Neoadjuvant Intratumoral Cytokine-Loaded Microspheres are Superior to Postoperative Autologous Cellular Vaccines in Generating Systemic Anti-Tumor Immunity

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Background: Sustained intratumoral cytokine release using poly-lactic acid microspheres (PLAMs) can induce a systemic immune response, shifting immunotherapy to the neoadjuvant setting.

Methods: C57BL6 mice with established B16 melanomas underwent a single intralesional injection of IL-12, TNF-α or GM-CSF PLAM, alone or in combination. Tumor draining lymph nodes (TDLN) and spleens were assessed for a specific anti-tumor response by IFNγ release assay and ELISPOT.

Results: Intralesional injection of TNF-α, alone or in combination, resulted in significant tumor ablation. The induction of tumor specific reactive T-cells in the TDLN was greatest with IL-12 and TNF-α. Only mice treated with IL-12 and TNF-α demonstrated a substantial T-cell response in cultured splenocytes. This combination resulted in a significant reduction in new tumors after re-challenge. Adjuvant therapy, using irradiated B16 cells in combination with equivalent doses of IL-12 and TNF-α, failed to generate a similar T-cell response or prevent re-challenge.

Conclusions: Intratumoral IL-12 and TNF-α loaded PLAM leads to both local eradication of tumor and the induction of specific anti-tumor T-cells in the lymph nodes and spleens, resulting in memory immune response. Neoadjuvant treatment was significantly superior to postoperative autologous cellular vaccines using IL-12 and TNF-α PLAM.


KEY WORDS: melanoma; microspheres; immunotherapy; intralesional; intratumoral; interleukin-12 (IL-12); tumor necrosis factor alpha (TNF-α); granulocyte-macrophage colony stimulating factor (GM-CSF); in situ vaccination

INTRODUCTION

Activation of the immune system to produce an anti-tumor response depends on the presentation of antigen to T-cells by antigen presenting cells (APC) and the production of cytokines that promote a Th1 cytotoxic response. Various formulations of vaccines designed to activate a Th1 response using cytokines as adjuvant immunostimulants are presently being studied. These include the use of either dendritic cells exogenously loaded with tumor-associated antigens or autologous tumor cells that have been transfected with genes encoding immunostimulatory cytokines and then delivered as a postoperative vaccine. Multiple cytokines have been investigated for this purpose with varying levels of success.

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Autologous cellular vaccines and dendritic cell vaccines are limited in both the amount of antigenic material that can be used and the levels and duration of cytokine release. An alternate approach is the sustained release of cytokines directly into tumors to provide not only local control, but also to stimulate a systemic immune response. This would shift the immunotherapy from an adjuvant setting to a neoadjuvant setting. We have recently described a novel method for delivering a local and sustained release of cytokines to the tumor microenvironment that does not require genetic manipulation [1]. Utilizing the B16 (murine melanoma) model, we sought to investigate the use of cytokine-loaded poly-lactic acid microspheres (PLAMs) as intrallesional therapy for melanoma, to identify the optimum cytokines for therapy, and to delineate the most successful timing of therapy (neoadjuvant intratumoral therapy versus adjuvant vaccine therapy). We report here that the intrallesional injection of the combination of IL-12 and TNF-α provides not only local control of the lesion, but generates a potent systemic anti-tumor immune response. This approach is superior to the use of a postoperative autologous cellular vaccine using these same cytokines as an adjuvant.

MATERIALS AND METHODS

Mice and Tumors

Six- to eight-week-old female C57Bl6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in specific pathogen-free conditions at the Animal Maintenance Facility of the University of Michigan Medical Center. B16-BL6 (B16) is a melanoma cell line of spontaneous origin that has been studied extensively [2]. MCA205 is a fibrosarcoma syngeneic to C57Bl6 mice induced by 3-methylcholanthrene and was used as a specificity control tumor [3]. Cell lines were maintained in complete media consisting of RPMI 1640 supplemented with 10% heat-activated fetal bovine serum, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 μg/ml streptomycin, 100 units/ml penicillin, 50 μg/ml gentamycin and 0.5 μg/ml fungizone, all from Life Technologies, Inc. (Grand Island, NY). In vivo generation of tumors was accomplished by s.c. injection of C57Bl6 mice with 2 × 10⁵ viable B16 cells. Tumors were measured every other day in two perpendicular dimensions (a = length, b = width) with a Vernier caliper, and the size recorded as a volume (mm³) as calculated by \(a \times b^2/2\). Statistical significance between groups was calculated using an unpaired Students t-test, with a P-value less than 0.05 considered statistically significant. Survival curves were generated using SPSS statistical software, version 13.0 (SPSS Inc., Chicago, IL). Statistical significance for disease-free survival was calculated using Log-Rank analysis with a P-value less than 0.05 considered statistically significant.

Microspheres

A phase inversion nanoencapsulation technique was used for encapsulation of cytokines as described previously. Briefly, BSA (Sigma Chemical Co., St. Louis, MO), PLA (Mr 24,000 and Mr 2,000 (1:1, w/w): Birmingham Polymers, Inc., Birmingham, AL), and recombinant cytokine in methylene chloride (Fisher, Pittsburgh, PA) were rapidly poured into petroleum ether (Fisher) for formation of microspheres. Microspheres were filtered and lyophilized overnight for complete removal of solvent. Four formulations containing 1% BSA (w/w) were produced: (a) control (no cytokines); (b) murine IL-12 (~5 μg/mg of PLA) (R&D Systems, Minneapolis, MN); (c) murine GM-CSF (~5 μg/mg of PLA) (Biosource Intl, Camarillo, CA); (d) murine TNF-α (~5 μg/mg of PLA) (R&D Systems).

Tumor-Draining LN Cells and Activation

At varying time points after inoculation, TLIN and splenocytes were aseptically removed. Lymphoid cell suspensions were prepared by mechanical dissociation with 25-gauge needles and pressed with the blunt end of a 10-ml plastic syringe in RPMI 1640. The cells were activated with 1 μg/ml anti-CD3 monoclonal antibody (mAb) immobilized in 24-well plates (4 × 10⁶ cells/2 ml/well) for 2 days. The LN cells were subsequently cultured in recombinant human IL-2 (Chiron Therapeutics, Emeryville, CA) at 60 IU/ml IL-2 for 3 days at 2 × 10⁵ cells/ml.

Splenocytes and Activation

Spleen cells obtained from the C57Bl6 mice were treated with ammonium chloride-potassium lysate buffer (0.83% ammonium chloride, 0.1% KHCO3 and 0.004% EDTA) for 1 min to deplete erythrocytes and were washed twice with HBSS. Splenocytes were then activated with 1 μg/ml anti-CD3 mAb immobilized in 24-well plates (4 × 10⁶ cells/2 ml/well) for 2 days. The LN cells were subsequently cultured in 60 IU/ml IL-2 for 3 days at 2 × 10⁵ cells/ml.

Measurement of In Vitro Cytokine Release

1 × 10⁶ activated TLIN or splenocytes were co-cultured with 1 × 10⁶ B16 or MCA205 tumor cells irradiated to 15,000 cGy in 2 ml of CM per well of a 24-well tissue culture plate. IL-2 (4 IU/ml) was added at the beginning of the cultures, for 48 hr at 37°C. The supernatants were collected and, after centrifugation, analyzed for interferon-γ (IFNγ) using commercially
available ELISAs from PharMingen (San Diego, CA). A standard curve starting at 1,000 U/ml was established and 11 serial twofold dilutions were performed. Experimental values were computed with the use of regression analysis.

**ELISPOT Assay**

The number of IFN-γ producing cells was measured using ELISPOT assay after intratumoral injection of cytokine-loaded PLAM. Briefly, 96-well plates were coated with anti-mouse IFN-γ antibody (PharMingen). Activated splenocytes (1 × 10^6 cells/well) were cultured for 48 hr at 37°C in a 5% CO2 incubator alone or in the presence of 4 × 10^5 irradiated B16 or MCA205 tumor cells. After that time, wells were washed and incubated overnight at 4°C with a different clone of biotinylated anti-IFN-γ antibody (PharMingen). Reactions were visualized and counted using anti-biotin-AP.

**RESULTS**

Treatment of Established Tumors With an Intratumoral Injection of TNF-α Loaded PLAM Leads to Complete Regression of B16 Melanoma

C57BL6 mice were inoculated subcutaneously with B16 melanoma in the right flank. Treatment groups comprised ten mice each. Six days after inoculation, when all mice had palpable subcutaneous tumors, mice were treated by a single intratumoral injection of cytokine-loaded microspheres at a dose of 2 mg PLA/tumor. Control mice were injected with a single injection of BSA-loaded microspheres. Treatment groups consisted of IL-12, GM-CSF or TNF-α either alone or in combination. Mice treated with combination cytokines were treated with 1 mg PLA/tumor for each cytokine. Intratumoral injection of B16 tumors with PLAM loaded with IL-12, GM-CSF or the combination caused suppression of tumor growth, but did not lead to complete tumor regression (Fig. 1). B16 tumors treated with TNF-α, either alone or in combination with IL-12 or GM-CSF resulted in statistically significant tumor regression. Average tumor volume in mice treated with TNF-α alone or in combination was 19.35 mm^3 compared to 163.6 mm^3 for mice treated with IL-12, GM-CSF or the combination (P < 0.001 by Students t-test).

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TNF-\(\alpha\), either alone or in combination, were sacrificed 7 days after treatment and tumor draining lymph nodes (TDLN) and splenocytes were harvested for anti-CD3/IL-2 activation. Lymphocytes were then co-cultured either alone or in the presence of irradiated B16 cells and serum IFN-\(\gamma\) levels were determined by ELISA. As a control for a tumor-specific response, activated lymphocytes were also co-cultured in the presence of MCA205, a sarcoma cell line. A tumor specific response is characterized as the difference in IFN\(\gamma\) production between cells co-cultured with B16 versus MCA205.

Intratumoral injection of IL-12 and TNF-\(\alpha\) loaded PLAM resulted in the greatest B16-specific response in both the TDLN and splenocytes (Fig. 2). The ratio of the specific to non-specific tumor response is demonstrated in Figure 3.

To confirm these results, ELISPOT assay of the splenocytes was performed to quantify tumor-specific IFN-\(\gamma\) producing cells. As with the IFN-\(\gamma\) release assay, splenocytes from mice treated intraleesionally with IL-12 and TNF-\(\alpha\) demonstrated the greatest increase in B16 specific T-cells, compared to either IL-12 alone, TNF-\(\alpha\) alone or GM-CSF alone or in combination. Mice treated with IL-12 and TNF-\(\alpha\) had 75/10\(^6\) ± 4.6 IFN-\(\gamma\) producing cells compared to 48.6/10\(^6\) ± 2.6 in the next highest group; TNF-\(\alpha\) and GM-CSF (\(P < 0.01\)). The results are shown in Figure 4.

**Intratumoral Injection of IL-12 and TNF-\(\alpha\) Loaded PLAM Prevents Re-Challenge After Treatment**

In a separate experiment, C57BL6 mice were inoculated subcutaneously with B16 melanoma in the right flank. Ten days after inoculation, when all mice had palpable subcutaneous tumors, mice were treated by a single intratumoral injection of IL-12 and TNF-\(\alpha\) loaded microspheres either alone or in combination. Control mice were treated with microspheres containing BSA. Seven days after treatment, any residual tumor was surgically excised. One week after surgery, all mice were re-inoculated on the left flank with an identical tumorigenic dose of B16. Six of seven mice treated with BSA grew second tumors after treatment. As seen in Figure 5, intratumoral injection of either IL-12 or TNF-\(\alpha\) alone did not prevent tumor growth when re-challenged. However, mice treated by the combination of IL-12 and TNF-\(\alpha\) rejected tumors in 5 of 8 mice (Log Rank \(\chi^2 = 4.111, P = 0.043\)).
Fig. 3. Ratio of specific to non-specific IFN-γ production by TDLN and splenocytes after intratumoral injection of cytokine-loaded PLAM.

Fig. 4. IFN-γ producing cells after intratumoral injection of cytokine-loaded PLAM as measured by ELISPOT assay. C57Bl6 mice (five mice per group) with B16 tumors were treated with a single intratumoral injection of poly-lactic-acid microspheres encapsulating IL-12, TNF-α, and GM-CSF alone or in combination. One week later, spleens were removed aseptically. Lymphoid single-cell suspensions were activated with anti-CD3 mAb for 2 days and then cultured in 60 IU/ml IL-2 for 3 days. Activated splenocytes were co-cultured with irradiated B16 or MCA205 tumor cells. A: ELISPOT assay demonstrates that splenocytes from mice with B16 tumor treated with PLAM loaded with the combination of IL-12 and TNF-α also showed the greatest number of tumor-specific T-cells. (P < 0.01 compared to each other group by t-test). B: Ratio of specific to non-specific IFN-γ production by splenocytes after intratumoral injection of cytokine-loaded PLAM.

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Neoadjuvant Treatment of B16 Tumors With IL-12 and TNF-α PLAM Leads to a Greater Systemic Anti-Tumor Response Than an Adjuvant Vaccine

C57BL6 mice were inoculated subcutaneously with B16 melanoma in the right flank. Eleven days after inoculation, all mice with palpable subcutaneous tumors underwent treatment. Mice in the neoadjuvant group were treated with either intratumoral injection of IL-12 or IL-12 and TNF-α loaded microspheres (2 mg PLAM/mouse). Mice in the adjuvant group underwent surgical resection. One week later, the mice who had neoadjuvant treatment underwent surgical excision and the mice in the adjuvant group received vaccination in the form of a subcutaneous injection of 2 × 10⁵ irradiated B16 cells in combination with IL-12, IL-12 and TNF-α or IL-12 and TNF-α loaded microspheres. The dose of adjuvant cytokine was identical to the neoadjuvant group (2 mg PLAM/mouse). One week later, all groups were sacrificed and TDLN and splenocytes were harvested (Fig. 6). Mice treated with neoadjuvant IL-12 and TNF-α had a significant, tumor-specific response demonstrable in both the TDLN and the splenocytes as compared to IL-12 alone in the neoadjuvant setting. IL-12 and TNF-α PLAM as adjuvants to a postoperative vaccine, however, failed to induce such a response.

To rule out whether the radiation altered the ability of the tumor cells to stimulate an immune response, naïve mice were vaccinated with live B16 cells in combination with IL-12, TNF-α or IL-12 and TNF-α loaded microspheres. Ten days after vaccination, TDLN and splenocytes were harvested and an IFN-γ release assay was performed. IL-12 alone led to no increase in tumor-specific response compared to non-stimulated lymphocytes in either the TDLN (171.07 ± 18.6 vs. 177.2 ± 20.3) or splenocytes (187.84 ± 27.0 vs. 167.71 ± 15.4). TNF-α also had no significant effect. IL-12 and TNF-α led to only a mild increase in IFNγ production (360.21 ± 27.9 vs. 270.34 ± 33.3 in the TDLN, 345.43 ± 35.0 vs. 284.09 ± 37.7 in the splenocytes). Although the combination was superior to either cytokine alone, using live B16 cells was no better than irradiated cells in generating a significant tumor specific response in either the TDLN or splenocytes.

Neoadjuvant Treatment of B16 Tumors With IL-12 and TNF-α PLAM is Superior to an Adjuvant Cellular Vaccine in Preventing Re-Challenge

C57BL6 mice were inoculated subcutaneously with B16 melanoma in the right flank. Eleven days after inoculation, all mice with palpable subcutaneous tumors
underwent treatment. Mice in the neoadjuvant group were treated with IL-12 and TNF-α loaded microspheres (2 mg PLAM/mouse). Mice in the adjuvant group underwent surgical resection. One week later, the mice who had neoadjuvant treatment underwent surgical excision and the mice in the adjuvant group received vaccination in the form of a dorsal cervical subcutaneous injection of 2 × 10^5 irradiated B16 cells in combination with IL-12 or IL-12 and TNF-α loaded microspheres (at an identical dose to the neoadjuvant group). One week later, TDLN and spleens were removed aseptically. Lymphoid single-cell suspensions were activated with anti-CD3 mAb for 2 days and then cultured in 60 IU/ml IL-2 for 3 days. Activated TDLN (Fig. 5A) or splenocytes (Fig. 5B) were co-cultured with irradiated B16 or MCA tumor cells. The supernatants were collected and analyzed for IFN-γ production. Only IL-12 and TNF-α in the neoadjuvant setting was able to generate a significant tumor-specific response (P < 0.01 compared to each other group by Students t-test).

**CONCLUSIONS**

There has been significant interest in vaccination strategies that aim to develop immune responses specific to tumor-associated antigens, primarily the use of either autologous whole-cell vaccines or dendritic cells pulsed with autologous tumor lysate. The advantage of using autologous tumor is exposure to the large repertoire of tumor-associated antigens specific to the patient. The immunostimulatory effects of cytokines can be used to further improve the efficacy of these vaccines by recruiting and activating APC to the vaccination site, increasing the uploading and presentation of tumor antigens, and escalating the differentiation and proliferation of effector cells. As higher cytokine levels can be sustained at the site by local production, without the toxicity associated with systemic administration, autologous cellular vaccines and dendritic cells can be genetically modified with the gene of the cytokine of interest.

While cytokine gene-modified cellular and dendritic cell vaccines show the most potential from an immunologic standpoint, they are greatly hampered clinically by the availability of sufficient tumor. Patients with adequate harvestable tumor (those with bulky lymph node metastases or multiple metastatic foci), while possessing enough tumor to create a vaccine, have the worst
prognosis and are least likely to respond to therapy. Immunotherapy is most likely to be effective in the adjuvant setting, but these patients rarely have an extremely limited availability of tumor. Therefore, new approaches to immunotherapy are needed, those that utilize autologous whole cells but do not require ex vivo manipulation. One such approach is in situ vaccination, which consists of delivering cytokines directly to the site of the tumor [4–11]. In this way, the tumor itself serves as the vaccine, precluding the cost and difficulty in establishing a cell line and transfecting it with the gene for a cytokine. We have previously described an alternate strategy for in situ cytokine delivery: the use of slow-release polymer microspheres [1,12,13]. Using the poorly immunogenic B16 (murine melanoma) model, we sought to identify the optimum cytokines (or combination) for in situ vaccination, and to compare this neoadjuvant approach to the adjuvant use of autologous cellular vaccines.

The result confirms the synergistic effects of intratumoral IL-12 and TNF-α in generating a systemic antitumor response. In this model, neither cytokine alone nor GM-CSF alone or their combination was effective. IL-12 exerts potent Th1-inducing effects and augments NK cell cytotoxicity [15,16]. IL-12 activates and mobilizes tumor-altered NK and T-cells in lymphoid organs, and seems to exert its greatest effect in those sites where T-lymphocytes have been activated and have accumulated [17,18]. Against a poorly immunogenic tumor such as B16 melanoma, intratumoral IL-12 alone may not be able to stimulate enough inflammation and tumor necrosis to initiate the activation of T-cells. Combining IL-12 with other agents may therefore enhance its anti-tumor efficacy. Previous reports have demonstrated increased activity when IL-12 is combined with GM-CSF [19], IL-18 [6,20], and IL-2 [10,21]. TNF-α is a multipotent cytokine that causes hemorrhagic necrosis in tumors, evokes apoptosis and activates immune cells [22]. As with IL-12, while some tumor cell lines are killed by TNF-α alone, others are not at all affected, and require the combination of TNF-α with other cytokines [23]. Our results confirm that the combination of IL-12 and TNF-α appears to be a particularly effective combination. Synergistic effects possibly include skewing towards a Th1 response [24], induction of the tumoricidal activity of macrophages [25], and increased necrosis and tumor infiltration of both PMN and CD8+ cells at the site of the tumor [13].

Given the synergy seen between IL-12 and TNF-α, we sought to examine whether the cytokines are better used...
intratumorally as opposed to adjuvants to postoperative vaccines. The use of intratumoral therapy would shift the timing of immunotherapy from postoperative to preoperative. These results clearly demonstrate that neoadjuvant therapy is superior to adjuvant therapy in both stimulating an anti-tumor T-cell response and preventing tumor growth when re-challenged. Postoperative vaccines are limited by both the amount of tumor (and tumor-associated antigens) available for uptake and the need to irradiate the tumor cells, which has been shown to induce less of an immune response, perhaps contributing to the ineffectiveness of postoperative vaccines [26]. However, using live tumor cells for vaccination in combination with IL-12 and TNF-α, failed to generate results similar to intratumoral therapy, suggesting that the irradiation was not the reason why the vaccine failed.

This makes a strong argument that in situ vaccination may be a better immunologic approach than postoperative vaccination. In addition, there are other potential advantages to the neoadjuvant approach. In situ vaccination precludes the need to harvest adequate amounts of tumor or manipulate these cells ex vivo, which is not only time consuming and cost-prohibitive, but not technically feasible for many solid tumors. Chemotherapy given prior to surgical resection of a solid tumor can offer an increased likelihood of complete resection and may increase the odds of cure by reducing or eliminating micrometastatic disease at an earlier time point. This same premise may be applied to immunotherapy as well.

In conclusion, intratumoral IL-12 and TNF-α loaded PLAMs results in not only tumor ablation, but also in the generation of a regional and systemic tumor-specific immune response capable of preventing re-challenge and improving disease-free survival after resection. The local and sustained release of these cytokines in the neoadjuvant setting is greatly superior to their use as an adjuvant to a postoperative autologous cellular vaccine. Based on these results, we are now examining the mechanisms behind the apparent synergistic effects between IL-12 and TNF-α at both the site of injection in the primary tumor and on established metastatic disease. This information will hopefully further guide the clinical applicability of these cytokines in the neoadjuvant setting and suggest additional methods to augment the resultant anti-tumor immune response. Furthermore, these results suggest there may be a clinical benefit to shifting away from postoperative adjuvant immunotherapy towards preparative intratumoral immunotherapy.

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