Protein Transfer of Glycosyl-phosphatidylinositol-B7-1 into Tumor Cell Membranes: A Novel Approach to Tumor Immunotherapy

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ABSTRACT

Modification of tumor cells with one or more costimulatory adhesion molecules has been proposed as a means to develop therapeutic cancer vaccines for use in human immunotherapy. Expression of B7-1 (CD80) in tumors by gene transfer creates an immunogenic tumor cell that induces antitumor immunity and protects mice from further challenge with wild-type tumor cells. In this report, we demonstrate that protein transfer of glycosyl-phosphatidylinositol (GPI)-anchored costimulatory molecules into tumor cell membranes could be used as an alternative to gene transfer for tumor immunotherapy. Incubation of isolated tumor membranes with purified GPI-anchored B7-1 results in stable incorporation of B7-1 on tumor cell membranes within a few hours. Immunization of C57BL/6 mice with EG7 tumor membranes modified to express GPI-B7-1 by protein transfer induces tumor-specific T-cell proliferation and CTLs. In addition, immunization with these EG7 membranes protects mice from parental tumor challenge. The protein transfer approach used here does not require foreign vectors or live tumor cells and is completed within a matter of hours. Irradiated cells or membrane preparations from fresh or frozen tumor tissue can be used. Therefore, protein transfer of glycolipid-anchored molecules provides an efficient and novel approach to modify tumor membranes for human immunotherapy. This approach is not limited to costimulatory molecules because any cell surface protein can be converted to a GPI-anchored form by recombinant techniques.

INTRODUCTION

For T cells to proliferate and respond to an antigen, two signals are required (1). Engagement of the T-cell receptor with antigen-MHC complexes provides the initial signal. The second signal, termed costimulation, is received from any number of adhesion receptor-ligand interactions between the antigen-presenting cell and T cell, such as B7/CD28 (2–4). The absence of costimulation during T-cell recognition of a specific antigen results in T-cell unresponsiveness (5, 6). Many tumor cells express antigen-MHC complexes while failing to express appropriate cell adhesion molecules necessary for T-cell costimulation (7–11). By lacking these costimulatory adhesion molecules, tumor cells may thus escape host immunity.

Expression of costimulatory adhesion molecules such as B7-1, B7-2, or intercellular adhesion molecule 1 on tumor surfaces by gene transfer techniques can create a more immunogenic tumor cell that induces protective immunity against a parental tumor challenge (7–11). Recently, we (12) and others (13) have shown that costimulatory molecules like B7-1 induce protective immunity against a parental tumor challenge (7–11). By lacking these costimulatory adhesion molecules, tumor cells may thus escape host immunity.

For incorporation of GPI-B7-1, frozen (−80°C) or fresh membranes were resuspended to 100 μl using serum-free RPMI 1640 containing 100 μg/ml ovalbumin. Purified GPI-B7-1 (10 μg/ml) was added to the membranes, and the mixtures were shaken for 4 h at 37°C in siliconized microfuge tubes. Nonincorporated control EG7 membranes were incubated at 37°C in the same buffer without GPI-B7-1. The membranes were washed two more times and either analyzed by ELISA or resuspended in HBSS or HBSS containing 2 ng of rIL-12, using a 20-gauge needle for mice immunizations. The GPI-anchored form of FcγR III, CD16B, was also used to modify tumor cell membranes by protein transfer. CD16B was immuno-affinity purified from CD16B-transfected K562 cells and diluted in the above-mentioned buffer to a final concentration of 20 μg/ml for incubation of the membranes as described. For ELISA, the membranes were coated onto microtiter plate wells overnight at 4°C. The wells were blocked with complete RPMI 1640 containing 10% FCS, and then the membranes were analyzed using X63 (negative control), PSRM-3 (antihuman B7-1 mAb), M1/42 (antimouse monomorphic class I), or CLB.C1.12 (anti-human B7-1 mAb). The absorbance of PSRM-3-binding wells was normalized to the absorbance of M1/42-binding wells, which was designated as 1.0.

Immunization of Mice and Tumor Protection Studies. C57BL/6 mice (five mice/group) were immunized i.p. with 100 μl of HBSS, EG7 membranes (100 μg of equivalent protein), or GPI-B7-1- incorporates EG7 membranes twice at a 2-week interval. Three weeks after the final immunization, the spleens were harvested, and T cells were purified using mouse T-cell enrichment columns (R&D Systems) to 95% purity as analyzed by flow cytometry. The T cells were used in either a MLTR or CTL assays.

For tumor challenge experiments, mice were immunized s.c. in the hind flank with HBSS, EG7 membranes, or GPI-B7-1-incorporated EG7 membranes, with or without 2 ng of rIL-12. Two weeks later, the mice were boosted. EG7 cells (106) were injected s.c. at a remote site 1 week after the boost. Mice were monitored daily for tumor growth and euthanized when tumors reached 2 cm in diameter.
T-Cell Proliferation and CTL Assays. For the MLTR, T cells (10^5) purified from immunized mice were cocultured with irradiated (10,000 rads) EG7 cells (2 x 10^5) for 5 days in a 5% CO_2 incubator at 37°C. T-cell proliferation was measured by pulsing the wells with 1 μCi of [³H]thymidine for the last 18 h of culture.

For CTL assays, T cells were restimulated in vitro for 5 days with irradiated (10,000 rads) EG7 cells. On the second day of the restimulation, 10 units/ml rIL-2 (PharMingen) were added. Live T cells were recovered by density sedimentation using Histopaque 1077 (Sigma) and resuspended to 10^7 cells/ml. The effectors and targets were mixed at various ratios, and a standard 4-h [³Cr] release assay was performed.

For T-cell depletion, T cells were pretreated with either 53.6 (anti-CD8) or 145-2C11 (anti-CD3) for 30 min. The coated cells were then incubated at 37°C for 30 min with the rabbit complement. Live cells were recovered as described above and used in the [³Cr] release assay.

RESULTS AND DISCUSSION

Cell membranes were prepared from EG7 cells (18) and modified to express GPI-B7-1 by protein transfer. GPI-B7-1 was stably incorporated in these membranes after incubation with purified GPI-B7-1 for 4 h at 37°C (Fig. 1A). These membranes were analyzed for B7-1 and MHC class I expression by ELISA. After incubation with 10 μg/ml purified GPI-B7-1, we obtained GPI-B7-1 expression at 50% of MHC class I expression (data not shown). Nearly 100% of the incorporated GPI-B7-1 was retained in the membranes for at least 4 days under culture conditions (Fig. 1A). To determine whether the GPI-B7-1-incorporated membranes could stimulate tumor-specific immune responses in vivo, we first measured the proliferation of T cells from mice immunized with either HBSS, EG7 membranes, or GPI-B7-1-incorporated EG7 membranes in a MLTR. As seen in Fig. 1B, T cells from mice immunized with GPI-B7-1-incorporated EG7 membranes proliferated fivefold over the background when cocultured with wild-type EG7 cells. T cells from the HBSS control- and buffer-treated EG7 membrane-primed mice were unable to mount a significant proliferative response to EG7 tumor cells.

Next, the ability to generate a CTL response against the parental tumor was investigated. After immunization, T cells were purified and restimulated in vitro with irradiated EG7 cells for 5 days and then assayed for cytotoxicity to EG7 cells. T cells from mice primed with GPI-B7-1-incorporated EG7 membranes had an increased cytotoxic response to the EG7 targets in comparison to the EG7 membranes or HBSS-immunized controls (Fig. 2A). Analysis of CTL activity from two independent experiments shows an average of 14% specific lysis of target cells at the 50:1 E:T ratio.

Many cytokines have been shown to augment antitumor immune response (19). In particular, IL-12 has been reported to augment the production of cytotoxic cells and work in concert with B7-1 in generating antitumor immunity (20, 21). Therefore, we investigated whether IL-12 could further enhance the CTL activity induced by tumor membranes modified with GPI-B7-1. IL-12 was administered i.p. at regular intervals during the membrane immunizations. As shown in Fig. 2B, IL-12 administration increased the specific lysis of the EG7 targets to 54% by the T cells primed with GPI-B7-1-reconstituted EG7 membranes as compared to 12.4% specific lysis from T cells of mice immunized with the same membranes in the absence of IL-12 treatment. IL-12 treatment of mice immunized with HBSS or EG7 membranes did not enhance CTL activity. In this assay, another GPI-B7-1 molecule constructed using the GPI signal sequence from LFA-3 gave a similar response, indicating that the origin of the GPI anchor signal sequence does not influence B7-1 function (data not shown).

To confirm that it is the specific incorporation of GPI-B7-1 and not the addition of any lipid-modified protein that confers immunogenicity, tumor membranes incorporated with CD16B, the naturally GPI-anchored form of FcγR III (22), were included as a control. As shown in Fig. 2C, no CTL responses were observed in mice immunized with CD16B-incorporated EG7 membranes, even with the coadministration of IL-12.

To determine the nature of the effector cells mediating antitumor cytotoxicity, CTL responses were analyzed after the depletion of CD8+ T cells in vitro. Treatment of effector cells with anti-CD8
antibody and complement eliminated nearly 83% of the cytolytic activity, indicating that CD8<sup>+</sup> cells are the major effector of cytotoxicity (Fig. 2D). Similarly, the in vitro depletion of CD3<sup>+</sup> T cells from mice primed with GPI-B7-1-reconstituted membranes completely eliminated cytotoxicity (data not shown), indicating that T cells and not natural killer cells were the effector of the immune response to EG7 tumor cells.

Although we have shown that EG7 membranes modified with GPI-B7-1 can generate CTLs against the parental tumor, it is necessary to determine whether these membrane preparations can immunize and protect mice against a subsequent challenge with the parental tumor. To determine this, mice were immunized twice s.c. in the hind flank with GPI-B7-1-modified or buffer-treated membranes in the presence or absence of IL-12. One week after the final immunization, mice were challenged s.c. with wild-type EG7 cells. After a few weeks, tumors developed and grew rapidly in mice immunized with HBSS, IL-12, or buffer-treated EG7 membranes (Fig. 3). In contrast, mice immunized with EG7 membranes modified with GPI-B7-1, with or without coinjections of IL-12, remained tumor free for the duration of the experiment.

As shown in Fig. 3, only mice immunized with GPI-B7-1-modified EG7 membranes were protected from the tumor challenge. Although tumors grew in mice immunized with EG7 membranes and IL-12, there was a delay in tumor growth (Fig. 3). This was reproducible, suggesting that IL-12 treatment may enhance immune activity to unmodified EG7 membranes. However, tumors still developed, indicating that immunization with EG7 membranes and IL-12 was unable to induce a sufficient antitumor immune response. The ovalbumin used during GPI protein transfer did not influence the immunity against tumors because mice immunized with tumors modified in presence or absence of ovalbumin showed a similar level of protection against tumor challenge (data not shown). These results demonstrate that EG7 membranes modified to express B7-1 by protein transfer can effectively induce antitumor immunity and protect mice from further tumor challenge.

Protein transfer techniques can also be used to introduce B7-1 onto
the surface of intact live tumor cells. We have observed, however, that intact live cells lose surface expression of GPI-B7-1 quickly under culture conditions (12). This is most likely due to the fact that the GPI-anchored B7-1 molecule is exogenously added to the membrane and therefore cannot be replaced after cell division, internalization, or shedding. Therefore, expression on live cells will be lost rather quickly. Live tumor cells, however, are an unlikely candidate for administration to human patients. Other preparations of tumor cells would need to be used, such as irradiated cells or cell membrane preparations. Preliminary studies in our laboratory on tumor cell lines such as P815, a murine mastocytoma, and K1735, a murine melanoma, show that immunization with GPI-B7-1-incorporated γ-irradiated tumor cells can induce a tumor-specific T-cell-proliferative response in mice.4 However, in humans, irradiated tumor cells may cause problems if not all cells have been killed by irradiation. In addition, some murine tumor cells, when irradiated, have been shown to be poor inducers of an immune response toward the parental tumor (23, 24).

As an alternative, we have used isolated tumor cell membranes. These preparations offer many advantages because they do not divide or have the metabolic functions of cells and can therefore provide a stable environment for incorporated GPI-anchored molecules. Membranes can also be easily stored in frozen aliquots or freshly prepared from frozen tumor tissue. These membranes can then be quickly incorporated with GPI-anchored proteins for convenient immunization protocols. Membrane preparations also retain the ability to interact with and stimulate cells in culture. Membranes of Chinese hamster ovary cells expressing GPI-B7-1 can polyclonally stimulate T cells in the presence of suboptimal doses of phorbol 12-myristate 13-acetate (data not shown). In other studies, membranes from T helper cells have been shown to stimulate B cells in vitro (25, 26).

Currently, gene transfer is the method of choice for the expression of new proteins in cells. However, gene transfer may present problems for human tumor immunotherapy in the clinical setting. This method introduces foreign vectors, some of which are of viral origin. At the site of incorporation, these vectors could introduce chromosomal mutations. Also, due to the immunity developed against vaccinia viral proteins, the vaccinia-based vectors can be used only once to deliver the desired genes (27). Other viral vectors, such as adenovirus, also increase cellular infiltration at the site of delivery, indicating an immune response to the vector that would prevent its subsequent use for gene therapy (28, 29). The protein transfer method described here could eliminate the problems associated with vector-mediated gene transfer and allow functional expression of B7-1 or other molecules for use in many experimental and therapeutic applications. Other desirable features of this protein transfer method are that live cells are not needed and incorporation can be completed within a short time, if the appropriate GPI-anchored molecules are available. The level of expression can also be easily controlled by varying the concentration of protein used, the temperature, and the time during protein transfer (30). Cell membrane preparations, RBCs, liposomes, or any amphiphilic/hydrophobic surface can be modified with lipid-anchored proteins by protein transfer. Therefore, isolated cell membranes, irradiated cells, or liposomes entrapped with proteins can be modified to express the appropriate GPI-anchored molecules for targeted delivery to antigen-presenting cells.

Many investigators are beginning to exploit the properties of GPI anchors by designing proteins to have these unique lipid tails (31, 32). MHC class I has been modified to be cell surface-anchored via GPI. Using in vitro assays, it has been shown that cells modified to express GPI-anchored MHC class I molecule-hepatitis B viral peptide complexes by protein transfer served as targets for CTLs (33). In this report, we have shown that GPI-B7-1-incorporated tumor cell membranes can induce tumor-specific T-cell responses and provide protective immunity from tumor challenge in vivo. To our knowledge, this report is the first demonstration of the use of protein transfer of GPI-anchored proteins to express new proteins on isolated cell membranes and induce immunity against a disease.

In addition to the incorporation of proteins that stimulate immune activity, proteins that down-regulate or modulate effector functions would need to be used, such as irradiated cells or cell membrane preparations. Preliminary studies in our laboratory on tumor cell lines such as P815, a murine mastocytoma, and K1735, a murine melanoma, show that immunization with GPI-B7-1-incorporated γ-irradiated tumor cells can induce a tumor-specific T-cell-proliferative response in mice.4 However, in humans, irradiated tumor cells may cause problems if not all cells have been killed by irradiation. In addition, some murine tumor cells, when irradiated, have been shown to be poor inducers of an immune response toward the parental tumor (23, 24).

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can be expressed on the cell surface by protein transfer. For example, using protein transfer, one could introduce an antigenic peptide presented by GPI-anchored MHC complexes on cells such as RBCs or liposomes, which lack costimulatory molecules, and use these vehicles to induce anergy in antigen-specific T lymphocytes. This may be beneficial for the treatment of autoimmune diseases or transplantation. Further exploitation and study of this method could enable researchers to create the optimal immunotherapeutic or immunomodulatory cell that can induce regression of established tumors, treat autoimmune disease, and potentially aid in the acceptance of tissue transplants.

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REFERENCES


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