REVIEW

Gene transfer approaches in cancer immunotherapy

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The idea of enhancing or establishing effective immune response against endogenously developed tumor cells is not novel. More than a hundred years ago, bacterial components were used to develop antitumor immune response. Later, when a number of immune system-affecting cytokines had been discovered, they were used for systemic treatment of cancer patients. However, systemic treatment often resulted in even negative outcome. Recent developments of genetic approaches of cell modifications allowed developing of modern techniques of targeted tumor cell elimination. In the present paper, we review modern trends of the antitumor response enhancement based on immunoregulatory gene transfer into different cell types both *in vivo* and *in vitro*. Almost all these approaches are based on the activation of the adaptive arm of the immune system in response to tumor cells. However, recent studies indicate that the innate arm of the immune system, as well as adaptive arm, is involved in tumor suppression. The innate immune system uses non-rearranging germline receptors, which could trigger cellular effector responses that are conditional (or instructive) to the

subsequent adaptive immune response. Last years' viewpoints on 'self' and 'non-self' recognition and primary induction of the immune response have changed. The key role of lymphocytes is pathogen recognition and, following immune response induction, switched on the central role of dendritic cells in 'non-self' recognition and induction of both innate and adaptive responses. Moreover, innate response is supposed to be an essential starting point in induction of successful and effective acquired response. Most cancer vaccines do not have 'non-self' marks presentation due to their endogenous origin, thus lacking their effectiveness in the induction of the specific long-lasting immune response. Taking this point into consideration, we can conclude that to make cancer vaccine more effective we have to present tumor antigens, together with the molecules that can potentially activate downstream 'non-self' recognition events not in parallel, but as a consequence of tumor antigen processing and presentation.

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Modern view on antitumor immune response

The last two decades were characterized by intensive studies in the fields of molecular and cellular immunology. A lot of new findings allowed scientists to take a look deeper into the Immune Surveillance Hypothesis. Today, the main role in immunological recognition phenomena, distinguishing 'self' from 'non-self' and activation of immune response, has been drawn aside from the key role of lymphocyte to the main role of dendritic cells (DCs) – professional antigen-presenting cells.^{1,2} It was shown that DCs could activate both arms of the immune system: innate³ and adaptive,⁴ via recognition of 'non-self' in the presence of the so-called 'danger signals' during DC maturation. Moreover, the evolutionary conservative innate immune response plays an important role in the induction of subsequent acquired immunity and successful memory formation.^{4,5}

Knowledge of the molecular mechanisms of immune response allowed investigators to develop a broad list of techniques based on the finetuning of the immune system to treat some not previously curable diseases. One of the promising fields is the control of malignant growth. The idea came out in the early 1900 when for the first time Paul Ehrlich proposed that tumor cells can arise

from the body normal cells, but usually neoplastic cells should be eliminated by the immune system. Later, Lewis Thomas has demonstrated that the main cells involved in tumor elimination are cytotoxic T lymphocytes (CTLs). Today's knowledge about the broad spectrum of tumor antigens, recognized by the immune system^{6,7} and main principles of immune surveillance, are actively used in development of antitumor immunotherapy.⁸ However, a lot of mismatches and contradictions were found in Ehrlich's hypothesis. The main problem is that the result of interaction between the immune system and new antigen at the periphery depends on the microenvironment of the meeting.^{9,10} Generally, when a pathogen injury (penetrate, invade) the body at the periphery, a lot of 'alarms' or 'danger signals' like reactive oxygen intermediates, extracellular-matrix components, heat-shock proteins, nucleotides, soluble mediators, and cytokines are released in inflammation site. These molecules induce activation and maturation of DCs, whereas antigens derived from pathogens are effectively presented to lymphocytes and the typical result is activation of the corresponding clones of effector cells.¹¹ In case of tumor formation, endogenous tumor cell growth occurs without any inflammation and tissue damage, at least in the initial stages. Accordingly, in the absence of 'danger signals', tumor antigens cannot be effectively presented by immature DCs and the typical outcome of this immune

system 'blindness' is tumor cell recognition as a 'self' and not dangerous.^{12,13}

Now, taking together all aspects of oncoimmunology, we can suppose that successful anticancer vaccination is possible, but it is difficult to find the combination of immunostimulatory signals, which would work well and overcome this 'blindness'. At the present moment, thousands of experimental models and hundreds of clinical trials aimed to induce antitumor immunity have been reported. The gross part of such investigations actively involved gene transfer approaches to activate antitumor response. These approaches can be classified in different ways.

In the present review, we attempted to classify them on the basis of the target for gene transfer to achieve immune system activation:

- immunization with genetically modified tumor cells;
- use of autologous or allogeneic genetically modified normal cells (eg fibroblast) for local secretion of immunostimulatory molecules;
- gene modification for antigen processing and presentation by DCs;
- application of gene-targeted effector cells like lymphocytes or natural killer cells (NKs);
- transfer of genes encoding antibodies to known tumor antigens *in vivo*;
- vaccination with DNA-encoding cancer antigens.

Over the last decade of the 20th century, the most popular vaccine strategy is based on the *immunization with the genetically modified tumor cells* and dates back to experiments of William Coley in the 1890s. At that time, he tried to activate the immune system with bacterial components, and supposed that immune response at least partially can be transferred to tumor cell rejection. Later, this approach was significantly improved by Lindenmann and Klein.¹⁴ They have demonstrated that immunization with virus-infected tumor cells representing the complex of noncharacterized tumor antigens on these cells can protect against noninfected parental cells. During such immunization, viral proteins attracted immunocompetent cells in the site of vaccination. T lymphocytes and DC recognized viral antigens in complex with tumor antigens became activated and rejected virus-infected tumor cells, followed by rejection of parental tumor cells. The important resulting component of such approach was cytokine production by activated antigen-presenting cells (APCs) and T helper cells. At a later time, the idea to mark tumor cells with 'danger flag' developed to compel tumor cells to produce immunostimulatory cytokines by themselves without the use of virus activation of APCs. This fruitful idea gave rise to the studies where tumor cells were modified not with the virus but with the genes encoding such cytokines as interleukin (IL)-2,^{15–17} IL-4,^{17–19} IL-7,^{17,20} IL-12,²¹ granulocyte colony-stimulating factor (G-CSF),²² granulocyte macrophage colony-stimulating factor (GM-CSF),²³ interferon (IFN)- γ ²⁴ and a wide list of other genes. It was not absolutely the best choice because an important step of innate immunity activation was missed and immunological mechanisms were directed straight up to CTL and DC activation to develop adaptive immune response, although it was very actively used during the decades. Almost all listed cytokines

were successfully tested in phase I–II of gene therapy trials in Europe and USA.²⁵

Use of autologous or allogeneic genetically modified normal cells for local secretion of immunostimulatory molecules

Bubeneik and co-authors proposed the idea to deliver biologically active cytokine at a tumor site without manipulating with tumor cells *ex vivo*. For this purpose, allogeneic or autologous fibroblasts genetically modified to secrete cytokines were used for injections at the site of tumor. These transfected cells functioned like a source of immunoactivating molecules. Peritumoral administration of retroviral transduced, IL-2-producing fibroblast cells into mice carrying subcutaneous transplants of carcinoma cells inhibited the growth of the tumor.²⁶ Later, this approach was successfully exploited in a lot of labs; for example, M Lotze and collaborators demonstrated similar results with the gene encoding IL-12. They also demonstrated that autologous genetically modified fibroblast injections were safe and nontoxic. No lymphoid hyperplasia or tissue necrosis was found in the liver, spleen, or lungs of mice receiving repeated inoculation of IL-12-engineered fibroblasts. Tests of liver and renal function were within the normal range. At the same time, long-term protective antitumor immunity was observed.²⁷

Gene modification for antigen processing and presentation by DCs

This was firstly discovered by Steinman.²⁸ DCs have become known as professional antigen-presenting cells and inducers of immune response. Moreover, at present, they are recognized as the most important cells in induction of innate and primary adaptive immune responses.^{1–5} Scientists were extremely excited by the possibilities of influencing these cells to enhance tumor antigen presentation.²⁹ There is a wide range of studies, which involved DCs targeting tumor antigens. We will focus only on those that involved gene transfer approaches. Pioneered work in this field belongs to the E Gilboa lab. Firstly, the phenomenon was shown for DCs loaded with RNA isolated from tumor cells. In stimulation of CTL response, DCs pulsed with unfractionated RNA (total or polyA+) from OVA-expressing tumor cells were as effective as DCs pulsed with OVA peptide. In the poorly immunogenic, highly metastatic B16/F10.9 mouse melanoma tumor model, a dramatic reduction of lung metastases was observed in mice vaccinated with DCs pulsed with tumor-derived RNA. It is worth mentioning that only total or polyadenylated RNA induced protective vaccination, whereas polyA(–) RNA fraction did not.³⁰ Later, the potential opportunity to activate immune response with mRNA-transfected DCs was demonstrated for a number of characterized tumor antigens, for example prostate-specific antigen (PSA),³¹ carcinoembryonic antigen (CEA),³² E6 and E7 proteins of human papillomavirus.³³ A clinical trial utilizing such approach also has been reported.³² Next modification of the approach was the use of DNA-transfected DCs. For modification, genes encoding cytokines, tumor antigens, and molecules involved in antigen presentation were used.³⁴

Application of gene-targeted effector cells like lymphocytes or NKs

Using effector cells directly targeted to tumors came from the fundamental papers of Rosenberg. In his brilliant work, Rosenberg *et al* for the first time have demonstrated that tumor-infiltrating T lymphocytes, that carried T-cell receptors specific to tumor antigens, can be cultured to achieve large cell mass *ex vivo*. Following injection of tumor-specific lymphocytes back to the mice bearing micrometastases has led to significant cure of advanced hepatic metastases and advanced pulmonary metastases.³⁵ Today, the genes encoding T- and B-cell receptors to antigens are cloned. Determination of the exact mRNA sequence of receptors to specific cancer antigens gave an opportunity to obtain a large quantity of tumor-targeted CTLs by *ex vivo* transfection of naive cells, with constructs encoding chimeric receptors to designed antigen. This is a promising approach, but injection of genetically modified effector cells targeted to tumor lacked an important step of tumor recognition by DCs and as a consequence misses memory formation. Nevertheless, some papers reported about successful tumor cell-specific targeting of T lymphocytes³⁶ and inhibition of tumor growth after adoptive transfer of T hybridoma cells expressing chimeric scFv/zeta gene composed of the variable regions of an HER-2/neu-specific monoclonal antibody joined to the TCR-zeta chain.³⁷

With regard to genetically modified NK cells, it looks like more preferable because NK-mediated tumor rejection is a part of innate antitumor response. Participation of NK cells in tumor lyses should induce local cytokine and chemokine secretion with the subsequent attraction of DCs, resulting in developing of memory cells and strong adaptive response. Development of a fast and simple technique of NK nucleofection³⁸ clears the way to the adaptation of the approach based on utilization of human NK cells modified with a chimeric immunoglobulin T-cell receptor.³⁹ After resolving the problem with the efficient NK cells genetic modification, this idea will be really attractive.

In vivo transfer of genes encoding antibodies to known tumor antigens

After the development of the technology of monoclonal antibody humanization,⁴⁰ investigators obtained the fine tool for antigen targeting. Genetically engineered antibody molecules composed of a constant part of human antibody molecule and variable domain of mouse monoclonal antibody against the antigen of interest have generally a long half-life in the human body due to the absence of neutralizing immune response and at the same time maintaining all biological functions of natural antibodies.⁴¹ A high level of such therapeutic protein can be achieved after *in vivo* electroporation of plasmid DNA encoding humanized antibody.^{42,43} There are different possible immunological mechanisms of tumor rejection following antibodies encoding gene transfer. First, the mechanism can be based on direct binding of recombinant antibodies with receptors on the surface of tumor cells and subsequent growth inhibition through the growth factor receptor blocking. The best example is Herceptin. Direct binding of this humanized monoclonal antibody to Her-2-positive tumor cells leads to signifi-

cant tumor growth inhibition. Second, tumor rejection strategy is based on the development of antibody-dependent cellular cytotoxicity. In this case, binding of NK cells Fc surface receptor with the opsonized tumor cells leads to the activation of killing mechanisms directly.

Vaccination with DNA encoding cancer antigens

The last 15 years were marked by rapid development of a new gene therapy approach termed DNA vaccination. It was started from studies of the early 90s,^{44,45} and after a short time became a quickly developing field of gene transfer-mediated immunostimulation with special emphasis on infections and malignances. The main advantages of DNA vaccination are relative safety (absence of attenuated or killed pathogens), low cost (missing step of protein preparation), and long storage without loss of activity. All these advantages are combined with very efficient immune response. The important fact defining the effectiveness of DNA vaccination is the presence of unmethylated CpG motives in bacterial DNA, which are one of the best immunostimulatory 'danger signals', involved in innate response activation.⁴⁶ As a consequence, DNA vaccines effectively induce both MHC I and MHC II pathways allowing induction of CD4⁺ and CD8⁺ T lymphocytes, leading to the development of cellular and humoral immunity.⁴⁷

The list of diseases cured by DNA vaccination is very broad.⁴⁸ Our interest is focused on cancer treatment. In this case, the immunostimulatory properties of unmethylated plasmid DNA are combined with the genes encoding well-characterized individual or a combination of tumor antigens. *In vivo* intramuscular injection, electroporation into muscle or skin, and particle-mediated delivery of plasmid DNA⁴⁹ could potentially induce both arms of the immune system, leading to the tumor antigen recognition and memory formation.

Immune system mediators in gene therapy of cancer

Coming back to the key role of DCs and to the necessity of innate response in acquired response activation,⁵⁰ we can divide all approaches to activate immune response against tumor in two not equal groups. Bearing in mind that successful immune response usually begins with activation of the innate arm in the presence of 'danger signals', followed by activation of the adaptive arm and memory formation, our division will be based on the level or on the starting point of immunological response. First, a small group of immunostimulatory gene transfer approaches are based on utilization of molecules involved in innate immunity mechanisms. In Table 1, we summarized such approaches. In this small group, molecules involved in the recognition of pathogen-associated molecular pattern or molecules representing pathogen-associated pattern or expressed in response to infection during the first fast step of the innate immune response were used. At the same time, all the above-mentioned molecules play the role of 'danger signals' simulating bacterial infection, and as a result strong immune response against tumor cells

was observed. All these approaches have at least the same or greater effectiveness than the use of molecules involved in regulation or activation of adaptive response. The second group is represented by the genes that encode a large variety of molecules starting from cytokines: IL-2, IL-12, IFN- γ , GM-CSF; molecules of major histocompatibility complex (MHC); co-stimulatory molecules such as B-7.1 and B-7.2; genes encoding T-cell receptor, and a lot of other mediators of adaptive immune response. All these molecules are usually involved in direct activation of lymphocytes, CTL induction, and often miss an important step of innate response, antigen processing, and presentation by activated DCs. The use of these genes to obtain local release of cytokines or to obtain expression of cell surface molecules does not consider 'danger signals' that might play an important role at the initial phase of immune response. Nevertheless, these approaches are effective in cancer treatment and they have potencies for future improvement. There is a large number of trials, in which mediators of acquired immunity were used; the best-known classical works are cited in the second part of Table 1.

There are also a number of studies involving a combination of immunoregulatory molecules. To our mind, they are unfortunately also based on a combination of cytokines and receptors from the second part of the table; in other words, on mediators of adaptive immunity,⁶⁴⁻⁶⁷ excluding rare exceptions, which combine molecules of both arms of immune response.⁶⁸ In most cases involving genetic modification, amplification of immune response induced by a combination of immunoregulatory molecules was usually reported. Explanation of immune response amplification is based on the

understanding of the molecular mechanisms of immune response and its regulation. For example, IL-2 has been shown as a T-cell growth factor. IL-2 is a main cytokine that induces T-lymphocyte cell cycle from the G1 to S phase, followed by T-cell proliferation. Thus, it is the most important cytokine in the induction of CTL immune response. INF- γ was discovered as a factor inhibiting virus replication. It was shown later that INF- γ is rather an immunomodulatory cytokine, then antiviral. It activates macrophages, increases the expression of MHC class I and class II molecules, and increases antigen presentation. Thus, a combination of IL-2 and INF- γ must activate both antigen presentation and activation of T-effector cells. This strategy proved to be very efficient.⁶⁴

The next example, GM-CSF, was first described as a factor supporting clonal growth and differentiation of bone marrow progenitor cells. GM-CSF effects hematopoietic progenitor cells, granulocytes, and monocytes. It also stimulates the antibacterial and antitumor activities of neutrophils, eosinophils, and macrophages, stimulating cytokine production (TNF- α , IL-1, M-CSF, and others). GM-CSF stimulates maturation of DCs and upregulates the surface expression of co-stimulatory molecules, thus enhancing the antigen presentation efficiency of DCs. IL-12 is a cytokine with a broad spectrum of activities and is produced by activated macrophages, B cells, and DCs. Among other activities, this cytokine stimulates the proliferation and cytotoxic activity of NK and T cells. It also induces secretion of a range of cytokines, including INF- γ , which has various secondary actions. Thus, a combination of these cytokines is a powerful tool, as it was reported.⁶⁵ The main cause of such amplifications of the immune

Table 1 Classification of cancer gene therapy trials by approaches directed to stimulate innate or adaptive immune responses

Therapeutic molecule	Treatment scheme	Refs.
<i>Approaches directed to stimulate innate immunity mechanisms</i>		
Adenovirus	Adenoviral vectors delay tumor growth in mice bearing melanomas, through activation of NK cells	[51]
Immunostimulatory CpG DNA	Mesothelioma model, anticancer effects of the lipid:DNA complex	[52]
Mannan-binding protein	Recombinant vaccinia virus carrying human MBP intratumoral or subcutaneous injection	[53]
Beta-defensin 2	DNA immunizations with fusion constructs elicit humoral, protective, and therapeutic immunity against two different syngeneic lymphomas	[54]
Tag7/PGRP protein	Vaccination by <i>ex vivo</i> transfected tumor cells	[55]
Macrophage inflammatory protein 3 α	Intratumoral adenovirus injection	[56]
<i>Mycobacterium tuberculosis</i> Ag38 gene	Retroviral vector transduced melanoma cells	[57]
RANTES	<i>Ex vivo</i> fibrosarcoma was genetically modified to express and produce the human RANTES chemokine stably	[58]
<i>Approaches directed to stimulate acquired immunity mechanisms</i>		
IL-2	Retroviral vectors poorly immunogenic tumor cells	[15-17]
IL-4,	Transfecting malignant cells to produce IL-4	[17-19]
IL-7	IL-7 gene transfer in patients with metastatic colon carcinoma, renal cell carcinoma, melanoma, or with lymphoma	[20]
IL-12	<i>Ex vivo</i> transformation of murine sarcoma using a polycistronic retroviral vector	[21]
INF- γ	Retrovirally transduced tumor cells	[24]
B-7.1 and B-7.2	Autologous transfected tumor cell lines	[59-61]
MHCI, MHCII	Plasmid DNA transfection in human melanoma	[59,62]
G-CSF	Retroviral vector delivery of the human G-CSF gene into the colon adenocarcinoma	[22]
GM-CSF	Mouse models and clinical trial of autologous irradiated tumor cells generated by <i>ex vivo</i> transfection	[23,63]

system response is the involvement of different and independent mechanisms of adoptive immune response.

Innate immunity pattern recognition molecules in gene therapy of cancer

Knowledge about the importance of innate immunity mechanisms allowed developing a more promising tool based on activation of innate response against tumor, followed by the development reactions of adoptive response such as CTL clonal proliferation and memory formation.⁵⁵ This strategy is based on the utilization of a member of a novel family of pathogen molecular pattern recognition molecules. The first member of this diverse family was cloned from mouse and designated as *tag7*.⁶⁹ Later, its insect homologue was cloned from insects and pathogen molecular pattern recognition was demonstrated,⁷⁰ thus leading to another name, PGRP, for peptidoglycan recognition protein. Intensive sequencing of mammalian genomes allowed us to identify the mouse *tagL* gene by EST database analysis.⁷¹ It was designated *tagL* because it encodes the protein almost twice larger than Tag7/PGRP-S. Later, a large family of genes was identified in *Drosophila* and human genomes using the same approach.^{72,73}

Mammalian Tag7/PGRP-S has been cloned from mouse,⁶⁹ human,⁷⁰ rat,⁷⁴ and cow.⁷⁵ There are small extracellular proteins (19–24 kDa), which structurally share homology with T phage lysozyme but do not possess any amidase activity. Apart from their ability to recognize oligosaccharides and particularly peptidoglycan^{70,75} cytotoxicity towards mammalian cells,^{69,75,76} bactericidal and bacteriostatic activities⁷⁵ were detected. Tag7/PGRP-S is expressed in the spleen, brain, intestine,^{69,74,77} bone marrow,⁷⁰ in lymphocytes⁷⁸ and neutrophils.^{70,75} Isoforms of mammalian TagL/PGRP-L are rather large proteins, which share strong C-terminal homology with Tag7/PGRP-S but also have transmembrane domain and intracellular N terminus.^{71,73} TagL/PGRP-L was found to be both secreted and membrane-bound proteins recognizing a wide variety of pathogens independently of the surface peptidoglycan.⁷⁹ Actually functions other than oligosaccharide binding are not well documented for the mammalian tag7/PGRP family. However, *Drosophila* studies allowed establishing important mechanisms of PGRP functioning in innate response in insects. One isoform of PGRP-S initiates activation of the prophenoloxidase cascade⁸⁰ not presented in mammals. Another *Drosophila* isoform PGRP-SA is required for the activation of the Toll receptor⁸¹ insect orthologue of mammalian TLRs. Interaction of extracellular PGRP-SA with bacteria activates *Drosophila* Toll via protease cleavage of Spaetzle. Toll activation initiates signaling that results in the activation of two members of the Rel family transcription factors (similar to mammalian NF- κ B), which upon translocation into the nucleus bind κ B sites and initiate insect immune response. Isoforms of the insect PGRP-L participate in the Imd pathway of *Drosophila*, somewhat similar to the mammalian TNF receptor activation of NF- κ B^{82–84} and mediate antimicrobial gene activation upon Gram-positive and negative bacteria recognition.^{82–85}

None of these cascades was demonstrated for mammalian immune system yet. However, our recent findings indicate that both human and mouse Tag7/ PGRP-S are able to induce lymphocyte chemotaxis both *in vitro* and *in vivo*, and also induce DC maturation via CD80, CD86 co-stimulatory molecule expression (unpublished results). All these data taken together allowed us to assume that presenting of tumor antigens in the context of the molecules that potentially activate innate immunity mechanisms, particularly dendritic cell activation after antigen uptake, may have an advantage in antitumor vaccine preparation. The use of genetic modification of tumor cells by the *tag7* expressing construct as a vaccine led to the establishment of long-lasting antitumor protective immunity.⁵⁵ Moreover, this effect was T-cell dependent and worked on a different tumor model. At the same time, we did not observe any signs of toxicity. Tumors shrunk due to apoptotic events without the neighboring tissue burden. In prophylactic scheme, more than 60% of animals remained free of tumors although the use of the GM-CSF gene in the same experiments allowed to keep only 40% of animals tumor free. Preclinical data encouraged initiating Phase I/II clinical trial of the autologous *tag7*-modified antitumor vaccine.⁸⁷

Future perspectives of anticancer vaccine development

The idea to enhance or establish effective immune response against endogenously developed tumor cells is not novel. More than a hundred years ago, bacterial components were used to develop antitumor immune response. Later, when a number of immune system-affecting cytokines had been discovered, they were used for systemic treatment of cancer patients. Recent developments of genetic approaches of cell modifications allowed developing of modern techniques for targeted tumor cell elimination. Nevertheless, the open question at the moment is the search for the most suitable molecules for activation of the immune system against cancer cells. Unfortunately, most cancer vaccines do not have 'non-self' marks presentation due to their endogenous origin, thus lacking their effectiveness in the induction of the specific long-lasting immune response. In our opinion, the most promising results may be obtained with genes encoding molecules, which are involved in activation of the evolutionary more conservative innate arm of immunity and which are shown to be a starting point in development of adaptive immune response and memory formation. Also, special interest should be focused on a combination of genes encoding regulatory molecules from both arms of the immune system.

Taking it into consideration, we can conclude that to make cancer vaccine more effective we have to present tumor antigens together with the molecules that can potentially activate downstream non-self recognition events not parallel but as a consequence of tumor antigens processing and presentation. It is hoped that these approaches can be developed into effective, safe, and practical treatments for patients with cancer and may be some other diseases.

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