



Neoadjuvant Immunotherapy in High Risk Patients with Cutaneous Melanoma: A Novel Approach

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Abstract

Cutaneous melanoma is an immunogenic tumor, but it seems to be very heterogeneous. Utilizing patient own tumor, before its excision, as the source for tumor-specific antigens, intratumoral administration of low dose of GM-CSF weekly in dermal and subdermal metastases did result in over 50% response rate. Failure to establish complete tumor response (CR), low weekly dose of IL-2 was substituted and resulted in CR. Therefore, it seemed that some melanoma lesions did therapeutically respond to intratumoral GM-CSF therapy, while other lesions required Intratumoral IL-2. Each of these two cytokines has different mechanism of action that may complement one another. Therefore, sequential administration of GM-CSF followed by IL-2, once at the primary site of deeply invasive primary melanoma, one week prior to its resection (Neoadjuvant Approach), did induce massive antitumor immune response at the injection sites. Such an immune response did result in complete tumor necrosis with massive histiocytosis. In addition, there was an overexpression of a great number of immune cells at the injection sites as well as in some regional lymph nodes. This autologous approach seemed to overcome tumor heterogeneity. The overall duration of response ranged from 31- over 60 months to the last date of contact. In conclusion, *in vivo* autoimmunization of melanoma sites (prior to its excision) by intratumoral administration of these two cytokines seemed to induce an immense antitumor response without major side effects, and such immune response was transmitted via the lymphatics. Such an approach seemed to prolong patient survival.

Keywords: Preoperative intratumoral cytokine therapy; Survival benefits

Abbreviations

GM-CSF: Granulocyte-Macrophage Colony Stimulating Factor (also known as Leukine) manufactured by Sanofi-Aventis Corporation; IL-2: Interleukin-2 (also called Aldesleukin) manufactured by Chiron Corporation

Introduction

Justification for the new approach

Tumor heterogeneity: Cutaneous melanoma is an immunogenic tumor as it expresses various melanoma-specific antigens. However, it seems to be very heterogeneous both clinically and biologically. Clinically; some melanomas develop in sun exposed skin areas, while others develop in none exposed sites. Furthermore, melanomas can present with various amount of pigmentation that ranges from black to none pigmented lesions known as “Amelanotic Melanoma”. In addition, some melanomas metastasize to the regional lymph nodes initially then systemically, while others do metastasize directly to the viscera and the brain. Biologically, melanoma expresses different specific antigens and has diverse genetic profiles among different patients. To overcome such heterogeneity, tumor-specific and autogenic therapeutic approach, for each patient, could be essential to obtain an antitumor immune response. It has been shown that patients with resected metastatic melanoma who have melanoma-specific infiltrating lymphocytes (TILs) in the resected metastases have statistically better survival than those who have melanoma-specific T cells in the peripheral blood [1]. Furthermore, the higher the number of TILs at the primary sites of melanoma carries better prognosis [2]. Therefore, the activation of these cells at the tumor site is a logic approach to obtain antitumor immune response.

Adjuvant therapy; past and present: Early trials with systemic adjuvant therapy, administered after resection of the melanoma, included non-specific immune stimulants such as BCG (bacillus Calmette Guerin), *Corynebacterium parvum*, levamisole or combinations of these agents with and without chemotherapy with decarbazine (DTIC) revealed no significant impact on the

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disease [3]. In addition, adjuvant vaccines trials were ineffective and sometimes harmful except with autologous melanoma vaccine [4]. High dose postoperative systemic administration of interferon α -2b as an adjuvant therapy for one year did initially show significant improvement in disease-free and overall survival [5]. However, the overall survival benefit was not sustained overtime [6].

The neoadjuvant approach: To utilize patients' own tumor as the source for melanoma-specific antigens, prior to its excision, and to activate the local immune response at the tumor site could overcome such heterogeneity.

In the meantime, two cytokines have shown activity in the management of dermal metastatic melanoma. These included granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-2 (IL-2).

GM-CSF is a multifunctional molecule administered as a single agent in dermal metastases can increase the number and activation of autologous dendritic cells (DCs), T cell infiltrate at the tumor site particularly helper cells and increases the expression of IL-2 receptors (IL-2R) on some T lymphocytes. DCs are very efficient antigen presenting cells (APCs) capable of processing tumor antigens and present the processed antigens by crosstalk to T lymphocytes in the context of major histocompatibility class I and II molecules. DCs are also rich in co-stimulatory factors such as B7-1 and B7-2 which are needed to complete the second immune signal to T lymphocytes which become committed to specific immune response. Its intratumoral administration at doses of 10-80 μ g has shown its biological effects but without major clinical benefit [7,8]. However, when the doses were increased to 400-500 μ g daily for 4-5 consecutive days and repeated every 21-28 days, it has given excellent clinical responses but with some side effects [9,10]. On the other hand, IL-2 is a glycoprotein immune modulator [11]. Its intratumoral administration as a single agent in in-transit metastatic melanoma at doses ranging from 0.6-6.0 million IU, 2-3 times per week or with escalating doses has resulted in complete tumor response (CR) in two thirds of the patients, but with grade I and II toxicity especially at the higher doses [12-14].

To investigate the effect of intratumoral administration of low dose GM-CSF and IL-2 in melanoma lesions, patients with dermal and subdermal lesions were chosen as these lesions were accessible to intratumoral therapy and could be repeatedly inspected, palpated and easily biopsied to confirm the effect of therapy. Low doses of both cytokines were utilized to avoid toxicity. GM-CSF was administered first, and in case of failure to obtain CR, intralesional IL-2 was substituted.

Patients and Methods

Patients with dermal and subdermal metastases, including those with in-transit metastases were initially studied. This was regardless to the extent of the disease, anatomic site of involvement or previous therapy. The study patient did not receive any other anticancer therapy while on the study. None of the lesions were evaluated for their tumor antigenicity or genetic expressions. However, the treated tumor sites had to have pathological confirmation for the presence of melanoma [15].

Each patient received intratumoral low dose of GM-CSF (500 microgram) once/week. Those who failed to respond to GM-CSF therapy were managed by intratumoral therapy with low dose IL-2 (11 million IU) weekly. All these patients were previously treated for their



Figure 1: A photograph of melanoma of the scalp:
A. Before intratumoral therapy: The patient presented with multiple in-transit metastases with unidentified primary site among the metastases.
B. After intratumoral therapy with low dose GM-CSF administered weekly for 4 weeks: Note the complete clinical response, proven pathologically by re-biopsy. The patient had no surgical resection or other treatment and is alive free of disease for over 5 years.

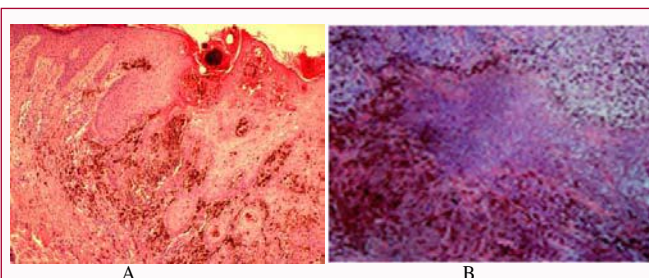


Figure 2: Pathological confirmation of the response:
A. Before intratumoral therapy, from the initial biopsy of the primary site. Please notice the depth of invasion.
B. One week after the administration of neoadjuvant therapy with GM-CSF and IL-2. Notice the complete tumor necrosis and massive histiocytosis at the injection site (primary site). X400, H & E staining.

metastases by various methods including repeated local excisions, intratumoral BCG, and hyperthermic isolated limb perfusion with melphalan, limb infusion, radiation therapy, systemic therapy and combinations of the above.

Among them were two patients who were failure to postoperative systemic adjuvant therapy with higher doses of GM-CSF and IL-2 administered subcutaneously. GM-CSF was given first at 125 μ g/m²/day for 14 consecutive days followed by IL-2 at 9 million IU/m²/day for 4 days, repeated every month for 2 years. They developed in-transit metastases; one within a year of initiating such adjuvant therapy, and the other 3 months after completing 2 years of the adjuvant therapy. Another two patients had distant metastases with palpable subdermal metastatic lymph nodes were included; one had palpable supraclavicular metastases (site of intratumoral therapy) with distant metastases to left iliac lymph node, and the other had palpable metastatic lymph nodes under the skin of the anterior axillary fold with lung metastases. Each received intratumoral therapy at the palpable masses.

A second group of patients consisted of those with invasive primary skin lesions with satellitosis or in-transit metastases and regional lymph node metastases, i.e., stage III disease, but remained to be surgical candidates with very guarded prognosis. Each received preoperative intratumoral sequential injection of 500 μ g GM-CSF at the primary site and at the dermal metastases on day # 1, followed by 11 million IU at the same sites on day #2, just one week before the planned surgical resection.

Results

There was over 50% CR to intratumoral therapy with GM-CSF, an example can be seen in Figure 1. All failures to GM-CSF therapy were successfully treated by intratumoral IL-2. This included the two patients who were previously treated with systemic adjuvant therapy. The other two patients with distant metastases, both responded to intratumoral cytokine therapy at the injection sites. One had CR at the distant metastatic iliac lymph node after receiving intratumoral therapy in the supraclavicular lymph nodes. Some of the treated sites with either cytokine were biopsied 6-8 weeks after cessation of therapy and the histopathological examination revealed no residual tumor cells or mononuclear cell infiltrates.

However, patients with large sclerotic coalesced skin lesions of over 3 cm each but without evidence of metastases failed to show response to either cytokine therapy. Furthermore, some of the previously treated patients who were successfully treated by intratumoral cytokines developed recurrences at 12-22 months in none of the treated sites with intratumoral cytokines.

It seemed that some melanoma lesions did respond to intratumoral administration of GM-CSF while other lesions require IL-2 intratumoral therapy. As each of these 2 cytokines has different mechanism of action that could complement one another, sequential administration of GM-CSF followed by IL-2 could be more effective.

The next group of patients, who were not previously treated and each had stage III melanoma, but remained to be surgical candidates but with very guarded prognosis. Each was managed by neoadjuvant immunotherapy that consisted of intratumoral administration of GM-CSF followed by IL-2 on two consecutive days, one week before the surgical resection. The clinical response could not be assessed because of the local reaction in the short time period of one week between the injection to the surgical resection. However, the histopathological examination of the resected tissues revealed complete tumor necrosis with massive histiocytosis at the injection sites, Figure 2. On the other hand, the enlarged regional lymph nodes harbored metastases. This was not a surprise, because of the short duration between the injections and the resection, after a single course of therapy. These patients were alive and free of disease for over 5 years.

Immunohistochemical studies were performed on the resected tissue, one week after the administration of neoadjuvant therapy, utilizing commercially available antibodies. This showed overexpression of several immune cells that included CD3+ (total T cells), CD8+ (cytotoxic T cells which are the actual tumor killer cells), CD4+ (helper cells) and CD83+ (mature dendritic cells) [16]. Figure 3 shows the effect of neoadjuvant therapy on CD8+ cells at the primary site. In addition, these immune cells were also detected in some of the resected regional lymph nodes, an example of CD8+ cells can be seen in Figure 4. It should be noted that all these immune cells were autologous to each patient. These patients are alive free of disease for over 5 years.

Discussion

It should be realized that these two cytokines have no direct cytotoxic effects on tumor cells. Their function is mediated through the induction and activation of immune cells *in vivo*.

Intratumoral therapy with low dose GM-CSF and IL-2 was safe, i.e., did not cause any tumor dissemination, well tolerated and seemed to be more effective than the more frequent intratumoral

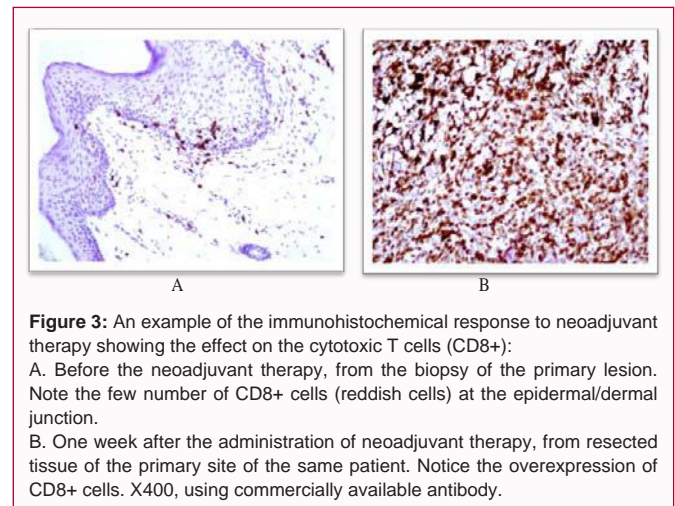


Figure 3: An example of the immunohistochemical response to neoadjuvant therapy showing the effect on the cytotoxic T cells (CD8+):
A. Before the neoadjuvant therapy, from the biopsy of the primary lesion. Note the few number of CD8+ cells (reddish cells) at the epidermal/dermal junction.
B. One week after the administration of neoadjuvant therapy, from resected tissue of the primary site of the same patient. Notice the overexpression of CD8+ cells. X400, using commercially available antibody.

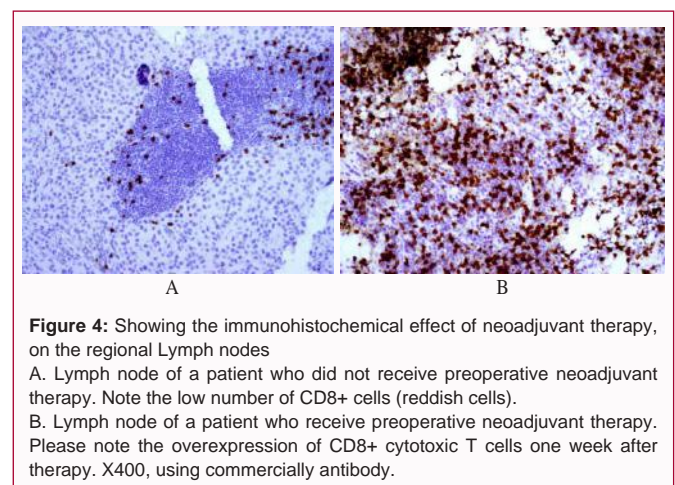


Figure 4: Showing the immunohistochemical effect of neoadjuvant therapy, on the regional Lymph nodes
A. Lymph node of a patient who did not receive preoperative neoadjuvant therapy. Note the low number of CD8+ cells (reddish cells).
B. Lymph node of a patient who receive preoperative neoadjuvant therapy. Please note the overexpression of CD8+ cytotoxic T cells one week after therapy. X400, using commercially antibody.

administration of higher doses of either cytokine. In addition, none of the treated patients had any systemic side effects in the form of fever, chills, fatigue, rash or any significant changes in their CBC or serum chemistry including the LDH. The only side effect was local skin reaction at the injection sites.

The neoadjuvant approach was effective and relatively cheaper than any futuristic adjuvant therapeutic approach such as the use of anti-cytotoxic T lymphocyte associated antigen-4 (anti CTLA-4) or anti-program cell death and its primary legend (anti PD-1 and PDL-1) which showed some survival benefits in patients with metastatic melanoma [17,18]. A recent adjuvant study by the European group proved this point utilizing anti CTLA-4 (Ipilimumab) after surgical resection of stage III melanoma showed some early success but with 48% recurrence rate at a median of 2.7 years, with grade 3 and 4 immune related adverse events that required the discontinuation of the therapy in 52% of the patients [19]. Intratumoral GM-CSF alone resulted in over 50% CR, and failure to secure CR was successfully rescued by IL-2 therapy. This again shows heterogeneity of melanoma. Therefore, it seemed that some melanoma lesions did respond to the activation of dendritic cells by intratumoral GM-CSF, while other lesions required the activation of the cytotoxic T cells by IL-2. Therefore, the sequential administration of both cytokines seemed to be justified.

Intratumoral therapy with these cytokines utilized each patient own tumor as the source for tumor-specific antigens. It was effective

in metastatic lesions as well as in primary invasive melanoma. The only failures were large sclerotic skin lesions, probably due to the large tumor load and the sclerotic nature of the lesions from previous therapy that could not be handled immunologically [15]. Hypothetically, such lesions could be excised followed by cytokines injections at the resection margins for two to three weeks prior to skin grafting.

It was of interest to notice the absence of any residual tumor cells or mononuclear cell infiltrates 6-8 weeks after complete clinical response to intratumoral cytokine therapy. This could indicate an immense autologous antitumor immune response to the therapy with complete washout of the local effects over such period of time. This was further confirmed by histopathological evaluating the resected tissue, one week after preoperative intratumoral administration of both GM-CSF and IL-2. This did clearly show an efficient anti-tumor response within days after the administration of both cytokines at the injection (tumor) sites, as seen in Figure 2. In addition, there was overexpression of various immune cells at the injection sites that included cytotoxic T cells (CD8+) as seen in Figure 3, as well as helper cells (CD4+) and mature dendritic cells (CD83+). Furthermore, these immune cells were also identified in some regional lymph nodes that contained no metastases. An example can be seen in Figure 4. This might suggest that such an immune response was taken-up by the lymphatics and could have possibly eliminated early micro-metastases in a patient with stage IIIC disease. Such findings confirm other reports that the administration of GM-CSF near the biopsy site of primary cutaneous melanoma can increase the number and activation of dendritic cells and tumor-specific cytotoxic T cells in sentinel lymph node [20,21].

The route of the administration of a vaccine can be a critical variable in determining the outcome of an immune response. In an animal model, when a vaccine with naked antigen-encoding RNA is being administered in the skin, subcutaneous tissue or near a lymph node, no significant immune response has been noted. However, when this vaccine is administered in a lymph node, it elicited potent prophylactic and therapeutic antitumor immunity [22]. Therefore, it was no surprise to obtain CR in two patients who failed systemic adjuvant therapy with both cytokines but responded to intratumoral therapy with low doses of the same cytokines.

Furthermore, the two patients with distant metastases who had palpable subdermal metastatic lymph nodes had CR at the injection sites, with one of them had CR in the distant metastatic iliac lymph node after intra-lymphatic cytokine therapy at the supraclavicular metastatic lymph nodes. This could suggest a possible role for intra-lymphatic injection of these cytokines. However, while intralesional therapy could initiate an antitumor immune response in patients with distant metastases, it would need systemic support as the injected sites (source of antigen) did dissolve after the initial intratumoral therapy, and therefore such therapy could not be continued. On the other hand, it could be speculated that patients with limited distant metastases could be treated with intratumoral cytokine therapy utilizing sonographic or CT control.

Recurrences did occur at 12 and 22 months in previously treated patients. This could be due to the development of some tolerance from previous systemic adjuvant therapy with both cytokines, and secondary to the use of immune suppressive therapy by chemotherapy and radiotherapy. Such recurrences could have been managed by repeated intralesional cytokine therapy at the recurrence sites rather

than by surgical excisions.

The durable response in primary melanoma lesions could suggest a role for preoperative intratumoral administration of both cytokines as a neoadjuvant therapy in high risk primary melanoma, and newer prospects of targeted cancer therapy. Furthermore, this study showed a promising anticancer therapeutic strategy which could be reliant on formation of an immune microenvironment at the tumor site, and it may be applicable to other solid malignancies.

This specific autologous approach had its limitation as it should not be used in infected lesions or with allogenic antigens as it may result in an immune deviation. An example can be seen in two reports of active immunization with two vaccines; one with multi-peptides and the other with allogenic whole melanoma cells +3 peptides in combination with GM-CSF that resulted in a negative outcome [23,24]. The authors blamed such negative results on the use of GM-CSF, but the fact is that their antigens that were used, whether peptides or allogenic cells, did not express patients' own tumor antigens and as a result the induced immune response by GM-CSF was directed to the administered antigens.

The response seen with intratumoral therapy with these two cytokines targeted the whole tumor cells that could be regardless to its antigenic or genetic profiles. It can also replace the repeated surgical excision of local recurrences in-transit metastases. It should be pointed out that combined (mixed) administration of these two cytokines has not been tried.

Conclusions

Intratumoral administration of GM-CSF and IL-2 prior to the surgical resection of the melanoma lesions as a Neoadjuvant Immunotherapy is non-toxic and effective in the management of patients with dermal and subdermal metastases as well as in patients who present with high risk primary melanoma. These new findings warrant the initiation of prospective controlled randomized studies.

This approach may be applicable to other small solid malignancies.

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