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Cancer Vaccine Development

Protein Transfer of Membrane-Anchored Cytokines and Immunostimulatory Molecules

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Abstract

Many tumor cells escape host-immune recognition by the down-regulation or lack of immunostimulatory molecules. Expression of immunostimulatory molecules on tumor cells by gene transfer can be used to induce an antitumor immune response. However, we have previously shown that protein transfer of glycosyl-phosphatidylinositol (GPI)-linked costimulatory molecules is a successful alternative to traditional gene transfer in preparing such a tumor vaccine. Vaccination with membranes modified by protein transfer to express GPI-linked B7.1 (CD80), a costimulatory adhesion molecule, induces protective immunity in mice and allogeneic antitumor T-cell proliferation in humans in vitro. Our goal is to develop an optimal tumor vaccine using tumor membranes modified by protein transfer to target and stimulate antigen-presenting cells (APCs) and T cells. We have investigated the efficacy of expressing GPI-anchored cytokine molecules on the surface of tumor cells. Expression of interleukin-12 (IL-12) on tumor-cell membranes in a GPI-anchored form induces a strong antitumor immune response that is comparable to the effects of secretory IL-12. Because many cytokines act synergistically, we are testing the membrane expression and immunostimulatory effects of cytokines individually as well as in combination to determine potential complementary effects of coexpression on the antitumor immune response. Ultimately, the protein-transfer vaccination may be used in humans alone or in multimodal combination therapies to induce tumor regression and to serve as a protective measure to prevent postsurgical secondary metastases.

Key Words

GPI
Cytokines
Costimulation
CD80
IL-12
IL-2

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Introduction

Tumor-specific immune responses play a major role in eliminating tumors from the body. Immunotherapy in oncology aims to supplement and stimulate a patient's immune system in the fight to recognize and eliminate cancer. Potentially promising immunotherapeutic approaches for cancer treatment have emerged from recent advances in the understanding of the requirements necessary for antigen-specific immune responses. Some of these strategies include: vaccination with dendritic cells (DCs) modified to express tumor antigens *(1–4),* peptide *(5,6),* or DNA vaccines *(7,8),* heat-shock proteins (HSPs) *(9,10),* hybrid tumor cells *(11),* and tumor cells transfected with costimulatory molecules *(12,13)* or cytokines *(14–16).* We have developed tumor vaccines based on a novel protein-transfer method using GPI-linked immunostimulatory molecules and isolated tumor-cell membranes *(17–19).* Here, we outline the recent developments in various therapeutic tumor-vaccine strategies using immunostimulatory molecules.

Systemic Administration of Cytokines and Ex Vivo Stimulation of Antitumor Cytotoxic Cells

Immunostimulatory molecules like cytokines play an important role in the antitumor response. Cytokines are a critical component in the modulation of the immune system in fighting cancer and are thus attractive candidates for immunotherapeutic interventions *(20–22).* Studies in murine tumor models have demonstrated, for instance, that antitumor immune responses can be stimulated postadministration of the cytokines interleukin (IL)-2 *(23),* IL-4 *(24),* IL-6 *(25,26),* or granulocyte-macrophage colony-stimulating factor (GM-CSF) *(27,28).* IL-2, one of the chemical mediators of the immune response, has been shown to have antitumor capabilities through its activation of helper *(29)* and cytotoxic T cells *(30),* natural killer (NK) cells *(31),* lymphokine-activated killer (LAK) cells *(23),* and macrophages *(32).* IL-12 attracts T cells, APCs, NK cells, and inflammatory cells to the site of secretion or vaccination and can also activate and enhance the maturation of antigen-specific cytotoxic T cells (CTLs) *(33).*

Systemic administration of cytokines to humans, particularly IL-2, initially appeared to have promising results *(34–36);* however, systemic administration of the IL-2 to humans is problematic, not only because of rapid degradation *(37),* but also because of severe toxic side effects owing to paracrine activity *(38).* Leonard and co-workers *(39)* found that systemic delivery of IL-12 is also highly toxic to patients, depending on the cytokine-administration schedule. To circumvent the negative side effects associated with systemic cytokine administration, researchers developed an ex vivo method of stimulating T cells *(23,36, 40,41).* High doses of cytokines such as IL-2 were used to produce LAK cells from T cells and NK cells, which were then administered to patients. This method was met with only minimal success, however, and it has recently been shown that neither the co-administration of systemic IL-12 nor GM-CSF improves the antitumor response *(42).* Alternative methods have therefore been developed to use cytokines in antitumor immunotherapy.

Development of Vaccines With Antigen-Presenting Cells

An alternative approach to stimulating an antitumor immune response is through the direct use of APCs. Initial methods relied peptide-pulsed macrophages *(43)* and on cell fusion of APCs with tumor cells, resulting in antigen-specific immunogenic tumor cells *(11).* Cell fusion with DCs in particular results

in the strongest antitumor responses *(44)*. More recent attention has been given to immunization with active DCs armed with tumor antigens on their cell surface *(45)*. Sources of antigens for DC loading include apoptotic cells, tumor cells, live cells, cell lysates, proteins, or antigens encoded by DNA or RNA *(46)*. It has also been demonstrated that HSPs isolated from tumor cells act as potent adjutants in inducing an antitumor immune response by stimulating DC maturation and antigen presentation *(9)*. DCs are attractive candidates for tumor-vaccine strategies because relatively few numbers of cells are able to potently stimulate T-cell activation *(47)*. Notably, DCs are able to prime both antigen-specific $CD4^+$ T cells and $CD8^+$ T cells *(46)*. Clinical studies have in fact demonstrated metastatic regression and increased T- cell immunity post-DC-vaccination *(48,49)*. However, further work is necessary to optimize and standardize the development, preparation, and administration of such vaccines *(45)*.

Gene Transfer of Immunostimulatory Molecules

Antitumor T-cell response is dependent not only upon interaction with the tumor-peptide antigen and major histocompatibility complex (MHC) *(50),* but also upon a second costimulatory signal that comes from the adhesion-receptor ligand binding between the APC and the T cell *(51–53)*. Many tumor cells, while expressing MHC molecules, lack the immune costimulatory or adhesion molecules necessary for T-cell activation and subsequent initiation of a host-immune response *(54,55)*. Without the second, costimulatory signal, clonal anergy will result in the tumor-specific T-cell population *(53,56,57)*. To counteract the downregulation or lack of many secondary stimulation signals, researchers have shown that the expression of costimulatory and other immunostimulatory molecules by gene transfer induces antitumor immune responses *(12,13)*.

Direct vaccination of mice with tumor cells transfected with IL-2 genes has been shown to provide protective immunity against parental tumor challenge *(58)* and to cause tumor regression in mice *(59,60)*. Tumors transfected with genes from other cytokines, such as GM-CSF and IL-12, can also induce antitumor immunity *(22,27,33)*. Many studies in the murine system have shown that the transfection of costimulatory molecules can induce an antitumor immune response *(54,61,62)*. Our laboratory has shown that after the expression of B7.1 in the human renal carcinoma line RCC-1 via gene transfer, RCC-1 stimulates strong proliferation and differentiation signals to autologous T cells *(63)*.

Gene transfer requires the use of viral vectors, however, which complicate the treatment strategy because antiviral host immune responses may prohibit multiple immunizations using the same vector *(64–66)*. Additionally, owing to the difficulty in transfecting primary tumor lines, gene transfer requires the establishment of tumor-cell lines, which is also a time consuming process. Phase III gene-therapy studies of immunostimulatory molecule transfection in humans have shown that the limiting factors in the process were the isolation of cells from the primary tumor and the low frequency of gene uptake. Gene transfection is ultimately impractical for a clinical setting *(67)*.

Protein Transfer of Immunostimulatory Molecules to Live Tumor Cells

Previously, our laboratory *(68)* and others *(69)* have shown that costimulatory molecules such as B7.1 can be inserted and expressed on the cell surface via a novel method of direct protein transfer. The proteins are recombinantly

linked to GPI lipid-molecule tails, which can spontaneously insert into amphiphilic structures, such as a cell membrane *(70).* Studies have since optimized conditions for the incorporation of GPI-anchored proteins onto the cell surface *(18,68,71–73),* and purified GPI-anchored molecules are able to incorporate into the cell membrane in just 2 h at 37°C *(18,68).* GPI-linked molecules can incorporate into nucleated cells *(74),* non-nucleated cells *(73),* and various types of tumors, including primary breast carcinoma *(68).* Notably, all the studies showed that the preparation and incorporation of the GPI-linked proteins does not affect the proteins' ligand-binding abilities *(75–77).* Thus, one can quickly express immunostimulatory molecules on tumor cells by this method without the use of time-consuming gene-transfer techniques for cancer-vaccine development *(78,79).* Our lab has demonstrated that human melanoma tumor cells (SKMEL28) expressing GPI- linked B7.1 from protein transfer are able to induce an allogeneic T-cell response in vitro *(68).* In subsequent protein-transfer studies, immunization of mice with other tagged or tailed immunostimulatory molecules, such as B7.1 and CD40 *(80)* or toxic shock syndrome toxin-1 *(81),* has also been shown to initiate demonstrable antitumor responses in vivo.

Protein Transfer of Costimulatory Molecules to Isolated Tumor-Cell Membranes

Protein transfer of costimulatory molecules to whole tumor cells has provided tumor vaccines that initiate promising antitumor immunity *(68,80,81).* However, this method has various limitations, because it is difficult to establish and maintain tumor cell lines from many primary tumors, and the tumor lines that are established gradually lose the GPI-linked proteins with progressive cell divisions *(68,72).* Additionally, the administration of live tumor cells to patients is improbable, and irradiation of cells may not be complete and may yield cells that are incapable of immunostimulation *(12,82).*

As an alternative method, our laboratory has demonstrated that protein transfer can be used to express GPI-linked immunostimulatory molecules in preparations of isolated tumor-cell membranes alone *(17,18).* B7.1-expressing membranes are effective in stimulating tumor-specific T-cell and CTL proliferation and providing complete immunity to parental tumor challenge with murine T-cell lymphoma *(17).* Additionally, we have shown that the cell membranes isolated from surgically removed human melanoma and renal-cell carcinoma tumor tissue can be modified to express GPI-linked B7.1 by protein transfer *(18).* These membranes are able to stimulate allogeneic T cells in vitro. At present, the mechanism by which these modified tumor membranes stimulate an antitumor response is not known. It is possible that the B7.1 molecules may be acting to directly prime T cells or to indirectly prime them through interactions with other CD28-expressing cells, such as NK cells and mast cells (Fig. 1). These cells in turn can stimulate the potent DCs to process and present antigens more efficiently to T cells.

Protein transfer to tumor-cell membranes, as opposed to live tumor cells, offers several advantages. Membranes do not divide or actively metabolize, thus eliminating the loss of GPI-linked molecules through cell divisions, and GPI-linked B7.1 is stably expressed for at least 7 d. Membranes prepared from patients' tumor cells can be frozen in aliquots for at least 2 yr and later modified to express the GPI-linked immunostimulatory molecules for immunization *(18).* Additionally, the membranes already modified to express the costimulatory molecules can also be frozen and thawed with little loss of

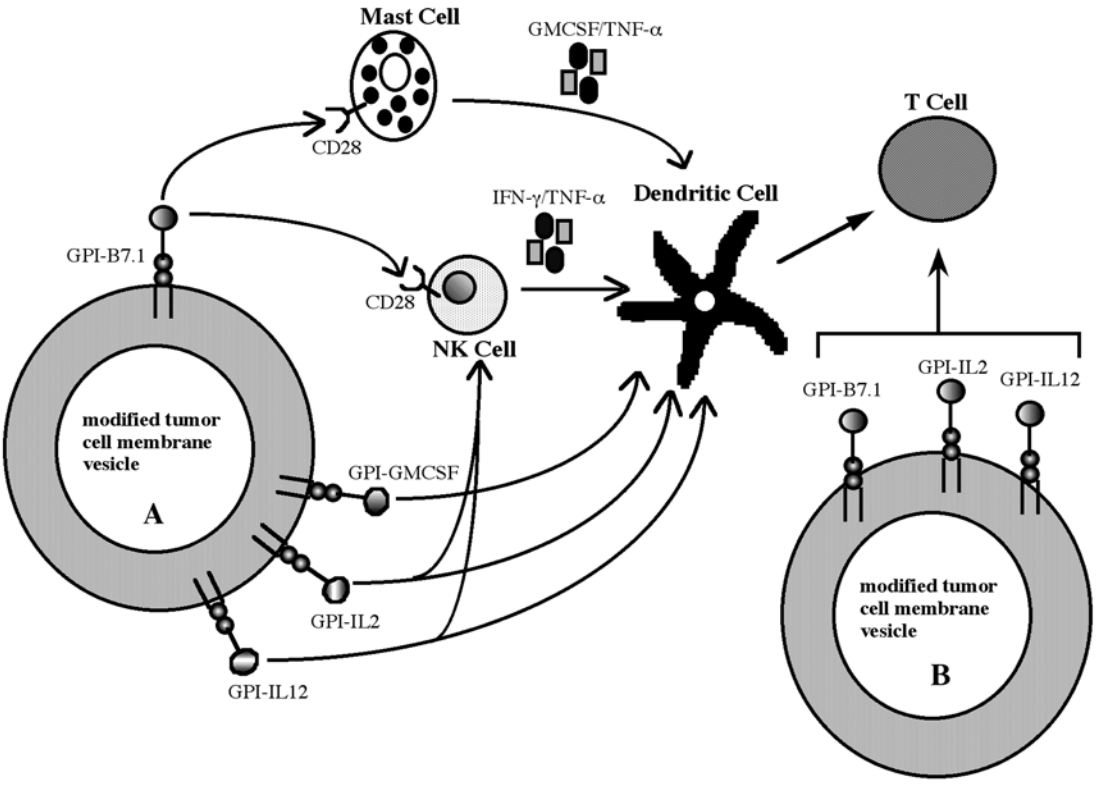

Fig. 1. A hypothetical model for stimulation of T-cell proliferation by modified tumor membranes. **(A)** Membrane-bound immunostimulatory molecules can indirectly stimulate T-cell production. B7.1 can bind to CD28 expressing mast cells and NK cells. After binding, these mast and NK cells release IFN-γ and TNF-α, which stimulate the DCs , resulting in further T-cell proliferation. Cytokines can also induce T-cell differentiation through DC stimulation. In addition, **(B)** membrane-bound cytokines and adhesion molecules can directly stimulate T-cell proliferation.

expression *(18).* Notably, membranes prepared from surgically removed tumor samples expressed both MHC class I and class II molecules *(18),* thus indicating that their use in a vaccine could possibly stimulate both $CD8^+$ and $CD4^+$ T-cell proliferation, which would augment the antitumor response *(28,83).*

Cytokines Expressed on Cell Membranes as Adjuvants

As described, cytokines also play a central role in the modulation of the immune system *(20–22).* Recently, we have shown that the expression of GPI-linked IL-12 molecules on tumor-cell membranes (Fig. 2) induces T-cell proliferation and IFN-γ production, as well as tumor immunity in a highly tumorigenic murine mastocytoma model *(19).* Immunized mice are protected for up to 55 d from tumor challenge. A secondary advantage of GPI-linked cytokine molecules may be the creation of an insoluble slow-release depot at the vaccination site, as opposed to a transient soluble cytokine depot. A major advantage of local administration is the lack of toxicity

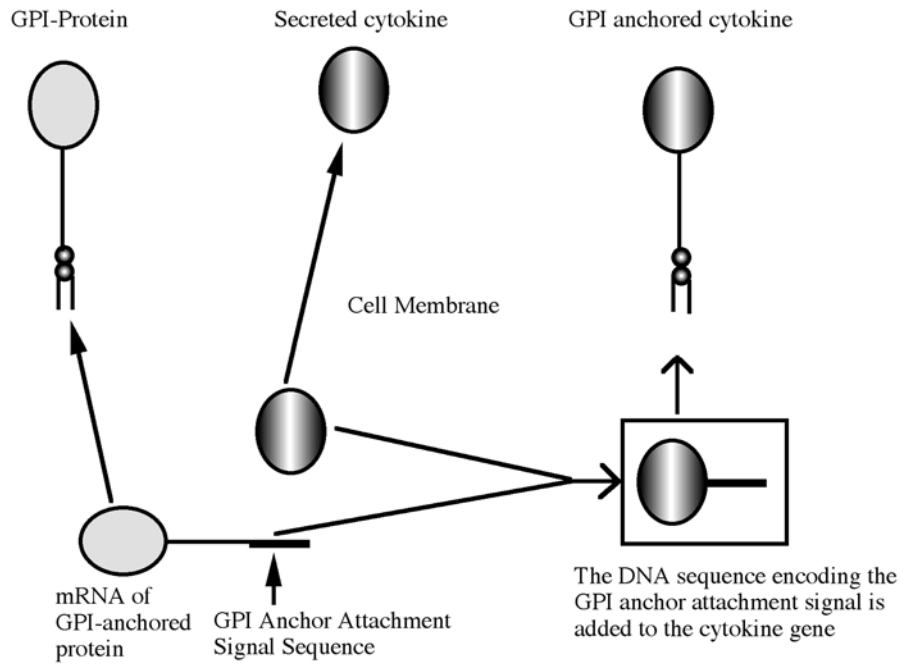

Fig. 2. Attaching a GPI-anchor to secreted cytokines. GPI-anchor attachment sequence and cytokine gene are recombinantly linked to form a GPI-modified cytokine that will be anchored to the cell membrane.

associated with systemic administration. GPI-linked cytokine molecules can also be used in protein transfer, allowing for a more rapid preparation of cancer vaccines *(18,19,84).* Finally, the presence of cytokines at the site of immunization will attract cells of the immune system, increasing the rate of antigen uptake and presentation, and thus increasing the efficacy of the tumor vaccine.

We have also engineered the GPI-linkage of the cytokine GM-CSF to the cell membrane *(85).* GM-CSF stimulates DCs, key initiators of the adaptive immune response *(86),* and potently induces antitumor immune activity *(27,28).* Our study has shown that GPI-linked GM-CSF can stimulate bone marrow-cell proliferation in vitro and can induce DC generation in vivo, thus maintaining stimulatory function while anchored to the cell membrane. Additionally, the GM-CSF molecules are partially shed from the cell membrane, likely through proteolytic cleavage, resulting in local cytokine release *(85).* This local cytokine release promotes the migration of APCs, such as DCs, to the site of vaccination, thus facilitating tumor-specific antigen uptake and presentation.

In current studies, we are evaluating the expression of transfected GPI-linked IL-2 cytokine molecules on the surface of murine mammary tumor cells. A recent study from our laboratory has shown that the expression of GPI-linked IL-2 molecules induces antitumor immunity in the EL4, P815, and EG7 murine tumor models *(87).* We are testing the expression and immunostimulatory effects of IL-2 alone, as well as in conjunction with B7.1 and IL-12 to determine the potential complementary effects of co-expression. IL-2 and IL-12 have been shown to have syner-

gistic effects on the activation of DCs and their production of IFN-γ *(88)* and well as on the generation of cytotoxic effector cells from normal human bone marrow *(89)*.

Conclusion

In summary, recent molecular and cellular advances have furthered the understanding of mechanisms necessary for immune-response initiation and thus the development of immunostimulatory cancer vaccines. In humans, the protein transfer vaccination may be used one day, alone or in multimodal combination therapies, to induce tumor regression and to serve as a protective measure postsurgery to prevent secondary metastases. Our laboratory has already shown that tumor membranes can be isolated from human primary tumors and that protein transfer of GPI-linked B7.1 to the membranes is possible and in fact effective in stimulating T-cell proliferation in vitro *(17,18)*. Clinical trials and comparison to other vaccine strategies are necessary to determine clinical efficacy. The protein-transfer method offers several advantages, including the elimination of the need for live cell culture in vaccine preparation as well as providing the possibility of rapid vaccine preparation. GPI-linkage provides a promising alternative for the transport and delivery of immunostimulatory molecules, such as adhesion molecules and cytokines, for the initiation of an antitumor response in tumor vaccines.

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