Glycoinositol Phospholipid-anchored Interleukin 2 but not Secreted Interleukin 2 Inhibits Melanoma Tumor Growth in Mice[1]

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Abstract

Whereas cancer immunotherapy with interleukin (IL) 2 and/or other cytokines has proved effective in activating immune responses against tumor cells, the major obstacle with the use of these cytokines in cancer patients is their severe side effects when delivered systemically at high doses. In an effort to overcome this problem, in the present study, a fusion protein containing human IL-2 and a glycoinositol phospholipid (GPI) anchor sequence of decay accelerating factor was generated. When expressed by transfected cells, these fusion proteins were presented on the cell surface in the GPI-anchored form as demonstrated by fluorescence-activated cell sorter and ELISA analyses. This GPI-anchored IL-2 is highly functional as indicated by significantly increased T-cell infiltration in tumor masses. Immunohistochemical analysis of tumor cells isolated from experimental tumors indicated that a local high level of IL-2 was achieved by GPI-anchored IL-2. More importantly, when injected into mice i.v., the growth of these B16F0 melanoma cells that were engineered to express this fusion protein was significantly inhibited. In contrast, the inhibition of secreted IL-2 on tumor growth was not observable in this study. These studies may provide a novel approach to locally deliver high doses of cytokines for cancer immunotherapy.

Introduction

IL-2[3] plays a vital role in activating immune responses because it is required for the growth of T lymphocytes, natural killer cells, and lymphokine activated killer cells (1, 2). Preclinical animal studies and human practice have shown that IL-2 alone or combined with other treatments has strong antitumor effects (3–8). However, the effect of systemic administration of IL-2 in human cancer immunotherapy is hampered by the short half-life of IL-2 and its severe toxicity because of necessary high doses (9, 10). Moreover, the severe side effects caused directly or indirectly by IL-2 have been an obstacle to the development of routine treatment protocols (11). A major advance in cancer treatment would be achieved if therapeutic methods could reduce the severity of side effects caused by IL-2 systemically delivered at high doses. Local or regional treatment instead of systemic application is an alternative approach to elicit a stronger immune response with fewer side effects. Additionally, immobilizing IL-2 or other cytokines on the tumor cell may enhance the effectiveness of current cancer treatments.

A wide range of cell-surface proteins, including enzymes, coat proteins, surface antigens, and adhesion molecules, are attached to the plasma membrane via GPI anchors (12). GPI is a posttranslationally added lipid anchor; therefore, unlike conventional polypeptide anchors, which have different transmembrane domains and connect to specific cytoplasmic extensions, GPI anchors use a common lipid structure to attach to the membrane, which is irrespective of the proteins linked with it (13). GPI anchor signal sequences have been identified for many proteins such as DAF and leukocyte function antigen-3 (14). The GPI anchor signals have been successfully engineered onto the COOH terminus of other un-GPI-anchored proteins, and these GPI-anchored proteins are coated on the cell surface and are functional (15, 16). Therefore, GPI anchor is a very useful technology to engineer proteins onto the cell surface.

To develop an approach for regional application of IL-2, in the present research a fusion gene was made by attaching a DNA oligo encoding human DAF GPI anchor signal in frame to the 3′ end of human IL-2 cDNA. Various *in vitro* assays confirmed that this fusion protein can attach to the cell surface and is functional. *In vivo* animal studies have demonstrated that this fusion protein significantly inhibits tumor growth without severe side effects.

Materials and Methods

Mice and Cell Lines. C57BL/6J female mice were purchased from the Jackson Laboratory (Bar Harbor, ME) at 4 to 6 weeks of age. All of the experiments were performed according to the NIH guidelines for care and use of laboratory animals. B16F0 murine melanoma cells purchased from American Type Culture Collection (CRL-6322) were cultured in DMEM containing 10% fetal bovine serum (HyClone, Logan, UT) and 50 μg/ml gentamicin (Life Technologies, Inc., Grand Island, NY).

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[3] The abbreviations used are: IL, interleukin; GPI, glycoinositol phospholipid; DAF, decay accelerating factor; FACS, fluorescent activated cell sorting; PI-PLC, phosphatidylinositol-specific phospholipase C.

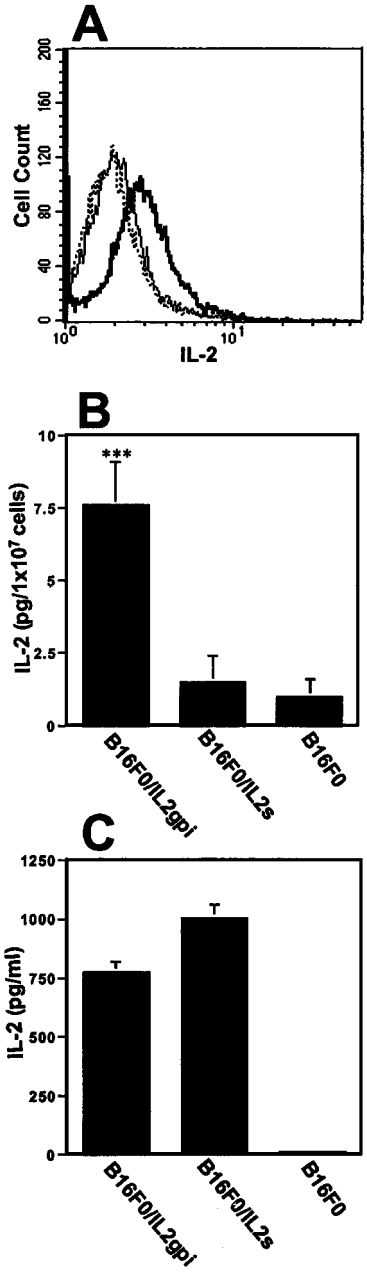

Fig. 1. GPI-anchored IL-2 expression. *A,* cell surface IL-2 was monitored by FACS after rat antihuman IL-2-FITC staining of B16F0 cells (– – –), B16F0/IL2gpi cells after treatment of PI-PLC (——), or B16F0/IL2gpi cells (▬). *B,* membrane-bound IL-2 was harvested from 1×10^7 B16F0 cells, B16F0/IL2s cells, and B16F0/IL2gpi cells by PI-PLC treatment, and measured with the human IL-2 ELISA kit. ***, $P < 0.001$. *C,* IL-2 activity in the culture medium of B16F0 cells, B16F0/IL2s cells, or B16F0/IL2gpi cells; *bars,* ±SD.

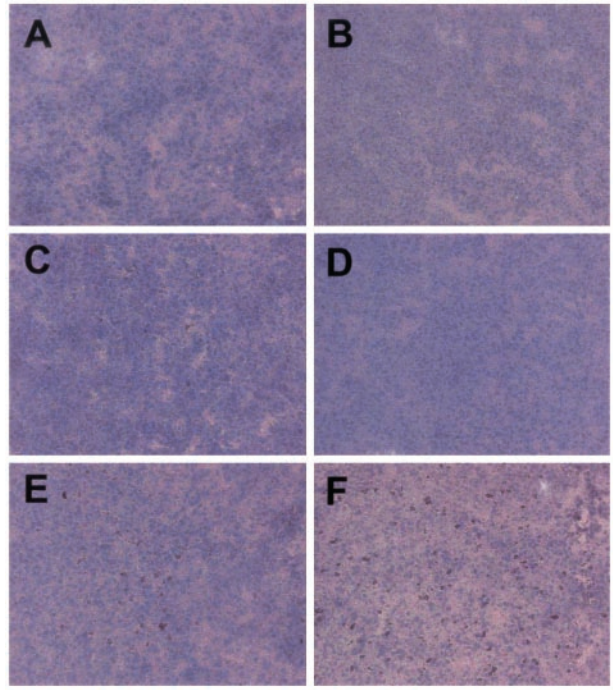

Fig. 2. Immunohistochemical analysis of tumors developed from B16F0 cells (*A* and *B*), B16F0/IL2s cells (*C* and *D*), or B16F0IL2gpi cells (*E* and *F*). The above cells (1×10^5) were s.c. injected into female C57BL/6J mice. Twelve days later, the tumors were recovered, and frozen sections were made. After fixation, slides were first stained with rat antimouse CD4 (*A, C,* and *E*) or rat antimouse CD8 (*B, D,* and *F*). The slides were then stained with goat-antirat IgG conjugated with horseradish peroxidase and developed using Vectastain Elite ABC immunohistochemical kit.

Construction and Transfection of GPI-anchored IL-2 Vector. A DNA fragment (114 bp) encoding the GPI anchor signal for DAF was generated by annealing two synthesized, cDNA oligos with XhoI site at the 5′ end and XbaI site at the 3′ end, and inserted into plasmid pcDNA3.1. Human IL-2 cDNA without the stop codon was generated by PCR from IL-2 cDNA (ATCC 59396) using primers 5′ GGGGTACCTAATCACTACTCACAGTAAC 3′ and 5′ CCGCTCGAGAGTTAGTGTTGAGATGATGC 3′, which was then inserted in front of the DAF fragment in frame, resulting in plasmid pcDNA3.1-IL2gpi. The entire sequence of the fusion gene was confirmed by an automatic DNA sequencer (Perkin-Elmer 310). As a control vector, pcDNA3.1-sIL2 encoding a secreted form of IL-2 was generated as well. pcDNA3.1-IL2gpi and pcDNA3.1-sIL2 were transfected into B16F0 tumor cells using LipofectAMINE transfection reagents according to the manufacturer's protocol (Life Technologies, Inc.). Stable cell lines were selected from DMEM containing 1000 μg/ml G418 (Sigma, St. Louis, MO) for 3–4 weeks, and named B16F0/IL2s for cells expressing secreted IL-2 and B16F0/IL2gpi for cells expressing GPI-anchored IL-2.

FACS Analysis. To determine whether GPI-anchored IL-2 is expressed on the cell surface, 1×10^6 B16F0/IL2gpi cells were stained with antihuman IL-2-FITC and analyzed using the FACScalibur (Becton-Dickinson, San Jose, CA). To additionally determine whether the plasma membrane binding of IL-2 is through the GPI anchor, 1×10^6 B16F0/IL2gpi cells were treated with PI-PLC, which cleaves protein from the lipid anchor. After washing, the cells were stained with antihuman IL-2-FITC and analyzed using the FACScalibur.

ELISA Assay. To quantitatively measure the amount of GPI-anchored IL-2 on the cell surface, 1×10^7 B16F0 cells,

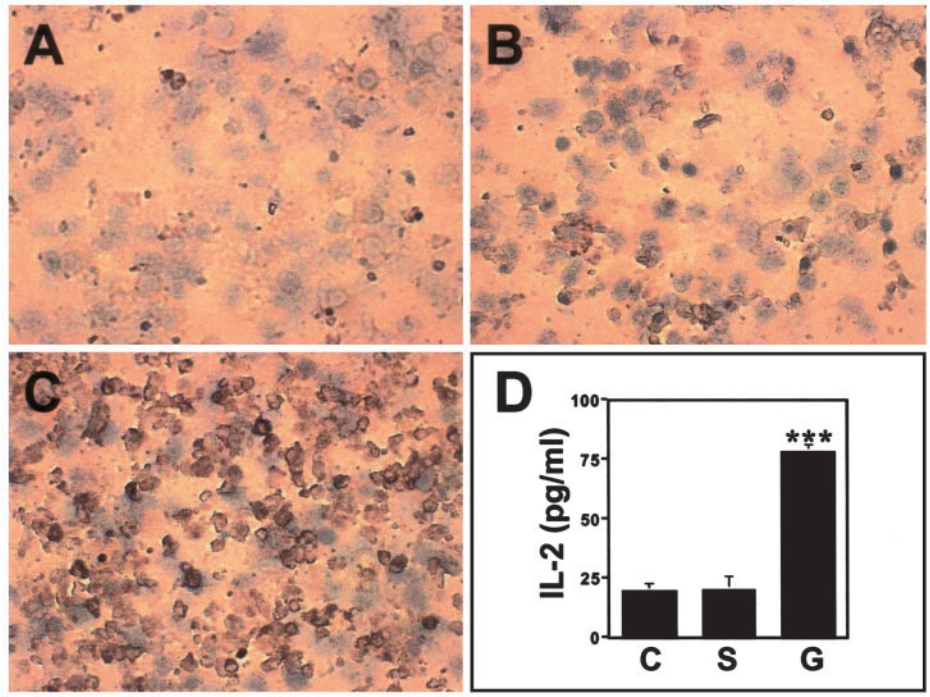

Fig. 3. Local high levels of IL-2 achieved by GPI-anchored IL-2. Tumor cells isolated from 2-week-old s.c. tumors were used to make slides by Cytospin. The slides were immunohistochemically stained for IL-2. *A,* tumor cells from regular B16F0 tumor; *B,* tumor cells from B16F0/IL2s tumor; *C,* tumor cells from B16F0/IL2gpi tumor; *D,* tumor cells were treated with PI-PLC, and the IL-2 level of the supernatant were measured by ELISA. Data are the average of four replicates; *bars,* ±SD. ***, $P < 0.001$.

Table 1 Pulmonary metastasis analysis of GPI-anchored cytokines

Tumor cells (2×10^5) were i.v. injected into C57BL/6J mice. Twenty-eight days after tumor injection, mice were sacrificed, and tumor nodules on lungs were counted.

Tumor cells	Lung metastasis (no. of tumor nodules on lung)
B16F0 cells	>200, >200, >200, >200, >200, >200, >200, >200
B16F0/IL2s cells	>200, >200, >200, 58, 158, >200
B16F0/IL2gpi cells	0, 5, 2, 4, 5, 7, 13, 0

B16F0/IL2s cells, and B16F0/IL2gpi cells were harvested. After two washings with PBS, each cell pellet was dissolved with 0.2 ml of PI-PLC solution (8 units/ml; Sigma) and incubated at 37°C for 1 h. The supernatants were collected, and the amount of IL-2 in them was measured with the QuantiGlo ELISA kit (R&D Systems, Minneapolis, MN). The same experiment was repeated three times, and the results were reported as an average.

Tumor T-Cell Infiltration Assay. Female C57BL/6J mice were each s.c. injected with 1×10^5 B16F0 cells, B16F0/IL2s cells, or B16F0/Il2gpi cells. Twelve days after tumor cell injection the tumors were removed and used to make frozen sections. After fixation, the slides were stained with anti-mouse CD4 or CD8 antibodies, followed by a secondary antibody staining using Vectastain ABC immunohistochemical kit (Vector Laboratories, Burlingame, CA).

***In Vivo* Tumor Study.** Three groups of female C57BL/6J mice (4/group) were i.v. injected with 2×10^5 B16F0 cells, B16F0/IL2s cells, or B16F0/IL2gpi cells. Four weeks later, the mice were sacrificed, and the tumor nodules on the lungs were counted.

Results

GPI-anchored IL-2 Expression. To determine whether GPI anchored IL-2 is expressed on the cell surface, B16F0 cells, B16F0/IL2s cells, and B16F0/IL2gpi cells were analyzed by FACS using FITC conjugated rat antihuman IL-2 antibody. Only B16F0/IL2gpi cells showed a significant amount of IL-2 on their cell surface. To additionally confirm that the IL-2 is attached to the plasma membrane through the GPI anchor, a GPI-specific lipase, PI-PLC, was used to remove the membrane bound IL-2. After PI-PLC treatment, there was no detectable IL-2 on the cell surface (Fig. 1*A*). To measure the absolute amount of GPI-anchored IL-2 on the cell surface, 1×10^7 B16F0 cells, B16F0/IL2s cells, and B16F0/IL2gpi cells were treated with PI-PLC. The amount of IL-2 in the supernatants was assayed by ELISA. As shown in Fig. 1*B*, B16F0/IL2gpi cells expressed significantly increased amounts of IL-2 on the cell surface compared with B16F0 cells or B16F0/IL2s cells. In addition, IL-2 was detected in the culture medium of B16F0/IL2gpi cells, indicating that GPI-anchored IL-2 was released from the cells (Fig. 1*C*). After coculture of B16F0 cells with B16F0/IL2gpi cells in a unique culture system, in which two different types of cells can be cocultured without physical contact but allows growth factors to be shared by both cells, IL-2 activity was detected on the B16F0 cell surface, confirming that the released IL-2 from B16F0/IL2gpi cells is still in the GPI anchored form and able to recoat the plasma membrane (data not shown).

T-Cell Infiltration in Tumors. To test whether the GPI-anchored IL-2 still keeps its biological function, B16F0 cells, B16F0/IL2s cells, or B16F0/IL2gpi cells were s.c. injected into mice, and 12 days later the tumors were recovered. Lymphocytes infiltrated into the tumors were detected by

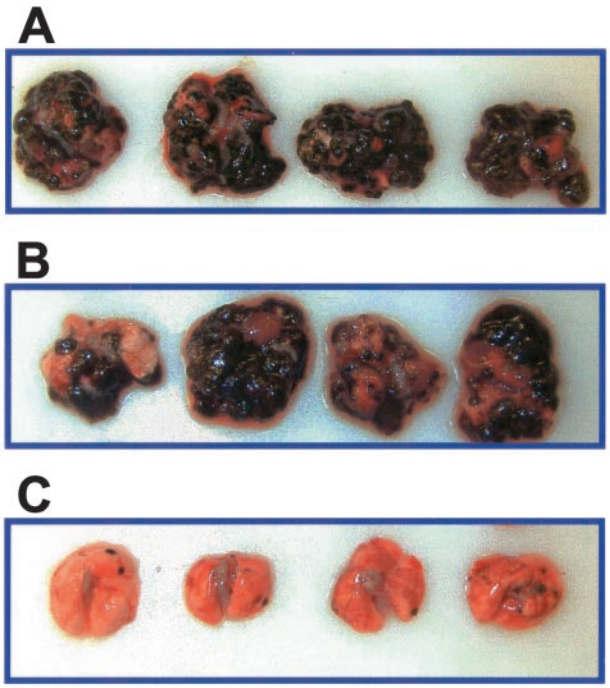

Fig. 4. Photos of the lungs from tumor injected mice. B16F0 cells, B16F0/IL2s cells, or B16F0/IL2gpi cells (2×10^5) were i.v. injected into female C57BL/6J mice. Four weeks later, the mice were sacrificed, and the tumor nodules on the lungs were counted and photographed. *A,* lungs from B16F0 cell injected mice. *B,* lungs from B16F0/IL2s cell injected mice. *C,* lungs from B16F0/IL2gpi cell injected mice.

immunohistochemical assay. The results demonstrated that the expression of GPI-anchored IL-2 significantly increased T-lymphocyte infiltration in tumors (Fig. 2), whereas the secreted IL-2 in this situation did not show any function.

Local High Level of IL-2 Achieved by GPI-anchored IL-2. To determine whether local high dose of IL-2 can be achieved by GPI-anchored IL-2, B16F0 cells, B16F0/IL2s cells, or B16F0/IL2gpi cells were s.c. injected into C57BL/6J mice. Two weeks later the tumors were excised and tumor cells were isolated. Tumor cell slides were produced by Cytospin. The cells were then stained for cell surface IL-2 by immunohistochemistry. Only tumor cells that express GPI-anchored IL-2 showed significantly high levels of IL-2 compared with tumor cells that express secreted IL-2 or regular B16F0 cells (Fig. 3). To be certain that the cell surface IL-2 on cells from the B16F0gpi tumor is in the GPI-anchored form, tumor cells from experimental tumors, after washing with PBS, were treated with PI-PLC. IL-2 in the supernatant was measured by ELISA. Only the cells isolated from the B16F0gpi tumor generated significantly high levels of IL-2 (Fig. 3*D*).

GPI-anchored IL-2 Inhibits Tumor Growth. B16F0 cells, B16F0/IL2s, or B16F0/IL2gpi (2×10^5) were i.v. injected into three groups of C57BL/6J mice (4 mice/group). Four weeks later, these mice were sacrificed, and the tumor nodules on their lungs were counted. The data were summarized in Table 1 from two independent experiments, and photographs were taken from one experiment (Fig. 4). GPI-anchored IL-2 dramatically inhibited tumor growth, whereas secreted IL-2, although expressed at the same level (Fig. 1*C*), did not show any antitumor effect. To rule out the possibility that this tumor growth inhibition effect is because of the variable levels of tumorigenicity of individual clones independent on any immune responses elicited, two experiments were performed. First, the tumorigenicity of the three clones, B16F0, B16F0/IL2s, and B16F0/IL2gpi, was tested in immunodeficient mice (SCID) by pulmonary metastasis assay. The results showed that there is no significant difference in tumorigenicity among the three clones (Table 2). Second, two more stable clones that express GPI-anchored IL-2 were selected and tested. Similar results were obtained (Table 3).

Table 2 Tumorigenicity of difference clones in SCID mice

Tumor cells (2×10^5) were i.v. injected into B6.Cg-Foxnl^{nu} mice. Twenty-eight days after tumor injection, mice were sacrificed, and tumor nodules on lungs were counted.

Tumor cells	Lung metastasis (no. of tumor nodules on lung)
B16F0	>200, >200, >200, 176, >200
B16F0/IL2s	187, 156, >200, 176, 134
B16F0/IL2gpi	174, 167, 195, 183, 173

Table 3 Tumorigenicity of different clones expressing GPI-anchored IL-2

Tumor cells (2×10^5) were i.v. injected into C57BL/6J mice. Twenty-eight days after tumor injection, mice were sacrificed, and tumor nodules on lungs were counted.

Clones	Lung metastasis (no. of tumor nodules on lung)
B16F0	180, >200, 191, >200
B16F0/IL2	157, 190, >200, 173
B16F0/IL2-2	>200, 156, 149, 167
B16F0/IL2gpi	10, 13, 23, 7
B16F0/IL2gpi-5	14, 27, 7, 15
B16F0/IL2gpi-8	5, 15, 0, 5

Immune Responses Generated by GPI-anchored IL-2 Inhibits Growth of Tumor Cells Not Expressing GPI-anchored IL-2. To determine whether the immune responses elicited by GPI-anchored IL-2 can inhibit regular B16F0 cell growth, the following experiment was performed. B16F0 cells (1×10^5) were mixed with 3×10^5 of B16F0/IL2s cells and B16F0/IL2gpi cells, respectively, and i.v. injected into mice (4 for each group). In the control group, only 1×10^5 B16F0 cells/mouse were injected. Four weeks later the lung nodules were counted. The results (Table 4) showed that immune responses generated by GPI-anchored IL-2 are effective to regular tumor cells.

Discussion

IL-2 was originally discovered as a growth factor for T cells *in vitro* and is one of the most extensively studied cytokines (17). It has been used clinically in several ways, and treatment of malignant melanoma and renal carcinoma has shown some efficacy (4, 5). A significant side effect of IL-2 is the vascular leak syndrome (10). Although IL-2 can be used

Table 4 Immune responses elicited by GPI-anchored IL-2 inhibits regular tumor cell growth

Tumor cells were i.v. injected into C57BL/6J mice. Twenty-eight days after tumor injection, mice were sacrificed, and tumor nodules on lungs were counted.

Tumor cells	Lung metastasis (no. of tumor nodules on lung)
B16F0 (1×10^5)	>200, >200, >200, >200
B16F0/IL2:B16F0 (3×10^5:1×10^5)	123, >200, >200, 160
B16F0/IL2gpi:B16F0 (3×10^5:1×10^5)	5, 23, 30, 8

for *ex vivo* expansion of lymphokine activated killer cells and tumor-infiltrating T cells (18), the severe side effects of IL-2 systemically applied at high doses has dramatically restricted its clinical uses. One of the ways to overcome this problem is to develop alternative approaches to deliver this cytokine locally or regionally. In the present study, a fusion protein containing human IL-2 and a GPI anchor signal from DAF was generated. IL-2 ELISA and FACS analyses demonstrated that the fusion protein expressed by cells that were stably transfected with the fusion gene is anchored on the cell surface. On the other hand, we did detect IL-2 activity in the culture medium, indicating some of the proteins are released from the cell. Previous studies demonstrated that the presence of G418 in the medium increases the release of GPI-anchored proteins; the released proteins, which are still in the GPI-anchored form, are able to anchor onto adjacent cells (19). Our coculture experiment confirmed this point.[4] This may represent an advantage over other forms of cytokines in gene delivery into target tissues, such as tumor masses, because up to now the most efficient gene delivery approach cannot deliver vectors into all of the cells or target tissues. By combining with the GPI anchor technology, the released cytokines from transfected (or transduced) cells can be reanchored onto adjacent cells so that in theory 100% of the target cells will have cytokines on their surface. Therefore, we speculate that through GPI anchors, cytokines can be efficiently delivered regionally.

Most unwanted side effects of traditional treatments of IL-2 were caused by systemic high-dose injection. It seems that the high dose is important for IL-2 to effectively induce host immune responses against tumors. To compare the antitumor effects of GPI-anchored IL-2 with secreted IL-2, a stable B16F0 line expressing secreted IL-2 (B16F0/IL2s) was generated. The expression level of IL-2 by this line is higher than that of GPI-anchored IL-2 including both anchored form and released form as indicated by ELISA assay (Fig. 1). Interestingly, whereas the secreted form of IL-2 did not show any antitumor effects in the T-cell infiltration assay or in the *in vivo* tumor growth assay, the GPI-anchored IL-2 at a very low dose compared with the high-dose therapy of recombinant IL-2 (5 μg/mouse) not only significantly increased T-cell infiltration into the tumor masses but also dramatically inhibited tumor growth *in vivo* (Figs. 2 and 4). Possible explanations for this phenomenon include: (*a*) because IL-2 is immobilized on tumor cells via GPI anchoring, compared with secreted or systemically applied IL-2, the local concentration is very high; and (*b*) the anchoring of IL-2 on the cell surface may block the process of IL-2/receptor complex internalization by T cells so that the GPI-anchored IL-2 may have a longer half-life. This speculation is confirmed by the immunohistochemical analysis of tumor cells from experimental tumors (Fig. 3).

In conclusion, we have developed an alternative approach to locally or regionally deliver cytokines through GPI anchoring. Because the cytokines are anchored on the cell plasma membrane, they are immobilized so that a locally high concentration of cytokines could be achieved, and their *in vivo* half-life could be elongated. Therefore, GPI anchored cytokines may be more effective than soluble versions. However, because GPI-anchored proteins can release from the cells and are able to reinsert onto the plasma membrane of neighboring cells, this technique could help gene therapy by increasing gene delivery efficiency, as well as allowing GPI-anchored proteins to be made in large scale and applied locally (such as to tumor masses) in high doses. One direct use of this technique is to inject GPI-anchored IL-2 directly into tumor mass of cancer patients. Because the IL-2 on injection will be anchored on regional tumor cells, a much higher dose could be used. Foreign MHC molecules and costimulatory molecules such CD80 or CD86 could be anchored onto tumor cells as well. Other potential uses of this technique include GPI anchorage of other cytokines or adhesion molecules onto tumor cells to mount immune responses against tumors. Therefore, GPI-anchoring technology represents an interesting approach of cytokine-based immunotherapy.

[4] Unpublished observations.

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