local antitumor efficacy while decreasing the dose of radiation for therapy. Our results following examination of the local response to the polymer after radiation suggest that the polymer is free from serious adverse events in this model and is well tolerated in vivo. Thus, the absence of observed toxic effects or significant acute inflammation in mice implanted with cisplatin-eluting polymer exposed to radiation warrants further investigation as a potential therapeutic modality through proposed clinical trials.

**Author Contributions**

Peter A. Pellionisz, acquisition, analysis, and interpretation of data; manuscript drafting; final approval; agrees to be accountable for all aspects; Yuan Lin, acquisition, analysis, and interpretation of data; manuscript drafting; final approval; agrees to be accountable for all aspects; Jon Mallen-St Clair, acquisition, analysis, and interpretation of data; manuscript drafting; final approval; agrees to be accountable for all aspects; Jie Luo, acquisition, analysis, and interpretation of data; manuscript drafting; final approval; agrees to be accountable for all aspects; Arnold Suwarnasarn, acquisition, analysis, and interpretation of data; manuscript drafting; final approval; agrees to be accountable for all aspects; Dorte Schaue, acquisition, analysis, and interpretation of data; manuscript drafting; final approval; agrees to be accountable for all aspects; David A. Elashoff, acquisition, analysis, and interpretation of data; manuscript drafting; final approval; agrees to be accountable for all aspects; Fernando Palma-Díaz, acquisition, analysis, and interpretation of data; manuscript drafting; final approval; agrees to be accountable for all aspects; Steven M. Dubinett, conception and design; critical revision for intellectual content; final approval; agrees to be accountable for all aspects; Sherven Sharma, conception and design; critical revision for intellectual content; final approval; agrees to be accountable for all aspects; Maic A. St John, conception and design; critical revision for intellectual content; final approval; agrees to be accountable for all aspects.

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