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STRATEGIES TO OVERCOME OBSTACLES TO SUCCESSFUL IMMUNOTHERAPY OF MELANOMA

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Summary

The immunogenicity of Malignant Melanomas has been recognized by the observed recruitment of tumor-specific cytotoxic T-cells (CTL), leading to the identification of several melanoma associated antigen (MAA). However, numerous strategies to treat melanoma with immunotherapy have resulted in only partial success.

In this review, we discuss recent data related to the ability of tumors to elude immune responses. In response, we discuss different strategies to induce a clinically effective immune response. These approaches include 1) immunostimulation: including peptide/protein based vaccines, dendritic cell vaccines, and adoptive cell transfer; and 2) overcoming immunosuppression: including targeting of checkpoint molecules such as CTLA-4, circumventing the activity of Tregs, and assuring antigen expression by tumor cells (thwarting antigen silencing). Finally, we discuss recent advances in gene therapy, including adoptive therapy with engineered TCRs.

These issues lead to the conclusion that successful immunotherapy in malignant melanoma will require a combination of strategies aimed at both inducing immunostimulation and blocking immunosuppression.

Keywords

Malignant melanoma; immunotherapy; cancer vaccines; gene therapy

INTRODUCTION

Melanoma, a highly malignant tumor of the pigmented cells, is a significant worldwide health concern. The 5-year survival for patients with involvement of regional lymph nodes is 35%, with fewer than 2% of patients with visceral metastatic disease alive at 5 years. Therapy with interferon alpha-2b (IFNalpha-2b), the only agent approved in the United States for adjuvant use in high-risk melanoma patients, has not shown consistent overall survival benefit in randomized trials and is associated with considerable toxicity. Conventional chemotherapy for unresectable metastatic disease does not improve survival, while patients with completely resected metastatic disease have a median survival of <20

months. Since there is clearly a need for more effective therapies in these high-risk patients and because steps towards new effective therapies against melanoma are currently being made, we will focus on melanoma therapies to highlight the new immunotherapy strategies.

While there is good reason to question the effectiveness of the host response against spontaneously arising tumors, there is clear evidence that specific immune cells are recruited to tumor sites. In fact, lymphocytic infiltration of tumors was first observed by Rudolf Virchow in 1863, before the full implications of lymphocyte function were recognized. The past 30 years have accumulated considerable evidence that many tumors elicit a significant immune response, and a more favorable prognosis is correlated with the intensity of tumor-infiltrating lymphocytes (TIL) (1). The relationship between the tumor and the infiltrating immune cells, including the finding of skewed tumor-specific TCR expression (2), has been instrumental in the identification of several melanoma associated antigens (3). Moreover, the demonstration of tumor-specific immune responses has raised considerable hope for the development of potent therapeutic tools against melanoma (4). However, it is clear that multiple obstacles remain. The fact that no single therapy has emerged in melanoma signifies the need to overcome the remaining impediments to clinical success.

The demonstration that melanomas contain tumor-specific T cells has led to the propagation and cloning of T-cell lines with potent ex-vivo CTL activity against autologous tumor cells. However, these cells inevitably come from clinically apparent tumors that frequently continue to grow despite the immune response. In this review, among the issues we shall try to address is the paradox, that the presence of significant numbers of tumor-specific TIL is insufficient to prevent tumor progression (5,6). Several mechanisms have been elucidated to explain the relative impotency of the immune response *in vivo*. The host-tumor interactions are complex and include regulatory activities that undermine the function of immune cells as well as down-regulation of target antigens on the tumors, including loss of both differentiation antigens (7) and antigen-presenting molecules (8,9). Both of these observations can help to explain the inability of tumor vaccines and ex-vivo expanded TIL to eradicate melanomas, even in cases where significant tumor reduction can be achieved by some of the more aggressive immunotherapy regimes. Even in protocols that have achieved a clinical response rate of more than 35%, complete curative responses have been achieved in a small subset of patients. Unfortunately, we do not have a clear understanding of what elements are critical to the success of therapy in responding patients. Even the frequency of specific T cells does not correlate with clinical responses as some responding patients have low frequencies of tumor-specific T cells (10), while high frequencies of specific T-cells have been detected in patients with progressing tumors (11). In this review we will attempt to identify some of the salient obstacles as well as the progress that has been made in addressing how best to implement successful immunotherapy.

1. BARRIERS TO EFFECTIVE IMMUNITY

Ineffective T Cell Signaling and Immune Inhibitory Activity Withing Tumors

When T cells interact with tumors there may be several potentially inhibitory signals generated, including lack of proper co-stimulatory activity by tumor cells and induction of immunosuppressive Tregs. Among the problems achieving effective immunity against tumor antigens is the relatively poor accessory cell function of most tumors. It has become clear that most melanoma cells are deficient in the expression of B7 receptors that are vital co-stimulants for T cell activation. Absence of B7-1 (CD80) and B7-2 (CD86) favors the escape of melanoma from immunosurveillance, while in pro-inflammatory situations B7-1 and B7-2 expression enhance T-cell-mediated anti-tumor immunity. When CTLA4 binds to CD80 and CD86 with higher affinity than CD28 it delivers an inhibitory signal for T-cell

activation. This observation has led to the proposition that blocking CTLA4 would be beneficial in treating malignancies.

Recent evidence has attracted interest in the role of regulatory T-cells (Treg), which are an immunosuppressive subset of CD4⁺ T cells expressing high levels of CD25 (IL-2R alpha), and FOXP3. Treg can inhibit the activity of cytotoxic T cells in a wide variety of tumor types (12).

Together, these regulatory events have played a role in hampering many immunotherapy strategies. However, as we will address further, understanding these barriers to immunity has also provided potential strategies for overcoming these obstacles, and now offers several points of attack that are being implemented in both animal and human trials.

Antigen Silencing

The key to specific immune responses is a specific molecular recognition process. Even if immune activation and selection have produced highly specific cytotoxic T cells uncompromised by auto-regulation, effector cells are impotent if the target antigens are not available (reviewed in (13)). Until recently, this elementary fact has been little heeded, despite abundant evidence that 'immunoselection' or 'immunoediting' are clinically relevant (14).

It has been demonstrated that the expression of melanocyte differentiation antigens in melanomas is heterogeneous, but that immune escape variants can be re-induced to express target antigens. Importantly, our studies have indicated that that antigen loss is rarely the result of genetic loss or mutation, but is more frequently the result of gene regulatory events. We discovered that melanoma cell lines produce factors that act to reversibly down-regulate the expression of their own melanocyte lineage antigens. For example, the silencing of Melan-A/MART-1 expression is mediated by soluble factors that act both on syngeneic and allogeneic melanoma cell lines through down-regulation of the gene promoter (15). The down modulation of Melan-A/MART-1 expression is accompanied by a loss of recognition CTL for the immuno-dominant peptide of Melan-A/MART-1 (peptide 27-35). It appears that a series of melanocyte differentiation antigens, including gp100 and tyrosinase, are simultaneously lost and restored, often together with the melanocyte master regulatory transcription factor, MITF-M, that can all be induced by several agents, including IFN-beta (16) and some MAP kinase pathway inhibitors (14). Of note, IFN-beta is already approved for human clinical use in other contexts, making it an excellent candidate as a co-treatment for augmenting tumor antigen expression during immunotherapy.

In addition to loss of differentiation antigens, such as Melan-A/MART-1, gp100 and tyrosinase, tumors also can lose MHC antigen as well as antigen-processing molecules and function, preventing the display of autologous antigens on the cell surface. In any event, if the antigen, or its presenting HLA molecule are not available for recognition at the cell surface, all the T cells the body can muster will be incapable of recognizing and destroying the tumor target cells. As for differentiation, it has been shown that most MHC antigen loss is also reversible by agents such as IFN-gamma (17) indicating that gene regulatory events are involved in expression of these molecules as well.

2. IMMUNOTHERAPY OF MELANOMA

In the past two decades the number of identified melanoma-associated antigens has increased rapidly, and there is ample evidence that many tumor structures are immunogenic (18). Indeed, a growing number of melanoma-associated antigens are known targets for T cell-mediated cytotoxicity. Thus, although there is no identified clonal marker in melanoma,

there are lineage-specific markers, that have limited non-tumor expression. The cellular immune response against melanomas is often characterized by the accumulation of tumor infiltrating lymphocytes (TIL), which include tumor-specific cytotoxic T lymphocytes (CTL) (5,6). In some patients, TIL show strong *in vitro* lytic activity that is often directed against targets expressing HLA-A2 and the immunodominant peptide Melan-A/MART-1 (peptide 27-35). However, as we have noted above, the presence of tumor-specific CTL within the tumor does not prevent tumor progression, although the better clinical prognosis associated with the presence of lymphocytes suggests that they might slow the growth of the tumor. Clearly, there remain numerous obstacles to the successful elimination of clinically-apparent tumors.

2a. IMMUNOSTIMULATION

Principally, there are two distinct approaches to exploiting the immune response to treat tumors. These involve active immunotherapy (or vaccination), which may include blocking of inhibitory pathways, and adoptive therapy including cell transfer, as detailed below.

1) Active immunotherapy—There are two main strategies in vogue for enhancing immunity to tumor antigens. The simpler involves administration of peptide/ protein based vaccines (with adjuvants, and cytokines) The second approach utilizes whole cells, or cell extracts instead of defined antigens. An additional strategy utilizes antigen-primed dendritic cells to enhance presentation of the vaccine.

Several melanoma-associated antigens, (MAA) including Melan-A/MART-1, gp100, and tyrosinase, as well as cancer-testis antigens such as MAGE-3 and NY-ESO-1 have been used as vaccines, either individually or in combination. The use of defined peptide vaccines and other non-cellular vaccines avoids the need for cell culture, and several studies have shown induction of both cellular immunity and antibodies to these vaccine antigens. However, the clinical efficacy of such vaccines has been limited. A detailed discussion of these trials is beyond the aim of the present paper and excellent reviews are available on this issue (19,20). It would appear, however, that the vaccines alone will not be useful for treatment of advanced disease, although they could play a role in therapies for early stage disease, and may prove useful in combination with other approaches. Most studies were not limited to the administration of peptides, but include the use of cytokines such as IL-2, IL7, IL-15 and GM-CSF to improve immune responses .

Dendritic Cell Vaccines: Dendritic cells, the most potent presenters of antigen, have been used to improve immunization in several different contexts. When antigen is introduced to a patient, dendritic cells will take-up, process, and present that antigen to lymphocytes: the dendritic cell is the final common pathway for the generation of tumor-specific immunity through the interaction of antigen-loaded MHC class I and II molecules with TCRs on CD8 and CD4 T cells. Thus, vaccination of melanoma with peptide- or tumor lysate pulsed dendritic cells has been implemented (21). This technique has the disadvantage of requiring harvest and isolation of autologous cells, involving leukopheresis and *ex-vivo* cultures. As with other vaccination strategies, dendritic cell vaccination has been shown to induce measurable tumor-specific immunity, but the rate of objective clinical responses has been disappointing, showing only about 7% responders (22).

2) Adoptive cell transfer—*Ex-vivo* selection and expansion of adoptively transferred cells has been extensively used in the treatment of immunodeficiencies and malignancies. Indeed, graft versus tumor activity may help in achieving successful remissions of cancers following immune reconstitution.

The transfer of immune cells provides a means to provide anti-tumor effector cells that are first manipulated *ex vivo* and subsequently administered with or without added cytokines to improve their *in vivo* survival and expansion (20). Most of these protocols require sophisticated infrastructures for each patient due to the need for extensive cell culturing. Furthermore, *in vitro* expansion leads to considerable variations in the results between clinical trials and among patients within a single trial.

Despite these difficulties, the clinical efficacy of these adoptive protocols show the most dramatic responses, and the obstacles to implementing them have not been insurmountable at large research-oriented facilities. Importantly, a recent strategy for providing large numbers of specific lymphocytes for adoptive transfer may overcome at least some of the variables in cellular therapies, as it is now possible to transfer specific T cell receptors using retroviral vectors, permitting the transfer of very large numbers of cells with much less *in vitro* manipulation. These newer protocols are still preliminary, but do provide a potentially important therapeutic modality.

With respect to adoptive immunotherapy of melanoma, some of the earliest studies involved the use of *in vitro* activated LAK cells (Lymphokine Activated Killer Cells). LAK cell therapy received intensive scrutiny when it was found that high levels of Interleukin 2 (IL-2) could stimulate broadly reactive anti-tumor cytotoxic activity in blood lymphocytes. Some dramatic results were achieved with LAK cells and *in vivo* infusion of IL-2, although a similar clinical response could be achieved using the IL-2 alone. Indeed, IL-2 infusion is still one of the first lines of therapy for advanced melanoma (18).

The use of activated blood cells has more recently included the propagation of antigen-specific CTLs to treat patients with refractory, metastatic melanoma. Unfortunately, the use of specific CTL can result in the selection of a tumor variant with loss of target antigens such as Melan-A/MART-1. On the positive side, survival and tumor localization of melanoma specific CTL has been shown (23), but the immunoselection noted emphasizes both the potential and the limitation in specific immunotherapy. When the target antigen is not essential for the malignant behavior of the tumor, elimination of antigen-bearing cells can render the immune response impotent, although killing of some tumor cells may induce immune responses to additional antigens not originally targeted in a process referred to as “epitope spreading.” (24).

Dramatic success at eradicating widely metastatic disease has been achieved using a combination of lympho-depleting chemotherapy followed by the adoptive transfer of autologous tumor reactive lymphocytes. These lympho-depleting drugs have multiple effects that seem to promote the anti-tumor activities of the adoptively transferred T cells. Among these effects are the killing of host Tregs that suppress immune responses (12).

In the largest study published to date, 18 (51%) of 35 treated patients experienced objective clinical responses, including three ongoing complete responses and 15 partial responses with a mean duration of 11.5 +/- 2.2 months (25). Higher response rates have been reported in more recent studies using aggressive pre-transfer chemotherapy, with clinical responses reaching as high as 70% (Yang, J reported at ISBTC meeting Boston, Nov. 2007). However, as impressive as these results are, only about half of the patients who are enrolled in this protocol as “intent to treat” were actually able to complete the protocol as planned, meaning that true response rates are closer to 35%, and the complete responders were again a small subset of these clinical responders.

3b. BLOCKING IMMUNOSUPPRESSION

The induction and manifestation of effective immunity against autologous cancers involves complex biological processes. As our understanding of the molecular and cellular components of the immune response has expanded, so have the various strategies for overcoming the obstacles to successful immunotherapy. One recent strategy involves CTLA4-blockade to reinforce cellular immunity against a variety of tumors by altering the balance of effector and regulatory mechanisms (26). While some dramatic clinical responses have been achieved, Attia et al showed only 2/56 complete remissions (CR) and 5/56 partial remissions (PR), an overall response of less than 15%. Significantly, these responses are strictly associated with the development of sometimes serious adverse auto-reactive reactions (27). In a recent study by Downey et al, only 3/159 CR were seen, with an accompanying 62% toxicity (28). Better results were reported when CTLA-4 blockade was combined with IL-2 administration (29), but a recent review (30) summarizes the overall relatively disappointing results obtained using this approach.

Targeting Tregs—Because of the role of Tregs in undermining the cellular response to tumors, strategies to avoid, or overcome, Treg suppression of immunity are under development. In human cancers, Dannull et al. showed that elimination of CD4+/CD25+ Tregs using a recombinant IL-2 diphtheria toxin conjugate, known as ONTAK, was able to enhance the immuno-stimulatory capacity of the tumor. They also showed that this product is able to selectively eliminate CD25-expressing Tregs from the PBMCs of cancer patients without inducing unwanted toxicity (31). More recently, a CD25-directed immunotoxin was shown to selectively mediate a transient partial reduction of Treg cells *in vivo* in patients with metastatic melanoma vaccinated with Melan-A/MART-1. These results suggest Treg cell elimination may be required to bolster anti-tumor responses in patients with metastatic melanoma.

3c. GENE THERAPY

The use of engineered cells is not new in melanoma immunotherapy however, its primary use has been to enhance the expression of cytokines or of melanoma antigens on dendritic cells (19). Gene transfer has also been used to engineer specific anti-tumor TCRs.

Adoptive Therapy with Engineered TCRs—As noted above, the ability to transfer specificity to a large population of naive T cells has been achieved using retro-virus transduced expression of melanoma-specific TCRs. Retrovirus-transferred TCRs are being used for therapy of melanoma and have shown modest success by investigators at the National Cancer Institute (NCI) (32). Adoptive transfer of these transduced cells in 15 patients resulted in durable engraftment of T cells expressing the transferred TCRs at levels exceeding 10% of peripheral blood lymphocytes for at least 2 months after the infusion. The NCI group observed high sustained levels of circulating, engineered cells at 1 year after infusion in two patients both of whom demonstrated objective regression of metastatic melanoma lesions. Again, these viral-engineered TCRs prove the principle that effective immunity can be transferred with this technology.

A cautionary note should be sounded regarding some retroviral vector gene transfer into hematopoietic stem cells, as the appearance of malignantly transformed cells could prove a dangerous sequela. An alternative approach has been safely used in HIV patients in the form of lentivirus-engineered gene expression. The reduced transforming capacity of the crippled lentiviral genomes used for expression of the TCRs suggest that lentiviral vectors could be safer vehicles (33) for transfer of antigen specificity. The utility of lentiviral vectors to transfer TCRs for therapy of cancer has also been demonstrated *in vitro* by Tsuji et al., who showed that they could produce tumor-specific Th1 and Tc1 cells containing TCR alpha and

beta genes obtained from an HLA-A24-restricted, Wilms tumor 1 (WT1) peptide-specific T cell clone. The gene-modified Tc1 cells showed cytotoxicity and IFN-gamma production in response to peptide-loaded lymphoblastoid cell lines, WT1 gene-transduced cells, and freshly isolated leukemia cells expressing both WT1 and HLA-A24 (34).

Engineered TCR transfer offers several theoretical advantages over other forms of autologous cell therapies. While the cells transferred are obtained from the blood of the patient to be treated, they only need to be cultured *in vitro* for a few days prior to re-infusion. The specificity can be derived from a ready-made product that is selected to allow recognition of tumor cells with matching antigen and HLA expression. It should be possible to produce a modest size library of TCRs with specificity for an array of tumor-associated antigens with a variety of common HLA types. Thus, for the treatment of melanoma patients expressing antigens such as Melan-A/MART-1, gp 100 or tyrosinase, or other defined antigens such as NY-ESO and MAGE antigens, a group of TCRs recognizing epitopes of such antigens as displayed on the most common HLA alleles would allow treatment of the vast majority of tumor patients using TCRs that recognize such antigens together with their restricting HLA allele. However, as with any highly restricted immune responses, these clonally-selected TCRs will react exquisitely with the target antigens, but will be impotent against tumors that do not express the relevant antigen or HLA alleles. Thus, the success of engineered TCR therapy will be highly dependent on strategies to maintain or re-induce antigen expression on the tumor targets which will otherwise evade destruction after immune selection.

CONCLUSIONS

More than a decade ago we noted a paradox in the host-tumor relationship, namely, that it was possible to identify specific cytotoxic T cells infiltrating melanoma tumor deposits that continued to grow. This finding suggested two contradictory facts: 1) There is sufficient expression of tumor-associated antigens to attract, activate, and clonally expand specific cytotoxic T-cells. However, the second fact suggested that there was either a defect in these T cells *in vivo*, or the tumor was somehow able to escape T-cell mediated killing, perhaps by selective down-modulation of target antigen expression. A possible explanation of this conundrum came with the seminal observation that Melan-A/MART-1 expression varied considerably during *in vitro* culture (35). This was followed by the observation of an autocrine pathway involving soluble factor secretion, which modulates antigen expression (36) (15). The reversible nature of antigen loss suggested that it should be possible to restore expression of antigen on loss variants, and indeed several agents and pathways have since been identified as inducing maturation and antigen expression in melanomas, including MAP-Kinase pathway inhibitors and IFN-beta (14). While by themselves, these maturation-inducing agents have not proven successful at inducing consistent clinical responses in patients, there is reason to be optimistic that antigen-enhancing agents could play a central role in combination with specific immunotherapy agents that are only as effective as their ability to recognize the target antigens.

While there are numerous immunotherapy strategies that have demonstrated the potential for immune destruction of tumors, there remain many unmet goals. We are tempted to speculate that the future of the field will be in a combination of techniques that will enhance tumor recognition and destruction, while blocking the many inhibitory mechanisms that prevent successful clinical responses. We are convinced that a combination of strategies is the avenue that will eventually lead to more consistent results. In this light, we suggest a possible new strategy to induce therapeutic immune responses to melanoma combining lentiviral expression of an anti-Melan-A/MART-1, (and/or other melanoma-expressed antigen-specificity) TCR to provide large numbers of specific CTLs, together with

administration of IFN-beta to enhance expression of target antigens on the tumor. It is important to recall that even in one of medicines's greatest therapeutic successes, that of antibiotic therapy, the successful eradication of chronic infections, relies on combination therapies that target different pathogenic pathways that can prevent outgrowth of resistant organisms. Likewise, success in tumor therapy will likely benefit from a multi-pronged approach that will focus not only on the induction and long term persistence of viable immune effector cells, but also on the availability of target antigens that will allow immune therapies to succeed.

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