A role of immunotherapy in Metastatic malignant melanoma

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Running Title: Treatment of malignant melanoma by immunotherapy.

Abstract

Worldwide incidence of malignant melanoma has been constantly increasing during the last years. Surgical excision is effective when primary tumors are thin with thickness of less than 1milli meter and there is no evidence of metastases. At later disease stages patients often succumb, due to failure of metastasis control. Therefore, great efforts have been made to develop improved strategies to treat metastatic melanoma patients. In the search for novel treatments during the last two decades, immunotherapy has occupied a prominent place. Numerous early phase immunotherapy clinical trials, generally involving small numbers of patients each time, have been reported; significant tumor-specific immune responses could often be measured in patients upon treatments. However, clinical responses remain at a dismal low rate. In some cases, objective clinical benefit was more frequently observed among immune responders than immune non-responders. This clearly calls for a better understanding of protective immunity against tumors as well as the cross talk taking place between tumors and the immune system. Here we discuss advances and limitations of specific immunotherapy against metastatic malignant melanoma in the light of the literature.

Key words: Immunotherapy, malignant melanoma, metastasis, T cells

Introduction

The prognosis for patients with metastatic melanoma is poor, with the median survival time of patients with metastatic melanoma is 8.5 months, with an estimated 5-year survival of 6% (1). Melanoma disseminates widely, and frequently involves sites that are unusual in other cancers, such as the GI tract, lung and the skin. Brain metastases are very common, and are associated with a median survival of less than 4 months. Malignant melanoma is the third most common cause of brain metastasis behind lung and breast cancer, with approximately 10,000 patients diagnosed yearly in the United States (2). While the incidence of brain metastasis in patients with melanoma is 9.6%, melanoma has the highest propensity to metastasize to the brain of all primary neoplasms in adults (3). Nearly 37% of patients with stage IV melanoma eventually develop clinically apparent brain metastasis, and autopsy series report the prevalence of brain metastasis at 55% to 75% of patients who died of melanoma (3-6).

Until recently, no treatment has been demonstrated to improve median survival in metastatic melanoma, and response rates to systemic therapies are low. Despite these grim statistics, there are occasional spontaneous regressions, and some long-term survivors, including up to 15% of patients with metastases to skin, subcutaneous sites and lymph nodes (7,8). A small and highly selected group of patients with advanced melanoma may achieve prolonged freedom from relapse after surgical resection of metastases. The patients that appear most likely to benefit are those with a solitary metastasis involving skin, lungs, distant lymph nodes, or the gastrointestinal tract, those with a long disease-free interval, and those in whom the metastatic focus can be completely resected. In these patients, 5-year survival rates of 4–35% have been reported (9-13).

Melanoma is refractory to most standard cytotoxic agents. Objective response rates to single-agent chemotherapy are in the range of 10–23%, and are typically of brief duration. Response rates to combination chemotherapy are somewhat higher, but toxicity is increased with the use of multiple agents, and no survival advantage has been demonstrated. Dacarbazine (DTIC) has been considered a standard treatment, and temozolomide, an oral methylating agent, is commonly used.

Several lines of evidence suggest that melanoma is immunogenic. Lymphocytic infiltration and regression of primary cutaneous melanoma is common. The concept of immunotherapy is based on the body's natural defense system, which protects the individual against a variety of diseases. Although we are less aware of it, the immune system also works to aid our recovery from many illnesses. For many years, physicians believed that the immune system was effective only in combating infectious diseases caused by such invading agents as bacteria and viruses. Growing evidence indicates that the immune system may play a central role in protecting the body against cancer and in combating cancer that has already developed. This latter role is not well understood, but there is evidence that in many cancer patients the immune system slows down the growth and spread of tumors.

Current approaches to cancer immunotherapy such as vaccine administration, oncolytic viruses, adoptive cell therapies (ACT), and cytokine administration are aimed at stimulating T cells to recognize cancer antigens and develop effector mechanisms that can destroy cancer cells. Given that many cancer antigens are derived from non mutated proteins, it may be necessary to break tolerance of T cells to self-antigens in an effort to unleash their antitumor properties.

Immunotherapy

Immunotherapy is a type of cancer treatment that targets specific molecules of the body's own immune system in order to disrupt the growth of cancer cells. It is frequently contrasted to chemotherapy, which interferes with all rapidly dividing cells, cancerous or not. For this reason, immunotherapy usually has fewer side effects than chemotherapy. The details of various immunotherapies available and their mechanism of action are described below.

1. **Anti–Cytotoxic T-Lymphocyte Antigen-4 antibody**

T-cell activation requires at least two signals. The interaction between the T-cell receptor and the specific antigen presented by a major histocompatibility complex (MHC) molecule on an antigen presenting cell (APC) , such as a dendritic cell (DC), conveys the first signal to the T cell (14). Engagement of co-

receptors on the surface of the T cell can either enhance or inhibit the T cell response. Failure of the T cell to receive a second signal can lead to anergy (15, 16). Thus second signals fine-tune the immune response.

The most well-studied T-cell receptors for co-stimulatory molecules are Cluster of differentiation 28 (CD28) and Cytotoxic T-lymphocyte Antigen 4 (CTLA-4), also called CD152 that react with ligands on the APC, CD80 and CD86 (also named B7-1 and B7-2, respectively) (17-19). CD28 and CTLA-4 molecules function antagonistically (20-21). Engagement by CD28 enhances T-cell activation, proliferation, and interleukin-2 (IL-2) production (22). CTLA-4 also binds to CD80 and CD86, but with greater affinity than it binds to CD28 (23), and antagonizes T-cell activation by interfering with IL-2 secretion and IL-2 receptor expression, and by inhibiting the expression of critical cell cycle components (24-27). CTLA-4 is not found on the surface of most resting T cells, but is up regulated transiently after T-cell activation (28-29). Few monoclonal antibodies target the inhibitory CTLA-4 resulting in T cell activation and tumor regression.

Ipilimumab is a monoclonal antibody that targets CTLA-4. Treatment with Ipilimumab (http://www.clinicaltrials.gov/ct2/results?term=ipilimumab) significantly increases median overall survival in both previously untreated and previously treated patients with metastatic or unresectable melanoma. Responses in patients treated with Ipilimumab may develop slowly and patients may have a transient worsening of disease before disease stabilizes or tumor regresses.

2. Cytokine administration

Cytokines are cellular products with the capacity to alter target cells functions in an autocrine, paracrine or endocrine manner. Many individual cytokines are produced by several different cell types like IL-1 that is produced by any leukocytes, endothelial cells or fibroblasts. These are often pleotropic, so they may act on many cell types and mediate many effects. These are also redundant and may stimulate the same or overlapping biological responses. They mediate their effects by binding to receptors on their target cells. These can be functionally classified into cytokines that medicate innate/natural immunity, cytokines that regulate lymphocyte growth, activation and differentiation, cytokines that activate inflammatory cells, chemokines and cytokines that stimulate hematopoiesis (30).

Interleukin2 is a T cell growth factor and also cause stimulation of B cells, NK cells and mononuclear phagocytes. It induces the lymphokine activated killer cell phenomenon in which lymphocytes are activated to kill tumor cell in antigen independent manner. This property of IL-2 is used pharmacologically to treat malignant melanoma. Interferon-alpha (IFN-α) is also known to have powerful effects on immune tumor cells, including enhancing NK cell tumor cytotoxicity (31), DC maturation, T-helper1 (Th1) skewing, enhancement of T cell survival, inducing immunological memory (32), and inhibiting the invasive ability of cancer cells (33). Therapeutic strategies using IFN-α in melanoma patients with leptomeningeal disease have included the direct intraventricular administration of IFN-α, which resulted in the clearance of malignant cells in the CSF (34) but was confounded by significant and sustained neurotoxicity (35).

3. Adoptive cell therapy:

Adoptive cell therapy (ACT) is with autologous antitumor lymphocytes infusion following a lymphodepleting preparative conditioning regimen. ACT has the potential to enhance antitumor and overall immunity.

Conditioning regimen

Conditioning regimens, also referred to as preparative regimens, are combinations of chemotherapy or radiation therapy designed to prepare the patient's body to receive the donor's bone marrow. The purpose of the conditioning regimen varies according to the type of stem cell transplant to be received by the patient (36). For autologous transplants, the conditioning regimen is designed so that increasing doses of chemotherapy with or without irradiation are administered to destroy more malignant cells. Usually for malignant melanoma, the regimen consists of chemotherapy with cyclophosphamide (60 mg/kg/d on days 7 and 6) and fludarabine (25 mg/m2/d on days 5 to 1). In some protocols, additional lymphodepletion with radiation may be added (2, 6, or 12 Gy) at 1 to 3 days immediately before cell transfer. Platelet counts will be generally maintained by transfusion at ≥20,000/μL for patients with known brain metastases. Patients were re-evaluated at all known tumor sites including the brain approximately 4 to 6 weeks after the initiation of treatment and subsequently at 4- to 8-week intervals (36).

Cell products used for therapy

Patients receive an autologous lymphocyte product manufactured in the noted Cell Production branch facility that has passed a protocol-specific certificate of analysis including potency, safety, and sterility criteria (37). Improved molecular biology techniques have also increased enthusiasm and feasibility for testing genetically engineered T cells**.** Usually the cell products used for therapy are tumor-infiltrating lymphocytes, Cytotoxic T lymphocytes (CTLs), Th cells, and Tregs. Infusion of autologous tumor-infiltrating lymphocytes or autologous peripheral blood lymphocytes retrovirally transduced to express a T-cell receptor for thereapeitic action. However the major challenge facing the field at present is to conduct randomized clinical trials demonstrating sufficient clinical benefit to justify the logistics and expense of customized cellular therapies.

In malignat melanoma tumorinfiltrating lymphocytes fail to destroy the tumor. Among various possible reasons for this occurrence are insufficient lymphocyte numbers, insufficient activation status, the existence of regulatory T cells which suppress the cytotoxic effect of TIL, and modulation of tumor and histocompatibility antigens (36). As a result, a new method to generate a very large number of activated, specifica cally reactive TIL has been developed (36). The patients receive highly potent TIL which, due to lymphodepletion, persist in the blood at > 70% of the total lymphocyte population for many months after transfer. Thus, the combination of anti-tumor activity of the T cells and the modified immunologic environment contribute to the effectiveness of the treatment (36).

4. Vaccine therapy and oncolytic viruses

Melanoma is an important model cancer for the development of cancer vaccines because of early identification of tumor-related antigens and the level of knowledge about their interaction with the immune system. The vaccine types that will be discussed here are multivalent cell culture-derived vaccines, autologous melanoma cell vaccines, peptide, DNA and DC vaccines. These are the major types of vaccines currently in use or under study.

Multivalent cell-culture derived vaccines are created by processing a number of different melanoma cell lines grown in vitro. These are non-autologous cell vaccines and the use of multiple cell lines helps ensure that at least some of the antigens in the vaccines are shared by the patient's own tumor. Autologous cell vaccines are prepared by harvesting melanoma cells from the individual patient to be treated. Autologous cell vaccines come in at least two general variants, killed cell vaccines and recombinant autologous cell vaccines. Killed cell vaccines require less in vitro manipulation than recombinant autologous cell vaccines. Melanoma tissue is harvested from the patient, processed, and then re-injected with an appropriate adjuvant (38). Recombinant DNA techniques can be used to alter autologous melanoma cell vaccines in ways that boost immune responses (39).

Peptide vaccines are based on peptide epitopes. The peptide response epitopes used in melanoma vaccines are amino acid subsequences from tumor-derived proteins that have been the target of successful immune responses in other patients (40). These epitopes have been determined through tedious efforts to sequence peptides displayed on MHC I receptors mediating responses in tumor infiltrating lymphocytes (TIL) for a schematic of a peptide response epitope presented on the MHC I receptors of tumor cells. Peptide response epitopes are potentially a more efficient means of immunization, since only the most immunogenic portions of tumor marker proteins are used for immunization. Naked DNA vaccines are one of the newest categories of melanoma vaccines (42). These differ from recombinant DNA vaccines in that the transfection of exogenous DNA occurs in vivo rather than in vitro, and no autologous tumor cells are required.

Lack of efficient tumor antigen presentation in DC in cancer patients has led to the use of DC based vaccines (41). DCs are collected from the blood of the patient by a process called leukapheresis and "loaded" with tumor antigens from the patient's own tumor cells. These DCs are then reintroduced into the patient and stimulate the immune system. The tumor antigens are taken up by DC, they are processed and presented to the T cells along with the appropriate co-stimulatory signal. Once activated by the DCs the cytotoxic T cells recognize and destroy the tumor cells expressing the tumor antigen (42).

Numerous viruses from a diverse range of virus families are being identified for use as oncolytic virotherapy agents. Many RNA viruses are displaying great promise in the field of oncolytic virotherapy. The underlying principle of oncolytic virotherapy is that the specificity of lytic viral infection can be harnessed to destroy malignant cells selectively, whilst leaving normal host cells intact. Coxsackievirus A21 (CVA21) can selectively infect and destroy in vitro cultures of malignant melanoma cells that characteristically over-express intercellular adhesion molecule-1 (ICAM-1) and/or decay accelerating factor (DAF) (43,44). Apart from this, there are may be a few other virus types that could be useful in oncolytic virotherapy.

OncoVEX (GM-CSF) is a second generation oncolytic virus. The virus consists of the herpes simplex type 1 virus backbone carrying the gene encoding human granulocyte macrophage-colony stimulating factor (GM-CSF), a potent immune stimulator. The herpes simplex viral genome has been modified to delete two herpes genes, ICP 34.5 and ICP47 making it non-pathogenic to normal cells and capable of selectively targeting and replicating in tumour cells, destroying them in the process. OncoVex (GM-CSF) may induce local tumour necrosis and inflammation, recruit antigen presenting cells and induce amplification of a systemic immune response. In phase III trials OncoVEX (GM-CSF) has been administered via intratumoural (IT) injection at up to 4ml of 108 pfu/ml per injection, every two weeks for up to one year (45).

5. A Novel Inhibitor of STAT3 Activation

Activation of the signal transducer and activator of transcription 3 (STAT3) has been identified as a key transcription factor that drives the fundamental components of melanoma tumorigenesis and metastasis (45). Growth factors and cytokines, including IL-6, activate Jak2 and activate STAT3 by phosphorylation of the tyrosine residue (Tyr705) in the STAT3 transactivation domain (p-STAT3) resulting in translocation into the nucleus and the expression of a variety of target genes. STAT3 is frequently over activated in most

cancers, including melanoma, and propagates tumorigenesis by preventing apoptosis (by increasing survivin, BCL-XL, and MCL1 expression) and enhancing proliferation (by increasing c-Myc and cyclin D1/D2 expression), angiogenesis (by increasing vascular endothelial growth factor [VEGF] and hypoxia-inducible factor [HIF]-1α expression), invasion (by increasing matrix metalloproteinase [MMP]-2 and MMP-9 expression), and metastasis (46,47).

The induction of the p-STAT3 pathway within the CNS may be particularly relevant because reactive astrocytes are a major inducible source of IL-6 (48) and these cells are often seen in direct contact with tumor metastasis (49). Furthermore, tissue microarray studies of human melanoma brain metastases have demonstrated higher levels of p-STAT3 in brain metastasis specimens compared with parenchymal tumors (45). Thus, the p-STAT3 pathway is a relevant therapeutic target for CNS melanoma metastasis. In addition to the direct tumorigenic properties of the STAT3 pathway, STAT3 is also a key regulator of immunosuppression in patients with cancer (50). The signaling of STAT3 is upregulated within the various immune cell populations upon becoming associated with the cancer microenvironment (50). In mice that had ablation of STAT3 in only the hematopoietic cells there was marked anti-tumor clearance by the immune system (51). Induced p-STAT3 has divergent functions within the various immune cell populations but the overall result is to shift the balance of cytokine production from IL-12, which activates T cells and NK cells, to IL-23, which activates regulatory T cells (52-54). For example, STAT3 activity in DCs reduces the expression of MHC II, CD80, CD86, and IL-12 in these cells, rendering them unable to stimulate T cells and generate antitumor immunity (14).

Additionally, the activation of STAT3 in macrophages, CNS microglia, and NK cells suppresses their activation and function (55-58). However, within immune suppressive cells, induced p-STAT3 enhances their functional activity. Specifically, STAT3 has been shown to be required for both TGF-β and IL-10 production by CD4+ T cells (59), factors necessary for the generation of tumor-associated Tregs and STAT3 binds to the first intron of the FoxP3 gene (60). Thus, STAT3 seems to be a key molecular hub for inhibiting immune surveillance and clearance of malignancy. So there is a hypothesis that the addition of a STAT3 inhibitor would enhance the therapeutic efficacy of other immunotherapies.

Immune related side effects

Treatment with immunotherapy is associated with autoimmune manifestations such as dermatitis, enterocolitis, hepatitis, uveitis, and hypophysitis. There was a strong correlation between the induction of tumor regression and grade 3/4 autoimmune toxicity (61). Histopathologic analysis of the sites involved in the toxicities of treatment often showed inflammatory changes that were difficult to distinguish from autoimmune changes.

Inflammatory cytokines induce a behavioral syndrome, known as sickness behavior that strongly resembles symptoms typically seen in depression. This resemblance has led to the theory that an imbalance of inflammatory cytokine activity may be a contributing factor in depressive disorders. Support for this is found in multiple lines of evidence, such as the effects of cytokines on the activities of the hypothalamic-pituitary-adrenal axis, serotonin and brain-derived neurotrophic factor, and hippocampal function, all of which are implicated in the etiology of depression. In addition, associations between inflammatory activity and depressive symptomology have been documented in a number of studies, and the depressogenic effects of cytokine therapy are well known. Accordingly, given that depression has a substantial genetic basis, genes involved in the regulation of inflammatory cytokine activity are strong candidates for involvement in genetic susceptibility to depressive disorders. (62)

Inflammation plays a role in neurological and psychiatric disorders (63) Inflammation and immune signaling can also have adverse affects in the brain leading to exacerbation of sickness and development of symptoms of depression in vulnerable individuals (64), this may be the reason for the increased prevalence of depression in the physically ill individuals. Cancer or hepatitis C patients who were treated with pro-inflammatory cytokines show depressive symptoms (65,66). The details of various immune related side effects in each system associated with immunotherapy in general are provided in table 1.

Discussion

In recent decades immunologic therapies have become increasingly important in the treatment of melanoma. This applies not only to nonspecific immune stimulation with, for example, IFNs, but especially for novel vaccines or antibody mediated therapies (e.g., anti-CTLA-4). The management of these patients is complicated by the need to treat both central nervous system (CNS) metastases as well as other systemic diseases. Interleukin-2 (IL-2) is a central regulator of the cellular immune response, inducing activation and proliferation of T-cells and NK-cells. The objective response rate to high-dose IL-2 was 16% and the complete response rate was 6% in an analysis of 270 patients (67). IL-2 has been evaluated in various contexts: with adoptive cellular immunotherapy using tumor-infiltrating lymphocytes, with other cytokines, and with chemotherapy agents and vaccines. However, to date, none of these approaches has demonstrated clear superiority compared to IL-2 as a single agent.

Ipilimumab, an anti-CTLA-4 antibody has recently been reported to cause the complete response (CR) of untreated melanoma brain metastases in two patients (68,69). A phase II trial that investigated the treatment of melanoma brain metastases with Ipilimumab reported that of 51 patients with melanoma brain metastases, 5 developed PR in the brain (70). In a study 26 patients received ACT with TIL. Seven of these patients (41%) achieved a CR in the brain, and six patients achieved an overall PR. In the nine patients that received T Cell Receptors (TCR)-transduced lymphocytes, two patients achieved a CR in the brain (22%) and one of these two achieved an overall partial response (PR) (36). Lymphodepletion with cyclophosphamide and fludarabine, followed by adoptive transfer of tumor-infiltrating lymphocytes with interleukin-2, was associated with a 51% response rate in 35 patients who had progressed after treatment with IL-2 alone (71).

However, melanoma continues to be a paradigm in the understanding of interactions between the immune system and developing metastatic tumours. Rapid progress continues to be made fuelled by novel insights derived from both mouse models and the detailed analysis of patients. Recent research has begun to elucidate the genetic abnormalities underlying dysregulated growth, resistance to apoptosis, invasion, and metastasis in melanoma. Substantial molecular heterogeneity has been discovered among melanomas, but a

number of mutations occur with high frequency and appear to be critical for survival and growth. Local immune tolerance appears to be the dominant outcome of tumor-host interactions. Thus, for immunotherapy to reach significant clinical efficacy, novel combinations including vaccination and appropriately tumor targeted immune modulating compounds remain to be identified and optimized (72).

Conclusion

Immunotherapy has assumed increasing importance in the therapy of malignant melanoma. The main reason is the high immunogenicity of the tumor itself, so that an immune response against the tumor often exists even without immune stimulation. The goal of modern immunotherapeutic approaches is to augment these anti-tumoral immune reactions to fight the tumor. This overview summarizes the various immnotherapies available for therapy of malignant melanoma.

List of Abbreviations

Adoptive cell therapy (ACT), Antigen presenting cells (APC), Cerebrospinal Fluid (CSF), Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4), complete response (CR), Partial response (PR), Dendritic cells (DCs), Interferon (IFN), Interleukin (IL), Janus kinase 2 (JAK2), Natural Killer (NK), Matrix metalloproteinase (MMP), Major histocompatibility complex (MHC), Stereotactic radio surgery (SRS), T cell receptors (TCR), Tumor infiltrating lymphocytes (TIL), whole brain radiotherapy (WBRT), Intercellular adhesion molecule(ICAM1), Granulocyte macrophage-colony stimulating factor (GM-CSF)

Conflict of Interest

This paper has been written without external financial funding. There is no conflict of interest.

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Table 1. Immune-related adverse events

Body System	**Adverse Event**
Blood and Lymphatic System Disorders	Aplasia pure red cell
Endocrine Disorders	Adrenal insufficiency, autoimmune hypophysitis/hypopituitarism, hypothyroidism, hypogonadism , hyperthyroidism, Graves disease, thyroiditis (autoimmune).
Eye Disorders	Conjunctivitis, episcleritis, ocular inflammation, retinal pigment changes, uveitis, iritis
Gastrointestinal Disorders	Colitis (including ulcerative and haemorrhagic), intestinal perforation, diarrhea (including haemorrhagic), duodenitis, enteritis, esophagitis,

	ileitis, pancreatitis, stomatitis
Hepatobiliary Disorders	Autoimmune hepatitis
Musculoskeletal and Connective Tissue Disorders	Arthritis/arthralgia, myasthenia gravis, polymyositis
Nervous System Disorders	Guillain-Barre syndrome
Renal and Urinary Disorders	Granulomatous tubulointerstitial nephritis, nephritis (autoimmune)
Skin and Subcutaneous Tissue Disorders	Alopecia, pruritus, rash/desquamation, erythema multiforme, Steven-Johnson syndrome, toxic epidermal necrolysis, vitiligo, urticaria,
Tumor	Pseudo progression or flare of underlying melanoma

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