MINIREVIEW

The Potential of DNA Vaccination against Tumor-Associated Antigens for Antitumor Therapy

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Conventional treatment approaches for malignant tumors are highly invasive and sometimes have only a palliative effect. Therefore, there is an increasing demand to develop novel, more efficient treatment options. Increased efforts have been made to apply immunomodulatory strategies in antitumor treatment. In recent years, immunizations with naked plasmid DNA encoding tumor-associated antigens have revealed a number of advantages. By DNA vaccination, antigen-specific cellular as well as humoral immune reponses can be generated. The induction of specific immune responses directed against antigens expressed in tumor cells and displayed e.g., by MHC class I complexes can inhibit tumor growth and lead to tumor rejection. The improvement of vaccine efficacy has become a critical goal in the development of DNA vaccination as antitumor therapy. The use of different DNA delivery techniques and coadministration of adjuvants including cytokine genes may influence the pattern of specific immune responses induced. This brief review describes recent developments to optimize DNA vaccination against tumor-associated antigens. The prerequisite for a successful antitumor vaccination is breaking tolerance to tumor-associated antigens, which represent "self-antigens." Currently, immunization with xenogeneic DNA to induce immune responses against self-molecules is under intensive investigation. Tumor cells can develop immune escape mechanisms by generation of antigen loss variants, therefore, it may be necessary that DNA vaccines contain more than one tumor antigen. Polyimmunization with a mixture of tumor-associated antigen genes may have a synergistic effect in tumor treatment. The identification of tumor antigens that may serve as targets for DNA immunization has proceeded rapidly. Preclinical studies in animal models are promising that DNA immunization is a potent strategy for mediating antitumor effects *in vivo*. Thus, DNA vaccines may offer a novel treatment for tumor patients. DNA vaccines may also be useful in the prevention of tumors with genetic predisposition. By DNA vaccination preventing infections, the development of viral-induced tumors may be avoided.

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Conventional treatment options for malignant tumors include surgery, chemotherapy, and radiation. Because these treatment approaches are highly invasive and sometimes have only a palliative effect, alternative options to prevent or to treat malignant tumors are currently under investigation. In recent years, increasing efforts have been made to use vaccination strategies, including genetically modified tumor cells, dendritic cells either pulsed or transduced with tumor-associated antigens, immunization with soluble proteins or synthetic peptides, recombinant viruses or bacteria encoding tumor-associated antigens, and naked plasmid DNA encoding tumor-associated antigens (1). All of these antitumor vaccination approaches aim to induce specific immunological responses to tumor-associated antigens, destroying tumor cells and protecting patients from relapses. A persistent antitumor immune memory is based on the induction of expanded populations of T or B lymphocytes, which first recognize and then react against tumor-associated antigens with specificity and high destructive potential (2). One novel and powerful strategy for antitumor vaccination is the direct inoculation of plasmid DNA encoding tumor-associated antigens. This tech-

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nique, called DNA immunization, is known to induce both antigen-specific cellular as well as humoral immune responses (3–6). The generation of T cell-mediated cytotoxicity or antibody-mediated cytotoxicity against tumor cells can inhibit tumor growth and lead to tumor rejection.

History of DNA Vaccination

The DNA-based vaccination approach to elicit antigen-specific immune responses has been rapidly developed since the early 1990s when Wolff *et al.* (7) observed that mouse muscle cells express foreign antigens encoded by naked plasmid DNA that was injected intramuscularly. The first demonstration of the protective efficacy of a DNA vaccine was made by Ulmer *et al.* (8) who showed that DNA immunization with influenza A nucleoprotein resulted in the generation of nucleoprotein-specific cellular immune responses and protection from a subsequent challenge with heterologous influenza strains. Yet, DNA immunizations in animal models have been used to elicit protective immunity against various infectious pathogens and malignancies (3, 6). The demonstration of generating a cellular immune response against malaria and HIV peptides in human beings by DNA vaccination indicated the possibility for clinical application of this immunization technique (9, 10).

Immune Responses following DNA Vaccination

The construction of DNA vaccines involves cloning of the gene of interest into a plasmid under the control of a viral promoter, e.g., cytomegalovirus immediate early promoter. In cell nuclei, the plasmids persist as circular nonreplicating episomes, and they are not integrated into the host's genome (7), resulting in long-term expression of the encoded proteins by the host's cells (7, 11). Gene expression in the skeletal muscle can be detected for up to 19 months after injection (11). Therefore, DNA vaccines provide a stable and persistent source of the encoded antigen leading to a permanent stimulation of the immune system and the generation of long-lasting immunity (7). This antigen persistence may contribute to the efficacy of DNA vaccination in antitumor immunotherapy. The major advantage of DNA immunization is that both cellular (including $CD4^+$ and $CD8^+$ T cells) and humoral immune responses can be induced because the encoded antigen is processed through both endogenous and exogenous pathways, and peptide epitopes are presented by major histocompatibility complexes (MHC) class I as well as class II complexes. The uptake of plasmid DNA containing the gene of interest by host cells results in the *in vivo* synthesis of the encoded protein (7, 11, 12). The endogenously produced protein is processed into peptides by the proteasome. Membrane-associated transporters of antigeneic peptides (TAP) move these peptides into the endoplasmatic reticulum (13) where they associate with MHC class I molecules. The MHC class I-peptide complex is transported to the cell surface where it can be recognized by $CD8^+$ T cells (Fig. 1). Once activated, $CD8^+$ T cells acquire antigen-specific cytotoxic functions. These $CD8^+$ cytotoxic T lymphocytes (CTL) can kill tumor cells through the recognition of antigeneic peptides presented by MHC I molecules on the surface of the tumor. CTL are known to play an important role in the protection against tumors and in the induction of antitumor immunity. Therefore, an important goal for the development of an effective antitumor vaccine is the generation of a specific CTL response. The induction of CTL responses following DNA vaccination depends on the presentation of the antigen of interest by antigen-presenting cells (APC) (14, 15) displaying costimulatory molecules on their cell surface. APC are the predominant cell type capable of inducing T cells to become effector cells that can recognize and kill tumor cells (16). The CD28 molecule on the T cell membrane can interact with costimulatory molecules like B7-1 on APC. This interaction appears to be crucial for effective T cell activation and proliferation. One attractive feature of DNA vaccines is provided by the fact that bacterial plasmid vectors contain immunostimulatory nucleotide sequences—unmethylated cytidine phosphate guanosine motifs (CpG)—capable of causing maturation and activaion of APC (17–21). Bone marrow-derived APC have been shown to be responsible for stimulating naive CTL following itramuscular DNA immunization and gene gun bombardement of the skin (14, 22, 23). After DNA administration, APC either acquire antigen by being directly transfected (14, 24–26) or by the uptake of antigens released from other transfected cells (23, 27). Lysis of transfected cells expressing an antigen or secretion of the antigen lead to the release of protein, which is taken up by APC. In lysosomes, the antigen is proteolysed into peptides. These peptides bind to MHC class II molecules and travel to the cell surface. The MHC class II-peptide complex is recognized by $CD4^+$ T helper cells secreting cytokines like interleukin-2 (IL-2) that may facilitate tumor cell destruction in the effector phase of immune responses (Fig. 1). There is now increasing evidence that $CD4^+$ T cells are an important component of a successful antitumor immune response. Tumor-specific $CD4^+$ cells can not only provide help for the induction of specific $CD8^+$ CTL, but they may also be critical in activating macrophages and eosinophils to produce nitric oxide and superoxides that participate in the destruction of tumor cells (28–30). However, neither macrophages nor eosinophils have an intrinsic capacity for tumor specificity. Instead, the tumor specificity of these effectors is based on their activation by neighboring tumor-specific $CD4^+$ T helper cells (30). In addition, $CD4^+$ T helper cells may provide help to activate B cell antibody production. Humoral immune responses result from the secretion of antigen from transfected cells or by release of antigens as a result of cell lysis.

Advantages of DNA Vaccination in Comparison with Other Vaccination Strategies in Antitumor Therapy

DNA immunization is capable of inducing humoral as well as $CD4^+$ T helper cell and $CD8^+$ cytotoxic T cell im-

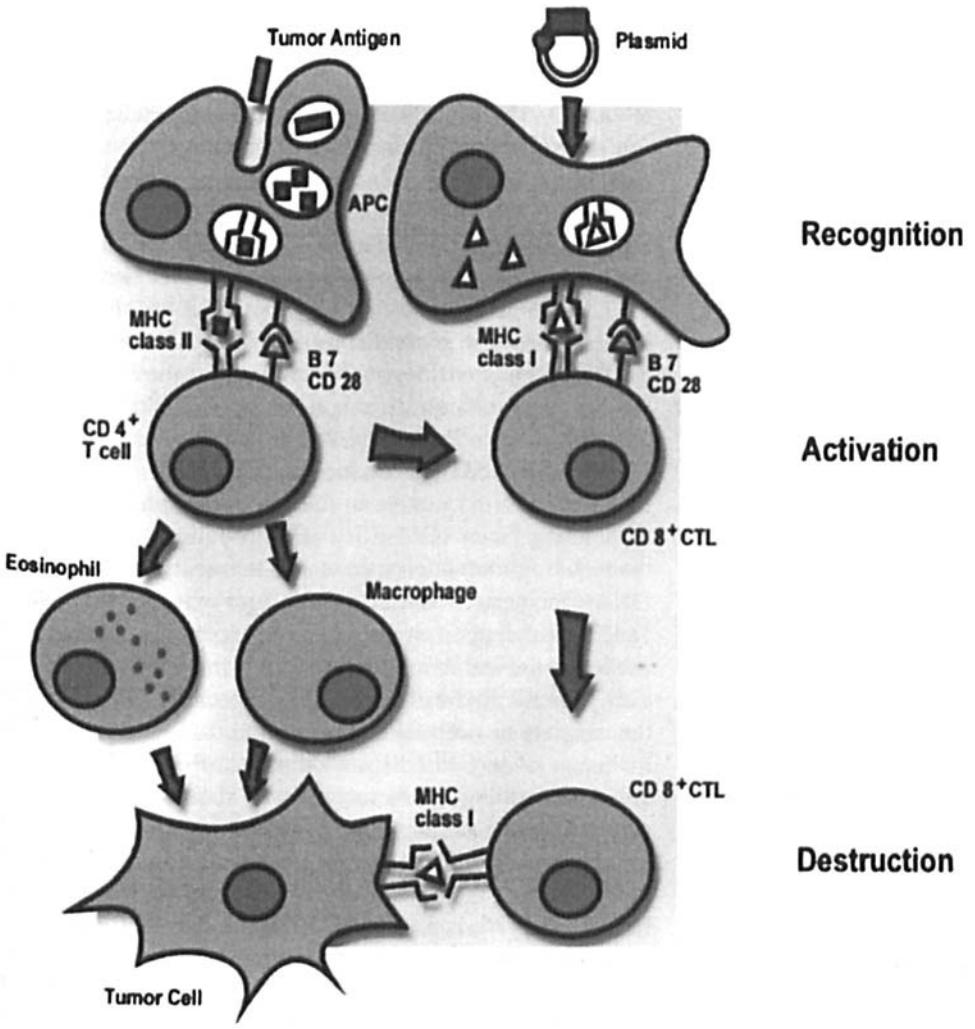

Figure 1. Priming of immune responses against tumor cells by DNA vaccination. The direct inoculation of plasmid DNA encoding a tumor-associated antigen into host cells, including professional APC, leads to the *in vivo* synthesis of the encoded antigen. The intracellular protein is processed into peptides that associate with MHC class I molecules. The MHC class I-peptide complex is displayed on the cell surface where it can be recognized by $CD8^+$ T cells. Once activated, $CD8^+$ T cells acquire cytotoxic functions and can specifically lyse cells expressing the target antigen. The predominant cell type capable of inducing T cells to become effector cells that can recognize and kill tumor cells following DNA immunization are bone marrow-derived APC. The CD28 molecule on the T cell membrane can interact with costimulatory molecules like B7-1 on APC. Lysis of transfected cells expressing the antigen or secretion of the antigen lead to the release of protein, which is taken up by APC. Internalized into lysosomes, the antigen is proteolytically degraded into peptides that associate with MHC class II molecules. The MHC class II-peptide complexes travel to the cell surface of APC where they can be recognized by $CD4^+$ T cells. These cells secrete cytokines that may facilitate tumor cell destruction in the effector phase of immune responses following DNA vaccination. Tumor-specific $CD4^+$ cells not only provide help for the induction of specific $CD8^+$ CTL, but may also be critical in activating macrophages and eosinophils to produce nitric oxide and superoxides that participate in the destruction of tumor cells.

mune responses. In contrast, by immunization with soluble recombinant protein, it is difficult to generate efficient CTL responses because exogenous soluble antigens are processed predominantly through the exogenous pathway. Nevertheless, it is possible to combine DNA and protein vaccination and, in some cases, this approach has been shown to significantly increase the antitumor efficacy (31). In contrast to peptide-based immunization approaches, which usually offer only a limited number of epitopes, DNA immunization allows the involvement of multiple different antigeneic epitopes and a broad range of MHC restriction. Thus, DNA vaccination does not require prior knowledge of host haplotypes. It is of interest to note that combining DNA and peptide vaccination strategies may have a synergistic effect in antitumor therapy (32). In addition, DNA vaccines offer a number of practical advantages. They can be easily and cheaply produced and purified in large quantities. They do not require special handling or storage conditions. In contrast to naked DNA, administrating vaccines based on viral vectors—most often adenovirus or vaccinia virus—may generate immune responses against viral antigens, which make booster immunizations less likely to be effective (33). Neutralizing antibodies may hamper the clinical efficacy of vaccines based on recombinant viruses. In addition, the use of viral vectors for vaccination poses risks for the recipient due to the potential pathogenicity, especially in immunocompromised hosts such as tumor-bearing patients with metastases (34, 35). DNA vaccines do not elicit undesirable immune responses to other components of the vaccine and, therefore, they can be used repeatedly to boost immune responses without the risk of provoking an immune attack against the vectors themselves (35). In addition, it has recently been shown that DNA vaccines can protect against tumors that are resistant to recombinant vaccinia vaccines containing the same gene (36).

Routes of Delivery of DNA Vaccines in Antitumor Therapy

Several techniques have been developed for the *in vivo* delivery of DNA vaccines. Possible routes of DNA delivery include direct intramuscular or intradermal injections of expression vectors (37, 38). Also, there exists the possibility to

admix plasmids with polymers and administration with a needle-free injection device (39). Furthermore, DNA vectors can be applied by gene gun using gold particles coated with DNA. To deliver the gold particles into the target tissue, they are accelerated e.g., by helium gas under high pressure. This method is highly effective, atraumatic (40–43), and offers the advantage of much smaller amounts of DNA being required for immunization than with direct intramuscular injection. Gene gun-mediated DNA immunization results in 10–100 times higher expression of the gene compared with intramuscular application (44). The ballistic delivery can introduce DNA directly into dermal APC, which subsequently migrate into local lymph nodes and prime immune responses (22, 24, 45). It has been demonstrated that priming of $CD8^+$ T cells by direct transfection of APC is the key event in DNA immunization with the gene gun (46, 47). It is of interest that gold particles without plasmid can have a modest effect on APC accumulation in tumor-draining lymph nodes and can increase the ability of lymph node cells to mediate tumor regression. This effect of gold particles is hypothesized to be related to both the local and regional accumulation of APC observed in skin samples and lymph nodes of animals treated by gene gun (48). Administrating plasmids that encode only a single epitope derived from mutated p53 by particle gun into living animals has been demonstrated to induce epitope-specific T cell immune reponse that produces significant protection against tumor cells expressing the epitope (49). A further promising new method in increasing transfection efficacy is the application of DNA by *in vivo* electropermeabilization (50). Tumor antigens can be delivered intramuscularly as polymer-based formulations of plasmid DNA encoding the antigen, followed by electroporation of the injected muscle (51). Another way of delivering genes is the use of aerosols. Aerosol delivery of plasmid DNA to the lungs represents a targeted and noninvasive approach of direct application of gene preparations to pulmonary surfaces as a potential means of treating lung tumors. Using DNA formulations containing polyethyleneimine, a polycationic polymer, results in a high level of *in vivo* transfection of lung tissue and stability during nebulization. Densmore *et al.* (52) suggest that persistent immune responses can be achieved with a single administration of plasmid delivering the formulations to pulmonary tissue (52).

Enhancement of Immune Responses Generated by DNA Vaccination

It is known that the route of application of plasmid DNA (53–55) as well as the immunization schedule (56) can determine the quality of the immune response generated. Therefore, attempts to increase immune responses following DNA immunization include varying the vaccination regime. Combining different routes of vaccination was shown to enhance the immunogenicity of encoded antigens (56). In addition, there exists the potential to influence the immune response generated by a DNA vaccine via codelivery of an adjuvant. A common strategy to further enhance DNA-based immunization is to employ cytokine genes as adjuvants (Table I). Several studies indicate that codelivery of vectors encoding cytokines such as IL-2, IL-12, interferon-γ (IFN-γ), or granulocyte macrophage-colony-stimulating factor (GM-CSF) is able to direct the nature of the resulting immune response and augments the efficacy of DNA vaccines (57–64). The benefit of cytokine gene adjuvants might depend on the intrinsic properties of the antigen used and the immunologic cell types involved (65). However, several studies confirm that especially GM-CSF has the capacity to potentiate DNA immunization (66–69). The inclusion of a GM-CSF encoding plasmid with a tumor antigen encoding DNA vaccine was shown to allow a reduction in the tumor antigen-encoding plasmid dose required for antitumor efficacy in animal model (70). It is suggested that GM-CSF enhances the initiation of immune responses by recruiting APC to the site where antigen is expressed (71, 72). GM-CSF stimulates the proliferation and the activity of APC (73), induces differentiation from immature APC to mature APC (74), and increases the expression of MHC class II molecules in APC (75), thus augmenting their antigen-presenting ability. It has been shown that the application of GM-CSF-encoding plasmid by gene gun results in APC accumulation within draining lymph nodes of tumors (48). Another cytokine that is important for the generation of APC and augmenting their function and quantity is Fms-like tyrosine kinase 3 (Flt3)-ligand. Recently published data indicates that fusion of a gene encoding the extracellular domain of Flt3-ligand to an antigen gene can greatly enhance the potency of DNA vaccines (76). It is remarkable that it is not only possible to coad-

Table I. Enhancement of Immune Responses Generated by DNA Vaccination by Coadministration of Cytokine Genes

Cytokine gene	Enhancement of immune response	Reference
GM-CSF	Cellular and humoral immune responses	116
IFN-γ	Th1 cells, CTL activity, and IgG2a antibody production	61
IL-12	Th1 cells, CTL activity, and IgG2a antibody production	61
IL-2	Th1 cells, CTL activity, and IgG1 and IgG2a antibody production	61
IL-4	Th2 cells and IgG1 antibody production	61
Flt3-ligand	CTL activity and antitumor immune response	76

ministrate cytokine-encoding vectors to antigen-encoding ones, but also to link the cytokine gene directly to the DNA vaccine or to insert DNA coding for an immunomodulatory peptide of a cytokine (77–79). A novel alternative possibility for enhancing the immunogenicity of DNA vaccines is the use of plasmid DNA vectors containing replicons derived from viruses. Recent experiments pointed out that these plasmids launch a self-replicating RNA vector that in turn can direct the expression of a model tumor antigen. Leitner *et al.* (80) have shown that plasmid DNA replicons induce stronger immune reponses than conventional DNA vaccines and effectively treated tumor-bearing mice. In this study, transfection was associated with the apoptotic death of host cells, which may increase the uptake of antigen by APC (80). In addition, attempts to enhance the efficacy of DNA vaccines include coexpression of costimulatory molecules. These approaches may counteract immune escape mechanisms of tumors because one feature of tumor cells explaining their failure to stimulate effective CTL responses is their lack of expression of the costimulatory molecules B7-1 and B7-2 (81). These molecules are ligands for CD28 and CTLA4, providing the second signal that is required for the activation of T cells (82). It has been shown that vaccination of animals with plasmids encoding an antigen and B7-1, but not B7-2, can induce immune responses against a transfected malignant tumor expressing the antigen (83). CD40 ligand (CD154) as well can serve as a genetic adjuvant capable of augmenting humoral and cellular immune reponses to antigens encoded by plasmid DNA expression vectors (84). Another strategy for increasing the potency of DNA vaccines represents the linkage of tumor antigen gene to *Mycobacterium tuberculosis* heat shock protein 70 gene or to the translocation domain of *Pseudomonas aeruginosa* exotoxin A gene. These fusions have been shown to increase the frequency of specific CTL by at least 30-fold and to convert less effective vaccines into ones with significant potency against tumors expressing the antigen (85, 86). Recently, You *et al.* (87) described a novel DNA vaccination strategy for enhancing uptake and presentation of antigens by APC. The authors developed a DNA vaccine including an antigen fused to an IgG Fc fragment. After DNA vaccination, the produced antigen-Fc fusion proteins are secreted and efficiently captured and processed by APC via receptor-mediated endocytosis. Using this strategy, a broad enhancement of DNA vaccine potency, including all arms of the immune system, could be achieved (87).

Strategies to Overcome Difficulties Associated with Antitumor DNA Vaccination

For antitumor immunotherapy, it is of importance that most tumor immunity is mediated by the recognition of self-antigens, because antigens expressed by tumor cells can also be found in normal host tissue (88). Therefore, in order to induce an effective antitumor immune response by DNA vaccination targeting such antigens, the immunological tolerance to self-antigens has to be overcome. Recently published studies have shown that xenogeneic DNA immunization exploiting small differences in expressed tumor antigen protein sequence can result in immune recognition of self-molecules in mice, whereas immunization with syngeneic DNA failed to do so. This approach of using a xenogeneic source of tumor-associated antigens is documented to break tolerance to the corresponding self-antigen and to induce tumor immunity (65, 89–92). As one example, immunization of mice with xenogeneic DNA coding for the human melanosomal membrane glycoprotein gp100 is able to break tolerance to mouse gp100 and to generate an effective antitumor immunity. Animals immunized with xenogeneic human gp100 DNA were significantly protected from tumors after a challenge with a syngeneic melanoma expressing gp100, whereas immunization with mouse gp100 DNA failed to induce antitumor immune response. DNA immunization with human gp100 decreased lung metastases by 50%, and long-term tumor-free survival of the animals was noted (65). Weber *et al.* (89) speculated that minor differences in protein sequences between xenogeneic human and syngeneic mouse protein elicit T cell help, possibly by providing MHC class II-restricted peptides in the human homologous protein that bind MHC class II with higher affinity (89). In contrast, in the study of Hawkins *et al.* (65) xenogeneic DNA vaccination was demonstrated to induce tumor immunity without $CD4^+$ T cell help (65). Yet, the precise mechanisms that are critical for overcoming immunological self-tolerance are not completely understood. It has become clear that the immune system contains autoreactive CTL, B cells, and T helper cells that are not necessarily deleted from the immune repertoire during development. Autoreactive T and B cells with high-affinity receptors against self-antigens may be deleted during the development of the immune system, whereas immune cells with intermediate or low-affinity receptors may remain in the repertoire (89). However, several mechanisms prevent the activation of these autoreactive cells, including anergy, ignorance, deletion, and the presence of regulatory T cells (91). The autoreactive lymphocytes may be triggered by cross-reactivity, which is based on homologies between an evolutionarily conserved inciting antigen and the ultimate target antigen. Cross-reactive immunity to a mouse self-antigen is shown to be induced by immune recognition of the corresponding human protein following xenogeneic DNA immunization (89). Clearly, for a beneficial use of DNA immunization as immunotherapy for tumor-bearing patients, the precondition to break immunological self-tolerance to human antigens is required, leading to a potent response against self-tumor antigens. Using xenogeneic DNA vaccines in human beings might be a way to by-pass this difficulty. Also, breaking the immunological self-tolerance to human tumor antigens may be supported by codelivery of appropriate adjuvants.

One additional difficulty associated with antitumor DNA vaccination is that many tumors escape immunological destruction. Tumors are often heterogenous and the ex-

pression of tumor-associated antigens may be downregulated or even lost (93). Different subpopulations of tumor cells, especially in metastatic sites, may express various tumor-associated antigens and different amounts of them. Thus, the repertoire of tumor antigens of cells of one tumor may be variable, even within the same patient. Hence, it is possible that a defined tumor-associated antigen targeted by DNA vaccination is capable of stimulating an immune response, but is not expressed by all cells of one tumor. As consequence, malignant cells not expressing this antigen may not be destroyed by immune cells stimulated by the DNA vaccine. For that reason, vaccination including more than one tumor-associated antigen may be advantageous over single antigen-based vaccines. It is likely that polyimmunization is more efficacious against tumors by leading to the elimination of a greater number of the diverse populations of malignant cells. By stimulating immune responses to different antigens expressed by tumor cells, more of the heterogenous cells may be killed, and the generation of tumor cell populations lacking the expression of the encoded antigens by immune selection pressure may be avoided (94). DNA immunization opens the possibility of applying several genes encoding antigens simultaneously in one vaccine, allowing cotransfections of genes encoding different tumor-associated antigens. Antigen-encoding plasmids can be mixed in the same formulation without the need of constructing polycystronic vectors. Previously, examples of polyimmunization in a therapeutic setting have been published. It was shown that immunization of mice with plasmids encoding either human gp100 or mouse TRP-2 antigens induced only partial rejection of melanomas, whereas immunization with a combination of these two antigens in the same vaccine formulation caused tumor rejection in 100% of the immunized animals. These mice developed CTL responses against both antigens. Most important, polyimmunization led to the generation of a therapeutic immune response that significantly improved the mean survival time of mice bearing established lung metastases (51). These experiences indicate that polyimmunization with a mixture of tumor-associated antigen genes can have a synergistic effect in the treatment of tumors.

Therapeutic DNA Vaccination against Tumors

The identification of novel tumor antigen genes suitable for DNA vaccination has proceeded rapidly. In general, all antigens being expressed tissue specificly could be possible candidates targeted by DNA vaccines if the expressing tissue is not essential for health and survival to avoid autoimmune destruction with negative consequences for the vaccinated individual. During the last years in animal models, DNA immunization has been demonstrated to provide tumor immunity and to elicit immune responses specific against a wide variety of tumor-associated antigens (Table II); examples are gp100, gp75, melanoma-associated antigen MAGE-1, and MAGE-3, which are associated with malignant melanoma (51, 89, 95–99), the folate receptor α as antigen associated with ovarian carcinoma (100), free human chorionic gonadotropin β subunit, which is expressed by different tumors (101), HER-2/neu, which is associated with breast cancer (102, 103), the paraneoplastic encephalomyelitis antigen HuD, which is associated with small-cell lung cancer (104), tyrosine hydroxylase, which is associated with neuroblastoma (105), and prostate-specific antigen (64, 106). Also, DNA vaccines seem to be useful not only for treatment of solid tumors, but for diverse hematologic malignancies as well. Several studies have demonstrated that immunization with DNA encoding the idiotypic determinants of a B cell lymphoma can generate tumor-specific immunity (107–111), which is suggested to be largely attributed to idiotype-specific humoral immune response (112). An additional, novel example is DNA immunization with antigens of human T cell leukemia virus type 1, which has been shown to induce specific CTL responses and antitumor immunity against the virus-induced human adult T cell leukemia (ATL) in a rat model (113).

Table II. Examples for DNA Vaccination against Tumor Antigens in Animal Model

Target	Tumor	Antitumor immune response	Reference
Human gp100	Melanoma	Decreasement of lung metastases by 50% and 50% long-term tumor-free survivors	65
Human gp75	Melanoma	Significant protection from lung metastases and 86% decrease in lung nodules	89
Human TRP-2	Melanoma	Significant tumor protection	91
HER-2/neu	Breast cancer	Significant reduction of tumor development	102
Folate receptor α	Ovarian carcinoma	Significant delay in tumor growth, enhancement of survival time, and reduction of number of lung metastases	100
hCGβ subunit	Myeloma expressing free hCGβ protein	Marked reduction of tumor size and 30% long-term survivors	101
Encephalomyelitis antigen HuD	Small cell lung cancer	Reduction of tumor size	104
Prostate-specific antigen	Prostate cancer	Specific CTL response	106
Tyrosine hydroxylase	Neuroblastoma	Protection from lethal tumor challenge	105

Prophylactic DNA Vaccination against Tumors with Genetic Predisposition

Several gene mutations have been described that predispose for the development of special tumors (114), e.g., BRCA1-gene mutation, which is associated with breast cancer. In persons carrying such mutations, a prophylactic immunization appears to offer promise to prevent tumor formation. In this field, DNA vaccines may provide one opportunity of prevention for people genetically at risk. One potential example of hereditary tumors that could be treated prophylactically by DNA immunization may be medullary thyroid carcinoma. This tumor occurs sporadically, as a familial form without associated endocrinopathies, or combined with other endocrinopathies in multiple endocrine neoplasia type 2. The familial forms are associated with mutations in the RET-gene, and in subjects of families where an index case has been investigated, RET-gene mutation analysis are performed to detect asymptomatic disease gene carriers (115). Medullary thyroid carcinoma arises from thyroid parafollicular C cells secreting calcitonin. Almost all of these carcinomas specifically express calcitonin. Therefore, calcitonin may represent a suitable target antigen for DNA vaccines. Previously, we were able to show that DNA immunization by gene gun with an expression plasmid encoding the human calcitonin precursor preprocalcitonin enables induction of antigen-specific cellular and humoral immune reponses in mice. Codelivery of a plasmid encoding GM-CSF increased the efficacy of this DNA vaccine (116). These findings may provide the basis for the generation of an efficient antitumor immune response against medullary thyroid carcinomas by DNA vaccination and may raise hope for a prophylactic or even novel therapeutic treatment option for people suffering from this tumor. Family members genetically at risk of developing medullary thyroid carcinoma may benefit from preventative DNA vaccination against (prepro)calcitonin by eliminating or at least postponing disease manifestation. If successful, DNA vaccination could obviate the need for prophylactic thyroidectomy that now has to be performed at an early age of gene carriers.

Prevention of Viral-Induced Tumors by DNA Vaccination

Potential clinical applications for preventative DNA immunization are also found in tumors associated with viral infections. In these cases, DNA vaccines directed against viruses could be of value in reducing the risk of tumor formation. As examples of such tumors having a viral carcinogenesis that could be prevented by DNA vaccination, the cervix carcinoma and tumors associated with Epstein-Barr virus (EBV) infection are currently under intensive investigation. Most cervical cancers are associated with an infection of human papillomavirus (HPV), predominantly HPV16 and HPV18. For vaccine development, HPV16 antigens are often chosen as targets. The HPV oncogenic proteins E6 and E7 are important for the generation and maintenance of cellular transformation and are expressed in most HPV-containing malignancies. Several studies demonstrated in different animal models that DNA immunization with plasmids encoding HPV oncogenic proteins can prevent or delay invasive carcinoma development of HPV-induced skin papillomas (117–119). In this case of virally induced malignancy, not only preventative immunization seems to be effective, but also vaccination in a therapeutic setting (76, 120). Recently, it has been shown that DNA-based multiepitope vaccination against HPV16 was able to significantly reduce the size of established tumors in mice, but the addition of defined flanking spacers between the epitopes was crucial for the tumor protection. Moreover, targeting the vaccine-encoded protein to the protein degradation pathway by linking it to ubiquitin lead to eradication of 100% of established tumors of vaccinated mice (121). Hung *et al.* (76) showed that a chimeric DNA vaccine containing the E7 gene fused to a gene encoding the extracellular domain of Flt3-ligand was capable of controlling lethal pulmonary metastases of established E7-expressing tumors in mice. However, the E7 gene of oncogenic HPV types is a real oncogene and, for safety concerns, should not be utilized for DNA vaccination of human beings. For that reason, Smahel *et al.* (122) introduced point mutations into the HPV16 E7 oncogene to eliminate its transformational potential. The experiment pointed out that this mutagenesis significantly enhanced the immunogenicity of HPV16 E7. Thus, this strategy may provide an opportunity to treat HPV-associated cancers in humans. Tumors that are associated with EBV infection include Burkitt's lymphoma, nasopharyngeal carcinoma, and AIDS-associated lymphoma, etc. Charo *et al.* (66) found that mice, even after 3 months from the last DNA immunization with a plasmid expressing the EBV nuclear Ag-4, were fully protected against tumors expressing this antigen. This finding provided evidence for long-term memory induced by DNA immunization that leads to the protection of tumor outgrowth (66). The authors suggested the application of this method to vaccinate at an early age to avoid encountering diseases later in life.

Concluding Remarks

This short review shows that DNA immunization against tumor-associated antigens may be an additional form of antitumor therapy in the future. The approach may provide a potential tool in fighting tumor cells irrespective of their distribution in the body. All tumor antigens being expressed tissue specificly could be possible candidates targeted by DNA vaccines if the expressing tissue is not essential for health and survival. Accumulating evidence suggests the usefulness of DNA vaccination for treating various tumors in animal models, but results from clinical trials are lacking and the therapeutic benefit in the prevention or treatment of malignancies in human beings remains to be proven. The prerequisite for a successful antitumor DNA vaccination is that the immunological self-tolerance to tu-

mor antigens also found on normal cells has to be overcome. In animal model, advances have been made in this field by using a xenogeneic source of the DNA. Improvements of vaccine efficacy may be realized by combining different tumor-associated antigens as targets for DNA immunization as well as by coadministrating appropriate cytokine genes to enhance immune reponses. It is most likely that an optimal DNA vaccine against tumors targets to more than one antigen to avoid escape mechanisms by the development of antigen loss variants and includes one or more immunostimulatory adjuvant(s). Vaccination with DNA in combination with proteins, peptides, or cell lysates may have a synergistic therapeutic effect against tumors and may be necessary to achieve complete protection. In conclusion, DNA vaccination is a promising strategy capable of inducing immune-mediated tumor reductions in animal model, but further studies are required to investigate the potential of DNA vaccination in antitumor treatment in human beings. In cases of hereditary tumors and tumors that are associated with viral infections, prophylactic immunization strategies should be considered to potentially reduce the tumor incidence of family members genetically at risk or persons susceptible for, or suffering from, viral infections associated with tumor formation.

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