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

Yuichiro Ohtake,1, 2 Jiabing Chen,1 Shinobu Gamou,3 Atsushi Takayanagi,1 Yukihiko Mashima,2 Yoshihisa Oguchi2 and Nobuyoshi Shimizu1, 4

1Department of Molecular Biology, 2Department of Ophthalmology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582 and 3Department of Biology, School of Health Science, Kyorin University, 476 Miyashita-cho, Hachioji, Tokyo 192-8508

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. 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. 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. 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.

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 exhibited excellent targeting ability. 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. This Fab fragment/DNA complex was designated as “Fab immunogene,” 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.

Melanoma cell lines often produce a large amount of transforming growth factor (TGF)-α and EGF receptor, which together stimulate the growth of cells through an EGF receptor-mediated autocrine mechanism. 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 produgs 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. Y79 is derived from a retinoblastoma. HMV-I and Y79 were obtained from the RIKEN Cell Bank (Tsukuba). G361 was obtained from the Health Science Research Resources.
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Preparation of Fab immunogene (Fab/pLys/DNA complex) Fab immunogene was prepared as described previously.8) B4G7 antibody was digested to produce Fab fragments. The Fab fragment was modified with sulfo-succinimidyl 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 immunporter) 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 immunporter was then mixed with various amounts of DNA and incubated for 30 min at room temperature. The resulting Fab immunporter/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 immunporter in 50 μl of 0.1 M HEPES pH 7.3, 150 mM NaCl) for 72 h in culture medium (37°C, 5% CO₂). 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 (1×10⁵ cells/well) and treated with TK or CD/Fab immunogene (2 μg of pSRD-TK or pSRD-CD DNA/2 μg of Fab immunporter). 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 pre-treated 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, 1×10⁵ cells in 100 μl of PBS with 10 μg pSRD-TK or pSRD-CD DNA/10 μg Fab immunporter) and injected subcutaneously into the back of Balb/c nude 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 volume was determined by measuring the major (L) and minor (S) axes of the tumor and calculated by using the formula \( W (\text{mg}) = 0.5 \times L (\text{mm}) \times S (\text{mm})^2 \).8)
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). 125I-EGF binding assay allowed us to estimate the receptor number per cell: 2–3×10^5 for HMV-I and HeLa, and 0.3–0.4×10^5 for G361 (data not shown).

β-Gal gene transfer to melanoma cells The EGF receptor-positive melanoma cells (HMV-I and G361) and receptor-negative retinoblastoma cells (Y79) were treated with the Fab immunogene 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 immunogene-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 immunopporter 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 immunogene 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 immunogene 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 µM (Fig. 3A). However, those melanoma cells became extremely sensitive to GCV after TK/Fab immunogene transfer: the 50% lethal dose (LD50) was 5 µM for HMV-I and 40 µM for G361 cells, an 8-fold difference (Fig. 3A).

Similar experiments were carried out using the Fab immunogene carrying CD gene and 5-FC (Fig. 3B). 5-FC itself was not cytotoxic to melanoma cells up to 10 mM. However, the CD/Fab immunogene transfer made those cells very sensitive to 5-FC; LD50 was 30 µM for HMV-I and 4 mM 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 immunogene system is apparently more effective than the TK/Fab immunogene 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).

5-FC treatment
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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 EGFR 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

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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 immunoprotein). 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 control 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 immunoprotein). 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.
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 condition, 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.

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 (1x10^7 cells in 100 µl of PBS with 10 µg of pSRD-TK or pSRD-CD DNA/10 µg of Fab immunporter) 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.
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.” 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. 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 (0.3–3×10^5). 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. 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. One approach utilized melanoma cells transfected with cytokine genes. Another approach utilized melanoma cells transfected with co-stimulatory molecules such as B7 to prime naive T cells or to restimulate resting T cells. 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 utilize dendritic cells or muscle transfected with DNA encoding melanoma-specific antigen to induce a systemic T- and B-cell activation.

Another important approach is a targeted delivery of therapeutic genes to melanoma cells to induce cell suicide effects. 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. 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. Thus, the antitumor effect of the metabolite takes place even in the neighboring tumor cells (so-called bystander effect). 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.

Our immunogene approach toward melanoma gene therapy has several advantages as compared to virus vector systems. 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. Besides the EGF receptor, melanoma cells can be targeted by antibodies against melanoma-specific membrane antigens such as MART-1 and gp100. 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.

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