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SYMPOSIUM IN WRITING

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Immunotherapy of cancer with dendritic-cell-based vaccines

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Abstract Animal studies have shown that vaccination with genetically modified tumor cells or with dendritic cells (DC) pulsed with tumor antigens are potent strategies to elicit protective immunity in tumor-bearing animals, more potent than "conventional" strategies that have been tested in clinical settings with limited success. While both vaccination strategies are forms of cell therapy requiring complex and costly ex vivo manipulations of the patient's cells, current protocols using dendritic cells are considerably simpler and would be more widely available. Vaccination with defined tumor antigens presented by DC has obvious appeal. However, in view of the expected emergence of antigen-loss variants as well as natural immunovariation, effective vaccine formulations must contain mixtures of commonly, if not universally, expressed tumor antigens. When, or even if, such common tumor antigens will be identified cannot be, predicted, however. Thus, for the foreseeable future, vaccination with total-tumor-derived material as source of tumor antigens may be preferable to using defined tumor antigens. Vaccination with undefined tumor-derived antigens will be limited, however, by the availability of sufficient tumor tissue for antigen preparation. Because the mRNA content of single cells can be amplified, tumor mRNA, or corresponding cDNA libraries, offer an unlimited source of tumor antigens. DC transfected with tumor RNA were shown to engender potent antitumor immunity in animal studies. Thus, immunotherapy using autologous DC loaded with unfractionated tumor-derived antigens in the form of RNA emerges as a potentially powerful and broadly useful vaccination strategy for cancer patients.

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Introduction

There are many outstanding issues in cancer immunotherapy. As directed by the editor of this symposium-in-writing, I will limit the discussion to three issues: first, a comparison between two promising vaccination strategies, vaccines based on genetically modified tumor cells and dendritic-cell-based vaccines; second, the pros and cons of vaccinating with defined tumor antigens compared to using tumor-derived material as source of undefined tumor antigens; third, the preferred form of antigen to load on dendritic cells: protein, DNA, RNA etc.

Gene-modified-tumor vaccines or dendritic-cell-based vaccines

The recognition that most, if not all, tumors, including non-immunogenic tumors, encode tumor-rejection antigens and are capable of inducing protective immunity has reinvigorated the field of cancer immunotherapy [35]. This, and the recognition that the cellular arm of the immune response, in particular the $CD8^+$ cytotoxic T cell (CTL) arm, is best equipped to recognize tumor cells as foreign and lead directly or indirectly, to their eradication, has shifted the emphasis in vaccine development from vaccines that favor the induction of humoral responses to vaccines that favor the induction of cellular responses.

One such approach, the use of genetically modified (autologous) tumor-cell-based vaccines (GMTV), has received much attention [6, 9]. The original working hypothesis of the GMTV approach was to provide cytokines to the CTL precursors as a means to circumvent the dependence on $CD4^+$ T helper cells [7, 8]. This was based on the notion prevailing at that time that, in addition to antigen presented by the tumor cells, full maturation of the tumor-specific

CTL required cytokines secreted by activated CD4+ T cells. There is now growing evidence that somatic cells, whether tumor cells or cell infected by pathogens as a rule do not present antigen to naive CD8+ CTL. Rather, the ability to activate naive CD8+ T cells is an exclusive property of a specialized type of cells called professional antigen-presenting cells (APC). Accordingly, antigen is transferred from cells expressing the antigen to the APC, a process that is triggered by inflammatory reactions that cause the degradation and release of antigen from the dying cells. This process has been referred to in the literature as cross-priming, re-presentation or indirect presentation and popularized as the "danger theory" [22]. A number of observations, old and recent, argue that indirect presentation is an important, if not a major pathway for induction of CTL responses in vivo. The current view is, therefore, that the role of cytokines or costimulatory molecules in GMTV is to enhance the transfer of tumor antigens to professional APC for activation of naive CTL precursors, as may be the case for interleukin-2(IL-2)- or granulocyte/macrophage-colony-stimulating-factor(GM-CSF)-secreting GMTV [2, 15], or to enhance the expansion of activated or memory CTL, as may the case for B7-1-expressing GMTV [16]. Compelling, though indirect, evidence suggest that the main form of professional APC is the dendritic cell [34]. Hence the working hypothesis underlying the use of dendritic cells in tumor vaccination is that the limiting factor in tumor CTL induction in vivo is the transfer of antigen from the tumor cell to the dendritic cell, and that direct loading of dendritic cells with the relevant antigen is an effective method to achieve that.

I see three main reasons why dendritic-cell(DC)-based vaccines are an attractive approach for cancer immunotherapy and may prove to be superior to GMTV:

1. First, DC vaccines "make sense". The emerging view, as discussed above, is that, regardless of the method of immunization, class-I-restricted antigens have to be funneled through the dendritic cell system to activate the CTL arm of the immune response. This is the essence of DC-based vaccination, loading DC with antigen and reinfusion into the patient. By contrast, the mechanism of action of most forms of GMTV is to activate the DC system in situ and enhance the transfer of tumor antigen to the dendritic cell system. Thus, the primary goals of DC vaccination and GMTV are the same, channeling antigen to the DC system, but the latter strategy is indirect.
2. Numerous studies have documented the exceptional ability of dendritic cells to activate naive T cells, CD4+ T-helper cells as well as CD8+ CTL [34]. Not surprisingly, animal studies have also shown that DC loaded with tumor antigens induce potent antitumor immunity in the experimental animal [10]. Interpretation of animal studies, however, is not always straightforward. To enhance the clinical relevance of animal studies we evaluated the potency of DC-based tumor vaccines in a murine model designed to mimic as closely as possible the conditions prevailing in the cancer patients. In this model, called the post-surgical metastasis model, vaccination is used to prevent the growth of preexisting micrometastasis in mice from which the primary tumor was surgically removed before initiation of treatment [29]. We have repeatedly observed that treatment of the tumor-bearing animals with various formulations of DC vaccines: DC loaded with tumor-derived antigens in the form of tumor extracts, peptides or RNA, was remarkably effective in this post-surgical metastasis model, more so than the most effective GMTV formulations [3, 24, 25]. While animal studies do not tell us what will work in the human patient, they serve as an important screening tool to compare and discard strategies that may not work in human patients. What we did learn from the animal studies is that DC-based vaccines may constitute a highly effective means of inducing protective tumor immunity, not less and perhaps more so than GMTV, and therefore should be evaluated in clinical trials.
3. GMTV and DC vaccines are forms of cellular therapies; they require ex vivo manipulation of the patients' cells. The complexities associated with cellular therapies notwithstanding, they have to be considered if they represent the only potential treatment for a terminal disease, as is the case for most forms of cancer. While GMTV were highly effective in animal studies, translation to clinical settings turned out to be a limiting factor. This is primarily due to the relative inefficiency of gene transfer techniques when applied to primary human tumor cells, the difficulty of obtaining sufficient number of tumor cells from the patient, and the overall complexity of the procedure. (It is less appreciated that GMTV also carry an inherent risk. The genetically manipulated tumor cells must be administered in an irradiated form, yet most human tumor cells cannot be cultured ex vivo to determine the effectiveness of the inactivation protocol. Since excessive irradiation adversely affects secretion of the transfected cytokine, the possibility that live tumor cells will be present in the vaccine preparation cannot be rigorously excluded.)
Compared to GMTV, preparation of DC vaccines is a "user-friendly" and a clinically manageable process. At present, DC can be generated from cancer patients in relatively simple and largely automatable protocols by culturing adherent peripheral blood mononuclear cells from the patients for 5–7 days in the presence of cytokines [32] (contrast this with the isolation or culture of sufficient tumor cells from patients). While the source of tumor antigen may be a contentious issue (see below), loading of DC with antigen is simple compared to transduction of freshly isolated human tumor cells. Additional simplifications of DC vaccine preparation are also anticipated. An exciting possibility stems from the observation that Flt3-ligand mobilization significantly augments resident DC in the blood, potentially eliminating the need to culture PBMC ex vivo for DC generation [21].

Vaccination with defined tumor antigens or with total-tumor-derived antigens

A number of tumor antigens recognized by CD8+ CTL have been identified and molecularly cloned [4]. Since CTL are an important effector arm of the antitumor immune response [11], such antigens are attractive candidates for use in tumor vaccines. It is noteworthy that many of the recently isolated tumor antigens are non-mutated cellular products and are expressed in many cancers, i.e. they could be the long-sought-after "universal cancer antigens". There are three main advantages of using defined tumor antigens in cancer immunotherapy. First, the use of defined antigens is logistically simple and obviates the need to isolate tumor antigens from each patient. Second, the purity of the antigenic preparation is likely to enhance the effectiveness of the vaccine. Third, the absence of irrelevant tumor material will minimize possible autoimmune reactions against "self antigen". There are also three major disadvantages of using defined tumor antigens. Foremost, immunization with defined tumor antigens is currently limited to a small number of cancers in which candidates for tumor-rejection antigens have been identified [4]. Second, it is unclear whether or which of the recently identified human tumor-associated antigens are the best choice to mount an effective antitumor immune response in vivo, an issue that must await clinical studies for resolution. This potential concern was underscored in a report by Anichini et al., who have shown that the majority of CTL present in HLA-A2.1 melanoma patients were not directed to the recently identified tumor antigens, Melan-A/Mart-1, tyrosinase, gp100, or MAGE-3 [1], suggesting that immunization with other, yet unidentified, antigens would be more effective in eliciting tumor immunity in these patients. Third, the use of vaccines consisting of single or a few tumor antigens carries the risk of generating antigen-loss escape mutants. This is not a theoretical concern. Selection of antigen-loss variants in the face of a vigorous immune response was shown to occur in murine models and was also seen in a cancer patient [17].

An alternative approach, not encumbered by these limitations, is to use unfractionated tumor material as a source of tumor antigens. There are two main advantages of using total tumor-derived antigens in vaccine formulations. First, the identity of the effective tumor antigen(s) need not be known, a fact that expands the type of cancers that can be treated to include the majority of cancers where effective tumor antigens have not yet been identified. Second, the (likely) presence of multiple tumor antigens reduces the risk of escape mutants and increases the likelihood that some of the antigens present in the tumor tissue used for vaccine preparation will be represented in the metastatic lesions.

There are, however, also three potential drawbacks to vaccination with unfractionated tumor-derived antigens. First, use of unfractionated tumor material as a source of tumor antigen will depend on the availability of substantial amounts of tumor tissue from the patient. (The prudent assumption is made here that important tumor antigens are patient-specific and are not shared with other patients suffering from the same form of cancer). The problem is that many candidates for immunotherapy, i.e. patients with minimal disease who are at high risk of relapse, cannot provide sufficient amounts of tumor tissue for isolating the necessary amount of antigens needed for vaccination.

Second, the concern has been voiced that vaccination with unfractionated tumor-derived antigens could induce autoimmune responses directed against self antigens [13, 28]. This may not be altogether bad for prostate cancer and would be tolerable for melanoma or pancreatic cancer. What is the likelihood of that happening? In view of the recognition that passive tolerance (ignorance) may represent a major pathway for averting autoimmunity against peripherally expressed antigens [26, 27], this possibility has to be considered. With one exception, the majority of animal studies have shown that effective tumor immunity can be established in the absence of any visible signs of autoimmunity. Interestingly, in several instances even the effector arm of the T cell response was capable of distinguishing between tumor cells and normal cells expressing the cognate antigen [14, 23, 36].While all this is encouraging, clinical studies using total tumor-derived antigens must carefully monitor for autoimmune manifestations.

Third, it is conceivable that immunization with unfractionated tumor material would be less effective because of the low concentration of tumor antigens in the mixture. This, however, does not seem to be the case. Zitvogel et al. have shown that vaccination of mice with bone-marrow-derived DC pulsed with unfractionated tumor peptides was capable of reducing the growth of subcutaneously established, weakly immunogenic tumors [37]. Likewise, we have shown that DC pulsed with proteins or peptides extracted from tumor cells were remarkably effective in a post-surgical murine metastasis model [24, 25]. Why is vaccination with total tumor antigen so potent? Johnston et al. have shown that improved immunogenicity of tumor cells engineered to express the B7-1 gene correlated with expansion of the antigenic repertoire of the tumor recognized by CTL, thereby implying that vaccination with multiple tumor antigens is additive or even synergistic [19]. Another mutually non-exclusive explanation stems from the observations that induction of CTL is generally dependent on concomitant presentation of class-II-restricted antigens and induction of a CD4+ T cell (helper) response [5, 12, 20, 31, 33]. Such class II antigens will be more readily provided in unfractionated tumor preparations.

Form of antigen: polypeptide or polynucleotide

The ideal formulation of a cancer vaccine would consist of a mixture of defined, commonly if not universally expressed, tumor antigens. When, or even if, such effective tumor ("rejection") antigens will be identified cannot be, predicted, however. As discussed above, general considerations supported by experimental evidence from animal

studies argue that, for the foreseeable future, vaccination with unfractionated tumor-derived antigens will be more effective than vaccination with defined tumor antigens. The most commonly used forms of antigen in immunotherapy are polypeptide-based, protein or peptides. Indeed, vaccination with DC pulsed with protein or peptide mixtures isolated from tumor cells was shown to be highly effective in murine models, more so than GMTV formulations [25, 37].

The major limitation of using proteins or peptides isolated from the patients' tumor cells as source of antigen is that the amount of tumor tissue or the purity of the tumor specimens will preclude the isolation of sufficient amounts of antigen needed for vaccination, especially if it will require repeated boosting. The use of nucleic acids, DNA or RNA, as the form of antigen loaded onto DC would overcome this major practical limitation because the technology exists to isolate and amplify the mRNA content of single tumor cells microdissected from fixed tissue sections to generate either a cDNA expression library or a mRNA-like population corresponding to the expressed genetic information of the tumor cell. The cDNA library or the mRNA-like product could then be transfected into the DC, transiently generating the functional equivalent of a fusion hybridoma between the DC and the tumor cell. A second potential advantage of using nucleic acids is that tumor-specific mRNA can be enriched by subtractive hybridization. This will not only increase the effective concentration of the relevant antigens in the vaccine preparation but, perhaps more importantly, reduce the concentration of common, non-turmor-specific, mRNA species and hence lessen the potential for autoimmunity. While subtractive hybridization will not eliminate mRNA species encoding tumor antigens generated by point mutations, it is interesting to note that the majority of tumor antigens identified to date consist of non-mutated tissue-specific normal gene products [4].

DC vaccines loaded with polynucleotide-based antigens: RNA or DNA?

We have shown that RNA-transfected DC are potent APC and can serve as effective tumor vaccines in animal models [3]. Murine DC transfected with RNA encoding the chicken ovalbumin (OVA) antigen were capable of stimulating potent primary CTL responses in vitro, and tumor-RNA-transfected DC were remarkably effective in the post-surgical metastasis model mentioned above. Recently we have also shown that human DC transfected with RNA encoding a variety of antigens were capable of stimulating primary CTL responses in vitro (Nair et al. submitted for publication). The ability to transfect DC with DNA has also been reported and the transfected DC were able to present antigen to $CD8^+$ CTL [30]. While the efficacy of DNA-transfected DC as vaccines has not yet been rigorously demonstrated, there is no reason to believe that loading DC with DNA will be less effective than loading with RNA.

Efficiency of transfection aside, what are the possible advantages and disadvantages of using tumor antigen in the form of DNA or RNA in vaccine formulations? DNA is, of course, more stable than RNA and hence simpler to handle, an important consideration especially in clinical settings. DNA-encoded antigens are also likely to persist and be presented longer than RNA-encoded antigens in the transfected DC. There is, however, mounting evidence that DC presenting foreign antigen in the lymph nodes disappear quickly, presumably because they are eliminated by the activated T cells [18].

A potentially significant advantage of using RNA-encoded antigens is safety. The half-life of stable mRNA species in the mammalian cell is less than 24 h, while unintegrated DNA can persist and function in non-dividing cells for extended periods of time, measured in months. Considerations of safety come into play when tumor antigens that are mechanistically implicated in the neoplastic process, such as *E6* or *E7* genes of human papilloma viruses, are considered. Of even more concern would be vaccination with total-tumor-derived antigens. Clearly the RNA-encoded transient expression of the expressed genetic information of a tumor cell in normal, growth-arrested cells poses less risk than a cDNA expression library. (This argument does not contradict the suggestion that antigen-presenting DC in the lymph nodes are quickly eliminated because in a vaccination setting only a fraction of the injected DC are likely to find their way to the lymph node.)

There is also an important technical advantage of using RNA compared to DNA-encoded antigens. Generation of a cDNA expression library from the tumor cell will require the cloning of the amplified cDNA product into an expression plasmid to place an appropriate promoter 5′ to the cDNA and a polyadenylation signal 3′ to the cDNA. The cloning step is labor-intensive and requires considerable skills to generate sufficiently large representative libraries comprising more than 10^6 members. By contrast, no cloning is needed to generate a mRNA-based library because a 28-nucleotide-long T7 promoter can be easily accommodated in the primers used for amplification. (Primers encoding mammalian promoters have to be over 100 nucleotides long, which at present is not practical.) Thus, generation of mRNA-based expression libraries involves simple largely automatable test-tube reactions, which are completed overnight and generate very large representative libraries.

General conclusions

Three issues were considered here: 1) tumor vaccination strategies: GMTV compared to DC-based vaccines; 2) the pros and cons of using defined tumor antigens versus unfractionated tumor-derived material; 3) the form of antigen to load onto DC: polypeptide-based or polynucleotide-based, DNA or RNA. The jury is out on any of these issues. Nevertheless, general considerations and experimental data suggest some recommendations.

1. A large body of evidence derived from animal studies suggests that GMTV- and DC-based vaccines are potent tumor vaccination strategies, more potent than, "conventional" strategies that have been tested in clinical settings with limited success. The complexities of these cellular therapies notwithstanding, they therefore deserve serious consideration. If one has to choose between GMTV and DC vaccines, DC vaccines are currently the method of choice. While DC vaccines are equally, if not more potent than GMTV, as judged from animal studies, they offer a significantly simpler, clinically manageable, widely applicable, and (relatively) cost-effective, treatment strategy.
2. The ideal formulation would consist of a mixture of commonly expressed tumor-rejection antigens, that is to say, antigens that are capable of inducing protective immunity (as opposed to simply inducing CTL). It is not known, and there is reason to doubt in some instances, which of the currently known tumor-associated antigens will serve as effective antigens in a vaccine formulation. It is impossible to predict when such antigens will be identified. For the foreseeable future, and for the reasons discussed above, vaccination with total-tumor-derived material as source of antigen may, therefore, be preferable to using single defined tumor antigens.
3. The major limitation of using protein- or peptide-encoded antigens derived from tumor cells is that the amount of tumor tissue available from patients is often limited or heavily "contaminated" with normal tissue. The use of nucleic-acid-encoded antigens offers a means of generating sufficient antigenic material from as little as one tumor cell, hence expanding the ability to treat patients with tumor-derived antigen preparations to practically everyone. At present we have shown that RNA transfection is an effective means of delivering antigen to DC. While there is no reason to think that similarly potent strategies cannot be developed with DNA-encoded antigens, the use of RNA offers several potentially important advantages over DNA, which include increased safety and technical ease.

Thus, if put into the difficult position of having to pick the most promising approach to cancer vaccination today, we are forced reluctantly, and with the near certainty that we will be proven wrong, to choose DC vaccines loaded with total-tumor-derived antigens in the form of RNA.

(One has to wonder if the fact that the major focus of our program at Duke is the development of DC vaccines loaded with total-tumor-derived antigens in the form of RNA had anything to do with this conclusion...)

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