Ex vivo Delivery of Suicide Genes into Melanoma Cells Using Epidermal Growth Factor Receptor-specific Fab Immunogene

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The Fab fragment of monoclonal antibody B4G7 against human epidermal growth factor (EGF) receptor was conjugated with cationic poly-L-lysine and the resulting conjugate was further complexed with reporter genes or therapeutic genes. This Fab/DNA complex was designated as "Fab immunogene." The Fab immunogene transfer *in vitro* was mediated through the EGF receptors in two melanoma cell lines. The frequency of cells expressing β-galactosidase (*β-Gal*) reporter gene was approximately 1%. The induction of suicide effects after Fab immunogene transfer of herpes simplex virus thymidine kinase (*TK*) or *Escherichia coli* cytosine deaminase (*CD*) gene was quite remarkable, and the growth of melanoma cells was inhibited for over 7 days in the presence of ganciclovir (GCV) or 5-fluorocytosine (5-FC). Similarly, when melanoma cells treated *in vitro* with the Fab immunogene carrying *TK* or *CD* were transplanted into the back of nude mouse, subsequent systemic administration of GCV or 5-FC effectively suppressed the growth of tumors, indicating the occurrence of *in vivo* suicide effects.

Key words: Gene therapy — Melanoma — Anti-EGF receptor antibody — Endocytosis — Immunogene

Malignant melanoma is one of the most aggressive neoplasms in skin and eye and is associated with a high incidence of metastasis. The survival of patients with malignant melanoma has improved during the past several decades, due to earlier diagnosis and surgical excision of primary malignant melanoma. Despite these advances, approximately 15% of patients with malignant melanoma die of this disease.[1] There is no effective therapy for metastatic melanoma. Many attempts have been made to evoke or amplify an immune response against melanoma, since it is one of the most immunogenic tumors.[2] Cytokine therapy and tumor-infiltrating lymphocyte immunotherapy were partially effective in inducing melanoma regression, but caused auto-immune problems such as vitiligo, poliosis, uveitis and meningitis as a result of melanocyte destruction.[2] Thus, a new therapeutic method such as gene therapy is needed, and attempts have been made to introduce genes encoding cytokines and a co-stimulatory factor into tumor cells by the use of viral and nonviral vectors.[3]

Previously, we reported experimental therapy of squamous cell carcinomas *in vivo* using an immunotoxin, and showed that a monoclonal anti-human epidermal growth factor (EGF) receptor antibody B4G7[4] exhibited excellent targeting ability.[5, 6] With this same antibody, we developed a novel gene delivery system, in which the Fab fragment of B4G7 antibody is conjugated to poly-L-lysine (pLys) to form an affinity complex with DNA.[7] This Fab fragment/DNA complex was designated as "Fab immunogene,"[8, 9] by analogy with immunotoxin. To date, we have demonstrated that the immunogene can deliver various reporter genes and therapeutic genes *in vitro* into EGF receptor-overproducing A431 tumor cells of squamous carcinoma origin.[7]

Melanoma cell lines often produce a large amount of transforming growth factor (TGF)-α[10–12] and EGF receptor,[13–16] which together stimulate the growth of cells through an EGF receptor-mediated autocrine mechanism.[17, 18] Thus, the targeted delivery of therapeutic genes into melanoma cells might also be accomplished by the Fab immunogene system. In this article, we present evidence that the Fab immunogene delivers the β-galactosidase (*β-Gal*) reporter gene into melanoma cells through EGF receptors. Moreover, the delivery of suicide genes by the Fab immunogene followed by treatment with prodrugs is effective in suppressing the growth of melanoma cells both *in vitro* and *ex vivo*.

MATERIALS AND METHODS

Cells and cell culture HMV-I and G361 are derived from human vaginal and cutaneous melanomas.[19, 20] Y79 is derived from a retinoblastoma.[21] HMV-I and Y79 were obtained from the RIKEN Cell Bank (Tsukuba). G361 was obtained from the Health Science Research Resources

Bank (Osaka). HMV-I was maintained in Ham F-12 medium plus 10% fetal calf serum. G361 and a cervical carcinoma cell line HeLa were maintained in Dulbecco's modified Eagle's medium plus 10% fetal calf serum. Y79 was maintained in RPMI1640 medium plus 10% fetal calf serum. Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

EGF receptor on melanoma cells EGF receptor on the melanoma cell surface was analyzed by immunofluorescent staining, immunoblotting and ^{125}I-EGF binding.

For immunofluorescent staining, cells were grown on a cover glass, fixed with acetone, treated with monoclonal anti-human EGF receptor antibody B4G7 at 4°C, and then treated with FITC-labeled goat anti-mouse IgG antibody.[22]

For immunoblot analysis of the EGF receptor,[23] cells were lysed with Tris-buffered saline (0.15 M NaCl, 50 mM Tris-HCl pH 7.4, 0.1 mM phenylmethylsulfonyl fluoride) containing 1% Triton X-100. Cell lysates were clarified by centrifugation and incubated with B4G7 antibody bound to protein A Sepharose (Pharmacia Biotech, Uppsala, Sweden). Immunoprecipitates were electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The EGF receptors on a blot were visualized by incubation with sheep anti-human EGF receptor antibody (Upstate Biotech. Inc., Lake Placid, NY), with biotinylated anti-sheep IgG antibody and avidin-biotin-alkaline-phosphatase complex (Vector Lab., Burlington, CA).

EGF binding assay was carried out using ^{125}I-EGF.[23] Cells in 24-well culture plates were placed on ice and incubated with ^{125}I-EGF in phosphate-buffered saline (PBS) for 1 h. The cell-bound EGF was solubilized with 0.5 N NaOH and the radioactivity was determined in Beckman 5500 gamma counter.

Plasmid preparation pCMV is an expression vector with the cytomegalovirus promoter, the *Nhe*I-*Spe*I-deleted plasmid of pBK-CMV (Stratagene, La Jolla, CA). *Escherichia coli* cytosine deaminase (*CD*) gene[24] was amplified from *E. coli* DNA by polymerase chain reaction (PCR) using a pair of primers (5′-GGAATTCGCCACCATGGTGTC-GAATAACGCTTTAC-3′ and 5′-GGAATTCAGTCGT-TCAACGTTTGTAAT-3′). The amplified *CD* gene was inserted into the *Eco*RI site of the plasmid pSRD,[25] affording the mammalian expression vector pSRD-CD driven by SRα promoter,[8] and the *Eco*RI site of pCMV. *E. coli* β-*Gal* expression vectors, pSRD-β-Gal and pCMV-β-Gal, were constructed as follows: a β-*Gal* gene fragment, *Hin*dIII-*Dra*I fragment of pSV-β-Gal (Promega, Madison, WI), was inserted into the *Eco*RI site of pSRD and the *Hin*dIII-*Sca*I site of pCMV. Herpes simplex virus thymidine kinase (*TK*) expression vector pSRD-TK was described previously.[8]

Preparation of Fab immunogene (Fab/pLys/DNA complex) Fab immunogene was prepared as described previously.[7,8] B4G7 antibody was digested to produce Fab fragments. The Fab fragment was modified with sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate (Pierce, Rockford, IL). pLys (average molecular mass 44 kDa, Sigma, St. Louis, MO) was modified with 2-iminothiolane (Sigma). The modified Fab fragment was mixed with an equal weight of modified pLys to form a conjugate via a thio-ether bond. The resulting conjugate (designated Fab immunoporter) was separated from non-conjugated Fab fragment by cation-exchange chromatography on a Mono S HR 5/5 column (Pharmacia Biotech.) using an NaCl gradient. The conjugate fractions were filtered through a 0.22 μm MILLEX-GV filter (Millipore) to ensure sterility. The Fab immunoporter was then mixed with various amounts of DNA and incubated for 30 min at room temperature. The resulting Fab immunoporter/ DNA complex (designated, for example, *β-Gal*/Fab immunogene) was applied to cells for measurement of reporter gene expression.

β-*Gal* gene expression in melanoma cells Cells were treated with the *β-Gal*/Fab immunogene (2 μg of pSRD-β-Gal DNA/2 μg of Fab immunoporter in 50 μl of 0.1 M HEPES pH 7.3, 150 mM NaCl) for 72 h in culture medium (37°C, 5% CO_2). *β-Gal* gene expression was detected as described.[8]

Suicide gene expression in melanoma cells Cells were grown in 6-well plates at a low cell density (1×10^5 cells/ well) and treated with *TK* or *CD*/Fab immunogene (2 μg of pSRD-TK or pSRD-CD DNA/2 μg of Fab immunoporter). After 6 h, various concentrations of ganciclovir (GCV) or 5-fluorocytosine (5-FC) were added to the transfected cells. Medium containing GCV or 5-FC was replaced every other day. For some assays, cells were pretreated with 20 μg/ml of Fab fragments of B4G7 antibody for 30 min and then treated with the Fab immunogene. Surviving cells were detached by trypsinization and counted with a hemocytometer.

***Ex vivo TK*/Fab and *CD*/Fab immunogene delivery** For *ex vivo* assay, HMV-I cells were harvested, washed to remove fetal calf serum and resuspended in PBS. The cells were mixed with *TK*/Fab immunogene or *CD*/Fab immunogene (finally, 1×10^7 cells in 100 μl of PBS with 10 μg pSRD-TK or pSRD-CD DNA/10 μg Fab immunoporter) and injected subcutaneously into the back of Balb/ c *nu/nu* mice (4- to 6-week-old females, Sankyo Laboservice Co., Tokyo). Then, GCV (100 μg/mouse) or 5-FC (5 mg/mouse) was injected intraperitoneally 6 h after the cell injection and again every other day. The tumor weight was determined by measuring the major (*L*) and minor (*S*) axes of the tumor and calculated by using the formula W (mg)=$0.5\times L$(mm)$\times S$(mm)^2.[6]

RESULTS

EGF receptors on melanoma cells We first examined how many EGF receptors are present in melanoma cells by three different methods. Immunofluorescent staining using anti-human EGF receptor antibody B4G7 revealed significant levels of EGF receptor in two melanoma cell lines (HMV-I and G361) and HeLa cells (Fig. 1A). Immunoblot analysis showed the mature EGF receptor of 170 kDa in both melanoma cell lines, as seen in HeLa cells, but not in the retinoblastoma cell line Y79 (Fig. 1B). ^{125}I-EGF binding assay allowed us to estimate the receptor number per cell: $2-3 \times 10^5$ for HMV-I and HeLa, and $0.3-0.4 \times 10^5$ for G361 (data not shown).

β-*Gal* gene transfer to melanoma cells The EGF receptor-positive melanoma cells (HMV-I and G361) and

A

HeLa   G361   HMV-I

B

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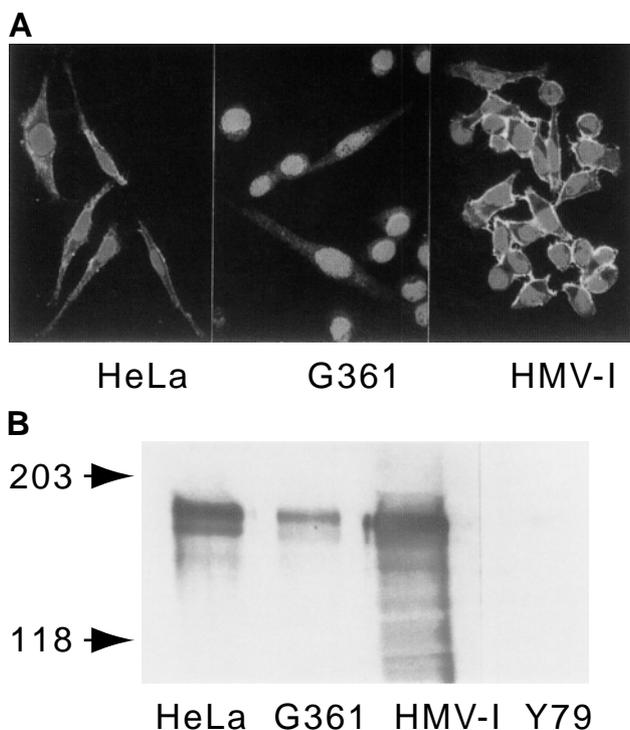
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HeLa G361 HMV-I Y79

Fig. 1. EGF receptors on melanoma cells. A: Immunofluorescent staining of EGF receptors. Cells on a coverslip were fixed, treated with anti-EGF receptor antibody B4G7 and then with FITC-labeled goat anti-mouse IgG antibody. Cell nuclei were counter-stained with propidium iodide. Magnification, ×600. B: Immunoblot analysis of EGF receptors. Cells were lysed and subjected to immunoprecipitation using B4G7 antibody bound to protein A-Sepharose beads. The immunoprecipitated EGF receptors were electrophoresed on SDS-polyacrylamide gel and transferred to a PVDF membrane. The EGF receptor was visualized by incubation with sheep anti-human EGF receptor antibody, biotin-labeled anti-sheep IgG antibody and then avidin-biotin-alkaline phosphatase complex.

receptor-negative retinoblastoma cells (Y79) were treated with the Fab immunogen carrying *β-Gal* gene under the control of SRα or CMV promoter. After 3 days, β-Gal enzyme-expressing cells were examined by histochemical enzyme staining. The β-Gal expression was detected in EGF receptor-positive HMV-I and G361 cells, but not in receptor-negative Y79 cells. The β-Gal expressing cells amounted to about 1% of the HMV-I melanoma cell population for SRα promoter and 0.2% for CMV promoter (Fig. 2A). On the other hand, the β-Gal expressing cells in the immunogen-treated G361 melanoma cell population amounted to approximately 0.5% regardless of promoter type (Fig. 2A). No β-Gal-expressing cells were found among the receptor-negative Y79 cells (Fig. 2A). In melanoma cells, the β-Gal-expressing cells appeared within a day and increased for 4 days (Fig. 2B). A non-specific immunoporter consisting of mouse non-specific IgG and pLys did not deliver the *β-Gal* gene into melanoma cells (data not shown). Interestingly, the liposome-mediated *β-Gal* gene transfer was equally effective (approximately 1%) in these three cell lines regardless of the numbers of EGF receptors (data not shown). These results are consistent with our previous observation using squamous carcinoma cell lines that the Fab immunogen transfer of *β-Gal* gene is processed through the EGF receptors.[7]

Suicide gene transfer to melanoma cells Melanoma cells were treated with the Fab immunogen carrying *TK* gene for 6 h and then with GCV for 4 days. GCV itself was not cytotoxic to melanoma cells even at the high concentration of 100 μ*M* (Fig. 3A). However, those melanoma cells became extremely sensitive to GCV after *TK*/Fab immunogen transfer: the 50% lethal dose (LD_{50}) was 5 μ*M* for HMV-I and 40 μ*M* for G361 cells, an 8-fold difference (Fig. 3A).

Similar experiments were carried out using the Fab immunogen carrying *CD* gene and 5-FC (Fig. 3B). 5-FC itself was not cytotoxic to melanoma cells up to 10 m*M*. However, the *CD*/Fab immunogen transfer made those cells very sensitive to 5-FC: LD_{50} was 30 μ*M* for HMV-I and 4 m*M* for G361 cells (Fig. 3B). It is noteworthy that HMV-I melanoma cells are more sensitive than the other cell line G361 and that the *CD*/Fab immunogen system is apparently more effective than the *TK*/Fab immunogen system.

The cell suicide effects induced by the *TK*/Fab immunogene and *CD*/Fab immunogene were significantly reduced when HMV-I cells were pretreated with excess amounts of Fab fragments ($P<0.01$, by Mann-Whitney's *U* test) (Fig. 4), again indicating the receptor specificity of gene transfer. As expected, pretreatment with non-specific IgG did not reduce the suicide effects (data not shown).

The suppression of melanoma cell growth by the *CD*/Fab immunogen transfer followed by 5-FC treatment

was effective for over 6 days (Fig. 5). As shown in Fig. 3B, the suppression of G361 cell growth was not as complete as in the case of HMV-I melanoma cells. This may reflect the fact that G361 cells are 10 times more resistant to 5-fluorouracil (5-FU), a toxic metabolite of 5-FC, than HMV-I cells (data not shown).
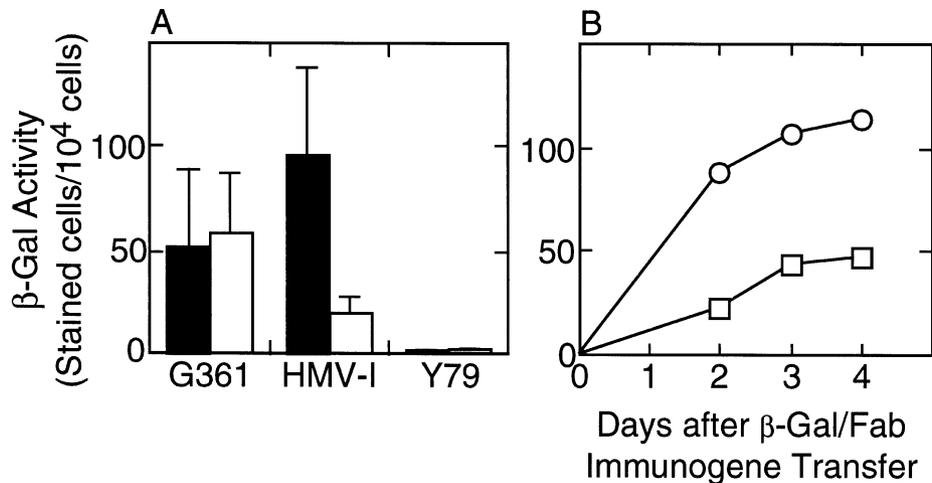

Fig. 2. *β-Gal* gene expression. A: Dependency on the EGF receptors and expression vectors. Cells in 6-well plates at low density (1×10^5 cells/well) were treated with the Fab immunogene carrying pCMV-β-Gal or pSRD-β-Gal plasmid DNA (2 μg of DNA/2 μg of immunoporter). After 72 h, cells were fixed and assayed for the β-Gal activity. Averages of three independent experiments with duplicate samples are shown with standard deviations. ■ pSRD, □ pCMV. B: Time course of *β-Gal* gene expression. Melanoma cells grown in 6-well plates were treated with the Fab immunogene carrying pSRD-β-Gal DNA (2 μg of DNA/2 μg of immunoporter). The cells were fixed and assayed for β-Gal activity at indicated times. Averages of duplicate samples are shown. ○ HMV-I, □ G361.
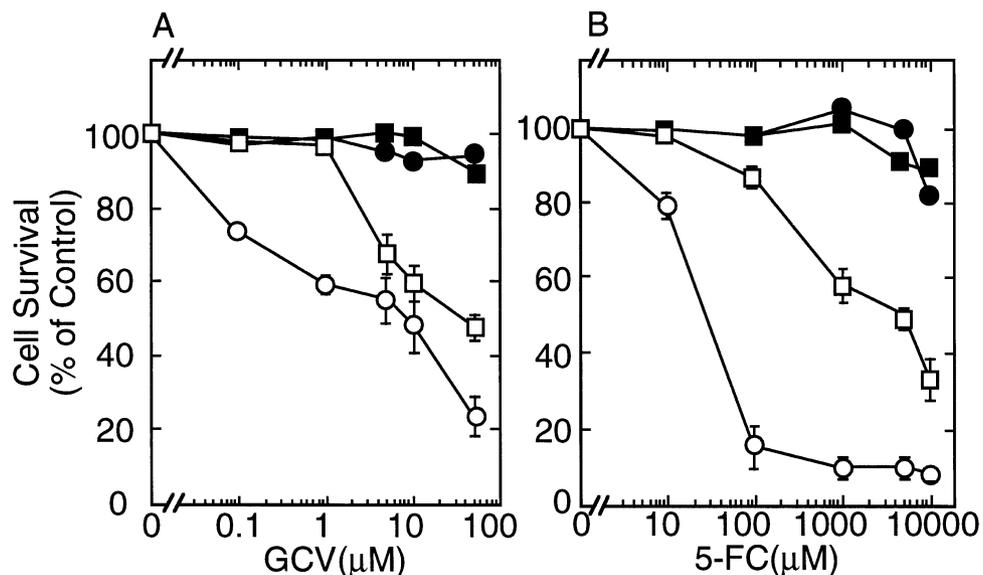

Fig. 3. Suicide gene transfer and induction of cell death. Melanoma cells grown in 6-well plates at low density (1×10^5 cells/well) were treated with the Fab immunogene carrying pSRD-TK or pSRD-CD DNA (2 μg of DNA/2 μg of immunoporter). After 6 h, various concentrations of GCV or 5-FC were added and incubation was continued for 96 h. Surviving cells were detached by trypsinization and counted with a hemocytometer. A: Treatment with the *TK*/Fab immunogene and GCV. ■ G361, □ G361/TK, ● HMV-I, ○ HMV-I/TK. B: Treatment with the *CD*/Fab immunogene and 5-FC. ■ G361, □ G361/CD, ● HMV-I, ○ HMV-I/CD. Circles indicate HMV-I cells, while squares indicate G361 cells. Open symbols indicate treatment with the immunogene and prodrugs, while closed symbols indicate treatment with prodrugs alone.

Thus, it is evident that significant cytotoxic effects are induced through EGF receptors when suicide genes are transferred.

***Ex vivo TK*/Fab immunogene or *CD*/Fab immunogene delivery** The *TK* or *CD*/Fab immunogene transfer quite efficiently induced suicide of HMV-I cells *in vitro* in the
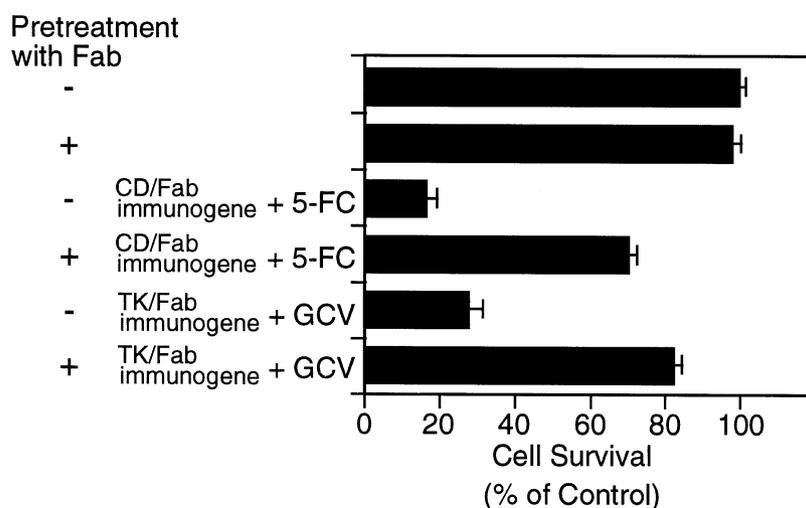

Fig. 4. EGF receptor-specific suicide gene expression. HMV-I melanoma cells grown in 6-well plates at low density (1×10^5 cells/ well) were pretreated with the Fab fragment of B4G7 antibody for 30 min and then with the immunogene carrying pSRD-TK or pSRD-CD DNA (2 μg of DNA/2 μg of immunoporter). After 6 h, GCV (10 μM) or 5-FC (1 mM) was added and incubation was continued for 96 h. Surviving cells were detached by trypsinization and counted with a hemocytometer. + or − indicates treatment with or without Fab fragments as a contol or before the immunogene transfer. Averages of triplicate samples are shown with standard deviations.
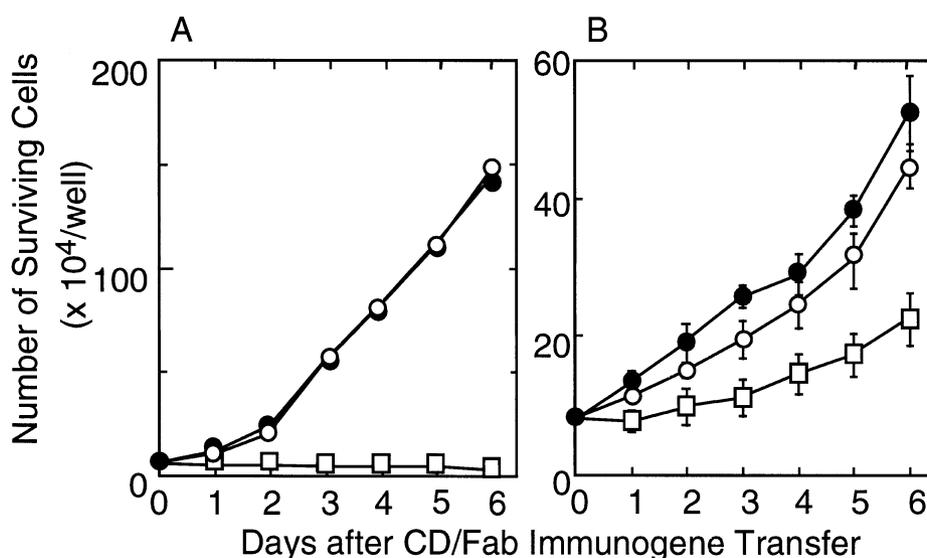

Fig. 5. Cell growth inhibition by suicide gene expression. Melanoma cells grown in 6-well plates at low density (2×10^4 cells/well) were treated with the immunogene carrying pSRD-CD DNA (2 μg of DNA/2 μg of immunoporter). After 6 h, 5-FC was added to a final concentration of 1 mM and incubation was continued for 6 days. Surviving cells were detached by trypsinization and counted with a hemocytometer. Closed circles indicate control cells without treatment. Open circles indicate cells which were treated with *CD*/ Fab immunogene alone. Open squares indicate cells which were treated with *CD*/Fab immunogene and 5-FC. Averages of triplicate samples of HMV-I (A) cells are shown; the standard deviations were <5%. Averages of triplicate samples of G361 (B) cells are shown with standard deviations.
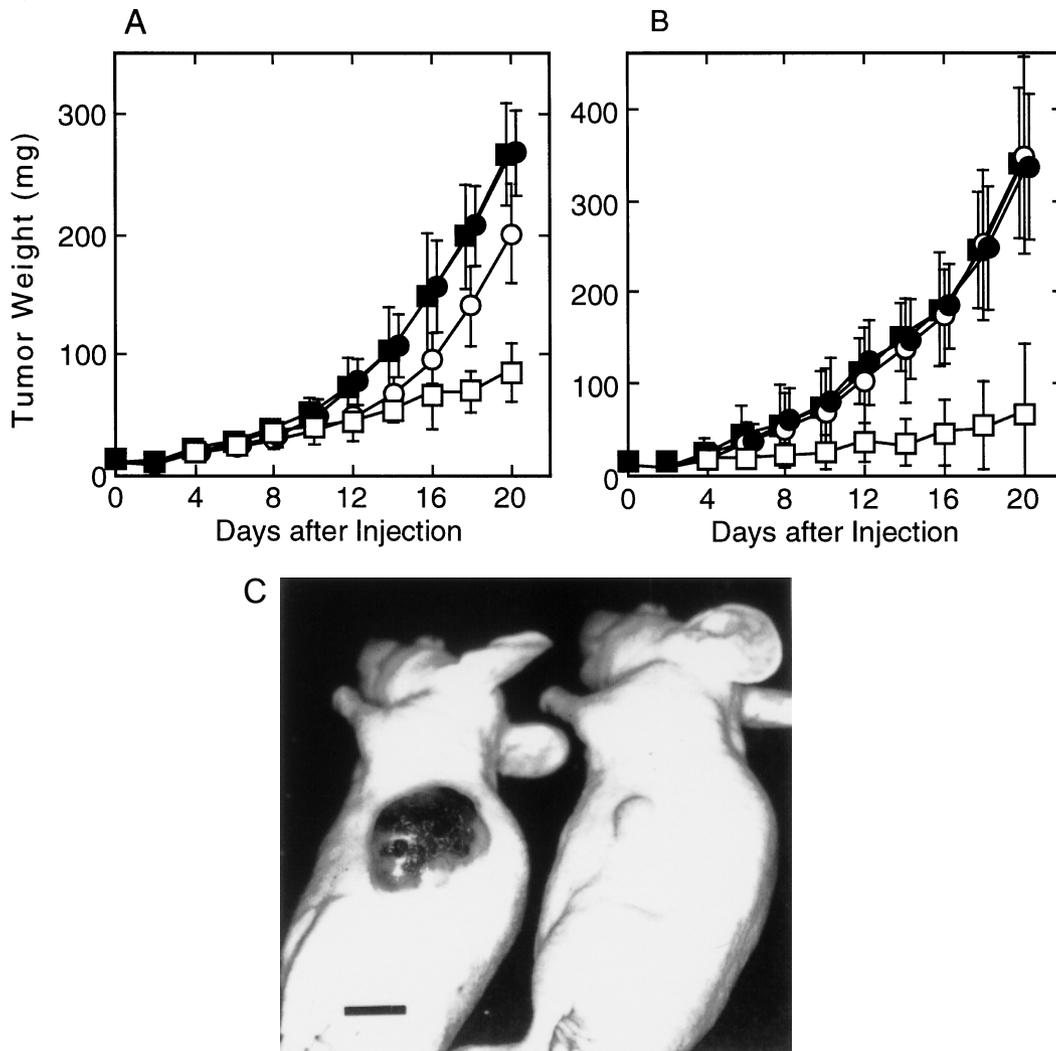
Fig. 6. *Ex vivo TK*/Fab immunogene and *CD*/Fab immunogene delivery. HMV-I cells were mixed with *TK*/Fab immunogene or *CD*/Fab immunogene (1×10^7 cells in 100 μl of PBS with 10 μg of pSRD-TK or pSRD-CD DNA/10 μg of Fab immunoporter) and injected subcutaneously into the back of Balb/c *nu/nu* mice. Then, GCV(100 μg/mouse) or 5-FC (5 mg/mouse) was injected intraperitoneally 6 h after the cell injection, and this was repeated every other day (open squares). As a control, mice were injected with HMV-I cells alone (closed circles), with a mixture of HMV-I cells and *TK*/Fab or *CD*/Fab immunogene but not GCV or 5-FC (open circles), or with GCV or 5-FC alone (closed squares). A: *TK*/Fab immunogene. Average sizes of four tumors (two tumors for GCV alone) are shown with standard deviations. B: *CD*/Fab immunogene. Average sizes of eight tumors (two tumors for 5-FC alone) are shown with standard deviations. C: Right, a mouse injected with a mixture of HMV-I cells and *CD*/Fab immunogene and then with 5-FC. Left, a mouse injected with a mixture of HMV-I cells and *CD*/Fab immunogene alone. Scale bar=10 mm.

presence of GCV or 5-FC. We examined if it is equally effective *in vivo* by performing an *ex vivo* study. HMV-I melanoma cells were mixed with *TK*/Fab immunogene or *CD*/Fab immunogene *in vitro* and injected subcutaneously into the back of nude mice. The effective dose and interval of injection were determined in preliminary experiments to be 3–8 mg of 5-FC (i.e., 300–800 mg of 5-FC/kg of body weight) and every day or every other day, respectively (data not shown). Under this *ex vivo* condi-

tion, the immunogene-bearing melanoma cells grew and developed tumors, whereas tumor growth was significantly suppressed if GCV or 5-FC was administered intraperitoneally (Fig. 6, A, B and C). Thus, transfer of the *TK* gene or *CD* gene as a form of Fab immunogene and subsequent systemic administration of GCV or 5-FC induced substantial suicide of HMV-I melanoma cells in nude mice.

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DISCUSSION

Previously, we developed a targeted gene delivery system using the Fab fragment of monoclonal antibody cross-linked with a cationic polymer (pLys) to which DNA binds. We designated this complex "Fab immunogene" by analogy with "immunotoxin."[8] We have documented the unique features of immunogene in terms of its receptor-specific binding and internalization, intracellular endocytic processing, and gene expression in the nucleus, using squamous carcinoma cells which overproduce EGF receptors.[7] In this study, we applied the Fab immunogene transfer system to human melanoma cells, which also produce a large number of EGF receptors.

The frequency of β-Gal-expressing cells was approximately 1%, which is as high as the lipofection method can achieve in those melanoma cells. The Fab immunogene transfer was dependent on the presence of EGF receptor. Interestingly, the Fab immunogene transfer was equally effective in two melanoma cell lines regardless of the number of EGF receptors ($0.3-3\times10^5$). This is consistent with our previous finding that the transfer efficiency of β-Gal/Fab immunogene is not directly related to the receptor number and the post-receptor processes are rate-limiting.[7] In this study, despite the relatively low transfer efficiency, we were able to induce substantial growth inhibition of melanoma cells by suicide gene and prodrug treatment under *in vitro* and *ex vivo* conditions. These observations justify further refinement of the Fab immunogene system, aiming at gene therapy for melanoma.

Melanoma is one of the most immunogenic tumors and therefore gene therapy studies so far have attempted to evoke anti-tumor immunity to the melanoma itself in the patient.[3, 26] One approach utilized melanoma cells transfected with cytokine genes.[27] Another approach utilized melanoma cells transfected with co-stimulatory molecules such as B7 to prime naive T cells or to restimulate resting T cells.[28] In these cases, therapeutic genes are introduced into melanoma cells *ex vivo* or by intratumoral injection using viral vectors or liposome/DNA complex. More recent approaches utilized dendritic cells or muscle transfected with DNA encoding melanoma-specific antigen to induce a systemic T- and B-cell activation.[3, 29]

Another important approach is a targeted delivery of therapeutic genes to melanoma cells to induce cell suicide effects.[30, 31] In the present study, we employed two cell suicide systems, TK/GCV and CD/5-FC, both of which effectively inhibited the growth of melanoma cells *in vitro*

and *ex vivo*. In these suicide systems, the transferred suicide genes produce enzymes which can convert a nontoxic compound (prodrug) to a toxic metabolite, and hence the toxic metabolite is selectively produced in transfected tumor cells. The enzyme TK converts a relatively nontoxic prodrug GCV to the highly toxic nucleotide analog, which is transported into neighboring cells through cell-cell gap junctions and eventually inhibits DNA replication.[31] The other enzyme, CD, catalyzes conversion of the nontoxic prodrug 5-FC to highly toxic 5-FU, which can freely diffuse across the plasma membrane into neighboring cells independently of cell-cell gap junctions and eventually inhibits their growth.[24] Thus, the antitumor effect of the metabolite takes place even in the neighboring tumor cells (so-called bystander effect).[24, 30, 31] Melanoma cells build only loose cell-cell junctions, and therefore the CD/5-FC system was expected to be more effective than TK/GCV, and in fact this was the case. The remarkable growth suppression *in vivo* despite the relatively low transfection efficiency of the Fab immunogene could be accounted for by the bystander effect.[7, 32]

Our immunogene approach toward melanoma gene therapy has several advantages as compared to virus vector systems.[9] The Fab immunogene can form affinity complexes with any genes under any control elements and thus it is easy to adapt for the delivery of cytokine and co-stimulatory factor genes. Fab immunogene is specific to EGF receptor-producing tumor cells but additional security might be provided with the use of a transcriptional control element such as the tyrosinase promoter, which is specific to melanocytes and melanoma.[29] Besides the EGF receptor, melanoma cells can be targeted by antibodies against melanoma-specific membrane antigens such as MART-1 and gp100.[33] We are now pursuing *in vivo* immunogene studies using melanoma-bearing nude mice and immuno-suppressed rabbits. It is our hope that the Fab immunogene system may eventually allow systemic treatment of inoperable metastatic melanomas.

ACKNOWLEDGMENTS

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