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Developing recombinant and synthetic vaccines for the treatment of melanoma

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Abstract

To develop new vaccines for the treatment of patients with cancer, target antigens presented on tumor cell surfaces have been cloned. Many of these antigens are non-mutated differentiation antigens and are expressed by virtually all melanomas, making them attractive components for a widely efficacious melanoma vaccine. These antigens are also expressed by melanocytes, however, and are likely to be subject to immune tolerance. A central challenge for tumor immunologists has thus been the breaking of tolerance to cancer antigens. We review recent clinical trials using experimental cancer vaccines, including recent evidence that therapeutic vaccines can induce objective responses in patients with metastatic malignant melanoma. We focus on the foundations of these approaches in new experimental animal models designed to test novel vaccines and report on what these new models predict for the future development of therapeutic vaccines for cancer.

Vaccines designed to treat patients with metastatic cancer have shown the first evidence of efficacy in the clinic. While a widely efficacious tumor vaccine is not yet available, a great deal of progress has been made in the development of effective cancer vaccines, especially those designed to treat metastatic malignant melanoma. This progress is due to a “reductionistic” approach which has made possible an understanding of the interactions between the immune system and tumor cells on a molecular level [1,2].

Recent attempts to develop recombinant and synthetic vaccines stand in sharp contrast to previous immunotherapeutic strategies such as the irradiation of tumor cells, their admixture with *Corynebacterium parvum*, Bacillus Calmette-Guerin (BCG), infectious viruses, or more recently, the insertion of immunomodulatory genes into tumor cells [3]. Whereas each of these strategies was aimed at increasing the *immunogenicity* of cancer cells, the rationale for the creation of new recombinant and synthetic vaccines is the presentation of tumor antigens on immune activating cells such as dendritic cells.

Tumor antigen identification

Central to the development of vaccines for the treatment of patients with cancer is the molecular identification of the antigens present on cancers that are recognized by the immune system. These antigens can then be used in their recombinant and synthetic forms in the development of anticancer vaccines [4,5]. The most progress in the identification of new tumor antigens has been made in the case of malignant melanoma [6]. One approach has been to start the cloning process using T cells with antitumor reactivity to screen cDNA libraries made from melanoma cell lines. In many cases, the antitumor T cells were derived from cultures of tumor infiltrating lymphocytes (TILs) that had been adoptively transferred to patients with cancer. Our group focused on the T cell reactivities that were associated with objective regressions of metastatic melanoma lesions after their adoptive transfer.

The cDNA libraries of expressed melanoma genes were screened by transfecting these genes, along with the gene for the restricting major histocompatibility complex (MHC) molecule, into an antigen-negative cell line which is then admixed with T lymphocytes that have antitumor

activity. If the T cells recognize the transfected cell line, they will lyse it and release cytokines such as granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor (TNF), and interferon (IFN), any of which can be measured. The process of cloning genes encoding tumor antigens is under constant improvement and has become considerably faster and more streamlined [7,8]. Many of the tumor antigens that have been identified are tissue differentiation antigens in melanocytes and include gp100, MART-1/MelanA, tyrosinase, and tyrosinase related proteins (TRP)-1 and -2. Interestingly, these antigens are involved in the synthesis of melanin and give both melanocytes and deposits of melanoma tumor their dark pigment (Table 1).

The fact that differentiation antigens are non-mutated in most tumors has two important implications. First, expression of these tissue differentiation enzymes are shared by the great majority of melanoma nodules in the great majority of patients, and thus an “off-the-shelf” vaccine strategy targeting these antigens is possible (a strategy that targets a mutated antigen may have to be individualized for every mutation). Second, the non-mutated nature of these antigens suggests that immunotherapies that target these antigens could elicit auto-reactivity. One consequence of this “auto-reactivity” may be vitiligo, the patchy and permanent loss of pigment from the skin and hair thought to result from the autoimmune destruction of pigment cells. Vitiligo has been correlated with objective shrinkage of deposits of metastatic melanoma in patients receiving high dose interleukin-2 (IL-2), a cytokine known to activate and expand T lymphocytes [9].

Thus, there is evidence that vitiligo can be coupled with tumor regression, and that adoptive transfer of antitumor T cells recognizing differentiation antigens is associated with objective shrinkage of melanoma deposits. Although the focus of this review is on melanocyte differentiation antigens, two other groups of antigens should be mentioned. Those in the first category are expressed by a diversity of tumor histologies but are not expressed by normal tissues, other than testis. Cloned in large part by Thierry Boon and his colleagues, these antigens are encoded by genes with family names like *MAGE*, *BAGE*, *GAGE*, *RAGE* and *LAGE* [10, 11]. *NY-ESO-1* also falls into this group and is expressed in a significant proportion of human melanoma cells as well as other tumor histologies including breast, ovary, bladder, prostate, and liver [12-15].

Another group of antigens includes those produced by mutations. Neoantigens produced as a result of mutation often are found to originate in ubiquitously expressed proteins. Examples include epitopes from [beta]-catenin, CDK4, FLICE (caspase-8), and most recently, a mutant gene from a bladder carcinoma that is recorded in databases under the name KIAA0205 whose function is unknown [16•,17•]. Mutated antigens may not lend themselves easily to “off-the-shelf” vaccines consisting of purely recombinant and synthetic components, because each neoantigen for each patient must be checked for sequence, and the sequence must be verified to be present on the surface of a tumor cell.

Animal models

The success of vaccines designed to target melanocyte differentiation antigens that are expressed by melanoma cells may require an understanding of the mechanisms behind breaking tolerance to “self” antigens that has for the most part been obtained from experiments in mice. Most experimental models used in the past by us and others have employed foreign antigens (*eg*, chicken ovalbumin, [beta]-galactosidase, or influenza nucleoprotein), or sequences from other species (*eg*, using human antigens in mice). These models have been used to optimize the factors like route of administration and prime-boost regimens [18,19] and to understand the basic biology of tumor-immune interactions [20••,21].

To explore the immunological effects of the “self” nature of melanocyte differentiation antigens, a new set of models has recently been developed that employ the mouse homologues for melanocyte differentiation antigens [22]. Like their human counterparts, the mouse homologues are expressed in normal melanocytes from unmanipulated C57BL/6 mice as well as in mouse melanomas. In one system designed to model the human situation with more fidelity, mouse gp100 was cloned from the spontaneous B16 mouse melanoma and the functional characteristics of mouse gp100-reactive T cells were evaluated in the recognition and destruction of B16 *in vivo*. [23,24••]. Autoreactive T cells have been induced and the epitope within the mouse gp100 molecule, as well as the restricting MHC class I molecule, have been identified. Using the mouse gp100 model, it is clear that immunogens containing a “self” peptide with enhanced binding to its restricting MHC class I molecule can be more effective in eliciting autoreactive T cells. A great deal of progress, summarized below, has been made in the development of cancer vaccines using animal models and many of the findings from these models have now been tested in clinical trials.

Molecularly-defined adjuvants

In the context of tumor vaccines, the word *adjuvant* (from the Latin “to help”) refers to an agent that can augment the quality or magnitude of the antitumor activity elicited by a vaccine. Mouse models have been used to evaluate large panels of molecularly defined adjuvants. They have revealed that IL-2 and IL-12 [25-27] are extremely potent in the ability to increase the efficacy of cancer vaccines. GM-CSF also plays an important role in the induction and proliferation of cellular immune responses [28]. Importantly, not all of the findings in murine models were confirmed in human clinical trials. When patients with metastatic melanoma were immunized with a modified immunodominant peptide derived from gp100, no increase in efficacy as measured by objective clinical response was observed using GM-CSF or IL-12 [29]. In this clinical trial, only peptide plus IL-2 was associated with an increase in clinical efficacy (this finding is described in more detail below).

Other key non-cytokine immunomodulators include key molecules involved in costimulation. One of these, CD40 and its ligand, a member of the TNF family, naturally forms homotrimers shown to be important in B cell activation, production of type 1 cytokines by T helper cells, and generation of cytotoxic memory responses. We have found that the addition of CD40L trimer to DNA vaccination can significantly increase antitumor efficacy [30]. The FLT3 ligand can induce the apparent growth and differentiation of functional dendritic cells and has been reported to have antitumor effects [31,32], but its role, if any, in the augmentation of recombinant and synthetic anticancer vaccines has yet to be demonstrated in experimental models (Restifo and Rosenberg, Unpublished data).

The most well-defined costimulators for T cells remain B7-1 (CD80) and B7-2 (CD86) which can be induced on antigen presenting cells (APCs). Whether B7 family molecules trigger the activation or inhibition is dependent on its interaction with molecules on the T cell: engagement of CD28 is associated with proliferation and differentiation whereas an encounter with cytotoxic T lymphocyte antigen-4 (CTLA-4) may trigger functional unresponsiveness [33••]. The blockade of the engagement of CTLA-4 has been reported to potentiate immune responses to tumor cells [34].

Recombinant vaccines

One group of vaccines that has been developed in the mouse include vaccines based on recombinant viruses and DNA. The creation of recombinant virus-based cancer vaccines involves the insertion of genes encoding tumor antigens into the viral genome. “Mini-genes” encoding immunodominant epitopes (*ie*, the fragments of antigens that are presented by MHC molecules) from cancer antigens can also be inserted into recombinant vaccines alone or in

combination with endoplasmic reticulum insertion signal sequences that can bypass the TAP transporters, in some cases profoundly enhancing the function of virus-based vaccines in animal models [35-37]. In addition, immunomodulatory molecules (such as cytokines and costimulatory molecules) can also be inserted into the viruses [25,38,39]. The inserted genetic material must be preceded by a promoter whose function is best optimized in dendritic cells [40].

The safety of recombinant viruses, always of paramount importance, can be insured in a number of ways. For example, some of the recombinant vaccines are composed of viruses incapable of replicating in mammalian cells because of their host range (*eg*, the avian poxviruses) [41], others are highly attenuated (like certain influenza A viruses [42] or the Wyeth and modified vaccinia virus Ankara strains [43]), and still others are made safe by the removal of viral genes that are critical to viral replication and virulence (such as adenoviruses) [44].

One important lesson that we have learned from our own clinical trials is that recombinant viral vaccines based on vaccinia and adenoviruses are likely to suffer from the problem of pre-existing immunity. Humans have high neutralizing titers to vaccinia virus because of its widespread use in the campaign to eradicate smallpox, and to adenovirus because of its ubiquitous presence in the environment in each of our upper respiratory systems [45]. Indeed, most patients' immune systems appear to be able to rapidly clear these viruses, making it difficult or impossible to effectively use cancer vaccines based on these viruses. One way of circumventing the problem of pre-existing immunity is the use of viruses whose natural hosts are non-mammalian, such as the avian poxviruses.

Reports dating from the early 1990s indicated the surprising finding that the intramuscular injection of "naked" plasmid DNA (*ie*, DNA without a viral coat) could result in an immune response. There is recent and clear evidence for a predominant role for directly transfected dendritic cells in antigen presentation to CD8⁺ T cells after nucleic acid immunization [46•]. Because they are relatively safe and easy to engineer and produce, but generally not as potent as recombinant viruses at eliciting immune responses capable of destroying tumors, there has been significant effort to improve the efficacy of DNA vaccines. Important innovations concerning the design of these vectors include promoter optimization, enhancement of polyadenylation sequences, the removal of 5' and 3' untranslated regions from the inserted gene, and the use of intronic sequences to improve nuclear export. Clinical trials using "naked" DNA vaccines encoding tumor antigens have been initiated. One of the first of these trials was recently initiated at the Surgery Branch of the National Cancer Institute using a plasmid encoding a modified form of the human gp100 melanoma antigen first alone then in combination with IL-2, previously shown in murine study to enhance vaccination with plasmid [26].

Synthetic peptide vaccines

Knowledge of the amino acid sequences of the epitopes presented by MHC molecules on the surfaces of tumor cells can be used to generate synthetic peptide immunogens for their use as vaccines. When admixed with incomplete Freund's adjuvant (IFA), these peptide immunogens have been shown to enhance antitumor T cell responses in cancer patients.

A promising new avenue toward the augmentation of synthetic peptide-based anticancer vaccines may be the use of peptides that have been altered to increase their ability to bind MHC class I molecules. Crystal structures of peptide MHC complexes, together with mass sequencing of peptides eluted from class I molecules, have revealed that peptides bind to their restricting class I molecules in large part by "anchor" residues. These residues can be modified to increase the peptide-MHC interaction without compromising the interaction of this complex with the T cell receptor [47].

In one study [47], we used a synthetic peptide immunogen corresponding to an epitope from the gp100 melanoma antigen. When the second amino acid from the amino terminus was modified from a threonine to a methionine to create a peptide called g209-2M, binding to the HLA-A*0201 molecule could be increased ninefold in an *in vitro* competitive binding assay [47]. *In vivo*, 91% of patients vaccinated with this peptide were successfully immunized on the basis of immunologic assays. Perhaps more importantly, when the T cell growth and differentiation cytokine IL-2 was added to the treatment regimen, 13 of 31 patients (42%) had objective clinical responses and four additional patients had mixed or minor responses (Table 2) [48•]. These results compare favorably, and are significantly different than results from clinical trials using high dose IL-2 alone, where objective response rates generally fall in the 15% to 17% range.

Future directions in the development of synthetic peptide vaccines may include the use of modified peptides embedded into microspheres, a maneuver that can target antigen for uptake and MHC class I-restricted presentation by professional antigen presenting cells. Other promising novel strategies developed in experimental animal systems involve the use of toxin-linked peptides [49•] and peptides linked to endoplasmic reticulum insertion signal sequences covalently attached to the amino-terminus of a peptide immunogen [50]. The enhanced hydrophobicity of the resultant peptide may be responsible for its increased immunogenicity, but the mechanism of action has not yet been fully elucidated.

Adoptive transfer studies

Previous efforts at adoptive transfer of anti-melanoma T cells used bulk populations of TILs expanded in high dose IL-2. It was these TIL cells that were successfully used for antigen identification described above, and can result in objective responses in about one third of patients with metastatic melanoma when used in combination with IL-2 [51]. Clinical trials of adoptive immunotherapy have now been reported for the prevention and treatment of cytomegalovirus (CMV), Epstein-Barr virus, and HIV [52-54]. Three new strategies in the adoptive immunotherapy of melanoma are now being explored.

The first of these strategies is the use of peptides that have been modified to increase their ability to make high-affinity stable complexes with MHC molecules. Indeed, the “anchor-fixed” gp100 peptide described above (g209-2M) has been used to sensitize peripheral blood leukocytes (PBLs) from HLA-A*0201⁺ melanoma patients *in vitro* [47]. In our current clinical trials, PBLs stimulated with the g209-2M peptide grow faster and have greater antitumor reactivity than cells grown with the native g209 peptide. It remains to be seen whether or not these cells have increased therapeutic efficacy upon adoptive transfer.

The second experimental strategy is the use of clonal populations of cells. Early in the growth phase, “bulk” populations of T cells can be cloned. Individual clonal populations of T cells can then be assayed for their antitumor activity and the culture with the highest apparent avidity can be expanded for adoptive transfer. This approach may have the advantage of generating therapeutic cells with a nearly uniform capacity to recognize tumor. Since clonal T cells have a unique rearrangement of their T cell receptor, they can be tracked *in vivo* using competitive polymerase chain reaction (PCR) technology. The genetic modification of T cell clones could be an important manipulation of these cells for future trials [52].

The third, and most preliminary, experimental thrust in efforts to enhance the therapeutic efficacy of adoptively transferred T cells is the use of helper CD4⁺ T cells. In studies using T cells specific for CMV, Walter *et al.* [55] have reported that the cytotoxic activity of adoptively transferred CD8⁺ clones declined in patients deficient in helper CD4⁺ T cells specific for CMV. These results suggested that CD4⁺ T cell function is needed for the persistence of transferred CD8⁺ T cells. The analysis of CD4⁺–CD8⁺ T cell interactions are being explored in

experimental animal models [56]. Attempts to identify the molecular targets of antitumor CD4⁺ T cells have already met with considerable success [57,58].

Tumor escape

Despite advances in the development of new immunotherapies, many patients do not respond favorably to vaccination leading many to speculate that tumors may actively evade immune destruction. Examples of tumor escape from treatment are well known from the field of chemotherapy and include the induction of the expression of the multidrug resistance gene.

It was recently reported that Fas ligand (FasL/CD95L) expression by melanoma cells was also involved in immune escape [59]. FasL expression has been reported in areas of immune privilege such as the eye and testis. Some authors argued that expression of FasL by melanoma cells indicated that the tumor bed was also an “immune privileged” site. To investigate the expression of FasL by melanomas, a panel of early passage cell lines were screened. None of the 19 human melanoma lines tested killed the Fas⁺ targets in a sensitive functional assay. Furthermore, none of the 26 human melanoma cell lines expressed FasL mRNA as evaluated by reverse transcription-PCR [60,61].

Thus, our unpublished data do not support a role for FasL expression in the escape of melanoma cells from immune destruction. Nevertheless, there is abundant evidence from our laboratory and others that melanoma cells do escape immune recognition by a variety of mechanisms. For example, there is the selective loss of [beta]₂-microglobulin, MHC class I expression, and loss of antigen processing capability [62,63]. The loss of some of these ligands, although it can hurt recognition by T cells, can result in increasing the susceptibility of tumors to lysis by natural killer (NK) cells [64]. As recombinant and synthetic vaccines become more effective, the selective pressure on the loss of particular target antigens may increase. Indeed, we have already begun to witness the loss of antigens (Marincola, Unpublished data) [65,66].

Conclusions

Great progress has been made in the identification of antigens recognized by T cells, not only in melanoma but in a variety of other cancers as well. Studies in experimental animal models have been used to preclinically test a variety of experimental vaccines. Knowledge of the peptide sequence together with a clearer picture of the way that a peptide antigen interacts with MHC makes it possible to tinker with peptide sequences in order to improve binding affinity and stability. Advances in the way antitumor T cells are expanded *ex vivo* may result in improvements in the efficacy of adoptively transferred T cells. Synthetic peptide vaccines and recombinant virus and “naked” DNA-based vaccines are being tried in the clinic. It seems likely that further innovation, based on a deeper understanding of the basic biology of tumor-immune interactions, will be required to develop widely efficacious tumor immunotherapies.

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Abbreviations

CMV	cytomegalovirus
CTLA	cytotoxic T lymphocyte antigen
GM-CSF	granulocyte macrophage-colony stimulating factor
IFN	interferon
IL	interleukin
MHC	major histocompatibility complex
PBL	peripheral blood leukocyte
PCR	polymerase chain reaction
TIL	tumor infiltrating lymphocyte
TNF	tumor necrosis factor

Table 1
Selected melanoma/melanocyte differentiation antigens recognized by melanoma-reactive T cells

Study	Gene	Restriction element	Peptide epitope
Kawakami <i>et al.</i> [67], Cox <i>et al.</i> [68]	<i>gp100</i>	HLA-A*0201	KTWGQYWQV
Tsai <i>et al.</i> [69]			AMLGTHTMEV
Tsai <i>et al.</i> [69]			MLGTHTMEV
Kawakami <i>et al.</i> [67]			ITDQVPFSV
Kawakami <i>et al.</i> [67], Tsai <i>et al.</i> [69]			YLEPGPVTA
Kawakami <i>et al.</i> [67]			LLDGTATLRL
Kawakami <i>et al.</i> [67]			VLRYGSFSV
Tsai <i>et al.</i> [69]			SLADTNSLAV
Kawakami <i>et al.</i> [70]			RLMKQDFSV
Kawakami <i>et al.</i> [70]			RLPRIFCS
Skipper <i>et al.</i> [71]		HLA-A3	ALLAVGATK
Kawakami <i>et al.</i> [70]			LIYRRRLMK
Robbins <i>et al.</i> [72]		HLA-A*2402 H-2D ^b	VYFFLPDHL
Overwijk <i>et al.</i> [24••]			KVPRNQDWL
			EGSRNQDWL
Wolfel <i>et al.</i> [73]	Tyrosinase	HLAA*0201	MLLAVCYLL
Woliel <i>et al.</i> [73]			YMDGTMSQV
Kittlesen <i>et al.</i> [74]		HLA-A1	DAEKCDKTEY
Kawakami <i>et al.</i> [70]			SSDYVIPIGTY
Kang <i>et al.</i> [75]		HLA-A*2402	AFLPWHLRF
Brichard <i>et al.</i> [76]		HLA-B44	SEIWRDIDF
Topalian <i>et al.</i> [58]		HLA-DR4	QNILLSNAPLGPQFP
Kawakami <i>et al.</i> [77]		HLA-A*0201	SYLQDSPDSFQD
	<i>MART-1/ Melan-A</i>		AAGIGILT
Schneider <i>et al.</i> [78]	<i>TRP-1</i>	HLA-*4501	AEEAAGIGILT
Wang <i>et al.</i> [79]		HLA-31	MSLQRQFLR
Parkhurst <i>et al.</i> , Unpublished data		HLA-A*0201	SVYDFFVWL
Wang <i>et al.</i> [80]		HLA-A31	LLPGGRPYR
Wang <i>et al.</i> [80]		HLA-A33	LLPGGRPYR
Bloom <i>et al.</i> [81]		H2-K ^b	VYDFFVWL

Table 2

Characteristics of the 13 patients with metastatic malignant melanoma who had objective responses to modified gp100 peptide (g209-2M) plus IL-2/CR—complete remission; IL—interleukin; PR—partial response. Adapted from Rosenberg *et al.* [48••], with permission

Patient	Age, yr/Sex	Tumor site	Type	Response duration, mo
a	48/M	Lung	CR	6
b	51/M	Lung, subcutaneous	PR	6
c	44/M	Lymph node, lung, subcutaneous, cutaneous	PR	2+
d	45/F	Lymph node, bone, subcutaneous	PR	4
e	42/F	Cutaneous, subcutaneous, liver	PR	7+
f	41/F	Lung	PR	5+
g	39/M	Lung	PR	6
h	22/F	Lung hilum	PR	5
i	48/M	Cutaneous	PR	6
j	43/M	Subcutaneous	PR	5+
k	42/F	Lung, lymph node, liver, brain	PR	2
l	47/F	Lung, lymph node	PR	5+
m	59/M	Subcutaneous	PR	3+

CR—complete remission; IL—interleukin; PR—partial response.

Adapted from Rosenberg *et al.* [48 ••], with permission.