Targeted gene delivery using humanized single-chain antibody with negatively charged oligopeptide tail

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We have recently developed the so-called recombinant immunoporter as a non-viral vector based on a single-chain antibody (scFv) derived from a monoclonal antibody B4G7 against epidermal growth factor (EGF) receptor. This immunoporter (mBBD20) was composed of single-chain antibody and negatively charged oligopeptide tail [5 units of Asn4Ser (D20)], and was expressed in yeast as a secreted protein. The purified mBBD20 was converted to an immunogene by mixing it with DNA and a cationic polymer, polyethyleneimine (PEI). The resulting complex, namely recombinant immunogene, exhibited gene transfer activity with EGFR-specificity *in vitro* (Suzuki *et al.*, Gene Ther. 2003). In this paper, we further improved various conditions necessary for the formation of the proper recombinant immunogene, retaining receptor specificity of its binding, intracellular processing of the receptor-bound gene, and efficient gene expression. Moreover, we provided evidence that the recombinant immunoporter made with humanized scFv could be used as a potent gene transfer vehicle to target particular tumor cells. This approach seems worthy of clinical trial. (Cancer Sci 2004; 95: 424–429)

We have been developing a non-viral cell-specific gene delivery system named immunoporter, using a modified monoclonal antibody.[1–6] The original immunoporter was a conjugate between a monoclonal antibody and a cationic peptide poly-L-lysine (pLys).[1] The Fab fragment of antibody was also conjugated with pLys.[3] These whole-antibody or Fab-fragment immunoporters readily bound to DNA and the resulting complex, named an immunogene, delivered a reporter gene or therapeutic gene(s) to target cells *in vitro* and *in vivo*.[5] Most importantly, the Fab immunoporter carrying *herpes simplex virus* (*HSV*) thymidine kinase (*TK*) gene together with ganciclovir treatment was effective in suppression of the growth of epidermal growth factor (EGF) receptor-hyperproducing tumor cells in nude mice.[5] Thus, the immunoporter/immunogene approach was considered as a promising alternative to viral vectors for targeted gene delivery. However, the chemical conjugation of monoclonal antibody or Fab fragment is laborious and unsuitable for large-scale preparation, which would be required for future clinical use. To overcome this potential problem, we exploited recombinant single-chain antibody (scFv) technology.[7] The scFv is regarded as the minimal structural component of an antibody required for antigen-binding activity,[7] and can be produced in high quantity by means of gene expression in *Escherichia coli*,[8] yeast[9] or mammalian cells.[10]

Recently, we were able to construct several modified scFv genes for use as immunoporter/immunogene systems.[11] One of these recombinant immunoporters consisting of scFv and a negatively charged oligopeptide tail was capable of delivering genes to A431 tumor cells through the EGF receptor.[11]

In this paper, we studied various conditions necessary for the formation of proper recombinant immunogenes. Moreover, we present evidence that recombinant immunogenes made with humanized scFv can be potent gene transfer vehicles to target particular tumor cells.

Materials and Methods

Cell culture. Human squamous carcinoma A431 cells over-expressing EGF receptors were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C and 5% CO_2.

B4G7 monoclonal antibody. B4G7 antibody is an IgG2b class murine monoclonal antibody and is specific to human EGF receptor.[20, 21]

Plasmid preparation. pSRK-GL3 encodes luciferase (*Luc*) gene and pSRD-TK encodes *HSV-TK* gene.[11] These recombinant plasmids were purified with Endfree QIAGEN Plasmid Purification Kit (QIAGEN).

Purification of recombinant immunoporters. The murine type recombinant immunoporter (mBBD20) and humanized immunoporter (hCaCD20) were purified as previously described.[11] In brief, the immunoporters secreted by yeast *Pichia pastris* into the medium were purified by successive use of anion-exchange chromatography on "STREAMLINE" Q-XL (Amersham Pharmacia Biotech), immobilized metal ion-affinity chromatography and gel filtration. Finally, the immunoporters were dialyzed against 600 m*M* NaCl. The yields of mBBD20 and hCaCD20 were 5 mg/l-medium and 4.5 mg/l-medium, respectively. The final preparation was subjected to gel electrophoresis and stained with 2D-Silver Stain II (Daiichi Pure Chemicals). The purity was over 95% (Fig. 1B).

Formation of recombinant immunogene. First, a complex was made with 25-kDa branched polyethyleneimine (PEI) and plasmid DNA (pDNA). For this, pDNA and PEI were mixed at N/P ratio=20 in either distilled water, 10 m*M* HEPES-NaOH (pH 7.3) or 10 m*M* $NaPO_4$ (pH 7.3), and incubated at room temperature for 30 min. The recombinant immunoporter was diluted with either distilled water, 10 m*M* HEPES or 10 m*M* $NaPO_4$, mixed with pDNA/PEI complex at a final salt concentration of 150 m*M* NaCl, and incubated for 1 h.

Gene transfer via recombinant immunogene and luciferase activity assay. The recombinant immunoporter carrying luciferase gene was added to A431 cells in 24-well plates (5×10^4 cells/well) in serum-containing medium, and after 24 h, luciferase activity was measured with a PicaGene kit (Toyo Ink, Tokyo) using a Lumat LB9507 instrument.

Binding assay for recombinant murine immunoporter. A431 cells in 96-well plates at 90–100% confluency were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 10 min at 4°C. Then, cells were treated with PBS containing 3% BSA (PBS-BSA) for 1 h at room temperature. They were exposed to recombinant immunoporters mixed with B4G7 in PBS-BSA for 1 h, followed by repeated washing with PBS. Then, they were treated with protein A-labeled horseradish peroxidase (ZYMED) for 30 min, washed repeatedly and incubated with *o*-phenylenediamine/hydrogen peroxide solution. Color developed after adding diluted sulfuric acid was measured as absorbance at 490 nm.

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Binding assay for recombinant humanized immunogene. A431 cells (5×10^4 cells/well) were treated with the medium containing B4G7 or mouse non-specific IgG (Sigma) and BSA at a concentration 1000-fold higher than that of hCaCD20. After 30 min incubation at 4°C, recombinant immunoporter hCaCD20 carrying the luciferase gene was applied and incubation was continued at 37°C. After 1 h, cells were washed with serum-free medium and incubated at 37°C in the growth medium with 150 μg/ml of gentamycin. After 24 h, luciferase activity was measured.

Uptake of recombinant immunogene into A431cells. A431 cells in 4-well culture glass slides (Becton Dickinson) at 20% confluency were treated with recombinant immunoporter carrying Rhodamine-labeled "pGeneGrip" (Gene Therapy Systems). At each time point, cells were rinsed with PBS and fixed at 4°C with 4% formaldehyde in PBS. The fixed cells were mounted with 90% glycerol containing 4,6-diamidino-2-phenylindole/diazobicyclo[2.2.2]octane and observed under an ECLIPSE E800 (Nikon) fluorescence microscope.

Cell suicide effects of *TK* gene transfer. A431 cells in 24-well plates at 4–5% confluency were treated with recombinant immunoporter carrying the *TK* gene. After 24 h, the medium was changed to a fresh medium containing various concentrations of ganciclovir (GCV) and this was further changed every day. After 6 days, cell viability was measured with AlamarBlue (AccuMed).

Results

Preparation of recombinant immunoporters (mBBD20, hCaCD20). We previously constructed the recombinant gene encoding a murine type single-chain antibody (scFv) against human EGF-receptor and connected it with DNA sequences encoding negatively or positively charged oligopeptide tails.[11] One of the negatively charged oligopeptide tails was composed of five repeats of a unit consisting of four aspartates and one serine (D4S×5, D20) (Fig. 1A). The resulting recombinant scFv gene was successfully expressed in the yeast *Pichia pastris* to produce a recombinant immunoporter (mBBD20) in large quantity and with high purity.[11]

The scFv gene used for this immunoporter was murine type, and it was converted to human type by the CDR-grafting method (Takayanagi *et al*., unpublished). The resulting humanized scFv gene was linked to the DNA sequence encoding a negatively charged oligopeptide tail (D20) and the humanized recombinant immunoporter (hCaCD20) was expressed in *Pichia* yeast and purified by means of the same procedure as used for mBBD20: consecutive use of anion-exchange chromatography, metal-affinity resin and gel filtration (data not shown). The purity of the final preparation of mBBD20 and hCaCD20 was over 95% (Fig. 1B). The molecular weights of both the recombinant immunoporter (mBBD20) and the humanized immunoporter (hCaCD20) were estimated to be 30.3 kDa. A difference of mobility in SDS-PAGE was also seen for scFv antibodies without the tail, but the reason for this apparent discrepancy is not known (data not shown).

Binding assay of humanized recombinant immunoporter to EGF receptors. Binding activity of humanized immunoporter hCaCD20 was compared with that of murine-type immunoporter mBBD20 by competition assay with binding of monoclonal antibody B4G7 to EGF receptors on A431 cells (Fig. 1C). Both murine and human recombinant immunoporters inhibited binding of B4G7 in a dose-dependent manner. Thus, the humanized immunoporter hCaCD20 maintains binding ability to the EGF receptor with high specificity.

The binding activity of immunoporter hCaCD20 was also compared with that of the Fab fragment of B4G7. The concentration of hCaCD20 required for 50% inhibition of B4G7 binding was 2-fold higher than that of Fab fragment (data not shown). This suggested that the binding activity of the hCaCD20 immunoporter is slightly lower than that of the original B4G7 monoclonal antibody.

We also reported that the recombinant immunogene (mBBD20/polyethylenimine/DNA complex) inhibited the binding of B4G7 antibody to A431 cells in a dose-dependent manner.[11]

Optimal conditions for the preparation of recombinant immunogene. Recombinant immunogene was prepared by mixing the recombinant immunoporter with a complex of PEI and pDNA encoding a reporter (or therapeutic) gene (Fig. 1A). We attempted to optimize the conditions for efficient luciferase gene delivery to EGF receptor-overexpressing A431 tumor cells by changing several parameters. These include the buffers for pDNA/PEI complex formation and the dilution buffer for immunoporter, the relative amount of immunoporter per assay, and the charge ratio of pDNA/PEI complex (N/P ratio) (Fig. 2, A–C).

The pDNA/PEI complex formation in distilled water (DW) and the dilution of immunoporter in PBS resulted in the highest expression of luciferase activity (Fig. 2A).

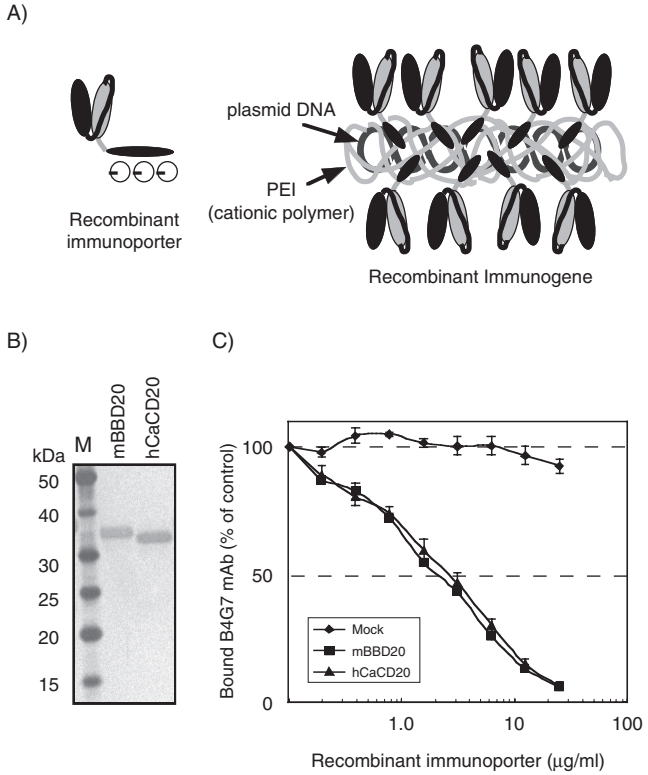

Fig. 1. Purification of recombinant immunoporters (mBBD20 and hCaCD20) and their binding to A431 cells. A) Schematic diagrams of recombinant immunoporter and recombinant immunogene. B) Recombinant immunoporter preparations (100 ng each) were electrophoresed on a 15% SDS-polyacrylamide gel and silver-stained. Purity determined by densitometric scanning of the gel was over 95%. The molecular weights of both recombinant immunoporter (mBBD20) and humanized immunoporter (hCaCD20) were estimated to be 30.3 kDa. The difference of mobility in SDS-PAGE was also seen in scFv antibodies without a tail, but the reason for this apparent discrepancy is not known (data not shown). M: Protein size markers. C) Competition between recombinant immunoporters and monoclonal antibody B4G7 for binding to A431 cells. Fixed A431 cells were treated for 1 h with a mixture of B4G7 (2.5 μg/ml) and different amounts of immunoporters: mBBD20 (■), hCaCD20 (▲), PBS as Mock (◆). The B4G7 antibody bound to the cell surface was measured by incubation with HRP-labeled protein A for 30 min. Each value is the average of six wells.

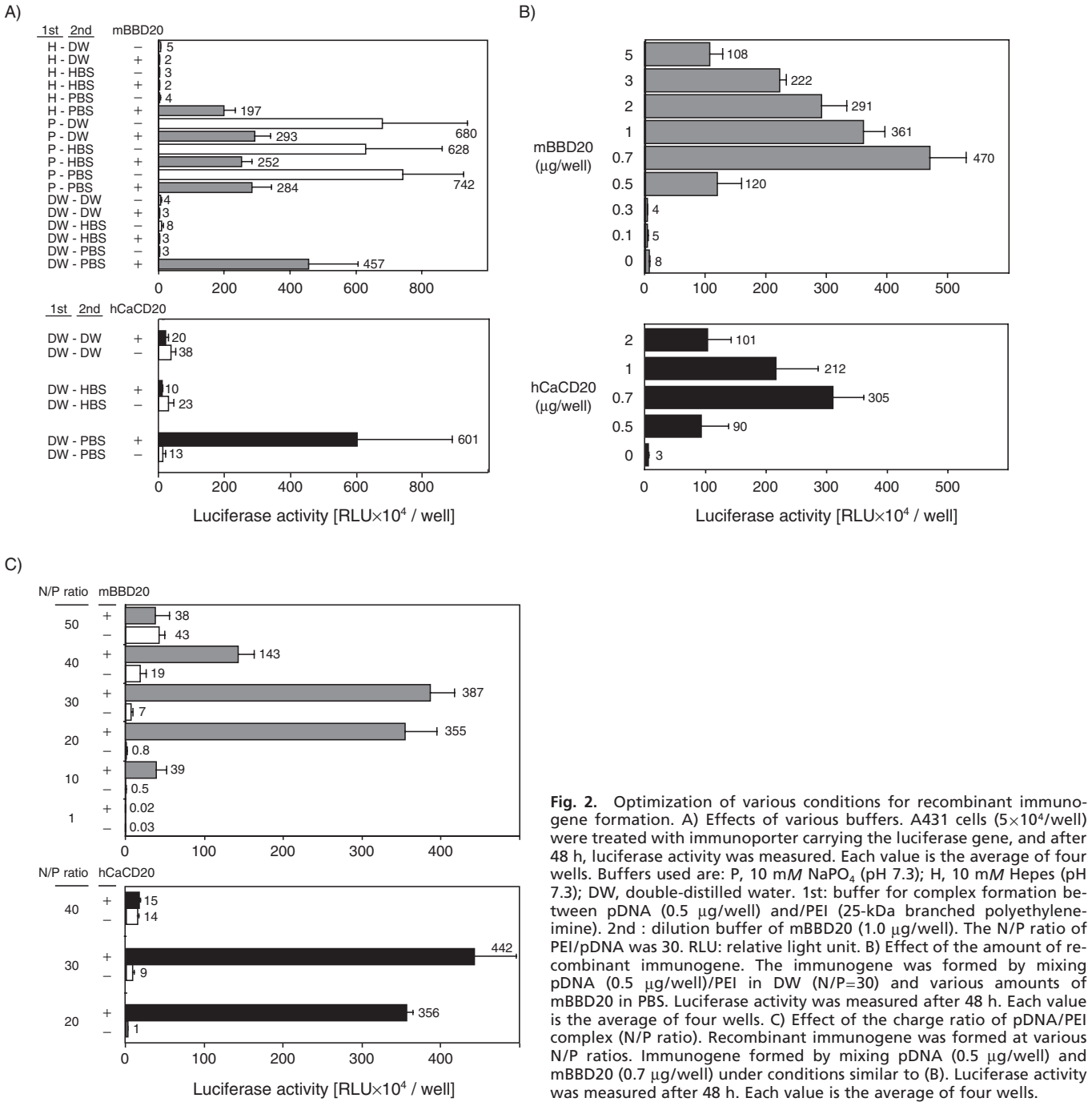

Fig. 2. Optimization of various conditions for recombinant immunogene formation. A) Effects of various buffers. A431 cells (5×10^4/well) were treated with immunoporter carrying the luciferase gene, and after 48 h, luciferase activity was measured. Each value is the average of four wells. Buffers used are: P, 10 m*M* $NaPO_4$ (pH 7.3); H, 10 m*M* Hepes (pH 7.3); DW, double-distilled water. 1st: buffer for complex formation between pDNA (0.5 μg/well) and/PEI (25-kDa branched polyethyleneimine). 2nd : dilution buffer of mBBD20 (1.0 μg/well). The N/P ratio of PEI/pDNA was 30. RLU: relative light unit. B) Effect of the amount of recombinant immunogene. The immunogene was formed by mixing pDNA (0.5 μg/well)/PEI in DW (N/P=30) and various amounts of mBBD20 in PBS. Luciferase activity was measured after 48 h. Each value is the average of four wells. C) Effect of the charge ratio of pDNA/PEI complex (N/P ratio). Recombinant immunogene was formed at various N/P ratios. Immunogene formed by mixing pDNA (0.5 μg/well) and mBBD20 (0.7 μg/well) under conditions similar to (B). Luciferase activity was measured after 48 h. Each value is the average of four wells.

The proper amount of immunoporter per assay was found to be 0.7 μg (Fig. 2B). The best N/P ratio of pDNA/PEI complex formation was found to be 20–30 (Fig. 2C). The ratio N/P=30 showed higher efficiency than N/P=20, but the antibody-specificity was less (data not shown), so N/P=20 was chosen for the immunogene formation. We also attempted to optimize conditions for humanized immunoporter (hCaCD20), like the murine-type immunoporter (Fig. 2, A–C). The results indicated no difference of optimum conditions between murine and humanized immunoporters for immunogene preparation. Moreover, a time course experiment showed the highest luciferase activity after 24 h of immunogene treatment (data not shown). Under the optimum conditions, the luciferase gene transfer via recombinant immunoporter was as efficient as conventional lipofection treatment. The recombinant immunoporter exhibited no obvious toxicity to A431 cells (data not shown).

Gene transfer to various human tumor cell lines with different numbers of EGF receptors. We examined if the number of EGF receptors on the cell surface is correlated with the efficiency of gene transfer via recombinant immunoporter using six different human tumor cell lines possessing various numbers (10^4–10^6) of EGF receptors (Fig. 3). The cell lines used were derived from human squamous carcinoma. The numbers of EGF receptors on the cell surface were estimated by means of Scatchard plot analysis of ^{125}I-EGF binding assay.[22, 23] There was a tendency that the level of luciferase activity was relatively higher

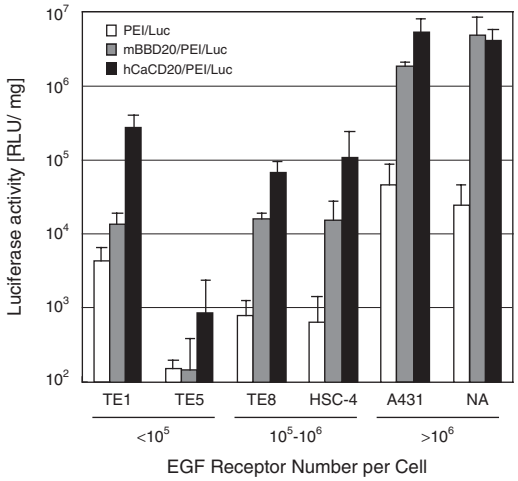

Fig. 3. Luciferase gene transfer into various human tumor cells *via* recombinant immunogenes. Various human tumor cells in 24-well plates (5×10^4 cells/well) were treated with recombinant immunoporter BBD20, or hCaCD20 carrying the luciferase gene. Luciferase activity was measured after 24 h. PEI/Luc: luciferase gene/PEI complex without immunoporter. mBBD20/PEI/Luc: immunoporter mBBD20 carrying the luciferase gene. hCaCD20/PEI/Luc: humanized immunoporter hCaCD20 carrying the luciferase gene. Each value is the average of four experiments.

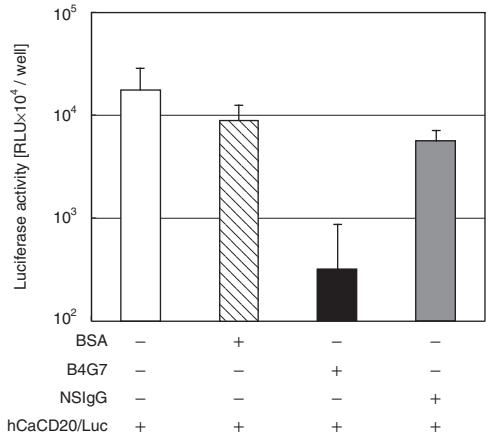

Fig. 4. Luciferase gene transfer via humanized recombinant immunogene and its inhibition by specific monoclonal antibody. A431 cells (5×10^4 cells/well) were treated with either B4G7 monoclonal antibody, NS IgG or BSA (70 μg/well) at 4°C for 30 min. Then, immunoporter hCaCD20 carrying the luciferase gene (0.05 μg pDNA/0.07 μg hCaCD20, N/P=10) was added. Luciferase activity was measured after 24 h. Each value is the average of four wells.

as the number of EGF receptors increased, but there was no clear correlation between EGF receptor number and luciferase activity.

EGF receptor-specific gene delivery via humanized recombinant immunogene. Next, we examined EGF receptor specificity of luciferase gene transfer *via* humanized recombinant immunoporter hCaCD20. For this, A431 cells were treated with either B4G7 antibody, mouse non-specific IgG (NSIgG) or BSA at 4°C for 30 min, and then immunoporter hCaCD20 carrying the luciferase gene was added to the medium. The luciferase activity in the immunogene-treated A431 cells was substantially reduced (to 2%) when cells were pretreated with B4G7 antibody (Fig. 4). Neither mouse NSIgG nor BSA affected the luciferase expression. These results support the idea that humanized recombinant immunoporter retained EGF receptor-specific binding ability even after complex formation with pDNA/PEI.

Uptake of pGeneGrip reporter gene via humanized recombinant immunoporter into A431 cells. We examined the fate of surface receptor-bound DNA. For this, A431 cells were treated with humanized recombinant immunoporter carrying fluorescence-labeled pGeneGrip DNA and observed under a fluorescence microscope at various time points (Fig. 5). At 1.5 h, significant fluorescent spots were detected on the cell surface, and the fluorescent clumps increased in the cytoplasm at 3 h. The increase in size may be caused by fusion of endosomes in which fluorescent pDNA was entrapped. At this time point, a few fluorescent clumps were observed in the nucleus. After 4.5 h, most fluorescence signals had been transported to and accumulated in the nucleus. The appearance of fluorescence was insignificant in A431 cells treated with pGeneGrip/PEI complex only. Thus, pDNA was taken into cells *via* humanized recombinant immunoporter hCaCD20, transported to nuclei and efficiently expressed to produce green protein after 24 h (data not shown).

Delivery of therapeutic *TK* gene and killing of A431 tumor cells. We examined the cell suicide effects of *HSV-TK* gene on A431 cells *via* the immunogene. For this, the plasmid DNA encoding the TK was transferred to A431 cells *via* humanized immunoporter hCaCD20, and then the cells were treated with various concentrations of GCV (Fig. 6).

The 50% lethal dose of A431 cells treated with GCV only or TK/PEI complex was found to be 300–400 μ*M* or 100 μ*M*, respectively. In contrast, A431 cells treated with the humanized recombinant immunogene showed a 50% lethal dose at 0.3–0.4 μ*M*. Thus, treatment with humanized recombinant *TK*-immunogene made A431 cells 1000-fold more sensitive to GCV. The killing of the transfected A431 tumor cells was more than 90% at 10 μ*M* GCV, at which little cytotoxicity was observed in untransfected cells. Thus, the humanized immunogene has the potential to be a non-viral vector for cancer gene therapy.

Discussion

The ideal vector for gene therapy requires efficient transfer of the therapeutic gene to the particular target cells with high specificity and efficiency. For this purpose, we developed the antibody-based immunoporter/immunogene system as a non-viral targeting vector.[1–6] This novel system has evolved into a recombinant immunoporter which is composed of a single-chain antibody (scFv) and various oligopeptide tails in tandem.[11] Here, we humanized the murine-type scFv to human type using CDR-grafting and constructed a gene which encodes humanized scFv and a negatively charged oligopeptide tail (D20) in tandem. The resulting humanized scFv gene (hCaCD20) was expressed in the *Pichia* yeast and the protein product (immunoporter) was purified in large quantity.

We optimized the conditions for receptor-specific binding, stable gene delivery and efficient gene expression *via* recombinant immunogenes made of murine-type and humanized recombinant scFv's. The DNA/PEI complex tends to aggregate in the presence of salt, forming large particles which come into close contact with the cell surface much faster *in vitro* and cause toxicity *in vivo*.[12] The highest gene transfer activity was obtained when DNA/PEI complex was formed in distilled water and the immunoporter was diluted in PBS.

We examined the optimal charge ratio (N/P ratio) of DNA/PEI complex for immunogene formation. In general, branched PEI of 25 kDa at N/P=4–9 is well tolerated for systemic application of therapeutic genes.[13] However, the immunogene with mBBD20 formed at N/P=20 was the best for gene transfer and expression with high EGF receptor-specificity. Immunogene formation requires excess positive charge to maintain electrostatic interaction between the negatively charged tail of the im-

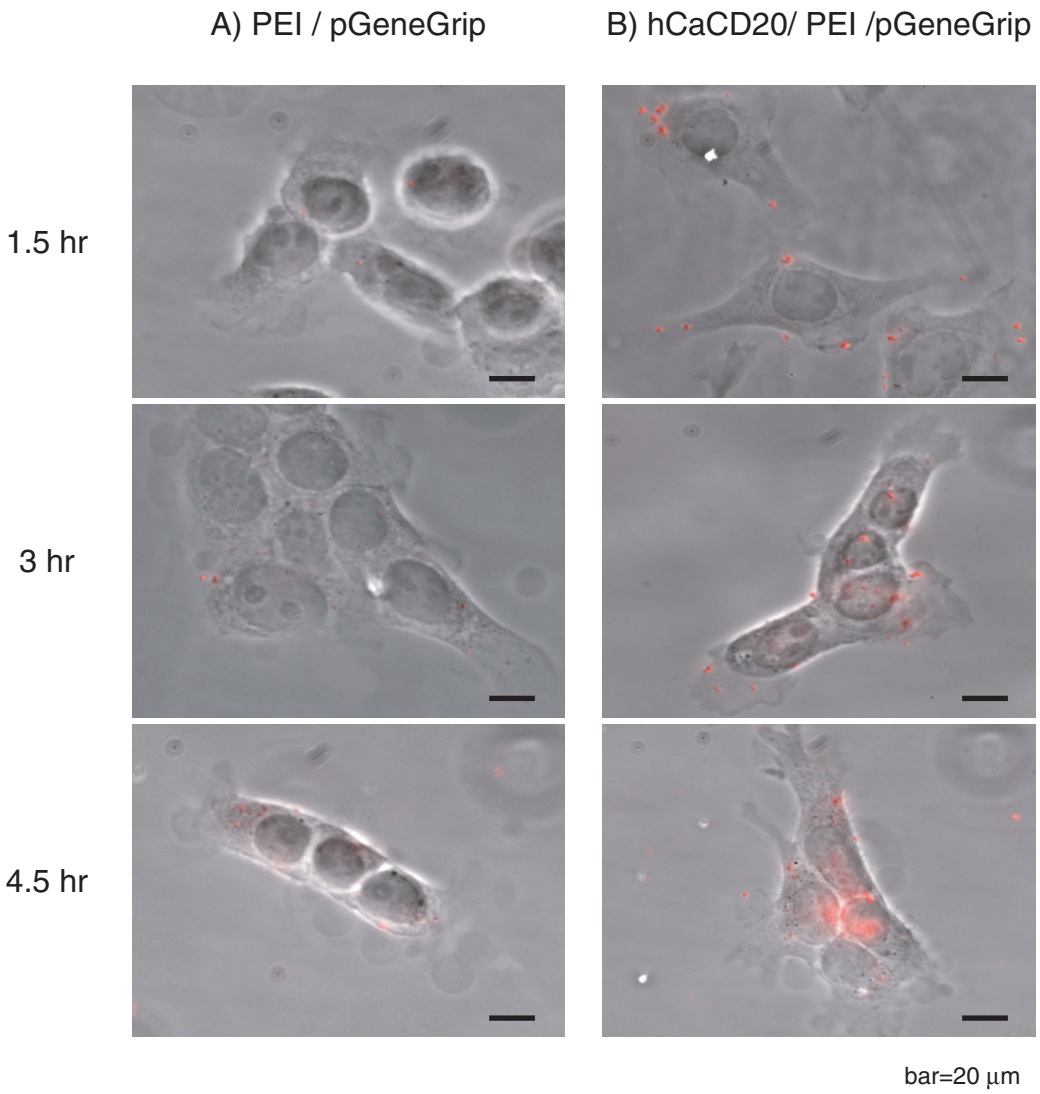

Fig. 5. Uptake of pGeneGrip DNA into A431 cells *via* humanized recombinant immunoporter. A431 cells (5×10^4 cells/well) were treated with hCaCD20-immunoporter carrying rhodamine-labeled pGeneGrip (0.25μg DNA/0.35μg hCaCD20, N/P=10) at 37°C. At the indicated times, cells were fixed with formaldehyde, and mounted with 90% glycerol containing 4,6-diamidino-2-phenylindole/diazobicyclo[2.2.2]octane and viewed under a fluorescence microscope. Bar=20 μm.

munoporter and PEI. The immunogene formed at over N/P=40 caused extensive aggregation and reduced gene transfer, whereas the immunogene formed at N/P=20 was effective and non-cytotoxic.

The highest gene expression of the recombinant immunogene was observed at 24 h, and it declined thereafter. This kinetics is different from that of the Fab immunogene, for which gene expression increased gradually up to 72 h.[3] The apparent difference may be caused by the nature of the cationic polymers (pLys and PEI) and the properties of Fab and scFv. The PEI/DNA complex is rapidly taken into cells[14] and may release bound DNA much faster than the pLys of Fab immunoporter. Moreover, the uptake of scFv is significantly faster than that of Fab.[15] These previous observations suggest that the immunogene utilizing scFv and PEI has favorable features for rapid uptake and expression in A431 cells.

The DNA/PEI complex using unmodified PEI is known to be trapped by negatively charged serum protein in the blood capillaries immediately after administration.[16] The recombinant immunoporter with a negatively charged tail will be protected from interaction with serum protein, prolonging its circulation in the blood, and this will be advantageous for *in vivo* use.

Gene transfer to various human tumor cells showed that the level of gene expression *via* recombinant immunogene does not correlate with the number of EGF receptors as a target. This is consistent with the previous observation using Fab immunogene,[4] whereas cytotoxicity generated by immunotoxin was dependent upon the EGF receptor number.[17] This apparent difference may arise because the immunogene must be transported from the membrane to the nucleus for its expression, and this process could be affected by many other cellular factors, whereas immunotoxin exhibits its extremely high toxicity by inhibiting protein synthesis immediately after receptor-mediated endocytosis.

The *TK* gene delivered to A431 tumor cells *via* humanized recombinant hCaCD20 immunogene increased the cytotoxic effect of GCV over 1000-fold. Since only 3–5% of the cell population was initially positive for gene expression, the highly effective cell suicide effect was considered due to a by-stander effect through the gap-junctions. This may be supported by the

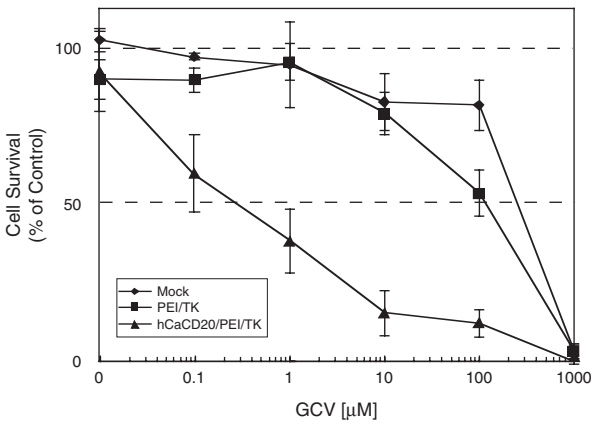

Fig. 6. Cell suicide effects of *TK* gene transfer *via* humanized recombinant immunoporter. A431 cells (1×10^4 cells/well) were treated with either hCaCD20-immunoporter carrying *TK* gene (0.25 μg pDNA/0.35 μg hCaCD20, N/P=20) or pDNA/PEI without immunoporter at 37°C. After 24 h, the cells were treated with GCV at various concentrations. Medium containing GCV was replaced every day, and after 6 days cell viability was measured with AlamarBlue (AccuMed). Each value is the average of six wells.

fact that A431 cells grow as tightly adhering clusters, and hence efficient drug transport through cell-to-cell communication takes place.[18] It should also be noted that the SRα promoter in the plasmid pSRD-TK is much more efficient than the CMV promoter in the plasmid pGeneGrip in A431 cells (data not shown).

The use of humanized scFv antibody with receptor specificity is ideal for targeting particular cell types and is expected to reduce immune response in the case of systemic application. Additional specificity will be provided by the use of tumor-specific gene promoters for the expression of therapeutic genes. The type of scFv can readily be changed to target other antigens on various tumor cells. The combination of *TK* gene and GCV treatment should be effective against many other cancer cells *in vitro* and *in vivo*. Moreover, the by-stander effect is greater *in vivo* than *in vitro*, even with immunocompromised mice.[19] Furthermore, combination of other therapeutic genes and prodrugs such as cytosine deaminase and 5′ fluorouracil will be possible.[6] Thus, we have made a step forward to clinical application of the recombinant immunoporter/immunogene system, and further improvement should provide a truly useful non-viral vector to target particular tumor cells.

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