Introduction

The incidence of melanoma in the United States is increasing at a faster rate than that of any other cancer. The prognosis for metastatic disease is poor, and more effective treatments for disseminated disease are needed. Since melanoma is one of the more immunogenic tumors, strategies have focussed on immune recognition. In vitro studies suggest that potent tumor-specific cytotoxic T cells can be induced against human melanoma. Melanoma specific T-cell activation depends on appropriate presentation to the immune system of recently defined melanoma-associated antigens presented in the context of self-HLA gene products. Full T-cell activation requires the co-stimulation by B7-Costar interactions at the T-cell surface and the elaboration of immune cytokines to promote T-cell growth. Data from animal models of tumor-specific immunization with tumor cells engineered to express immune cytokines or the B7 co-stimulatory molecule suggest that gene therapy for human melanoma may be an effective means to treat disseminated disease.

Mechanism of Immune T-Cell Activation

T-cell immunity is important for immune recognition and destruction of growing tumors.[11,12] An understanding of the mechanism of T-cell activation that follows stimulation by specific antigen is therefore necessary before addressing how tumor cells might be modified genetically to promote effective T-cell activation.

The two basic types of T-cells - CD4+ helper T cells and CD8+ cytotoxic T cells - both use a heterodimeric T-cell receptor (TCR) for recognition of target antigens presented at the target cell surface. The actual antigenic epitope recognized by the TCR is a combinatorial product of processed peptides (usually of nine amino acids in length) that are presented at the cell surface in association with gene products of the major histocompatibility complex (MHC), designated HLA in humans.[13] This HLA complex is comprised of two major regions, each containing an array of alternative alleles. One region codes for HLA class I proteins, and the second region codes for HLA class II proteins. The antigen receptors on CD8+ cytotoxic T cells recognize peptide antigens presented in the context of HLA class I proteins, whereas CD4+ helper T cells recognize peptide antigens presented by the HLA class II gene products.[14]

The peptides presented by class I proteins to CD8+ cytotoxic T cells are derived from normal or sometimes mutated intracellular proteins that carry out the everyday metabolic housekeeping functions of all cells. In addition, specialized gene products that are expressed as part of the differentiation program characteristic of a particular cell type or that may be derived from proteins coded by an infecting virus also can contribute to the array of peptide-HLA complexes presented at the cell surface. The peptides to be presented are generated within the cell through a complex processing pathway that selects short peptide sequences from degraded proteins. These peptides are tailored to the specific binding requirements of individual MHC gene products. The potential peptide antigens must fit into a three-dimensional cleft present in the outer surface of the MHC gene products. Since the HLA system is polymorphic, particularly in this cleft region, a wide variety of peptide antigens with their varied side chains may be bound. Each peptide, however, must conform to the spatial constraints in and around the cleft of each HLA allele. Thus, a particular peptide sequence derived from a degraded intracellular protein may fit best into the cleft of a particular HLA class I-encoded gene product. In essence, the peptide must conform to a "binding motif" determined by the three-dimensional conformation of each MHC allele.[15] Since a large number of alternative HLA alleles exists, and since each individual has the potential to express up to six different alleles, it is likely that most peptides can accommodate the binding motif of at least one of the HLA class I alleles of an individual and thus can be presented.

Peptide antigens presented by class II to CD4+ helper T cells are generated in a similar fashion, although it is thought that these peptides are derived most frequently from exogenous (noncellular) sources. However, evidence exists to indicate that endogenous self-peptide antigens may also be presented to CD4+ T cells in the context of HLA class II.[16,17] The binding constraints of the HLA class II proteins are not as well defined as for those of class I.

Thus, the highly polymorphic gene products of the many alleles comprising the HLA system serve as a "bulletin board" on which to display clues to the immune system concerning the ongoing activities within a particular cell. For example, in a virus-infected cell, peptides derived from virally encoded proteins will be displayed in the "groove" of HLA gene products. The bound peptides contribute to new combinatorial and potentially immunogenic epitopes against which the immune T cells may become activated and subsequently lytic for the infected cells. Under appropriate conditions, the immune system may be similarly activated when confronted with potential tumor-associated peptide antigens presented in the context of self-HLA gene products on the surface of growing tumor cells.
Fig 1. Antigen-presenting cells (APCs) process and present peptides from intracellular proteins or from proteins obtained from degraded tumor cells or virus-infected cells to CD8+ cytotoxic T cells and CD4+ helper T cells in the context of self-HLA class II gene products. Concurrently, the APCs provide co-stimulatory signals to both CD8+ and CD4+ cells via B7-CD28 interactions. Secreted cytokinons from triggered CD4+ cells help activated CD8+ T cells grow and differentiate into cytotoxic T cells that kill target cells expressing the peptide in the context of self-HLA class I gene products.

T-cell activation is controlled at several levels but may be simply represented as a two-signal model a shown in Fig 1. In this model, signal #1 is generated within the T cell when the antigen-specific TCR binds to appropriately presented antigen (peptide bound in the cl GET of self-HLA). The most effective presentation of peptide antigens occurs at the surface of an antigen-presenting cell (APC), which is capable of displaying immunogenic peptides in the context of both class I and class II HLA proteins to both CD8+ cytotoxic T cells and CD4+ helper T cells, respectively. For full activation, the T cell also requires a co-stimulatory signal (signal #2) resulting from the interaction of the CD28 molecule on the surface of the T cell with its ligand designated B7.1 or B7.2 Once again, professional antigen-presenting cells express high levels of B7 antigens and can provide highly effective co-stimulatory signals to the T cells. In an intact immune system these two events occur simultaneously in lymph nodes that drain the sight of antigenic insult. Generally, both CD4+ helper T cells and CD8+ cytotoxic T cells are activated at the same time.

Following appropriate co-stimulating by signals #1 and #2 provided by the APC, the CD4+ cells produce and release a number of cytokinons including interleukin-2 (IL-2) and IL-2, a T-cell growth factor. Both triggered T-cell populations (CD4+ and CD8+) up-regulate their IL-2 receptors (CD25). The secreted IL-2 cytokine promotes cell proliferation and clonal expansion. Secreted gamma-IFN alpha also may promote differentiation of CD8+ T cells into mature cytotoxic effector cells capable of recognizing and specifically killing target cells that present the original antigen that elicited the response. Once triggered, activated effector T cells are no longer dependent on co-stimulation with the B7 antigen but may be effectively restimulated following binding of the TCR alone to the peptide-HLA complex.

The sequence of events, from initial antigen presentation to the final B7 triggering of the responding T cells, generally occurs within the draining nodes and with an immunologic architecture that has evolved for the purpose. The proximity of the responding T cells to one another at or near the surface of the APC allows full and effective action of multiple-secreted cytokinons. When all of these elements are in place, the immune system can be effectively mobilized. However, tumors often present only a few unique and problematic situations to the immune system. Among their tactics to evade the immune system may be the tumor cell's low-level expression of HLA gene products that are specifically required for presentation of their endogenous peptide antigens. In the absence of significant levels of antigen-HLA complexes at the tumor cell surface, the tumors becomes almost invisible to the immune system, and no significant response can be initiated. Those tumor cells that continue to express detectable levels of HLA may be "seen" or detected by the immune system, but in the absence of co-stimulation by B7, a state of anergy or unresponsiveness often is induced in the antigen-bound T cells. The design of successful immunotherapeutic strategies will address these and other potential problems in immune recognition of growing tumors.

T-Cell Recognition of Autologous Melanoma

Many in vitro studies using recombinant IL-2 to promote T-cell activation and growth have demonstrated the ability to generate potent cytotoxic T-cell responses specific for autologous melanoma. Vose et al [23] demonstrated both cytotoxic and proliferative T-cell responses in peripheral blood following stimulation with autologous melanoma. Subsequently, a number of other investigators demonstrated in vitro T-cell responses to melanoma. Further analysis of cytotoxic T-cell responses in patients whose HLA type included the HLA-A2 allele revealed that melanomas presented at least one common melanoma-associated antigen in the context of the HLA-A2 "restricting" allele. In other studies, the HLA-A2 gene was inserted into melanomas that did not normally express HLA-A2, thereby providing a common presenting allele to HLA-A2 restricted T cells. These HLA-A2-expressing melanomas were rendered sensitive to killing by HLA-A2-restricted, melanoma-specific T cells. This indicates that the as yet unidentified melanoma tumor peptide antigen was already present in the melanoma and represented a truly shared tumor-associated antigen. The development of immunotherapeutic strategies was impacted by the discovery that allogeneic melanomas that expressed HLA-A2 could substitute as surrogate stimulator cells to induce in vitro HLA-A2-restricted, melanoma-specific T-cell responses in lymphocytes from HLA-A2 patients. It also has been demonstrated that in vitro induced, human melanoma-specific T cells can mediate dramatic inhibition of human melanoma growth in a xenograft nude mouse model of human metastatic melanoma, indicating that tumor-specific cytotoxic T cells can mediate tumor destruction in vivo.

In the past four years, a number of laboratories identified genes expressed within melanoma cells that code for melanoma-associated tumor antigens defined by cytotoxic T-cell responses specific for autologous melanoma. Vose et al [23] demonstrated both cytotoxic and proliferative T-cell responses in peripheral blood following stimulation with autologous melanoma. Several laboratories thereafter reported multiple melanoma-associated genes coding for proteins that could provide peptide antigens presented in the context of the HLA-A2 allele. In one study, a number of melanoma-associated peptides that are presented in the context of other HLA alleles. The distribution of these T-cell-defined tumor antigens is mostly limited to melanoma tumor cells. However, normal melanocytes also are killed by many of these melanoma-specific, HLA-A2-restricted cytotoxic T-cell responses, which indicates that these HLA-A2-associated tumor peptides are derived from melanoma differentiation antigens and appear unrelated to the neoplastic changes that resulted in the generation of the melanoma. In addition, two laboratories [39,40] have isolated antigenic peptides dissociated from HLA-A2 gene products at the tumor cell surface. Some of these isolated peptides are capable of sensitizing nonmelanoma cells to lysis by melanoma-specific T cells.

Given the in vitro immunogenicity of many human melanomas, the growing knowledge regarding the identity of melanoma-associated, T-cell-defined antigens, and the current understanding of the mechanism of T-cell activation, theoretically ideal immunotherapeutic strategies for this tumor would seem to be close at hand. Examinations of immunologic approaches to cancer therapies that apply multiple strategies have been performed in animal models. Those strategies that employ genetic modification of potentially immunogenic tumor cells are addressed.

Animal Models of Cancer Gene Therapy

Well-studied strategies for immunotherapy using gene-modified tumor cells in murine models have led to the application of gene therapy for human melanoma. While multiple immune cytokine genes have been studied, most of the studies have involved the genetic modification of murine tumor cells with the genes for IL-2 or gamma-IFN, two of the more critical cytokines necessary for T-cell activation and growth. Studies in which IL-2-producing tumor cells were injected into naive animals demonstrated that the tumoricidity of the gene-modified tumor cells was reduced or completely eliminated. Furthermore, animals that rejected the modified tumor cells were rendered immune to subsequent challenge with unmodified tumor cells. The effector cells in these studies appear to be CD8+ T cells that are cytotoxic even for the unmodified tumor cell.

Tumor cells modified with gamma-IFN were similarly effective in reversing tumorigenicity and inducing long-lasting tumor immunity. In one model, gamma-IFN gene-modified tumor cells secreting high levels of cytokine were able to induce the rejection of previously established tumors in a single therapy. Other cytokine genes that have demonstrated therapeutic efficacy when transduced into murine tumor cells include the gene for interleukin-4 (IL-4), the gene for granulocyte macrophage colony-stimulating factor (GM-CSF), and the gene for tumor necrosis factor-alpha (TNF-alpha). The co-stimulatory molecule B7 also has been transfected into murine melanoma cells, rendering them effective as immunogens to promote tumor-specific immunity.

Although many of these cytokines secreted by the tumor cells appear to promote the generation of CD8+ T cells, their mechanisms of action may be quite different. Secreted IL-2 may directly drive T cells that have been stimulated following binding of their receptors to tumor cells. The expression of MHC gene products and their associated tumor peptide antigens can be up-regulated by gamma-IFN. Thus, gamma-IFN secreted by gene-transduced tumor cells may increase the level of expression of tumor antigens at the tumor cell surface and therefore increase the antigenic stimulation of tumor-specific T cells. The transfected B7 molecule expressed by tumor cells may directly provide the second triggering signal to fully activate T cells whose TCR has bound the MHC-tumor peptide antigen complex.
Each of these cytokines has demonstrated efficacy to promote potent, protective tumor immunity in murine models. A number of in vitro human studies have led to clinical trials using gene-modified melanomas as immunogens in therapeutic phase I and II studies.

Three gene modifications for human melanoma of special note are represented in Fig 2. Transduction of tumor cells with the gene for B7 (Fig 2A) has the potential for increasing the number of tumor cells that can present peptide antigens to autologous T cells. Tumor cell lines modified with the gene for B7 can provide both signals to trigger T cells (A). Tumor cells modified with B7 can provide both signals to trigger T cells (A). Tumor cells modified with B7 can provide both signals to trigger T cells (A). Tumor cells modified with B7 can provide both signals to trigger T cells (A). Tumor cells modified with B7 can provide both signals to trigger T cells (A).

Human Immunotherapy With Gene-Modified Melanoma Tumor Cell Vaccines

One factor to be considered in the design of protocols for immunotherapy of human melanoma using gene-modified tumor cells as vaccines involves the selection of the immunogen. Although previous immunotherapy protocols for melanoma have employed whole autologous or allogeneic cell vaccines, [7, 8] tumor cell lysates, [9] or even purified antigens derived from melanomas, [10] our recent understanding regarding tumor peptide presentation in the context of self-HLA antigens suggests that the ideal antigen for immunization would be intact autologous tumor cells. The autologous tumor cells could be expected to provide all or most of the potential peptide tumor antigens associated with the patient's own tumor. These tumor antigens also would be presented in the context of all possible HLA class I and II genes that produce the patient's own immune system has learned to use. This approach theoretically maximizes the number of tumor peptide antigen self-HLA complexes displayed to the patient's immune system and would be expected to optimize the antitumor response.

A drawback in using autologous tumor cells is the difficulty in obtaining from each patient the number of viable autologous tumor cells usually required for gene modification and immunization. In fact, the success rate for obtaining autologous tumor cells sufficient for transduction and immunization is no greater than 30%. Thus, in 70% of cases, the patient could not be immunized. The use of allogeneic HLA-A2-matched (or perhaps HLA-A1-matched) tumor cells is an alternative to the use of autologous tumor cells for transduction and immunization. As previously noted, a number of shared melanoma-associated antigens expressed in the context of HLA-A2 or HLA-A1 gene products have been identified. [30-34] HLA-A2-matched allogeneic melanomas may be substituted in vitro for autologous tumor cells to induce potent melanoma-specific cytotoxic T cells. [26] Therefore, cultured HLA-matched melanomas may be substituted in the absence of autologous tumor cells for immunization of patients who are HLA-A2- or HLA-A1-matched. The advantages include immediate availability of already modified tumor cells expressing shared melanoma tumor antigens and, because the tumor cells are cultured, very high numbers of tumor cells can be produced. The single disadvantage may be that the surrogate tumor cells will present to the patient's immune system the shared HLA antigen only in the context of the shared HLA allele. Other potential tumor peptide antigens (shared or unique) that may be a part of the patient's own tumor cells and may be expressed in the context of the patient's own array of HLA alleles will not be available as immunogens. Approximately 50% of the population carries the HLA-A2 allele, and of the shared melanoma antigens that have been defined for HLA-A2 presentation, 80% to 100% are expressed by melanomas. Therefore, at least for HLA-A2 patients, allogeneic melanomas are an ideal substitute for autologous tumor cells. As other shared melanoma tumor antigens are identified and their presentations by multiple HLA alleles are defined, the selection of allogeneic tumors for immunization may be increased to include virtually all patient HLA types and tumor antigens.

Another factor to be considered in designing gene therapy is the choice of gene with which to modify the tumor cells. The selection of the employed cytokine has varied, with a strong bias toward the use of the IL-2 gene. This preference has resulted in part because this cytokine is central to the activation and growth of tumor-specific T cells. Tumor cells modified in vitro to express this gene generally continue to grow well in vitro and secrete IL-2 following modification. The cells also continue to secrete IL-2 following irradiation prior to injection. IL-2 secreted in vivo into the microenvironment by the modified tumor cells following injection may circumvent the need for activated helper T cells and may promote the full activation of reactive T cells following binding of their TCR to tumor antigen on the surface of the gene-modified tumor cells. IL-2 also has been shown to induce the secretion by other T cells of gamma-IFN, IL-1, IL-4, GM-CSF, and TNF-alpha, all factors that can promote the immune response. [51] Secreted IL-2 may activate other effector cells in vivo, including nonspecific lytic cells called natural killer cells. The IL-2 also may activate antigen-presenting cells, which can then participate more effectively in the immune response to tumor antigens.

Table 1. Level of HLA Class I and Class II Expression by Parental or gamma-IFN Gene-Modified Melanoma Tumor Cells

<table>
<thead>
<tr>
<th>Tumor Cell Line</th>
<th>HLA Class I</th>
<th>HLA Class II</th>
<th>IFN Secreted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Parental</td>
<td>17.6</td>
<td>27.5</td>
<td>NA</td>
</tr>
<tr>
<td>Control IFN</td>
<td>53.2</td>
<td>187.7</td>
<td>15</td>
</tr>
<tr>
<td>Modified Parental</td>
<td>11.3</td>
<td>11.0</td>
<td>18</td>
</tr>
<tr>
<td>Modified IFN</td>
<td>32.4</td>
<td>32.4</td>
<td>14</td>
</tr>
</tbody>
</table>

Nine of the more than 15 currently approved or active cancer gene therapy protocols employ the gene for IL-2. [51] Osanto et al. [52] in The Netherlands and Gansbacher et al. [53] Das Gupta et al. [54] and Economou et al. [54] in the United States all have initiated active immunization protocols using allogeneic, HLA-A2-matched melanoma vaccines that have been modified with the gene for IL-2. Rosenberg et al. [54] and Economou et al. [54] have initiated studies using allogeneic melanomas modified with the IL-2 gene.

The gene for human gamma-IFN has been employed in an autologous melanoma vaccine protocol by Seigler et al. [55] Dramatic increases in expression of the important HLA class I and class II genes required for antigen presentation have been demonstrated by gamma-IFN-transduced human melanomas (Table 1). [56] Expression of the HLA class I gene products (CD8+ T-cell recognition structure) following gamma-IFN gene modification is routinely increased from four to seven times or even 20 times in some tumor cell lines. Expression of HLA class II gene products (CD4+ T-cell recognition structures) may change from no detectable class II to extremely high levels that may elicit increased CD4+ helper activity. Secretion of gamma-IFN by modified cells ranges between 10 and 50 U/mL/10^6 cells per day. Introduction of the gamma-IFN gene also up-regulates the activity of the peptide antigen processing pathway to provide peptide antigens for display by the additional HLA gene products. Theoretically, the cells are capable of presenting much higher levels of HLA-associated melanoma peptide antigens to autologous T cells. In vitro studies indicate that stimulation of melanoma-specific T-cell lines with gamma-IFN gene-modified HLA-A2-matched melanomas in vitro results in significant increases in T-cell activation compared to stimulation with unmodified tumor cells (Table 2). Tumor cells modified with the gene for gamma-IFN induce a T-cell response that is two to four times that induced by parental tumor cells. Published data [56] also
Fenten et al.[57] have initiated a recent gene therapy protocol, in which a panel of three allogeneic HLA-A2 melanomas (one of which also expresses HLA- A1 and the MAGE-1 genes) have been modified with the gene for the co- stimulating molecule B7. When tested in vitro, these B7-modified tumor cell lines induced expression of the IL-2 receptor CD25 on allogeneic CD4+ and CD8+ T cells and induced a five- to ten-fold increase in T-cell numbers compared with the parental unmodified tumor cell line.[57] The B7 transfectants also induced cytokotic T cells, whereas the unmodified tumor cells did not. These potent stimulating tumor cells will be used as immunogens in an active immunization protocol of patients whose HLA type includes the A2 gene or the A1 gene. As in the animal studies by Townsend et al.[50] the expectation is that the B7- expressing tumor cells will present shared tumor antigens in the context of HLA-A2 and/or HLA-A1 in combination with the important co- stimulatory signal provided by the B7 gene product to fully activate tumor specific T cells in vivo. If effect, the tumor cells themselves become professional APCs to present their own array of endogenous tumor- associated peptide antigens.

IL-4 also has been used in gene therapy of human melanoma in a protocol by Lotze et al.[58] This cytokine can promote the in vitro activation of cytotoxic T cells,[59] the release of IL-2 by lymphocytes,[60] and the activation of macrophages[61] into potent antigen-presenting cells. In addition, IL-4 may play a critical role in the ability of immune T cells and eosinophils to home to the site of the tumor.[67] Thus, the multiple effects of this cytokine released by gene-modified cells make it a potentially effective immune modulator. The protocol design employs fresh-frozen autologous tumor cells thawed just prior to immunization. The cytokine gene will be introduced into cultured fibroblasts derived from patient biopsy material, rather than into the tumor cells. The IL-4 gene-modified fibroblasts are then irradiated and administered in combination with the thawed, irradiated tumor cells. This strategy avoids the problem of establishing autologous melanoma cultures and modifying them with the cytokine gene and requires a minimum of tumor for immunization. Fibroblasts are easily grown in vitro and are readily modified with genes for cytokines. The combination of transduced fibroblast- secreting IL-4 and tumor antigens provided by autologous tumor cells is expected to create a microenvironment in vivo in which secreted IL-4 can optimally modulate the immunologic events following contact of potentially responsive immune cells with tumor.

Conclusions

The current phase I and phase II protocols are designed to evaluate the safety and efficacy of immunotherapy with gene-modified tumor cells in patients with disseminated melanoma. Due to extensive tumor burden, these patients comprise the most difficult clinical situation in which a significant response rate can be expected. As early protocols allow us to establish the safety and efficacy of gene therapy, studies of patients with limited disease or no measurable disease may proceed. This population of patients eligible for adjuvant therapy may benefit most from gene therapy approaches. However, in these same patients, tumor would not be available for use as immunogen. In such cases, the use of allogeneic HLA-matched melanomas expressing shared tumor antigens would be most appropriate. Similarly, the immunotherapist could use gene- modified autologous cells such as patient derived fibroblasts to provide an in vivo source of secreted cytokine.

Several genes that code for melanoma-associated antigens have been identified and cloned.[30-37] Autologous patient fibroblasts are readily available and easily cultured and gene modified.[58] One or more (ideally all) of the genes for melanoma-associated tumor antigens conceivably could be introduced and expressed in an autologous cell such as a fibroblast. The gene- modified fibroblasts would then process the tumor antigen gene products into peptides to be presented at the cell surface in the context of all available HLA alleles, thus providing a tailored vaccine available to any melanoma patient. Furthermore, these immunogens might be modified even more to include the genes for IL-2, gamma-IFN, and B7 to become most potent immunogens.

Each of these vaccine approaches attempts to promote tumor-specific immune activation by providing an essential component selected from the array of cytokines and T-cell activating structures necessary for the induction of potent tumor specific immunity. Tumor immunotherapy using gene- modified melanoma vaccines, therefore, is a rational strategy, but progress in understanding the mechanisms of immune activation and the nature of tumor T-cell interactions is necessary for this approach to be translated into effective treatment for melanoma.

References


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