[Frontiers in Bioscience 6, d760-775, June 1, 2001]

DESIGNER CANCER VACCINES MADE EASY: PROTEIN TRANSFER OF IMMUNOSTIMULATORY MOLECULES FOR USE IN THERAPEUTIC TUMOR VACCINES

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1. ABSTRACT

Advances in the understanding of the immune response to tumors has led to the development of new strategies to design therapeutic vaccines. One of these strategies is the development of protein transfer of immunostimulatory molecules onto the surface of tumor cells, thereby directing the immune response to the tumor antigens carried by the modified tumor cells. This strategy has been developed as an alternative to gene transfer, the more classical technique of introducing immunostimulatory molecules onto tumors. In this report we briefly review current strategies for immunotherapy and then focus on several approaches to protein transfer and their historical basis. Finally, the application of these protein transfer approaches to develop cancer vaccines are reviewed and discussed.

2. INTRODUCTION

Tumors have been shown to express unique tumor antigens that can be used to immunize a host against tumor cells bearing those antigens (1,2). Knowing that tumor cells possess antigens that can be recognized by T cells as "foreign", immunotherapy for the treatment of cancer has become an area of intense investigation. These immunotherapeutic theories/approaches are not recent. Hypotheses by both Paul Ehrlich and William Coley, proposed in the late 1800's, suggested the use of anticancer antibodies or bacterial toxins to stimulate the immune system for anti-tumor therapy.

Tumor specific immunity is mostly cell-mediated, with humoral immunity playing a minor role in most cases. This cell-mediated immunity can be the result of both CD8+ and CD4+ effector T cells (3,4). In many cases, CD8+ cytotoxic T lymphocytes (CTLs) are the effectors recognizing intracellularly-expressed tumor antigens presented by class I major histocompatibility complex (MHC) molecules at the tumor surface. However, CD4+ T cells also play an important role, as tumor antigens can be ingested by professional antigen-presenting cells (APC) and presented in the context of MHC class II molecules. The role of CD4+ T cells has been shown to

enhance or regulate the CTL response and work in concert with macrophages for cytokine production, while some CD4+ cells have a direct cytopathic effect (5-8).

3. TUMOR ESCAPE MECHANISMS

Although some tumor antigens have been characterized and anti-tumor CTLs can be isolated and shown to be effective, many tumor cells still fail to induce a strong immune response. Many factors contribute to a tumor's ability to evade an immune response. These factors include the ability to modulate antigen, down regulate surface molecules involved in T cell recognition of tumor cells, or secretion of immunosuppressive factors (9-11).

3.1. Antigen modulation

Tumor cells can escape the immune response by antigen modulation or by selective pressure of the immune response (12-13). In the presence of specific antibodies or CTLs, tumor cells can down-regulate or alter antigen expression. Alternatively, tumor cells may express a heterogeneous array of tumor antigens at any given time. Therefore, when a response to one antigen is mounted only some of the tumor cells are destroyed. The tumor cells expressing different antigens survive the response by not being recognized.

Similarly, tumors cells can evade the immune system by down-regulation of MHC molecule expression, or a defect in the antigen processing and presentation pathway (9). MHC molecules are highly polymorphic glycoproteins controlling T cell immune responses by binding and presenting certain peptides that have been processed from either exogenous or intracellular antigens. MHC class I is essential for recognition of antigens by CD8+ cytotoxic T cells, the main effectors of tumor immunity. Without the expression of MHC class I, tumor cells cannot be recognized by CD8+ cytotoxic T cells. Therefore, tumors can go undetected by tumor-specific effectors. Many tumors of both human and murine origin, such as human colon carcinoma (14) and the murine D122 Lewis lung carcinoma (15), have been shown to have altered surface expression of MHC class I. The relationship between a loss in MHC class I expression and tumor growth has been studied most rigorously in the AKR strain of mice (16). These mice have a high incidence of spontaneously arising leukemias. These leukemias have significantly reduced levels of H-2K, one of the MHC class I molecules. Determined through many *in vitro* studies, the immune response against these leukemias is directed toward tumor antigens presented by H-2K molecules. Therefore, lack of H-2K expression severely inhibits T cell immune responses to these leukemias.

3.2. Immunosuppressive factors

One way that tumors may escape recognition by the immune system is through the release of soluble factors. Some of these factors are tumor antigens released by the tumor cell. When these antigens are taken up by resident APCs in the absence of an inflammatory response, tolerance is promoted (17-19). In addition tumor cells have also been implicated to suppress the generation and activity of APCs in general, and more specifically dendritic cells (DC), by secretion of vascular endothelial growth factor (VEGF) (20-23). These DCs, which are the most potent activators of T cells, are altered in their migratory, homing, and maturation patterns (20-24). In addition to secreting VEGF, tumors have been reported to secrete IL-10 (10,17), TGF-β (25), both anti-inflammatory cytokines, and MUC-1, which has been reported to induce apoptosis of activated T cells (11).

3.3. Lack of costimulation

By interfering with APCs, most specific T cells are anergized during contact with tumor-specific peptides either presented by the tolerizing APC, or by the tumor cells themselves. During a normal immune response, antigen specific T cells require and receive two specific signals through surface receptors in order to proliferate and respond to antigen (26). Tumor cells, when presenting antigens on MHC molecules, can provide the first signal. However, since most tumor cells lack costimulatory molecules, such as B7-1 or B7-2, that are needed to provide the second signal for development of full effector function of T cells, they are anergized instead (27). This prevents an anti-tumor immune response from developing, thereby leading to the escape of tumors from the immune system.

4. IMMUNOTHERAPEUTIC TREATMENTS

Many immunotherapeutic strategies have been designed for the treatment of tumors in mouse models. However, these treatments or therapeutics have achieved limited success in clinical trials, unless used in combination with chemotherapy.

4.1. Adjuvants

Some mechanisms of inducing anti-tumor immunity involve non-specific stimulation of the immune system. Adjuvants, such as BCG (28-30), or mitogens including anti-CD3 antibodies (31) or superantigens (32,33), have been used to treat established tumors or have been mixed with tumor lysates to create a tumor vaccine. Although these treatments have had some effect, generally immune activity is not heightened enough to induce full tumor rejection.

4.2. Cytokine treatment and TIL therapy

Another therapy, which received much attention in the 1980's, is systemic administration of cytokines, mainly IL-2 (34). IL-2 has been shown to be the major T cell growth factor, and large doses of IL-2 *in vitro* stimulate NK cells and CTL to kill normally resistant targets (35,36). Use of IL-2 *in vivo* has had limited success, and systemic treatment often has severe side effects. Due to the toxic effects, ex vivo stimulation of immune cells with cytokines has been developed (36-39). This therapy involves the generation of lymphokine activated killer (LAK) cells by stimulation of T cells and NK cells with high doses of IL-2 or other cytokines. These LAK cells are then readministered to the patient. Lymphocytes isolated from tumor tissue, tumor-infiltrating lymphocytes (TIL), are also stimulated *in vitro* and readministered. This therapy has had limited success and still requires systemic cytokine

treatment. Other cytokines administered systemically, such as IL-12 and GM-CSF, have also met limited success. IL-12 has been found to be highly toxic when delivered systemically, depending on treatment schedules (40). In a recent study by Rosenberg and co-workers (41), co-administering IL-12 or GM-CSF in tandem with tumor specific peptide had no effect on the generation of anti-tumor immunity. The side effects of delivering cytokines systemically continue to outweigh the ultimate benefits for tumor immunotherapy.

Recently, researchers have been looking at the delivery of cytokines locally by genetically engineered cells (42). Here, cytokines can be delivered locally by tumor cells transduced with cytokine genes. IL-2 (43-45), IL-4 (46,47), IL-12 (48,49) and GM-CSF (50,51), have been stably transfected into tumor cells. These tumor cells, when administered to syngeneic hosts, have a lower tumor incidence and in some cases induce rejection of established tumor. Administration of cytokines locally can still prime anti-tumor responses without adverse side effects. Cytokines have also been administered locally by several other methods. Some investigators have genetically engineered fibroblasts to secrete cytokines such as IL-12 (52) and IL-2 (53). Mice immunized with tumor cells mixed with transfected fibroblasts have a higher resistance to tumor growth (53). Moreover, mice treated with IL-12 secreting fibroblasts mixed with tumor cells can reject established tumors (52). Alternatively, cytokines can be delivered by biodegradable polymer microspheres, which slowly release cytokines into the surrounding area (54). Pardoll and co-workers have found that if IL-12 is encapsulated in microparticles, which time releases IL-12, and is co-injected with wild-type tumor cells, a strong anti-tumor response develops which is equal to that of transfected tumor cells (55). Many of these techniques for cancer vaccination show promise in protecting mice from further tumor challenge (44,46,52,56).

Both IL-12 and GM-CSF are potent activators of not only acquired immunity, but innate immunity as well (57,58). IL-12 augments the development of CTLs *in vivo* (59-61). Tumor cells transfected with IL-12 or co-administered with fibroblasts transfected with IL-12 have been shown to attract macrophages (Mϕ) and NK cells (48,62,63). This facilitates uptake of tumor antigens in an inflammatory environment, thus leading to powerful anti-tumor immunity. IL-12 has been reported to work in concert with B7-1 in generating strong CTL responses, as well as tumor regression (64-69). GM-CSF has been shown to be a growth factor and activator of dendritic cells (70). GM-CSF alone or in combination with B7-1+ tumor cells can elicit strong anti-tumor responses (50,51,71-74).

4.3. APC and DC-based vaccines

Another method of turning a tumor cell into an APC is to fuse the tumor cell directly to APCs. This is usually accomplished by incubating the tumor cells and APCs together and using a fusogen, such as polyethylene glycol. Introduction of B7-1 or B7-2 molecules onto the surface of hepatoma cells by fusion with activated B cells has been shown to induce tumor-specific immunity (75). Recently approaches using the more potent T cell activator, DC, as the fusion partner has resulted in induction of strong anti-tumor immune responses (76-79). In one report it was shown that merely pre-mixing DCs and tumor cells prior to immunization resulted in induction of anti-tumor immunity (80).

Current trends in cancer vaccines are being designed to present tumor antigens on professional APCs, such as dendritic cells (81-83). Dendritic cells are the most potent activators of T cell responses. DCs or precursors are recovered from hosts and stimulated *in vitro* to proliferate and differentiate. They are then pulsed with peptides, tumor antigens, tumor lysates, or tumor specific mRNA (84-86). Immunization with DCs under these conditions can be used as a vaccine to successfully prevent tumor development and therapy to treat established tumors. One drawback of this therapy is that DCs are relatively low in number and somewhat procedurally difficult to obtain in large enough numbers to provide for routine administration as a vaccine. Recently, a cytokine known as FLT3 ligand (FLT3L) has been described to elicit large numbers of DC *in vivo* (87-91). FLT3L has also been shown to induce regression of established tumors (92-94). This may obviate the need to generate DC *in vitro*, for subsequent manipulations. This therapy, however, is most effective when the identity of the tumor antigens responsible for recognition by T cells is known. Although great strides have been made in determining tumor antigens and antigenic epitopes, many are still unknown (2,95). Other cancer vaccines under investigation do not require the identification of the tumor antigen. These approaches rely on the tumor itself to present antigen to the T cell (96,97). However, tumor cells may not express all the necessary molecules needed to induce a protective immune response, such as costimulatory molecules and cytokines produced by normal host APCs. Therefore, the tumor cells will be unable to induce a protective immune response. Thus, methods are available to introduce new proteins onto the surface of cells. These methods include: gene transfer, in which the gene of the cell surface molecule is transfected or the tumor cells are transduced to produce new protein; cell fusion, as discussed above, tumor cells are fused to antigen presenting cells (APCs); and protein transfer, in which the protein is coupled to a lipid tail and can be inserted into the lipid bilayer of tumor cell membranes.

4.4. Gene transfer of tumor cells with costimulatory molecules

As mentioned above, studies have shown that tumor cells lacking costimulatory/cell adhesion molecules, such as B7-1, are poorly immunogenic. Expression of B7-1 (98-105) and other adhesion molecules, such as ICAM-1 (106,107), on the tumor cell by transfection result in induction of tumor immunity and subsequent tumor rejection in animals.

Previous results from our laboratory showed that the human renal carcinoma cell line, RCC-1 does not express B7-1 and does not stimulate autologous T cells *in vitro* (104). After transfection of B7-1, RCC-1 induces strong proliferative and CTL responses *in vitro* (104). This has also been shown for tumor cell lines of other

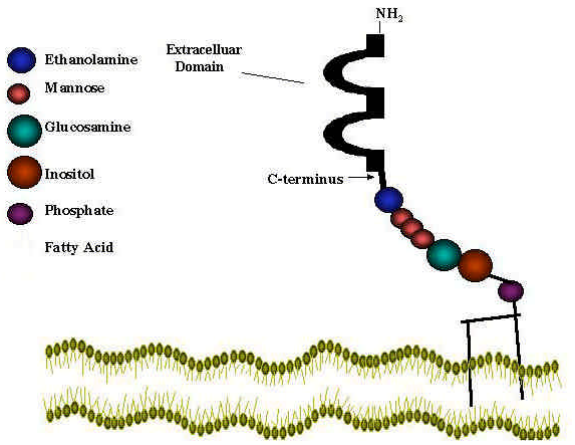

Figure 1. The schematic of a recombinant glycosyl-phosphatidylinositol anchored protein. The extracellular portion of the recombinant molecule is linked to the GPI-anchor through an ethanolamine at the C-terminal end of the protein. The glycan moeity connected to the ethanolamine consists of three mannose residues and a glucosamine which links to a phospholipid, phosphatidylinositol with two fatty acids.

histological origin (108). These results indicate that tumor specific immunity can be generated by expressing costimulatory molecules on tumor cells. Apart from B7-1, other cell surface adhesion molecules, such as ICAM-1, ICAM-2, ICAM-3, VCAM-1, LFA-3 have shown to provide co-stimulation for T cell proliferation (109-113). These molecules have also been demonstrated to stimulate T cells at different stages of activation (110), indicating that perhaps a combination of various costimulatory molecules on the surface of tumor cells will create a potent tumor vaccine.

The method of choice for introduction of new proteins is gene transfer, by either transfection or viral transduction of tumor cells. This technique can introduce many disadvantages when initiating human clinical trials. Transfection of primary tumor cells is difficult, therefore the establishment of tumor cell lines is needed. In addition, transfection of cells is time consuming, requiring weeks for selection of homogeneous cell populations expressing transfected molecules. Co-transfection, for the expression of several genes, can also prove to be difficult. The use of viruses to transduce cells has eliminated most time constraints, but this technique also has its disadvantages. This method utilizes vectors of viral origin that may introduce mutations at the site of DNA integration. In addition, it has been shown that the host can develop strong immune responses to the vector, making it difficult to immunize more than once with the same vector (114-116).

4.5. Protein transfer of tumor cells with immunostimulatory molecules

We have investigated an alternative method for the introduction of costimulatory molecules onto the surface of tumor cells to eliminate the problems encountered with gene transfer (117-119). This method, termed "protein transfer" can be used to anchor proteins to surface of the cell membrane, one of which uses unique proteins that are anchored to cell membranes via a glycosyl-phosphatidylinositol (GPI) linkage (120-122).

5. GLYCOSYL PHOSPHATIDYLINOSITOL-ANCHORED PROTEINS

The majority of known cell surface proteins are anchored to the membrane by a transmembrane domain that spans the entire lipid bilayer, followed by a cytoplasmic tail. Some cell surface glycoproteins are anchored to the cell membrane by utilizing lipids attached co-translationally to the protein. The most well characterized is the GPI-anchor (123-125). This anchor is composed of an ethanolamine and three mannose residues, a nonacetylated glycosamine and a phosphatidylinositol (Figure 1). The GPI-anchored precursor protein contains a hydrophobic signal sequence at the C-terminus. When the precursor protein enters the ER, the C-terminal hydrophobic sequence is cleaved and the attachment to the ethanolamine moiety of the pre-formed GPI-tail occurs by a transamidase through the ethanolamine residue (126,127). These proteins are then glycosylated and transported to the cell surface. Many proteins such as decay accelerating factor (DAF) (128), CD59 (129), LFA-3 (130,131), neural cell adhesion molecule-1 (NCAM-1) (132-134) and Fc gamma receptor III (CD16B) (135,136) are anchored to the cell surface by a GPI-linkage.

Membrane anchoring via a GPI-anchor has been associated with many unique properties. These proteins have a higher lateral mobility within the cell membrane (137) and are targeted to the apical cell membrane (138,139). Moreover, purified GPI-anchored molecules can spontaneously incorporate into membranes through their lipid tail (120). Observations by Medof *et al*. (140) have shown that incubation of cells with the complement regulatory protein, now known as DAF, results in its incorporation onto the surface of erythrocytes and subsequent inhibition of complement activity. Since then, most of the known GPI-anchored molecules have been shown to reincorporate onto erythrocytes and nucleated cells after a short incubation with cells (120). GPI-anchored mediated protein transfer has even been reported to occur *in vivo* in transgenic mice expressing CD59 under an erythroid restricted promoter (141). Endothelial cells lining the blood vessels in transgenic mice acquire CD59 from circulating erythrocytes (141). Most importantly, after incorporation onto the cell surface, these molecules retain their function.

The property of GPI-anchored proteins to transfer to foreign cell membranes has evolved into a simple and useful technology to express novel proteins on the cell surface without resorting to gene transfer. This has been proposed as an alternative to gene transfer to develop cancer vaccines where gene transfer is not desirable or feasible (118,119). Subsequently, other approaches to protein transfer, have been described and tested in the development of cancer vaccines (142,144). In the following sections we discuss the evolution of the protein

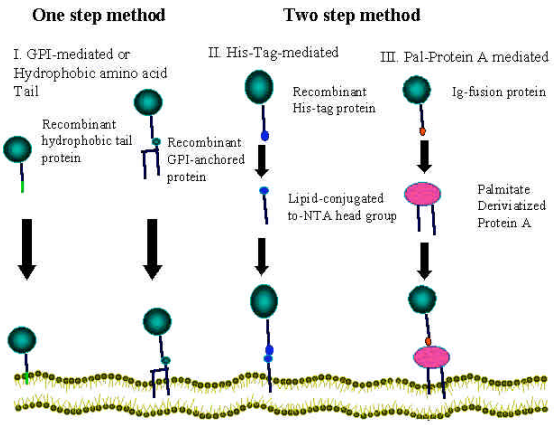

Figure 2. Single or two step methods of protein transfer. Proteins which are engineered with hydrophobic amino acids or the signal sequence for GPI attachment can be expressed by direct incubation in a single step (I). Proteins which do not directly incorporate into lipid bilayers need a scaffolding or platform to anchor them onto the cell membrane (II, III). The advantage of the one step process is the transfer of multiple proteins is tightly controlled. The scaffolding or platform approach reduces the need to engineer purify proteins from membranes of mammalian cells and instead soluble proteins can be anchored through the metal chelating NTA-lipids (II) or lipid-modified scaffolding molecules, such as palmitate-protein A (III).

transfer technology and current applications of GPI-anchored and engineered lipid-linked proteins of clinical interest in the development of therapeutic cancer vaccines.

6. STRATEGIES OF PROTEIN TRANSFER

There are many proposed strategies for protein transfer, however they all fall into one of two categories, a one step method or a two step, scaffolding method (Figure 2). The one step method utilizes recombinant proteins which are directly linked to a GPI-anchor or a stretch of hydrophobic amino acids. The two step method requires a scaffolding protein or metal chelator directly linked to lipids which can incorporate into the cell membrane (first step). The recombinant protein is then linked to the scaffolding protein or metal chelator (second step). In the following sections, four approaches of protein transfer are described which fall into these two categories.

6.1. Transmembrane proteins engineered into GPI-anchored forms

Although mature GPI-anchored proteins expressed on the cell surface do not have a transmembrane polypeptide domain, their mRNA sequence predicted that the precursors of GPI-anchored proteins have a hydrophobic stretch of amino acids at the C-terminus resembling TM domains. Site directed mutagenesis and recombinant DNA techniques revealed that the C-terminal hydrophobic domain and 15-20 amino acids of the extracellular domain proximal to the C-terminus posses the signal for GPI-anchor attachment. Analysis of the GPI-anchor attachment signal sequences of many cloned GPI-anchored proteins revealed a lack of consensus sequence for GPI-attachment (145-147). In the case of Qa-2 protein, the TM domain carries the signal for the GPI-anchor addition. Extensive studies by Udenfriend and coworkers have established the amino acid requirements for GPI-anchor attachment (145,148-150).

TM-proteins can be converted into GPI-anchored forms by replacing the TM and cytoplasmic domains with a GPI-anchor (127,139,151-159). This manipulation involves ligating the cDNA encoding the extracellular domain of a TM-anchored protein to the cDNA coding for the anchor attachment signal of a GPI-anchored protein. The chimeric construct is transfected into cells, which are then analyzed for surface expression of the protein and its susceptibility to phosphotidylinositol-phospholipase C (PI-PLC). Initial studies using this recombinant DNA methodology were focused on constructing chimeric molecules to identify the signal sequence for GPI-anchor attachment on endogenous GPI-anchored proteins. In a series of experiments, Caras *et al* (139, 158, 159) and Tykocinski *et al* (146) assigned the GPI-anchor addition signal of DAF to the last 37 amino acids at the C-terminus. Subsequently, GPI-anchored forms of type I and type II integral membrane proteins and secretory and viral envelope proteins were constructed to characterize the structural requirements of their extracellular domains to carry out specific functions.

The immunologically important CD4 protein was converted to a GPI-anchored form to map the domains required for HIV-1 binding and infection (160,161). Further investigation found that incorporating GPI-CD4 onto CD4 negative cells could render them susceptible to HIV infection (162). Other immunologically important molecules such as CD16A (163,164), CD8 (165), mouse MHC class I (154,166,167), MHC class II (152), TCR (168), ICAM-1 (153,169), B7-1 (119,170), B7-2 (170,171) and LFA-1 I domain (172), were also converted to GPI-anchored forms for investigating the functional consequences of this new mode of membrane association. Studies from our laboratory (164) and others (173) on membrane isoforms of CD16 have shown that GPI and TM-anchored forms differ in their signal transduction. GPI-anchored T cell receptor efficiently recognized antigen presented by MHC class II molecules (168). GPI-anchored mouse MHC class I molecule H2-D^d conferred protection from NK cell lysis *in vivo* and *in vitro* (166). However, in other studies GPI-anchored H2-D^b was not able to load endogenously processed antigenic peptides, though it bound to exogenously added antigenic peptides as efficiently as its TM-counterpart (174). Studies with Thy-1 and CD16A also suggest that membrane anchor can induce subtle conformation changes in the extracellular domain of a receptor (175,176). DAF protects cells from complement mediated damage equally well whether in GPI- or TM-anchored form (177). Recently, we have demonstrated that GPI-anchored B7-1 can bind to CD28 and induce T cell proliferation as efficiently as the transmembrane B7-1 (119,120). These results suggest that the type of membrane anchor can influence function of extracellular domain in some receptors, but not in all.

Table 1. Applications of protein transfer using GPI-anchored proteins

Molecule	Application	Reference
DAF	Reconstitution of complement regulation *in vitro*	140, 181, 182
CD59	*In vitro* and *in vivo* reconstitution of complement-deficiency	182-184, 211
CD16B	Ligand binding and endocytosis	118
Human B7-1	Stimulation of allogeneic T cells, Stimulation of anti-tumor immunity *in vivo*	119, 207
CD4	HIV-mediated gene transfer of CD4- cells	162
Mouse B7-1	Stimulation of anti-tumor immunity *in vivo*	205
MHC class I	Sensitize MHC class negative cells to CTLs	154

6.2. GPI-mediated protein transfer

Purified proteins that contain a GPI-anchor are able to spontaneously incorporate into the lipid bilayer of nucleated (117-119,178) and non-nucleated cells (130,140). Reconstitution of GPI-anchored proteins into cell membranes is a specific process, mediated by hydrocarbon chains of the lipid moiety as chemical or enzymatic removal of the acyl chains completely abolished the incorporation. The GPI-mediated protein transfer, has become an attractive strategy to express new proteins on cell membranes. Both naturally occurring and engineered GPI-anchored proteins transfer equally well. The membrane incorporation process is dependent on temperature and duration of incubation and concentration of the purified protein (117,118,140). Fatty acid binding serum proteins such as BSA and orosomucoid inhibit the transfer (118,140). Under serum free conditions, genetically engineered, affinity purified GPI-anchored proteins incorporate maximally after 2 h incubation at 37^{o}C (119,179). A number of tumor cell lines including primary breast carcinoma cells have been modified with GPI-B7-1 and show similar kinetics of incorporation (119).

Initially, the GPI-protein transfer was used to determine the functional consequence of defective expression of GPI-anchored receptors in Paroxysmal Nocturnal Hemoglobinuria (PNH) patients' erythrocytes. PNH is an acquired abnormality of hematopoietic cells affecting GPI-anchor biosynthesis or attachment, thus selectively affecting the membrane expression of GPI-anchored proteins (125,180). The complement regulatory activity of erythrocytes from PNH patients could be reconstituted by incorporation of GPI-anchored DAF and CD59 (140,181-183) by protein transfer. Other cell types that are sensitive to complement mediated lysis can also be rescued by incorporation of CD59 (184). Apart from complement regulatory proteins, PNH erythrocytes also lack the cell adhesion molecule LFA-3 and therefore, do not adhere to T cells expressing CD2, a natural ligand for LFA-3 (185). Expression of LFA-3 by protein transfer reconstituted the ability of PNH erythrocytes to adhere to T cells (130) suggesting that adhesion function of a cell can be manipulated by protein transfer.

In all these studies, purification and incorporation of GPI-anchored proteins did not alter their ligand binding capacity (154,160,161). Also, modification of tumor cells with GPI-B7-1 led to the stimulation of allogenic T cells (119). Applications in which GPI-anchored proteins have been tested for treatment of diseases are shown in Table 1.

6.3. Chemical modification of proteins with palmitic acid

The chemical modification of antibodies by palmitic acid has been well described (186-189). The advantage of this process is the ease of which it can be accomplished. Derivatization by palmitic acid covalently couples the protein to the *N*-hydroxysuccinimide ester of palmitic acid (186). The derivatized protein can be inserted into the plasma membranes of cells. This method has been used to study *in vitro* cell-cell interactions, receptor-mediated events, and to dissect distinct receptor pathways through a more natural interaction (190-195). The primary drawback of this methodology is that the palmitate derivatization of functionally active amino acids on the protein can lead to loss of functional activity of the protein. Moreover, the random nature of the palmitate derivatization also results in random orientation of proteins or antibodies, that when incorporated onto the surface of cells, may not be best to facilitate interactions with its ligands. More recently, a method to use palmitate derivatization in a two step process to add proteins to the surface of cells was shown. Peacock and co-workers (196) have shown that it is possible to modify protein A by palmitic acid. They showed that the resulting pal-protein A could incorporate onto the surface of cells and retained its ability to bind the Fc portion of antibodies (196). This method allows the construction of a platform, palmitate-protein A (pal-protein A) on which one can then assemble any number of antibodies or Ig-fusion proteins. The advantage of this design is that once cells were coated with pal-protein A, antibodies could be coated or expressed in the correct orientation, thereby maximizing interactions with its ligands and retaining Ag-binding affinity. This could be useful in studying receptor-ligand interactions *in vitro* using Ig-fusion proteins as well. Recently, Chen *et al* (143) using pal-protein A and B7-1-Ig-fusion protein showed that by varying the level of costimulatory signal, i.e. by level of B7-1-Ig-fusion protein onto cells, they could dissect differences in cytokine release and T cell proliferation depending on the level of costimulation. Historically, these responses and studies were conducted using cells modified by gene transfer.

6.4. Protein transfer using metal chelator lipids

Immobilized metal chelators, such as iminodiacetic acid and nitrilotriacetic acid (NTA), have been used routinely for the purification of recombinant proteins by metal-ion affinity chromatography (197). These chelators, in the presence of Ni^{2+} or Zn^{2+}, facilitate the binding to polyhistidine tags (his-tag) of the recombinant proteins. Some recent studies have shown that

NTA can be covalently linked to lipids, which can be used to anchor his-tag proteins onto planar lipid membranes (198). A recent study by van Broekhoven et. al. described the incorporation of a novel-chelator lipid (NTA-DTDA) and the anchoring of recombinant B7-1 and CD40 extracellular domains fused to a his-tag (142). In this study they show the expression is strictly dependent on the incorporation of chelator-lipids into the plasma membrane of cells. This is because this technique uses a two-step process, instead of just a one-step method like protein transfer by GPI-anchored proteins (Figure 2). The advantage in this system, is that soluble proteins which are significantly easier to produce and purify can be anchored to the cells as well. GPI-anchored proteins due to their surface expression must be purified from cell membranes which requires significant labor and the protein yields are limited (143). Incorporation of chelator lipids is concentration dependent and can be enhanced by helper lipids, such as DMPC and POPC, and also fusogens like polyethylene glycol. van Broekhoven et. al. showed that anchoring of B7-1 and CD40 his-tag fusion proteins could costimulate an allogeneic T cell response *in vitro* (142). Their studies also extended to generating an anti-tumor response *in vivo*, which is discussed in section 7.

6.5. Protein transfer using proteins with engineered hydrophobic tails

Recently another approach to protein transfer was described. Wahlsten et. al. (144) described a novel way to attach the super-antigen toxic shock syndrome toxic-1 (TSST1) to tumor cells using hydrophobic amino acids. In their report, they created a genetic recombinant form of TSST1 by fusing TSST1 cDNA to the transmembrane region sequence of c-erb-B-2 called TSST1-TM. The protein was produced in E.coli and purified by metal affinity chromatography. Wahlsten *et al*. utilized the hydrophobic tail from c-erb-B-2, instead of a lipid tail, to facilitate incorporation of TSST1-TM (144). Studies in E.coli as well as eukaryotic cells have demonstrated that hydrophobic protein sequences can facilitate membrane insertion (199,200). Incorporation of TSST1-TM onto tumor cells led to a polyclonal stimulation of human PBMCs. They also tested this protein transfer method in tumor studies *in vivo*. (See section 7).

7. DESIGNING CANCER VACCINES USING PROTEIN TRANSFER

Most murine and human tumors develop despite being antigenic. This lack of immunogenecity even in the presence of unique tumor antigens has been attributed to three factors: the lack of costimulatory molecules on most tumors, immunosuppression by tumors, and T cell ignorance or anergy to the antigens displayed by the tumor. The lack of costimulatory molecules, especially B7-1 (98,105,201) and B7-2 (202,203), necessary for T cell activation, render tumors unable to provide costimulatory signals to tumor specific T lymphocytes. However, they can provide the first signal, TCR recognition by presenting the tumor antigen on MHC class-I molecules. In the absence of a costimulatory signal these TCR stimulated tumor-specific T cells become anergic and may eventually die. In this way, tumor cells can incapacitate the T cell population specific for their antigens and escape from any immunosurveillance. Expression of B7-1 (98-104) and other costimulatory molecules such as ICAM-1 (106,107) on the tumor cell by gene transfection can induce specific anti-tumor immunity and subsequent tumor rejection in animal models. Gene transfection of tumor cells, as previously stated, has many disadvantages. Thus, protein transfer has been pursued as an alternative method to create therapeutic cancer vaccines.

7.1. Protein transfer modified tumor cells as a vaccine

Protein transfer provides an alternative method for the introduction of immunostimulatory molecules onto the surface of cells and eliminates most of the problems encountered with gene transfer (117-119,122,204). GPI-protein transfer, as well as the other described protein transfers, is fast and requires only a short incubation of the cells with the purified protein. This technique also allows for simultaneous incorporation of a number of molecules, virtually on all cell types including primary tumor cells, at any stage of their cycle.

Studies conducted by our laboratory have shown that GPI-B7-1 coated onto tumor cells augments an allogeneic response (119). GPI-protein transfer studies, by Tykocinski and co-workers and our laboratory, have shown that more than one molecule can be delivered to the cell surface simultaneously without interference and both molecules retain functional activity (205, Poloso et. al. unpublished observations). Using the his-tag system, van Broekhoven *et al*. was able to simultaneously incorporate mB7-1 and CD40 (142). There are some differences in the ability of both mB7-1 and CD40 to incorporate between the two proteins, which they attributed to difference in the molecular mass of B7-1 and CD40 (45 kDa and 25 kDa, respectively). Because the proteins are not directly anchored with lipid or hydrophobic tail, his-tag proteins compete for a limited number of chelator lipids which must be incorporated into the plasma membrane prior to his-tag binding (142). Nevertheless, the incorporation of mB7-1 and CD40 onto tumor cells used to immunize mice resulted in the priming of CTLs as measured by an *in vitro* CTL assay (142). Immunization with what the authors call 'engrafted tumors' with mB7-1 and/or CD40 resulted in a significant delay in the development of tumors after a parental tumor challenge. In this study, mice immunized with tumor cells engrafted with both mB7-1 and CD40 had the largest delay in tumor development, justifying the idea that more than one immunostimulatory molecule may be necessary for an optimal anti-tumor response. These results agree with previous studies using gene transfected tumor cells with multiple molecules, such as B7-1 and ICAM-1 (107).

Wahlsten *et al*. has described the construction of a superantigen TSST-1-TM, which contains a hydrophobic tail allowing for the insertion into the membrane of cells (144). Using this method to express a superantigen on tumor cells, these investigators have simultaneously lowered the toxicity of super-antigen treatment and linked it directly to the tumor cells that need to be lysed in order to

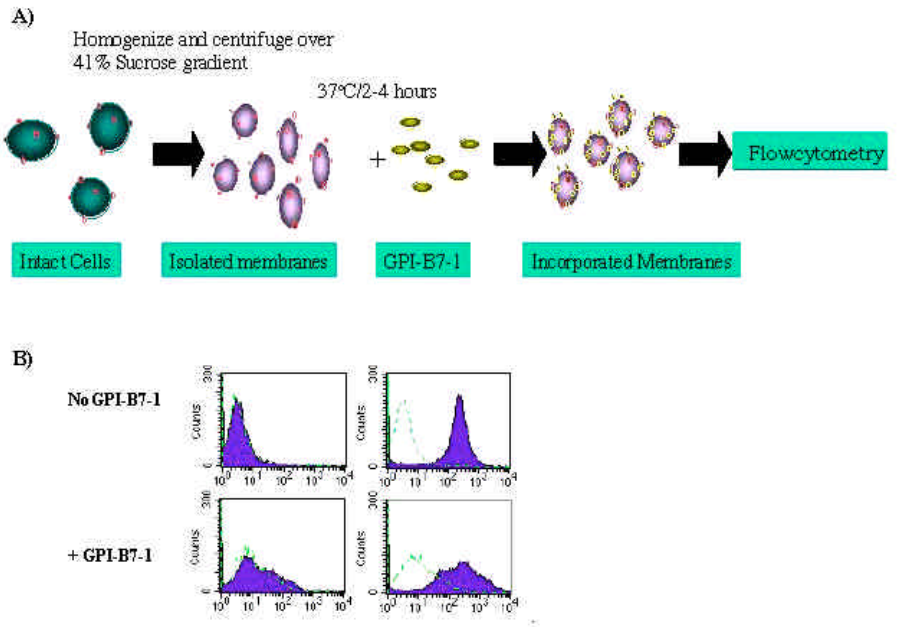

Figure 3. Preparation and modification of isolated membranes with GPI-B7-1 by protein transfer. A) Intact cells are suspended in a hypotonic buffer and homogenized. The lysate is then centrifuged over a sucrose gradient and the interface (containing cellular membranes) is harvested and washed. Recombinant GPI-anchored proteins, such as B7-1, can then be incubated with these membranes and the resulting membranes incorporate B7-1 into their lipid bilayers. B) Flowcytometric analysis of isolated membranes from MDA-231 breast cancer cells (upper panels) and SKMEL melamona (lower panels) after incubation with (right panels) or without (left panels) GPI-B7-1. Filled histograms represent staining by PSRM3, an anti-B7-1 antibody, while the open histogram represents staining by an isotype control.

facilitate uptake of tumor antigens from these cells. Immunization with tumor cells coated with TSST-1-TM or TSST-1-TM lacking the MHC class II binding domain resulted in significant anti-tumor immunity. Immunization with this vaccine induced regression of established parental tumors (144).

7.2. Protein transfer modified isolated tumor membranes as a vaccine

One of the limitations of protein transfer is related to the stability of the incorporated molecule on the cell surface. Live or irradiated cells gradually lose surface expression of the incorporated protein upon multiple cell divisions (118,119,142). However in clinical settings where live cells are undesirable to use, non-proliferating, irradiated cells or cell membrane preparations can be modified by protein transfer and for subsequent use. Isolated tumor cell membranes offer many advantages over intact cells. Since membranes do not have the metabolic functions of cells and do not divide, they provide a stable environment for protein transfer of GPI-anchored molecules. Cell membranes are isolated from intact cells by homogenization and centrifugation over a sucrose gradient (Figure 3a, (206)). Isolated membranes can then be modified by protein transfer, washed, and analyzed by flowcytometry or ELISA (Figure 3b). Stability studies using GPI-B7-1 have shown that this protein is stable on isolated membranes from a mouse thymoma for at least 4, and up to 7 days on isolated membranes from various human tumor cell lines (179,207). Moreover, membranes can be prepared from fresh and frozen tumor tissue and the membranes can also be easily stored in frozen aliquots. The fresh or frozen membranes can be modified equally to express GPI-anchored costimulatory molecules by protein transfer, for immunization protocols (207). The costimulatory molecule modified membranes can also be stored as frozen aliquots with minimal loss of the incorporated molecules (179). The optimal conditions for protein transfer of GPI-proteins onto isolated membranes is much the same as cells. Optimal expression is seen at 37^{o}C for 2-4 hours (179).

To our knowledge, protein transfer is the only method available to add new cell surface receptors on isolated membranes (207). Isolated membranes can be made directly from tumor tissue obtained from patients and subsequently modified by protein transfer (179). This is advantageous since the establishment of cell lines from surgically removed or frozen tumor specimens is difficult and often not successful (179,208). It has also been determined that a major limiting factor in gene transfer based vaccines in clinical studies is the limited number of successfully transfected tumor cells (209).

Vaccination with GPI-B7-1 modified isolated tumor membranes results in the generation of tumor specific T cells and also CTL generation *in vivo*, which can be measured *in vitro* (207). The responding cells in the

cytotoxicity assay were determined to be CD8+ T cells by antibody depletion studies. Vaccination also completely protects mice from a parental tumor challenge in this thymoma tumor model (207). Vaccination of GPI-B7-1 modified membranes in other tumor systems have resulted in either partial protection or a significant delay in tumor development (unpublished observations). Furthermore, our collaborative work has shown that tumor liposomes reconstituted with mB7-2 can protect mice from parental tumor challenges (210).

Our data showing protein transfer of GPI-B7-1 onto isolated membranes as a vaccine in combination with soluble IL-12 suggests that the addition of IL-12 greatly enhances the cytolytic activity of tumor specific T cells recovered from the spleen of immunized animals (207). Whether this is due to the actual activity of individual T cells or due to eliciting a larger number of CTLs is unclear.

8. CONCLUDING REMARKS

Understanding the mechanism of GPI-anchor modification of proteins has resulted in techniques to create GPI-anchored forms of cell surface glycoproteins (119,154,170). The special property of naturally occurring and engineered GPI-anchored molecules to incorporate spontaneously onto cell membranes has been utilized in a simple, rapid technique for transient expression of foreign molecules on virtually any cell type. This technique has overcome several of the limitations of gene transfer techniques and thus offers many advantages in consideration of human clinical trials. Using this technique we have demonstrated that GPI-anchored B7-1 can spontaneously incorporate onto many tumor cell lines (117-119) and provide them with the capacity to stimulate tumor specific T cells. Also, we have demonstrated this approach can result in the complete protection of mice from developing tumors after a wild-type tumor challenge (207). Beyond the GPI-method of protein transfer there are several other attractive alternative methods of protein transfer which have been successfully applied to cancer vaccines in mouse models (142-144). An important next step will be to see if these methods can prove to be effective in the clinical setting and compare to other strategies currently undergoing trials. We have demonstrated that protein transfer of GPI-immunostimulatory molecules can also be applied to human isolated tumor membranes from human tumor specimens (179). Whether these strategies are as efficacious as tumor transfected or DC-based vaccines still needs to be determined.

9. ACKNOWLEDGEMENTS

This work was supported by NIH grant # RO1 CA 70833

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Keywords: Tumor Immunotherapy, Gene Transfer, CD80, GPI, Lipid Anchor, Transmembrane Anchor, Costimulatory Molecules, Tumor, Review

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