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A Polymer that Delivers Cytokines and Cisplatin is Effective in Reducing Tumor Burden in HNSCC

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Abstract

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This data will be presented at the 2013 AAO-HNSF annual meeting.

Objective—To evaluate the therapeutic efficacy of a novel polymer platform delivering cisplatin and cytokines in the treatment of HNSCC.

Study Design—in vivo study.

Setting—Academic research laboratory.

Subjects and Methods—Mice were randomized to receive implantation of (1) no polymer; (2) plain polymer; (3) plain polymer with local cisplatin injection; (4) cisplatin polymer. The two groups of mice implanted with cisplatin polymer or no polymer were further randomized to receive (1) 4 Grays external beam radiation for 4 days; (2) no radiation. For cytokine studies mice were grouped into: (1) no polymer; (2) plain polymer; (3) plain polymer with intratumoral injection of recombinant CCL21 twice a week; (4) polymer containing parental dendritic cells; (5) polymer containing dendritic cells secreting CCL21 (DC-CCL21).

Results—The cisplatin secreting polymer effectively reduced tumors in the mice by over 16-fold ($P < 0.01$). We also observed a statistically significant lower tumor weight among mice treated with cisplatin polymer and concomitant radiation compared to control groups. The DC-CCL21 polymer reduced SCCVII/SF tumors in the C3H/HeJ mice by over 41% ($p < 0.01$).

Conclusion—Herein, we demonstrate the efficacy of a novel polymer platform in delivering cisplatin and cytokines. We also demonstrate that we can effectively grow dendritic cells in the polymer that can actively secrete CCL21 for a minimum of five days. This polymer may represent a new therapeutic modality for patients with HNSCC. Once this polymer platform is optimized we will plan to pursue prospective trials in patients with HNSCC.

INTRODUCTION

Patients with HNSCC are at considerable risk of mortality, with more than 300,000 deaths attributable to the disease annually.¹ Aggressive surgical resection, with or without adjuvant chemoradiation (CRT) is the cornerstone of treatment for early disease. In many patients, the necessary surgery can be disfiguring, with a profound impact on quality of life.² During the past 30 years, the 3-to 5-year survival rate of patients with advanced T3 and T4 HNSCC has remained poor (20–30%) despite considerable advances in surgical techniques and irradiation delivery and improvement in chemotherapeutic strategies. Because 50% of the patients with advanced and unresectable disease fail primary management, salvage these patients is of paramount importance.³ Many of these patients receive radiation (RT) as definitive or as adjuvant therapy, which makes retreatment a challenge. Currently, the standard of care for recurrent disease is surgical salvage. Unfortunately, many advanced head and neck cancers are unresectable due to their proximity to vital structures such as the carotid artery or the skull base. Although palliation by chemotherapy is often attempted, systemic toxicity and its impact on the quality of life of patients prevents its wider clinical application⁴. Given these dismal figures, new advances are needed in the effective treatment of HNSCC.

The science of polymer technology for drug delivery has evolved considerably since 1990.^{5,6,7,8} Because most head and neck cancers and their cervical metastatic nodes are

clinically accessible, local treatment with a polymer matrix may have significant clinical applications.

We have developed a modular drug delivery device that reproducibly reduces tumor growth *in vivo*. In this study, we have used a partial tumor resection model in the mouse, replicating the difficult situation we see in our patients in which the entire tumor is not resectable. The platform we have developed is a flexible sheet that is designed to be applied intraoperatively to the surgical bed after removing or debulking the tumor, and is engineered to adapt and adhere to the surgical resected tissue contours. Cisplatin has been widely used in combination with radiation as a radiosensitizer in preclinical and clinical studies.⁷ We hypothesized that the local delivery of cisplatin is expected to maximize therapeutic index, minimize systemic side effects, and enhance post-operative radiation treatment.

Another attractive addition to our modular platform is the concept of polymer delivery of immunomodulators, which will increase the efficiency of tumor cell killing by the host's immune system. Patients with HNSCC have been well documented to exhibit local immunosuppression with depressed T-cell-mediated responses as well as depressed NK cell and antibody-dependent cell cytotoxic effects⁹. CCL21 (C-C motif cytokine ligand 21) is an immune active chemokine involving in immune cell chemotaxis by binding to its CCR7 receptor¹⁰. Our group previously reported that CCL21 immunotherapy mediates T cell dependent anti-tumor effect and reduces tumor burden in a lung cancer model. Both innate natural killer and specific T-cell antitumor responses are significantly increased following dendritic cell (DC)-based CCL21 therapy.

Gene therapy approaches include replacement gene therapy, suicide gene therapy, and immunotherapy. Despite these many approaches, gene therapy still has been limited significantly^{11,12}. The major limitation for the clinical use of cytokines is the lack of an effective protocol for the local and sustained delivery of cytokines to the tumor milieu. We hypothesized that our polymer platform would provide a safer and simpler alternative to current cytokine immunotherapies designed to deliver cytokines into the tumor microenvironment in a sustained manner. After resection of head and neck cancer, the surgeon frequently finds a situation where part of the tumor is judged to be unresectable. In these cases, implanting the polymer system in proximity of the tumor may provide benefit to these patients, by increasing the dosage of local radiation, and by serving as a platform for immunomodulation/drug therapy.

METHODS

Polymer Fabrication

A 70:30 ratio of PLCL:PCL was found to offer the optimal flexibility.¹³ Cisplatin (4mg/kg) was added to the polymer solution. For the DC-CCL21 delivery, a well was made of fibrin gel on top of the PCL-PLCL. Material selection was limited to those that are used in FDA-approved devices.

Mouse Model

The mice used in this study were 6-week-old C3H/HeJ mice (Jackson labs, Bar Harbor, Maine). Animals were maintained in the University of California, Los Angeles vivarium according to NIH guidelines, and all procedures were conducted under institutionally approved animal protocols.

Animal Model Surgical Procedure

4×10^5 cells from the C3H/HeJ mouse SCCA cell line SCCVII/SF¹⁴ were injected into 6-week-old C3H/HeJ mice. Eight mice were injected in each group. Tumor growth was assessed three times/week. When tumors reached an average size of 0.5–1 cm³, all animals underwent surgery to debulk their tumors by 50% to approximate the surgical situation when a patient's tumor is unresectable, and some tumor is left behind prior to polymer therapy. Animals were then randomly assigned to the different treatment groups: (1) no polymer; (2) plain polymer; (3) plain polymer with local cisplatin injection; (4) cisplatin polymer. For cytokine studies mice were grouped into: (1) no polymer; (2) plain polymer; (3) plain polymer with intratumoral injection of recombinant CCL21 twice a week; (4) polymer containing parental dendritic cells; (5) polymer containing dendritic cells secreting CCL21 (DC-CCL21). Each tumor bed was completely covered with the polymer according to the treatment group.

Radiation therapy

For the RT experiments, the treatment groups were: (1) No treatment (no polymer addition, surgical debulking only); (2) No treatment (no polymer addition, surgical debulking only) + RT; (3) cisplatin polymer alone; and (4) cisplatin polymer + RT. Tumors were irradiated with a total dose of 16Gy given in 4Gy fractions on 4 consecutive days^{15,16, 17}.

ELISA

CCL21 concentrations in 1cc of culture medium were determined by ELISA as recommended by the manufacturer (R&D Systems, MN). 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, CA).

Flow Cytometry

Tumor leukocytes were isolated by digesting tumor tissue in collagenase IV (Sigma). Flow cytometric analyses were performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) in the UCLA Jonsson Cancer Center Flow Cytometry Core Facility.

Statistics

Tumor growth curves were compared between treatment arms using repeated measures analysis of variance (ANOVA) models. Tumor weight was compared between groups with a two-way ANOVA model containing terms for cisplatin polymer and radiation. In both types of tumor models the log transformation was used to reduce the influence of outliers. $P < 0.05$ was considered significant.

RESULTS

Cisplatin Secreting Polymer Reduces Tumor Burden in Head and Neck Cancer

We evaluated the antitumor efficacy of the chemotherapeutic layer of the polymer platform in murine models of head and neck cancer. Our results using this novel polymer platform demonstrate a significant reduction in tumor growth. The cisplatin secreting polymer effectively reduced SCCVII/SF tumors in the C3H/HeJ mice by over 10-fold on day 25 ($P < 0.01$) (Figure 1) as compared to control (surgical debulking only), plain polymer, and plain polymer + intratumoral cisplatin injection groups ($P < 0.01$). We were able to confirm this decrease in tumor burden by excising the tumors that had been treated with the cisplatin polymer (Figure 1). Our data shows that our cisplatin-secreting polymer is more effective against head and neck cancer than the plain polymer plus cisplatin given as an intratumoral bolus injection.

Cisplatin Secreting Polymer Enhances the Efficacy of Radiation Therapy

Mice treated with radiation, with or without cisplatin polymer implantation, demonstrated significant reduction in tumor regrowth compared to the control group (radiation alone, 22% the size of control, $P = 0.005$; radiation with cisplatin polymer 12% the size of control, $P = 0.003$).

A closer comparison between the two different radiation treatment groups revealed lower tumor burden in the group also implanted with cisplatin polymer (Figure 2) (53% the size of the radiation alone group). An overall analysis of the four treatment arms found that both cisplatin polymer and radiation resulted in significantly reduced tumor size over time ($p = 0.03$ and 0.001 , respectively). This observation was corroborated by the statistically significant lower tumor weight noted among mice treated with cisplatin polymer and concomitant radiation compared to the radiation alone group and the control group (Figure 2) (32% the weight of the radiation alone group, $P = 0.05$; 9% the weight of the control group treated with debulking alone, $P = 0.0002$).

Dendritic cells overexpressing CCL21 (DC-CCL21) can grow and successfully Secrete CCL21 from the Polymer

To investigate the biocompatibility of our polymer in culturing dendritic cells in vitro, we initially investigated the capacity of dendritic cells (DC) to grow and survive in vitro while in the polymer. Cells were plated at different initial densities within the polymer or directly onto plates (without the polymer) in vitro. Five days later, dendritic cells in the 10^6 /well group grew to 2×10^6 in the polymer, or to 3×10^6 without polymer (Figure 3A). Although we noted a less robust growth rate in the DC-polymer group, DC grown in the polymer exhibited similar morphology as those cultured without polymer.

To investigate the efficacy of CCL21 production from polymer-cultured dendritic cells (DC-CCL21), ELISA was performed on medium collected from the in vitro plates after 5 days of culture. Time dependent experiments demonstrated continuous release of CCL21 from 10^5 dendritic cells (Figure 3B). Our data revealed a reproducible correlation between initial cell density and final CCL21 production (Figure 3C). The maximum yield of CCL21 was

2058±203 pg/ml from 10⁶ cells over 5 days, and this is therefore the dose used in all subsequent experiments.

DC-CCL21 Secreting Polymer Reduces Tumor Burden in Head and Neck Cancer

We evaluated the antitumor efficacy of the DC-CCL21 polymer platform in murine models of head and neck cancer. Our results using this novel polymer platform demonstrate a significant reduction in tumor growth. Animals were randomly assigned to the following five treatment groups: (1) no polymer; (2) plain polymer; (3) plain polymer with intratumoral injection of recombinant CCL21 twice a week; (4) polymer containing parental dendritic cells; (5) polymer containing dendritic cells secreting CCL21 (DC-CCL21).

The DC-CCL21 secreting polymer effectively reduced SCCVII/SF tumors in the C3H/HeJ mice by 41% as compared to control groups ($p<0.01$) (Figure 4). Plain polymer or polymer +DC treatment showed no significant difference in tumor volume as compared to the control group ($p>0.05$). Our data shows that our CCL21-secreting polymer is more effective against head and neck cancer than the plain polymer plus CCL21 given as an intratumoral bolus injection twice weekly.

DC-CCL21 recruits DC, T cells and inhibits Treg cells to the tumor site

Animals treated with DC-CCL21 polymer exhibited a significant increase in the frequency of CD4⁺ T cell and CD11c⁺ dendritic cells, as well as a marked decrease in CD4⁺CD25⁺ regulatory T cells infiltrating the tumor sites (Fig. 5). These data suggest the antitumor effect of polymer-based DC-CCL21 treatment may, at least partially, be due to enhanced immune activity around tumor site.

DC-CCL21 polymer inhibits epithelial-to-mesenchymal transition (EMT) in HNSCC tumors

We sought to investigate the effect of DC-CCL21 polymer on epithelial-to-mesenchymal transition (EMT), a potential mechanism of metastasis. Tumor samples were harvested 12 days after implantation and were analyzed by immunoblotting against EMT markers. Epithelial markers, including E-cadherin, beta-catenin and gamma-catenin, were increased, whereas the mesenchymal marker vimentin was decreased in the DC-CCL21 polymer treated tumors (Figure 6). These data suggest that DC-CCL21 polymer treatment may lead to a less mesenchymal status that attenuates tumor invasion, and by promoting an epithelial phenotype, can also sensitize tumors to other forms of therapy.

DISCUSSION

We have developed a novel modular drug delivery device that reproducibly reduces tumor growth and enhances the efficacy of RT *in vivo* after partial tumor resection. We have used this partial tumor resection model in the mouse, replicating the difficult situation we see in our patients in which the entire tumor is not resectable. The device we have developed is a flexible sheet that is designed to be applied intraoperatively to the surgical bed after removing or debulking the tumor, and is engineered to adapt and adhere to the surgical resected tissue contours.

The combined use of radiotherapy and chemotherapy has been effective in improving the therapeutic index of radiation therapy for a variety of human cancers^{17,19,20}. Our data herein shows that our cisplatin-secreting polymer is more effective against HNSCC than the plain polymer plus cisplatin given as an intratumoral bolus injection. This enhanced antitumor activity is likely due to a more durable sustained release of cisplatin from the polymer platform increasing the interaction time with the tumor cells.

Some advantages of this polymer system over conventional brachytherapy are: better control of dose distribution, elimination of radioprotection and safety issues for the patient and the patient's family, and treating personnel. An important psychological factor is that the patient's daily activities are not restricted during the entire treatment time. An additional benefit of this polymer system includes prophylaxis against tumor recurrence following resection. Viable squamous cell carcinoma (SCC) 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 recurrence²⁰. Therefore, exposing such cells to this polymer system in combination with EBRT, before they implant (while they are isolated and fragile), may decrease the chance of implantation.

Another attractive addition to our modular platform is the concept of polymer delivery of immunomodulators, which will increase the efficiency of tumor cell killing by the host's immune system. Patients with HNSCC have been well documented to exhibit local immunosuppression with depressed T-cell-mediated responses as well as depressed NK cell and antibody-dependent cell cytotoxic effects²¹. The role of cytokines in tumor regression is now well established¹². The major limitation for the clinical use of cytokines is the lack of a simple and effective protocol for the local and sustained delivery of cytokines to the tumor milieu.

We are aware of the results of other groups who have reported that secretion of CCL21 by HNSCC cells and by other paracrine sources can combine to promote activation of CCR7 pro-survival signaling associated with tumor progression^{22, 23}. We feel that the seeming disparity in the data may be explained by physiologic vs. pharmacologic doses of CCL21. Our efforts to produce effective cancer therapy focus on methods to address the deficits in the tumor microenvironment. To restore tumor antigen presentation and antitumor effector activities we are utilizing CCL21 that is known to recruit DC, T, NK and NKT cells^{24, 25, 26, 27}. The recruitment of NK and NKT cells is advantageous because these effectors can recognize tumor targets in the absence of MHC expression^{28, 29}. CCL21 may be distinctly advantageous because of its capacity to elicit a type I cytokine response *in vivo*³⁰. Our group has demonstrated previously that CCL21 administered intratumorally elicits potent antitumor responses in murine cancer models^{30,31,32,33}. Vicari et al³⁴ substantiated our findings in a colon cancer model. They demonstrated that CCL-21 transduced colon cancer cells had reduced tumorigenicity that was attributed to both immunological and angiostatic mechanisms³⁴. Arenberg et al³⁵ have also reported that CCL21 inhibits human lung cancer growth and angiogenesis in a mouse model.

On the basis of these dual capacities and our prior data, we studied the efficacy of CCL21 in the immunomodulator layer of our polymer platform. We demonstrate for the first time that

DC are able to survive and secrete functional CCL21 when grown in our polymer. The DC-CCL21 secreting polymer significantly reduces tumor burden; recruits DC and T cells while inhibiting Treg cells at the tumor site; and effectively inhibits epithelial-to-mesenchymal transition (EMT) in HNSCC tumors.

We have designed and synthesized a novel biocompatible modular polymer platform that will improve the outcome for patients with advanced or recurrent HNSCC. The surgical demand in such a setting is for wider resection or, in some instances when the tumor is fixed to the underlying vital structures, to debulk large tumors. Unfortunately, local failure in these cases is at least 40% or greater. Our polymer wrap is biocompatible; is slowly degradable; and can serve as a platform to deliver immunomodulators and chemotherapeutic agents so as to most effectively kill tumor cells in the proximity of the polymer application. This polymer wrap is designed to be applied intraoperatively to the surgical bed after removing or debulking the tumor, thus allowing for enhanced post-operative radiation treatment, and also functioning as a platform for the delivery of immunomodulators. Our work demonstrates that the polymer system is well tolerated and that CCL21, and cisplatin therapy combined with RT generates a potent antitumor immune response against HNSCC.

Although the polymer described is currently used in FDA-approved devices, and both the intact polymer as well as its degradation products are known to be well tolerated in humans, the exact polymer-tissue interactions in the vicinity of a dynamic tumor environment is unknown. In this current work, we did not explicitly evaluate biocompatibility in such an environment, and instead focused on demonstrating the overall utility of this modular approach. Now that feasibility has been demonstrated, the next step of our research program is to optimize the polymer platform to improve handling, cell delivery, and drug delivery.

The modular nature of this polymer platform also provides an elegant approach to future investigations, allowing us to seek out specific molecular targets. This will enable us to dissect underlying mechanisms of immune activation and expansion, which will in turn help us design additional strategies to block the inactivation and death of the cytotoxic effectors in patients with aggressive HNSCC. We will also be able to build polymers that specifically target each patient's tumor as we can profile the tumors and then target them selectively on the polymer platform. As more combinations of the polymer platform are developed, direct polymer therapy may play an important role in the armamentarium against oral and head and neck SCCA, as well as many other cancers and human diseases. Once this polymer platform is optimized in an *in vivo* model, we will plan for the ultimate validation in the context of a prospective trial in patients with unresectable advanced or recurrent HNSCC.

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Figure 1. Cisplatin Polymer Effectively Reduces the Growth of Mouse SCCVII/SF
 4×10^5 SCCVII/SF tumor cells were inoculated in C3H mice. The implanted cisplatin polymer significantly reduced tumor growth by 16 fold compared to controls. n=8 mice/group,** p<0.01 compared to surgery only.

Figure 2. Time-series Plot of Tumor Size in the Four Treatment Arms

The effects of treatments were assessed with a repeated measures ANOVA model.

Treatment with cisplatin polymer and radiation resulted in significantly reduced tumor size over time ($p=0.03$ and 0.001 , respectively).

Figure 3. DC-CCL21 cultured in the polymer is capable to producing CCL21 in vitro
 (A) DC-CCL21 were grown directly on plates or in polymer at different initial densities as indicated. Growth curve were made using cell numbers counted five days later (open bar).
 (B) Time dependent continuous release of CCL21 from DC-CCL21 in polymer. (C) Density dependent release of CCL21 from DC-CCL21 in polymer.

Figure 4. Polymer-based DC-CCL21 treatment inhibits tumor growth in a partially resected HNSCC model

Established SCCVII/SF tumors were partially resected and were treated with control, polymer, polymer+CCL21 injection, polymer+DC and polymer+DC-CCL21. Polymer+DC-CCL21 treatment exhibited a significant antitumor effect, starting from day 6 to day 12 ($p < 0.05$). In contrast, polymer with CCL21 injection only demonstrated a weak and insignificant decrease in tumor growth, compared to control group or plain polymer group ($p > 0.05$). Plain polymer or polymer+DC treatment showed no significant difference in tumor volume compared to the control group ($p > 0.05$). Results are representative of three independent experiments; bar, $\pm$ SD.

Figure 5. DC-CCL21 polymer recruits dendritic cells and T cells and inhibits regulatory T cells in vivo

Flow cytometry was performed to evaluate single cell suspensions of tumor nodules. Animals receiving DC-CCL21 polymer therapy exhibited a significant increase in the frequency of CD4⁺ T cell and CD11c⁺ dendritic cells, as well as a marked decrease in CD4⁺CD25⁺ regulatory T cells infiltrating the tumor site.

Figure 6. DC-CCL21 treatment inhibits EMT in squamous cell tumors

Epithelial markers, including E-cadherin, beta-catenin and gamma-catenin, are increased, whereas the mesenchymal marker vimentin is decreased in the tumors from the DC-CCL21 polymer treatment group, as compared to tumors from the plain polymer control group.