Review article
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Vaccine therapy in melanoma today

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Melanoma is the most significant malignant tumor of the melanocyte system. It is characterized by a high malignant potential and an outstanding possibility for giving metastasis. Despite all investigations and progress concerning molecular genetics and immunology melanoma is a therapeutic problem even today. Vaccines are being developed with an intention not only to prevent but also to cure the disease, and the most important aim of clinical trails is to develop corresponding antitumor immunotherapy based on vaccine.

KEY WORDS: *Melanoma; Immunotherapy, Active; Vaccines; Adjuvants, Imunologic*

INTRODUCTION

The most recent meaning of the term vaccine involves usage of any antigen or a fragment of antigen, or any group of antigens, which are used separately or together in order to modify the immune response. It is significant that experimental vaccines are now being developed with an intention to treat a disease and not only to prevent it (1)

From the early '90s, many clinical trials on the development of cancer vaccines have been initiated. There are a growing number of pharmaceutical and biotechnological firms that participate in development of cancer vaccines. It was shown that following decisive factors have an important role in clinical investigation: an explosive growth of understanding the antigen presentation and T-cell activation, better understanding of the factors important for antitumor immune response, the easiness with which genes may be introduced in malignant cells in order to modify their immunogenicity, and the achievements in immunology and in molecular biology which enable the creation of defined antigen vaccine (2).

Maybe the most important influence on the development of cancer vaccine so far has been the recognition of the crucial role that T lymphocytes have in antitumor immune response. Activation of both CD4+ and CD8+ T cells is critical for generating the most potent antitumor immune responses, which are initiated by "professional" antigen-presenting cells (APCs). At the same time any protein in the tumor cell is a potential tumor antigen (3).

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It appears that in both animal and human malignancies T cells that gather around tumor specifically make lyses with autologous tumor cells *in vitro* and proliferate and specifically secrete cytokines, such as IL-2, IFN-gamma, granulocyte macrophage colony-stimulating factor (GM-CSF), and TNF in answer to the stimulation by autologous tumor cells. The observed antitumor activity with the systematic application of T-cell growth factor IL-2 in animal models, and possibly in people, is mediated to large extent by tumor antigen-specific T lymphocytes (2,4).

In carcinoma, the genetic look of tumor is changed and leads to changes in expression and structure of different molecules. Patients with carcinoma are not able to reject their tumors so that the clear antigen presentation is suboptimal, and some form of vaccine is necessary (5). In the last 5 years, a large number of cancer vaccines in clinical trials are designed to induce cell-intervened immune responses, based on following key discoveries in the field of cancer immunology: 1) proving that immunotherapy, including cancer vaccines, has an antitumor activity, both in the animal models and in patients; 2) recognition of the crucial role which T-cells play in these anti-cancer immune response; 3) understanding that antigens recognized by T-cells are in fact peptide fragments of intracellular proteins which are transported in endoplasmic reticulum, bounded to MHC molecules and then taken to cell surface; 4) comprehension that these tumor antigens may originate from normal proteins with limited normal tissue expression, or from proteins which are exprimated only in tumor, such as mutated oncogenes and virus transformed proteins; 5) proving that these tumor antigens can be mutual (exprimated in tumors of different patients); 6) development of techniques for cloning and manipulating of these mutual antigens in order to make defined antigen vaccines (6,7).

A key moment, which created a completely new approach in vaccine development, is the easiness with which antigens may be introduced in carcinoma cells in order to modulate their immuno-

genicity. These techniques enabled a generation of immunogenic cancer vaccines from patient's tumor or from the continually preserved allergenic cell lines of carcinoma, which show mutual tumor antigens (7).

The main deficiency of defined antigen vaccines is connected with the problem that immune response against narrowly defined group of antigens enables tumor cells to evade immunologic recognition mainly because they very often mutate. Finally, it will be perhaps necessary to carry out combinations of defined antigens in order to span the problem of tumor heterogeneity (6,8).

GENE MODIFICATION OF TUMOR CELLS

Recombining DNA techniques enabled introduction of genes, which code specific immunomodulatory molecules in tumor cells. By supporting such approach, a significant progress was made during the 90s in the work on experimental animals. Tumor cells were genetically modified *in vitro* or *in vivo*, in order to exprimate several different classes of genes. Gene transfection can be significantly more immunized than a mixture of tumor cells or cell lyses with unspecific immunostimulants because gene modified tumor cells can continue to produce immunostimulants as long as the tumor cells are able to live (7).

A prerequisite for effective immune response to carcinoma cells

To reject carcinoma cells by using immune system, carcinoma cells should be presented as pathogens. Carcinoma cell itself is not a "professional" APC, and although it might be a target for antibodies and cytotoxic T lymphocyte (CTL), it cannot effectively present the antigens T and B to the cells (8). Because of that, any tumor-specific or tumor-associated antigen demands interaction with APC in such a way that they are presented to the immune system. Although these genes can be antigenically separated from molecules exprimated on normal cells, in majority of cases they do not look like proadrenomedullin peptides (PAMPs) of microorganisms (9).

A strategy of using DNA vaccines, which contain genes that code certain proteins of the targeted organisms, has recently been developed. It turned out that it is not only possible to construct and transport DNA vaccines, but that they can also be introduced into a cell in a similar way as an infective virus. That means that all paths of the immune system are activated. Preventive vaccines against cancer-associated viruses will be developed in future, but they will be applicable on only 10% to 20% of the carcinoma. A challenge for majority of carcinomas is to activate the immune system against already present tumor cells, although they could be in a small number in a minimal residual illness. DNA vaccines may have a power to induce immune system in such a situation. Maybe a big problem is in the immune status of a patient, which is often damaged both by the illness and the chemotherapy. There are new approaches in treatment and transplantation, which may help in reconstructing the immune system. Of course, for at least some tumors, we are in position to test vaccines on patients, with a hope of success (10).

MELANOMA VACCINE

The development of the therapeutic tumor vaccine in melanoma is under way. It is supposed that vaccines can enlarge the immune response of the patient to melanoma and thus to postpone or prevent the progression of the illness. The earliest studies from the 80s used tumor cell lyses (11). Since then many different approaches have been investigated and most of them have been directed to the therapeutic effect of vaccines on the patients with a progressed illness, and their obvious low toxicity suggest that they could be used as an adjuvant therapy in the early melanoma stadiums. One of current approaches combines tumor vaccines with the adjuvant IFN-alfa therapy in order to increase the benefit of this treatment. It is expected that a synergistic interaction will be produced between these treatments according to the effects of interferon-alpha on the treatment of antigens and the immune system (12,13).

Majority of all the conducted cancer vaccine studies were done on patients with melanomas. Almost all studies done before 1990 used preparations of autologous or allergenic cell line. Several researchers, who conducted the recent vaccine studies, treated many patients in metastatic disease, and in the post-surgical adjuvant treatment they followed the results for several years. The first defined antigen vaccines, which were presented in the early 80's, included anti-idiotypes and purified gangliosides that produce serologic answer against antigen tumors. These vaccines are observed separately from the modern melanoma vaccines which are primarily based on induced T-cell answer against tumors and include both the defined antigen composition and gene-modified tumor cells. Defined T-cell antigen vaccines came into clinical usage only four years ago. Vaccines with genetically modified cell lines have existed in clinical studies for almost the same time period (13).

Neoangiogenesis appears in horizontal and vertical phase of primary melanoma growth, but lymphatic genesis does not. Nevertheless, the existing lymphatics show signs of activation, and intraluminal malignant cells are present, which may contribute to the local tumor spread. Tumor-associated hemangiogenesis can force hematogenous spread by the way of melanoma-associated adhesive molecules sialyl-Lewis X (sLeX), sialyl-Lewis A (sLeA) whose expression grows with the thickness of tumor. In fact, sLeX and sLeA bind with endothelial E- and P-selectin, and MUC 18 reacts with MUC 18 ligand, which is exprimated both on melanoid and endothelial cells, inducing homotypical adhesion of melanoma cells and their heterotypical interaction

with endothelium. Contrary to melanocytes, which answer only to a limited number of growth factors, melanoma cells, as a rule, secrete cytokines excessively with stimulative or inhibitory activity on tumor growth, or cytokines such as IL-10, which has immunosuppressive features. Because of that, immunotherapy with cell or synthetic-peptide based vaccines can be inclined to cytokines which are liberated during vaccination or on the tumor spot. Uncoordinated liberation of cytokines also influences angiogenesis, adhesion or invasion and transition of melanocyte cells from the commonly acquired to dysplastic nevus (IL-8 and melanoma protein 1), or from radial to vertical phase of melanoma growth [platelet-derived growth factor (PD-GF)]. The suppression of tumor growth by using tamoxifen and quercetin, which bind with the type II estrogen bonding places on melanoma cells, is mediated by liberating transforming growth factor beta 1, which binds to endoglin exprimated on melanoma cells (14,15).

Availability of antigenic peptides melanoma associated antigens (MAAs), which would be used for T-cells-based immunotherapy, imposes a question how they should be given. One strategy is to vaccinate patients with melanoma with *in vitro*, peptide-pulsated autologous APCs. In fact, APCs show high levels of HLA class I and II, and/or co-stimulatory molecules (CD54, CD58, CD40, CD80 and CD86); they secrete cytokines, and migrate to lymphoid organs where optimal clones of T cells may appear. Vaccination of HLA-A1 or HLA-A2 melanoma patients with autologous GMCSF-activated and MAGE-1- or MAGE-3-pulsated monocytes induces peptide-specific CTL answer (15,16). As IL-10 under modulates inflammatory answers, its liberation on the tumor spot, stirred by vaccination with dinitrofenyl-conjugated autologous melanoma cells, can prevent destruction of the tumor through immunity (15-17).

Melanoma-defined antigen vaccines and genetically modified tumor cells for induction of T-lymphocyte immune responses

From 1989, several groups of investigators showed that CTL isolated from one patient could kill corresponding HLA class I-matched melanoma cell lines from other patients. At the same time, enough data were accumulated from the preclinical and clinical studies, which point to the importance of T-cell responses in the immunologically mediated regression of tumor. These discoveries stimulated further investigation, which led to cloning and production of several melanoma T-cell antigens and development of defined antigen vaccines that were applicable on patients with corresponding HLA phenotype (18,19). The first identified antigen was tyrosinase, a central enzyme of melanin synthesis. Others, including MART-1/Melan A, go100, TRP-1 and TRP-2 came subsequently (20).

Using autologous CTL from patients with melanoma and complex treatments which include cloning genes from antigen-loss variant of melanoma cell lines established in patients, van der Bruggen and associates discovered the first melanoma-associated T-cell antigen. T-cell antigen presentation referring to MAGE-1, is expressed exclusively through HLA-A1, which is present in about 25% of white (Caucasian) population. Peptide epitope recognized by CTL in the context of HLA-A1 was identified, followed by other CTL peptide epitopes limited to HLA-Cw1601. MAGE-1 is determined as a family member of normal proteins whose genes are located on X chromosome (19, 20). Its expression is limited to melanoma (in about 40% cases) and other malignancies, such as glioma, breast tumor, head and neck tumor, and non-microcellular lung carcinoma. MAGE genes are also exprimated in normal testicles. After discovering of MAGE-1, the other member of the family, MAGE-3, was discovered and exprimated in around 70% cases of melanoma. Both HLA-A1 and HLA-A2.1-restricted epitopes are recognizable for MAGE-3. Other two related tumor-antigen genes, BAGE and GAGE, have recently been isolated and show similarity with MAGE genes and in their way of expression in tumor, and in a poor expression in all normal tissues except testicles. Peptide epitopes, identified for BAGE and GAGE, are limited to HLA-Cw and HLA-Cw6 (21).

After discovering of MAGE gene, more effective techniques of antigen cloning were developed. Consequently, the series of HLA-A2.1- restricted CTL antigens and their peptide epitopes became available for immunizing approaches, including MART-1, GP-100, and tyrosinase (21,22). These genes probably contain other epitopes bound by different alleles of class I, and perhaps by alleles of class II. It seems that tyrosinase contains HLA-A24- and HLA-B44-restricted epitopes and it has recently been shown that the first melanoma antigen contained a common epitope with CD4+ helper cells (limited by HLA-DRBI*0404). At least two more normal genes appear in melanoma, GP75 (brown locus) and p15; they have recently been shown to contain peptide epitopes recognized by humane tumor-infiltrating lymphocytes and bounded by HLA-A31 and HLA-A24 (22, 23).

It appears that so far discovered melanoma antigens have wide distribution in tumor cells from the fresh biopsy material, including tumors of the patients with different MHC phenotypes of class I. Majority of the normal antigens emerge only on melanocyte line or retina, although p15 was also found in other normal tissue (21). Recently the researchers also discovered a unique mutation on CDK4 gene, which is present in melanoma tumors and can be recognized by cytotoxic T lymphocytes. It remains to be determined whether these unique mutations are more relevant for individual patients as rejecters of the tumor antigen in comparison with the mutual melanoma antigens which can be more easily used for making the vaccine (24).

Almost at the same time, the discovery of the common melanoma antigens gave an impulse to the development of defined antigen

vaccines, several preclinical studies documented the ability of gene-modified cytokines in tumor cells to cause protective immunity against the challenge with the wild-type of tumor cells. Under certain circumstances, gene-modified cells may cause regression of the already established micrometastatic illness. On the basis of these data, numerous clinical studies of autologous melanoma vaccines transfected with cytokines or other molecules, which can enforce antigen presentation, have been started or they will soon be started (25, 26). Techniques for inserting genes of interest vary, although in majority of cases transfection demands short-lived *in vitro* tumor cultures or placing a cell line. To evade *in vitro* tumor manipulation, several clinical studies in the USA and Europe try to insert genes in tumor by direct injecting *in vivo*, also including efforts for xenogenesis of melanoma tumors with extraneous HLA gene or to insert cytokines or T-cell co-stimulatory genes. Preliminary clinical experiences are achieved with plasmid DNA contained in HLA-B7 gene, which was created in cationic lipid vector. Such structure was applied to the melanoma lesion of 5 HLA-B7-negateive patients. HLA-B7 expression was demonstrated in injected tumors and 1 patient had a partial regression of both injected lesions and distant visceral metastases in lungs. The phase I of studies conducted with plasmid containing beta-2 microglobulin included patients with colon and kidney carcinomas. Among 11 melanoma patients treated in the recent studies, 5 demonstrated partial regression in injected lesion. According to the present data, the observed clinical responses including periodical regression of distant metastases are not much different from the description of the effect of locally injected BCG or virus antigen in studies which were conducted in the 70s (24,25,27).

Melanoma vaccine in adjuvant treatment

There is a great interest in the possibility of melanoma vaccine being used in adjuvant treatment to lower the risk of relapse. Various tumor vaccines have so far been created and tested initially on patients with progressed illness or in the adjuvant treatment of high- or medium-risk groups. Next step will be the combination of the vaccines with IFN-alpha therapy and other immunopotentiators to examine their usage in adjuvant treatment (25). Although it has been showed that IFN-alpha lowers the risk of relapse in the patients with stadium II or stadium III of the illness, the risk remains high and more effective therapies are necessary. It is expected IFN-alfa and vaccines to work united because IFN-alfa improves the handling of antigens. Low toxicity of the vaccines makes them highly suitable for usage in adjuvant treatment in combination with IFN-alpha (26,27).

At the moment, majority of the vaccines have been tested on patients with advanced illness and only limited efficiency was received, i.e. as a response rate and/or relapse-free or overall survival. In the sense of their development, additional agents are necessary for improving of relapse-free period and overall survival (27).

Vaccinia viral lysate

Vaccinia viral lysate (VVL) is under investigation as melanoma vaccine from the early 1980s. As Hersey described (Newcastle, Australia), promising results in phase II, led the Australian researchers towards randomized study, comparing immunization with vaccinia melanoma cell lysates during two years period versus no therapy in adjuvant treatment. This study started in 1999 and so far has recruited 648 patients. Hersey reported a little shift in disease-free survival and overall survival for patients who received the vaccine, in comparison to the control group (median survival: 96 months versus 80 months). However, the difference is not statistically significant. Hersey explained that this reflected the fact that the level of relapse and mortality in control group was much lower than the expected from the previous studies. The study will recruit and follow the patients in the following 3 years, too. The researchers hope that this can contribute to the benefit of the therapy with vaccine and become significant (26, 28).

GM 2 ganglioside vaccine

The second of the developed vaccines is GM2 ganglioside vaccine, GM2-KLH/QS-21, which proved to be well tolerated by the patients with the stadium III or IV of the illness, which was reported by Israes from New York, USA (29). This vaccine has now been examined on less advanced stadiums of illness in the phase III of the study: EORTC study of vaccination during 3 years versus observing in the patients of stadium IIA of illness; Australian and European study of vaccination versus placebo in the patients of stadium III of illness; North-American Inter-group study (ECOG 1694) of vaccination versus high dosage of IFN-alfa in the patients in the stadium III of illness.

Possible interactions between IFN-alpha and this vaccine are now under investigation in the phase II of study (ECOG E 2696) in the operated patients with stadium IIB, III or IV of the illness, which was discussed by Kirkwood. The patients received any of these: -GM2 vaccine alone; -GM2 vaccine + IFN-alfa concurrently; -GM2 vaccine followed by IFN-alfa and therapeutic treatment one month after vaccination (30,31).

Melacine

Melacine is another vaccine that is being tested in combination with IFN-alfa. This vaccine is a mixture of lysates from two humane cell lines of melanoma, which is introduced with an immunologic adjuvant (31). It has been evaluated as an adjuvant therapy for stadium II of melanoma (SWOG 9035) and it is currently tested on patients in the stadium IV of illness. Von Eschen showed the results of randomized study comparing Melacine to

chemotherapy (decarbazine, cisplatin, carmustine, tamoxifen) in patients with stadium IV of illness. All patients (70) were divided in two treated groups. There was no difference in median survival between these two groups of patients (Melacine: 9.4 months; Chemotherapy: 12.3 months). More objective responses were seen in the patients who received chemotherapy (7 versus 5) but the vaccine was endured better than chemotherapy (86patients with grade 3 or 4 in the group which received chemotherapy, compared to only one in the melacine group). This combination is now examined in the patients with progressed illness (32,33).

Dendritic cells

They are the most potent APCs that have been identified so far (originally isolated in 1973). Several dendritic cell growths factors have been identified that have facilitated their *in vitro* expansion and activation, allowing for the *in vitro* priming of dendritic cells with specific antigens (34). An excitingly new development in immunotherapy is the usage of autologous dendritic cells, which should present melanoma peptides. This approach was discussed by Lotze and associates (Pittsburgh, USA). In the initial studies, the researches defined optimal conditions for *in vitro* expansion of dendritic cells taken from peripheral blood. The cocktail containing cytokines, GM-CSF, IL-4, TNF-α and FLT-3 ligand, was found to give the best results (35,36).

The Pittsburgh group is now examining this form of therapy in the patients with progressed illness together with the preparation of vaccine HLA-A2 vaccine with restricted peptide (9,34). The therapy was well tolerated and it will be further investigated. This is only one of many new approaches in highly vigorous investigation of melanoma vaccines. In the next few years a significant improvement should be expected in our understanding how tumor vaccines may be used in an adjuvant treatment to prevent or lower the risk of relapse (37,38).

OUTLOOK

It is essential that neoplastic cells and the existing repertoire of tumor specific T cells be carefully analyzed before starting treatment of each patient with melanoma, although this requires an exceptional effort. With such an approach the best candidates for T-cells-based and humoral therapy could be selected. In the future, the accessibility of specific reagent, which may be used alone or with a suitable adjuvant, will allow conduction of vaccinal strategy suitable for each patient.

Obstacles in successful immunotherapy with vaccine

In spite of constant progress which in the development of anticancer vaccine several main obstacles must be overcome in order to make this type of treatment efficient for patients. First, induction of immune response on tumor antigens may be difficult because of the emphasized antigen specificity, or because of general immune deficit in tumor affected patients before chemotherapy, or other not yet identified factors. General deficiency of T-cell signalization may be present in some mice with tumor and in some patients with advanced malignity, including melanoma, but this is still controversial. Second, the possibility that immunization will not cause an immune response, strong enough, to lead to tumor regression. In later cases, it may be necessary to expand vaccine induced antigen-specific T cells *in vitro* for adoptive immunotherapy. Third, tumor heterogeneity in the expression of antigens and the possibility that a successful immunotherapy in majority of the patients demands immunization against more antigens may also be possible obstacles.

Tumor cells may evade the immune recognition through numerous different molecular mechanisms. These mechanisms may be histologically specific. Human melanoma may lose functional expression of beta (2)-microglobulin, deletion of allele for specific alpha 2-chain, and to decrease expression of a particular MHC locus.

Finally, the tumor itself can activate numerous mechanisms and allow tumor cells to evade or pass by an immune detection and destruction. Some tumor cells have the ability to produce cytokines or other mediators, which locally or systematically work as immunosuppressors. What complicates the subject further more is the fact that a high blood pressure, which in tumors is caused by some metastatic lesions, or inadequate pathological vascularization can prevent the entering of T cells or other effectual cells. Relative significance of any of these mechanisms, which can cause failure in immunotherapy, is still unknown. Nevertheless, it is extremely important to be aware of these obstacles and develop corresponding interventions in clinical studies in order to understand the complete potential of antitumor immunotherapy which is based on vaccine, and which is so well demonstrated on murine models (36,38).

FUTURE DIRECTIONS

Data that are expecting to become available in the next 12 months will give answers to many important questions that are currently asked. It is still expensive to produce vaccines and to treat large number of patients. In case of melanoma, it has been demonstrated so far that many tumor antigens can serve as T-cell targets expressed by tumors. Molecular definition of tumor-specific antigens that are recognized by activated T cells will allow the development of targeted antigen-specific vaccines for the treatment of cancer patients. Promising fact is that it is possible to design vaccines based on rational principles of immunology.

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