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Cancer vaccine development: Designing tumor cells for greater immunogenicity

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1. ABSTRACT

Cancer vaccine development is one of the most hopeful and exhilarating areas in cancer research. For this reason, there has been a growing interest in the development and application of novel immunotherapies for the treatment of cancer with the focus being on stimulating the immune system to target tumor cells specifically while leaving normal cells unharmed. From such research has emerged a host of promising immunotherapies such as dendritic cell-based vaccines, cytokine therapies and gene transfer technology. These therapies seek to counteract the poor immunogenicity of tumors by augmenting the host's immune system with a variety of immunostimulatory proteins such as cytokines and costimulatory molecules. While such therapies have proven effective in the induction of anti-tumor immunity in animal models, they are less than optimal and pose a high risk of clinical infeasibility. Herein, we further discuss these immunotherapies as well as a feasible and efficient alternative that, in pre-clinical animal models, allows for the expression of specific immunostimulatory molecules on the surface of tumor cells by a novel protein transfer technology.

2. INTRODUCTION

Despite growing knowledge of cancer biology in the last decade and a host of potential therapies for cancer, in the United States approximately 3400 people are diagnosed with cancer and another 1500 people die from cancer each day(1). Tumor cells are characteristically unique from normal cells within the human body. Through mutation of their regulatory growth mechanisms, tumor cells acquire the ability to grow uncontrollably. In addition, developing tumors acquire sustained angiogenesis, metastasize to other tissues, resist apoptosis and anti-growth signals, all while having a self-sufficiency of pro-growth signals (2). Moreover, tumors have also evolved numerous ways to evade immune surveillance including the downregulation of the costimulatory molecules B7-1 (CD80) and B7-2 (CD86), antigen modulation and the release of immunosuppressive factors (3). Naive T cells that interact with tumor cells become anergic or undergo apoptosis due to the lack of costimulation (4); this ultimately leads to a diminished repertoire of T cells capable of eliciting anti-tumor responses. Many tumors also modulate the surface

expression of MHC class I molecules to varying degrees including total deficiency of MHC, allelic and locus downregulation and loss of MHC haplotype (5, 6). This altered MHC expression prevents proper antigen presentation and recognition to T cells resulting in a deficiency of $CD8^+$ T cell-mediated immunity while making the tumor more susceptible to NK cell-mediated lysis (7, 8). Studies have also shown that tumors tend to upregulate a number of inhibitory molecules such as programmed death ligand (PDL-1) that further leads to immune dysfunction by inhibiting the effector functions of T cells which express PD-1 and subsequently inducing T cell apoptosis (9). Lastly, the immunosuppressive nature of the tumor microenvironment caused by the release of immunosuppressive factors and cytokines such as transforming growth factor β (TGF-β), interleukin-10 (IL-10), tumor necrosis factor (TNF) and vascular endothelial growth factor (VEGF), as well as the presence of regulatory T cells (Tregs), contributes to tumor immunotolerance and tumor evasion from immune surveillance (10).

Most tumors express antigens that are recognized to varying extents by the immune system. Accumulating evidence suggests that both the innate and adaptive immune systems are capable of recognizing and responding to an emerging tumor (11). This anti-tumor response is largely T cell-mediated, although an antibody-mediated response proves effective in some cases (12). Innate, in addition to cellular and humoral responses, contribute to the efficacy of immune surveillance, a concept shown to be responsible for controlling tumor development under normal circumstances. However, poorly immunogenic tumor cells manage to escape this immune surveillance. The host is subsequently unable to mount an adequate immune response that would otherwise control the development and metastasis of the tumor without being detrimental to normal cells. Thus the ability to target and stimulate immune cells specifically against tumor cells is paramount in the development of an effective cancer vaccine.

The recent identification and characterization of several MHC-restricted tumor-associated antigens (TAAs) such as human epidermal growth factor receptor (HER2/neu), melanoma antigen 1 (MAGE-1) and glycoprotein 100 (gp100) has enabled more targeted immunotherapies to be developed (13). While there are several therapeutic options geared toward cancer vaccine development currently under investigation, few such options demonstrate the dual potential to not only stimulate a robust anti-tumor immune response but to translate to human clinical trials as well. Herein, we will briefly discuss some of these current vaccine-based immunotherapies and focus on the many applications of protein transfer as an alternative immunotherapy that enables the expression of novel immunostimulatory proteins on the surface of tumor cells quickly while effectively stimulating an appropriate anti-tumor immune response.

3. CURRENT IMMUNOTHERAPIES

Current immunotherapies offer an exciting and fresh perspective to cancer vaccine development. The goals of cancer immunotherapy are to harness and to augment the immune system's natural ability to eliminate emerging or established tumors. In order for this goal to be realized, advances must continue to be made in tumor immunology such that we gain a better understanding of how the immune system naturally responds to a tumor. The current immunotherapies seek to target and to boost specific components of the host's immune response to a developing or established tumor. The primary targets of current immunotherapies include enhancing antigen targeting to antigen presenting cells (APCs), enhancing T cell activation and removing the inhibitory signals that diminish the effectiveness of the anti-tumor immune response (14). Pulsing dendritic cells (DCs) with tumor antigens, administering cytokines and using gene transfer technology to express various proteins on the surface of tumor cells have been found to be successful in eliciting an effective immune response. However these therapies have been met with numerous clinical limitations including limited specificity, partial responses and systemic toxicity. Additionally, these therapies are often cumbersome and expensive to implement.

3.1. Dendritic cell-based cancer vaccines

A cancer vaccine approach that is currently being evaluated involves the use of DCs, the most potent professional APC, that have the complete machinery for efficient antigen processing and presentation along with an array of costimulatory molecules (15-18). Due to their ability to prime naïve T cells, DCs have received growing attention as a potential adjuvant for cancer vaccines (19). DCs not only interact directly with T cells and B cells (20) of the adaptive immune system, but also with natural killer (NK) cells (21) and proinflammatory factors (22). These DC interactions provide the necessary and critical cross-talk between the adaptive and innate immune systems.

Researchers are pursuing numerous strategies involving the use of DCs as cancer vaccines. One such strategy involves the use of "loaded" DCs. To achieve this, a population of DCs would first be genetically manipulated *ex vivo* to express tumor antigens prior to injection into the cancer patient. In theory these "activated" DCs would be able to present the tumor antigens, through MHC molecules, to $CD4^+$ and $CD8^+$ T cells and thus elicit a robust immune response. In several models, vaccinating tumor-bearing mice with DCs loaded with autologous tumor-derived antigens in the form of peptides (23), heat shock proteins (24), tumor lysates (25) or mRNA (26) has proven to be highly effective. However, the complications arise initially from the difficulty of properly activating the DCs *ex vivo*, as well as from determining the form, dose or types of antigens to load (19). Such complications limit the overall efficacy and consistency of this approach. The tumor antigen peptide-pulsed DCs would also only be capable of activating a peptide-specific repertoire of T cells. As mentioned above, due to the high mutation rate of tumor cells, the antigens presented by the tumor may differ greatly from those to which the immune cells have been previously primed upon vaccination thus leading to immune evasion. This approach is also limited only to

tumor antigens that have been identified and characterized (16, 27).

Despite these limitations, DC-based cancer vaccines have been used as a treatment option in several human clinical trials such as those for breast and prostate cancer (28, 29), gliomas (30), melanoma (31) and renal cell carcinoma (32). While tumor regression, epitope spreading and proliferative immune responses were seen in some cases, most cases involved partial immune responses relating to peptide-specific T cell responses and low incidence of clinical responses (33). The most promising of these, currently in phase III clinical trials, is the prostate cancer vaccine Provenge that consists of autologous DCs pulsed with a fusion protein of GM-CSF and the prostate antigen prostatic acid phosphatase (PAP) which is expressed solely in prostate tissue and in 95% of prostate cancer cells (34,35).

Because the identity of many tumor antigens remains unknown, heightened interest in developing more effective methods to deliver tumor antigens to DCs have emerged. Recent studies have demonstrated the efficacy of generating fusions of DCs with tumor cells in order to induce anti-tumor immunity (36). The objective of this hybrid-cell vaccination is to combine the antigen-presenting capacity of DCs with a wide range of TAAs made available by the tumor itself in order to stimulate helper ($CD4^+$) and cytotoxic ($CD8^+$) T cells within the host effectively. DC-tumor fusion cells effectively process and present tumor antigens, stimulate host T cells, and prevent tumor growth *in vivo* in a variety of mouse tumor models including lung carcinoma (37), melanoma (38) and colon (39). These favorable results were partially recapitulated in preclinical and phase I clinical trials with melanoma patients (40). While this approach appears to circumvent some of the issues associated with exogenously loading tumor antigens onto DCs, more work must be done in order to improve upon the overall quality and potency of these fusion cell vaccines, particularly in their induction of innate immune responses (41).

3.2. Cytokine therapies

The cytokine milieu present in the tumor microenvironment is critical to the establishment and progression of tumors. As mentioned previously, tumors have been reported to secrete a number of immunosuppressive cytokines such as TGF-β and IL-10 (42, 43). This illustrates the significant role that cytokines play in suppressing the innate as well as adaptive immune responses. To overcome this immunosuppression, the systemic administration of certain cytokines such as IL-2, IL-12, and IFN-α seeks to alter the tumor microenvironment in a way that will mediate proper tumor recognition by APCs and tumor elimination by immune effector cells. Additionally, these cytokines can enhance the functionally of NK and CD8 T cells as well as inhibit tumor angiogenesis (44). In doing so, these cytokine therapies seek to augment the host's overall anti-tumor immune response.

IL-2 has been used in several clinical trials and approved by the FDA for the treatment of metastatic melanoma and renal cell carcinoma (RCC) (45-48). In a recent randomized clinical trial of patients with advanced RCC, intravenous administration of low and high doses of IL-2 yielded both partial and complete responses. Those receiving high dose IL-2 had a higher response rate compared to those receiving low dose IL-2 (21% to 13%), as well as a longer response duration; no difference, however, was seen in the overall survival rate between the two groups (49). Previous trials by Rosenberg, *et al* reported that in patients with advanced RCC and melanoma, overall survival increased in melanoma patients who received high-dose IL-2 along with lymphokine activated killer (LAK) cells compared to those who received IL-2 alone (47). Taken together, these clinical trials demonstrate the therapeutic potential of IL-2 for cancer treatment. IL-12 and IFN-α have also been used alone or as adjuvant therapy for the treatment of a variety of cancers and have yielded modest clinical benefits (50-53).

A common trend throughout most cytokine therapy clinical trials is the detrimental occurrence of a myriad of side effects ranging from nausea, vomiting and hypotension to more severe side effects such as systemic toxicity (45,49). The elevated cytokine levels within the host can induce a cytokine storm which can lead to organ dysfunction and, in the most severe cases, death (54).

In an attempt to circumvent the risk of systemic toxicity and to provide a more targeted therapy strategy, several approaches have been investigated including the intratumoral administration of cytokines (55), modification of tumor cells to secrete cytokines (56-58) as well as the fusion of cytokines with antibodies (reviewed in(59)). These cytokine therapeutic approaches seek to concentrate the administered cytokine at the tumor site for maximal anti-tumor effect. Upon release from the tumor cell, however, the cytokines may act systemically which increases the likelihood of toxicity. The intratumoral administration of cytokines requires that the tumor be accessible, and this is impossible for micrometastases. Additionally, this approach involves using replication-deficient viral vectors encoding cytokine genes delivered to the tumor via gene transfer which introduces a new range of clinical issues that will be addressed in subsequent paragraphs.

3.3. Gene transfer technology

Gene transfer-based therapy, a technique used to modify defective genes that are responsible for disease development, was initially seen as a treatment for single gene disorders (60). More recently, a growing number of gene transfer clinical trials have involved the treatment of cancer, infectious diseases and cardiovascular diseases (61). Previously, gene transfer methods were based on the modification of cells *in vitro* or the introduction of recombinant genes *in vivo* using cell-mediated gene transfer. This procedure ultimately failed in a clinical setting due to the difficulty in establishing a tumor cell line for most tumors (62, 63). Current gene transfer techniques are based on the use of highly efficient targeted gene delivery vectors such as replication-deficient retroviruses and adenoviruses (61). For generating tumor cell vaccines,

Table 1. Comparison of protein transfer technology and gene transfer technology

	Protein Transfer Technology	Gene Transfer Technology
***Ex vivo* manipulation**	Minimal	Cumbersome; Time Consuming
Time required to express immunostimulatory molecules	Hours	Days to Months
Breadth of cells that can be modified	Tumor cells, rapidly dividing cells, inflammatory cells, erythrocytes, isolated cell membranes, differentiated cells	Tumor cells, rapidly dividing cells
Expression of multiple molecules	Yes	Yes
Requires the establishment of a cell line	No	Yes
Specificity of expression	High	Variable
Effective in inducing anti-tumor immunity	Yes	Yes
Requires the use of viral vectors	Never	Typically

several initial experimental studies have demonstrated the feasibility of introducing cytokines such as IL-2 and IFN-γ into explanted tumor cells using retroviruses (64, 65). The clinical use of retroviruses raises a number of safety issues such as the risk of insertional mutagenesis and oncogene activation because retroviruses can integrate randomly into the host genome (66). A prominent example of this risk involved a clinical trial in 2000 of patients diagnosed with X-linked severe combined immunodeficiency disease (SCID). Three years after the gene therapy treatment, two of the patients developed T cell leukemia due to the activation of the LMO2 proto-oncogene promoter by the integrated retrovirus (67).

Replication-deficient adenoviruses are capable of infecting both dividing and non-dividing cells, have a large insert capacity, can be produced at high titers and unlike retroviruses, do not incorporate into the host genome (68). It has recently been reported that adenovirus- mediated gene transfer of the p53 upregulated modulator of apoptosis (PUMA) gene can efficiently and specifically target human breast cancer cells and enhance their radiosensitivity (69). Despite use in clinical trials for the treatment of head and neck cancer (70), prostate cancer (71) and colorectal cancer (72), adenovirus vectors are highly immunogenic (73, 74) which poses a potential health risk to the patient. These vectors typically activate the innate immune system and in doing so can mediate unwanted inflammatory responses. The immunogenicity of these vectors may also expedite their clearance from the host resulting in less effective vaccines. Due to the immune system's response to this "foreign" gene product from initial treatment, the utilization of these vectors for multiple immunizations to the host will also be limited (75, 76).

Due to the number of safety concerns associated with viral delivery systems, several non-viral delivery systems have emerged such as the direct introduction of therapeutic DNA into target cells via electroporation or ultrasound (77, 78), the transfer of DNA carried within a liposomal core (79), and covalently attaching a DNA-containing polymer to a ligand that will be internalized by receptor-mediated endocytosis (80, 81). The major drawback with the use of non-viral vectors is their poor efficiency of gene delivery to non-proliferating cells (82). In the hopes of providing more efficient tumor targeting, tumor infiltrating lymphocytes (TILs) have been studied as a vehicle to deliver a gene product specifically to tumor tissue. Adoptive transfer of tumor-reactive TILs has shown some functional activity towards cancer regression *in vivo* in clinical trials (83). At present, patients eligible for cytokine gene transfer tumor therapy are those with cancer that has failed all standard effective treatment and for which no other effective treatment options are available. Because most human tumors do not trigger an efficient host immune response, the introduction of a functional cytokine gene provides a strategy with potential application for the development of immunotherapies for non-immunogenic tumors. It has been shown that the enhanced expression and secretion of cytokines by altered tumor cells enhances specific immune responses, for example by inducing the activation of T cells, and thus provides a modality for the treatment of these tumors and their metastases (56). Various studies have also indicated the significance of tumor suppressor genes, such as p53, in the regulation of cell replication and suggest that the restored expression of these genes via gene transfer can be utilized as a potential anti-tumor therapy strategy (84-86)

Despite its promise as a cancer immunotherapy, several hurdles to the effective clinical application of gene transfer technology remain. Two such hurdles are the inability to deliver nucleic acids to their appropriate intracellular sites efficiently and the effect of toxicity induced by some viral-based vectors (87). In a clinical setting, the appropriate expression of the target gene as well as specific tumor targeting must also be appropriately addressed with the use of viral vectors. Finally, the possibility exists that the viral vector, once inside the patient, may revert to its replication-competent state and thus be capable of causing additional disease.

4. CANCER VACCINE DEVELOPMENT USING PROTEIN TRANSFER TECHNOLOGY

The overwhelming clinical limitations of gene transfer have propelled researchers to investigate an alternative that allows for the expression of specific proteins on the surface of tumor cells while avoiding the before-mentioned problems. One such attractive alternative to gene transfer is a novel protein transfer approach employed to express new molecules on tumor cells to develop cancer vaccines (88) (Table 1). This approach, which allows a variety of exogenous proteins to be incorporated onto the membrane of a tumor cell, was initially developed utilizing a glycosylphosphatidylinositol (GPI) anchor. Subsequently, several other protein transfer methods have been developed using palmatic acid and

biotin-avidin linkages. Herein, we will discuss these protein transfer strategies.

4.1. GPI-anchored protein transfer method

Various proteins commonly expressed by cells are attached to the cell membrane via glycosylphosphatidylinositol (GPI) anchors. Naturally occurring GPI-anchored proteins lack transmembrane and cytoplasmic domains that otherwise anchor membrane proteins. The GPI-anchor consists of a glycosylated moiety attached to phosphatidylinositol containing two to three fatty acid chains (89). The phosphatidylinositol portion, as well as an ethanolamine attached to the C-terminus of the extracellular domain of the membrane protein, anchors the molecule to the cell membrane lipid bilayer. These GPI-anchored molecules are widely distributed in mammalian cells and serve a host of different cellular functions such as cell adhesion, enzymatic activity and complement cascade regulation (90).

Previous studies have shown that purified GPI-anchored cell surface proteins can be spontaneously incorporated onto cell membranes (88, 91-93). Interestingly, these GPI-anchored proteins can be purified from one cell type and incorporated onto different cell membranes within hours. Thus this technology enables the customization of the tumor cell membrane as a cancer vaccine and provides the opportunity to incorporate any GPI-anchored protein quickly without the need for gene transfection. Protein transfer technology can also be exploited to incorporate multiple molecules simultaneously onto the same cell membrane to test their effectiveness in inducing anti-tumor immunity. Another promising feature of this protein transfer technology is the ability to control the level of expression by simply varying the concentration of the GPI-anchored molecules to be incorporated. The most significant implication of this technology will be the reduction of time required for cancer vaccine preparation from months to hours. These combined features make the protein transfer approach a more viable choice for the development of a cancer vaccine for clinical settings. The molecules incorporated by means of protein transfer have been shown to retain their functions associated with the extracellular domain (91, 93-96). These studies suggest that the tumor cells can be modified to express immunostimulatory molecules which will mediate the induction of anti-tumor immunity.

Essentially any immunostimulatory molecule can be modified to be a GPI-anchored protein. Using recombinant DNA techniques, the transmembrane and cytoplasmic domains of a transmembrane surface protein need only be replaced by the carboxy terminal end of the GPI-anchored precursor protein (Figure 1). The signal sequence for GPI-anchor attachment is found at the hydrophobic C-terminus of the GPI-anchored protein precursor (97). This method of genetic manipulation to generate GPI-anchored proteins is not limited to membrane proteins; attaching GPI-anchor signal sequences to secretory proteins also converts them to GPI-anchored forms.

Our lab has pioneered and extensively investigated this GPI-anchored protein transfer approach for the development of cancer vaccines (88,97-99). The first study was conducted using a purified recombinant GPI-linked B7-1 molecule. Using a variety of tumor cell lines including human T lymphoma (Jurkat), mouse and human melanoma (K1735 and WM115) and human Burkitt lymphoma (Ramos), the GPI-linked B7-1 molecule was found to incorporate spontaneously into the isolated tumor membrane after a short incubation; GPI-B7-1 maintained its costimulatory function demonstrated by its binding to cognate ligand CD28 on T cells *in vitro* (88). Additionally, tumor membranes from surgically removed tissues taken from cancer patients can be modified by this protein transfer technology to express GPI-anchored molecules (98). Tumor protection studies in the EG7 thymoma model demonstrated that anti-tumor immunity can be induced *in vivo* using tumor membranes modified to express GPI-B7-1 by the protein transfer approach (100).

This GPI-anchored protein transfer approach has also demonstrated stability of protein expression as well as longevity of storing the membrane vaccine with GPI-anchored molecules. Our results show that the GPI-B7-1 expression on isolated cell membrane fragments was stable up to 7 days at 37^0C, and frozen membranes can be used for up to 3 years when stored at -80^0C (98,100). Studies also suggest that the membrane vaccines are more suited for the stable expression of the GPI-anchored molecules than intact cells which lose the expression within 24 hours (88,100).

This straightforward approach for introducing novel proteins onto tumor membranes provides numerous advantages over gene transfer. This approach allows for practically any protein to be added either alone or in a combinatory manner to the tumor membrane surface and navigates around the necessity to establish tumor cells. Even cells that are difficult to transfect can be modified to express a particular GPI-linked protein. As the conditions for the incorporation of GPI-linked molecules were optimized, our lab, as well as others, has extended this approach to include the addition of cytokines on the surface of tumor cells (97,101). Studies have shown that soluble cytokines can be modified to be GPI-anchored to a tumor cell membrane and can be used for protein transfer to prepare cancer vaccines. Cytokines such as IL-12 and GM-CSF, when attached to the tumor membrane via the GPI-anchor, may exert their effector functions locally at the vaccination site without the risk of systemic toxicity (97, 99). Tumor cells and tumor membranes modified to express these GPI-anchored cytokines were shown to induce potent anti-tumor immune responses via the stimulation and proliferation of T cells (101).

4.2. Palmatic Acid mediated Protein Transfer Method:

Huang, *et al* described the initial application of using palmatic acid to couple molecules to the surface of cells (102). This study demonstrated the ease with which antibodies could be derivatized by palmatic acid and incorporated into liposomes in order to study liposome targeting to specific cell types. Peacock and Kim later developed a two step approach that allowed for the delivery of intact IgG to cell membranes (103). The first step

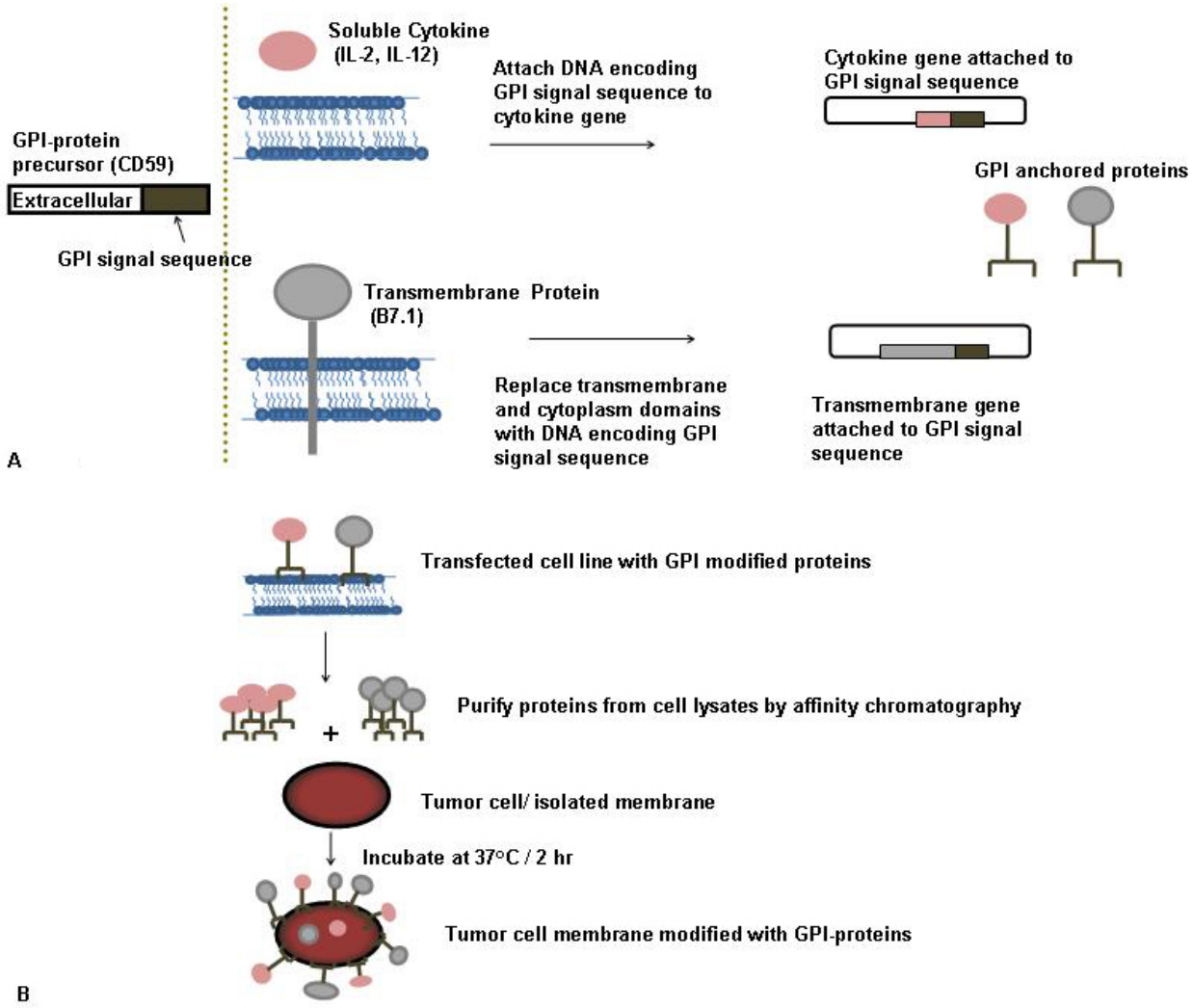

Figure 1. GPI-anchored protein transfer method. (A) Generation of GPI-modified cytokines and transmembrane proteins. (B) Protein transfer mediated expression of GPI-proteins on the surface of tumor cells.

entailed coating the cell membrane with a chemically-palmitated derivative of protein A, a protein that binds the Fc domain of antibodies with high affinity. This palmitated-protein-A (PPA) is incorporated at varying concentrations onto tumor cells and used to bind intact IgG (103) or a Fc fusion protein (Fcγ1) coupled to a costimulatory molecule such as B7-1 (104). Using the latter application provided evidence that the coupled molecule retains its costimulatory function.

To enhance the induction of anti-tumor immunity, this technique has been utilized in combinatorial tumor therapy studies that included the chemotactic molecules secondary lymphoid-tissue chemokine (SLC) and Fas ligand (FasL) in addition to the costimulatory molecules 4-1BBL and tumor necrosis factor-related activation-induced cytokine (TRANCE) (105). The expression of these four immunostimulatory proteins on the surface of L5178Y lymphoma and EG7 tumor cells lines via conjugates of PPA and Fc fusion proteins increased the number of immune cell infiltration at the tumor site, most notably neutrophils, DCs and T cells, and enhanced the overall cytokine milieu at the tumor site. Other protein combinations such as B7-1, 4-1BBL, CD48 and CD40L induced systemic anti-tumor immunity and tumor regression (106); this "tetra-costimulator" combination maintained stable expression on the tumor membrane. These applications speak to the ease of expressing multiple molecules via protein transfer which is in direct contrast to current gene transfer technology.

In more recent studies, this protein transfer approach has been extended beyond the modification of tumor cells to include increasing the potency of APCs (107) and the anti-tumor efficacy of T cells (108). Using the EG7 and TRAMP-C2 tumor models, DCs were modified to express three immunostimulatory molecules, SLC, 4-1BBL and TRANCE. When injected intratumorally into mice, these modified DCs migrated more efficiently to the draining lymph nodes and increased

T cell infiltration and Th1 cytokine production *in vivo*. In addition, this approach was used to link the B7-1 molecule to naïve OT-1 T cells which allowed the T cells to costimulate themselves and induced a robust anti-tumor response *in vivo*.

4.3. Biotin-Avidin platform for protein transfer

Darling, *et al.* first reported that by utilizing the high affinity binding of biotin and avidin, that tumor cells could be modified *ex vivo* to express activating antibodies via a biotin-avidin bridge (109). This bridge was established by biotinylating murine thymoma cells (TIB232) prior to treating them with avidin. These modified cell membranes served as a binding site for antibodies, such as anti-CD28, complexed with biotinylated protein-G; this complex remained stable for 6 hours and functioned as a costimulator when cultured with Jurkat cells expressing the CD28 receptor. This costimulation led to an increase in IL-2 production. The use of a biotin-avidin bridge proved to be effective among two primary acute myelogenous leukemia (AML) patient samples as well as other murine and human myeloid cell lines. However, following biotinylation certain proteins such as B7-1 had altered surface expression on transfected tumor cells which could translate to a less than optimal induction of anti-tumor immunity *in vivo.*

The biotin-avidin strategy, coined as ProtEx technology, was later simplified and its application extended to allow the expression of rat FasL on splenocytes (110) and cardiac vasculature (111) as well as human B7-1 on the surface of tumor cells (112). The recombinant B7-1 was modified to consist of a chimeric strepavidin (SA) core and then incubated with biotinylated tumor cells. As a result, not only did the surface expression of B7-1 on these decorated tumor cells persist with a half-life of more than 10 days ($t_{1/2}$ >10 days) *in vivo,* but these tumor cells were also effective in preventing tumor growth in mice challenged with a lethal dose of aggressive B cell lymphoma cells (112). More recently, the efficacy and ease with which this approach can be used to convert tumor cells to effective APCs was demonstrated by using primary tumor cells from patients and decorating them with B7-1-SA. Significant proliferative responses to autologous tumor cells were observed *ex vivo* (113).

Due to the high affinity non-covalent interaction between biotin and SA (K_d= 10^{-15}), this protein transfer approach allows for the durable, stable expression of exogenous proteins on the surface of tumor cells. This method allows for the rapid incorporation of immunostimulatory molecules (<2 hours) and was shown to be non-toxic to cells. Moreover, this approach demonstrated powerful immunostimulatory efficacy and caused complete tumor regression in lethally challenged mice (112). The optimal combination of rapidity and simplicity of preparation, persistence of expression and efficacy of induced anti-tumor immunity makes this technology for decorating cells with immunostimulatory molecules quite promising as a clinical cancer immunotherapy. However, as the host may mount immune responses to foreign proteins such as strepavidin and protein A/G, the long-term use of these vaccines is greatly limited.

5. CONCLUSION

Tumor cells have evolved multiple mechanisms by which they evade immune surveillance such as disruption of antigen presentation, antigen modulation, cytokine secretion that contributes to an immunosuppressive environment and lack of costimulatory molecules. The focus of many of the before-mentioned approaches to cancer vaccine development has been to modify tumor cells such that they will provide the necessary costimulatory signals to T cells, thus allowing them to become fully activated and exert their effector functions.

The key to an effective cancer vaccine is not only in its ability to induce a multi-faceted, robust immune response but also in its ability to be clinically feasible. While all approaches detailed here posses clinical limitations, we propose that the technique of protein transfer eliminates these issues while providing effective elicitation of an anti-tumor response. This approach can be used as an immunotherapy for any malignancy that allows for the extraction of tumor tissue which would serve as the source material for the vaccine. Therefore a limiting factor with this protein-transfer approach clinically is the availability of tumor tissue from patients. However, taking into consideration the predominant strategies of protein transfer that were highlighted herein, protein transfer poses several advantages over gene transfer and other current vaccine-based immunotherapies. Protein transfer is a relatively easy method of expressing immunostimulatory proteins on the surface of tumor cells, APCs and even T cells. This approach enables the modification of cells that are difficult to transfect, can be applied to a wide range of proteins and allows for the expression of a novel protein alone or in combination with others in order to induce a robust anti-tumor immune response.

Lastly, modifications to the membrane of tumor cells, APCs and T cells via protein transfer is a very promising, targeted immunotherapy effective in its induction of anti-tumor immunity leading to tumor regression, protection against secondary challenges and tumor clearance in a number of *in vivo* mouse tumor models. Despite this approach being solely tested in pre-clinical animal models and will in the future undergo evaluation for safety and immune efficacy in humans, the clinical feasibility of this approach appears to far surpass that of current immunotherapies, primarily due to the ease of molecule incorporation onto the membrane of various cells. In summation, protein transfer can effectively improve the immunogenicity of tumor cells and increase the anti-tumor efficacy of T cells, thus ramping up the immune system to respond appropriately to a tumor. Taken together, these characteristics extend the efficacy of this approach as a potential cancer vaccine.

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