Biotechnol. Bioprocess Eng. **2002**, 7: 303-310

Construction of Glomerular Epithelial Cells Expressing Both Immune Tolerance and GFP Genes and Application to Cell Therapy by Cell Transplantation

Masahiro Ohga, Mariko Ogura, Mastoshi Matsumura, and Pi-Chao Wang*

Institute of Applied Biochemistry, University of Tsukuba, 1-1-1 Tennodai, Tsukuba City 305-8572, Japan

Abstract Cell therapy applied to wound healing or tissue regeneration presents a revolutionary realm to which principles of gene engineering and delivery may be applied. One promising application is the transplantation of cells into the wounded tissue to help the tissue repair. However, when cells are transplanted from *in vitro* to *in vivo*, immune rejection occurs due to the immune response triggered by the activation of T-cell, and the transplanted cells are destroyed by the attack of activated T-cell and lose their function. Immune suppressant such as FK506 is commonly used to suppress immune rejection during transplantation. However, such kind of immune suppressants not only suppresses immune rejection in the periphery of transplanted cells but also suppresses whole immune response system against pathogenic infection. In order to solve this problem, we developed a method to protect the desired cells from immune rejection without impairing whole immune system during cell transplantation. Previously, we reported the success of constructing glomerular epithelial cells for removal of immune complex, in which complement receptor of type 1 (CR1) was over-expressed on the membrane of renal glomerular epithelial cells and could bind immune complex of DNA/anti-DNA-antibody to remove immune complex through phagocytosis [1]. Attempting to apply the CR1-expressing cells to cell therapy and evade immune rejection during cell transplantation, we constructed three plasmids containing genes encoding a soluble fusion protein of cytolytic T lymphocyte associated antigen-4 (CTLA4Ig) and an enhanced green fluorescent protein (EGFP). The plasmids were transfected to the above-mentioned glomerular epithelial cells to express both genes simultaneously. Using the clone cells for cell transplantation showed that mice with autoimmune disease prolonged their life significantly as compared with the control mice, and two injections of the cells at the beginning of two weeks resulted in remarkable survivability, whereas it requires half a year and 50 administrations of proteins purified from the same amount of cells to achieve the same effect.

Keywords: cell therapy, cell transplantation, immune tolerance, CTLA4Ig, GFP, CD28, CD80/86

INTRODUCTION

Cell therapy is a widely applicable therapeutic approach using cells and bioengineering cell elements. The application of cell therapy for wound healing is already reported as a proven concept [2,3]. For example, cells engineered for optimal skin wound healing may help to minimize scarring following surgery or to enhance the rate of healing of chronic wounds [4]. However, when cells are transplanted from *in vitro* to *in vivo* system, the problem of immune rejection occurs due to the immune response triggered by the activation of T-cell. Therefore, the transplanted cells are usually destroyed by the activated T cells and lose their function. Immune suppressant such as FK506 was commonly used to suppress immune rejection during transplantation [5,6], but such kind of suppressant not only suppresses immune rejection in the periphery of transplanted cells but also suppresses the whole immune system and causes the infectious tolerance [7,8].

Previously, we have reported the construction of glomerular epithelial cells for removal of immune complex *in vitro* [1]. Our final goal is to apply the cells *in vivo* to cure autoimmune disease of systemic lupus erythematosus (SLE). SLE is a typical autoimmune disease due to deposition and inflammation of immune complexes on the organs, particularly, the renal glomerulus. For patients with SLE, transplantation and dialysis have been adopted as the optimal therapies to prolong patients' life in the past several decades. However, the former encounters problems of insufficient donor and the latter requires complicated equipments, high cost and long period. Since nephritis of SLE has been demonstrated a loss of CR1 antigen on glomerular epithelial cells [9], we had reported a method transfecting a full length of human tonsillar CR1 cDNA to the cultured

***Corresponding author**
Tel: +81-298-53-6447 Fax: +81-298-53-4605
e-mail: cogitate@sakura.cc.tsukuba.ac.jp

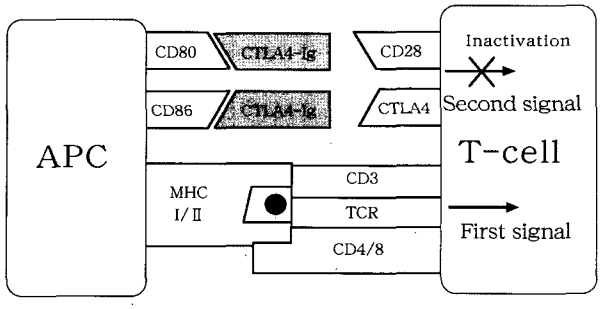

Fig. 1. The mechanism of activation and inactivation of naive T-cell requires two signals. The first signal is antigen-specific and based on T-cell receptor (TCR) recognition of a peptide-MHCI/II complex as well as CD3 and CD4/8. The second signal is antigen-nonspecific and triggered by T-cell receptors after binding to their ligands of co-stimulatory molecules expressing on antigen-presenting cells (APCs). The CD28 on T-cell interacting with CD80/86 on APCs serves as a positive co-stimulator in the context of TCR engagement by MHC/ antigen complex. In contrast, CTLA4 on T-cell interacting with CD80/86 on APCs serves as a negative co-stimulator to regulate T cell activation and triggers the induction of immune tolerance by attenuating cell cycle progression, IL-2 production, and proliferation of T cells after activation.

rat glomerular epithelial cells *in vitro* and investigated the mechanism of the removal of pathogenic immune complex of DNA/anti-DNA antibody through CR1. It was clarified that complement factors in serum mediated the binding of immune complex to CR1 and then CR1-expressing cells removed the immune complex through phagocytosis [1]. In order to apply such kind of cells to cell therapy, it is necessary to protect these cells from immune rejection and/or induce immune tolerance during cell transplantation *in vivo*. Several studies have been undertaken to address the mechanism of immune rejection [10-13]. Recently, It has been clarified that immune rejection is triggered by the activation of T cell through two signals shown in Fig. 1. The first signal is provided by the interaction between the T cell receptor (TCR) and antigenic peptides in the context of class I or II of major histocompatibility complex antigens (MHC I/II) on antigen presenting cells (APCs) as well as CD3 and CD4/8 [11,14]. The second signal, named as co-stimulatory signal, is provided by the cross interactions between CD28/CTLA4 (Cytolytic T Lymphocyte associated Antigen-4) on T-cell and CD80/86 of B7-family on APCs. It is suggested that blocking the co-stimulation signal will lead to the induction of immune tolerance and allograft survival [15,16]. Since CTLA4 is known to bind CD80 and CD86 with a 100-fold higher affinity than does CD28 and blocks co-stimulatory signal [17], a soluble form of CTLA4Ig fusion protein composed of the extracellular domain of CTLA4 linked to an immunoglobulin Ig Cγ2a chain was generated to block the binding of CD28 to B7 family (CD80/86), and inhibit the co-stimulatory pathway and T cell activation [14]. We had transfected the CTLA4Ig gene to macrophage and investigated its activity *in vitro* [18]. However, to detect the expression of CTLA4Ig requires complicated steps such as protein purification from cell culture, SDS-PAGE and Western blotting. Moreover, expensive equipments and reagents such as flowcytometer and antibodies are also necessary. To circumvent this problem, green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is adopted as a reporter for monitoring gene expression. The GFP-expressing cells can yield green fluorescence when excited by ultraviolet (UV) or blue light and can be detected simply by fluorescent microscope [19]. In this study, we reported constructions of three plasmids containing genes encoding CTLA4Ig and EGFP (the enhanced GFP with an expression of 35 times brighter than wild-type GFP). The plasmids were transfected to the above-mentioned glomerular epithelial cells to express both genes simultaneously. The expression of three constructed plasmids was investigated and the best one was chosen for further experiments. Limiting dilution was conducted to select the high light clones, and duration of gene expression was also investigated. Furthermore, cell transplantation to mice with autoimmune disease was performed to distinguish its effect from those administrated with and without proteins purified from cell culture.

MATERIALS AND METHODS

Cell and Culture Conditions

Rat renal glomerular epithelial cell expressing CR1 was derived from SGE1 cells [20] and reported previously [1]. Cells were cultured in DHSF medium (per liter: DMEM (Dulbecco's modified Eagle's medium), 5 g; Ham's F12, 5.3 g; $NaHCO_3$, 1.9 g; ITS (Collaborative Res. Inc. MA, USA), 6.25 mg; EGF, 1 μg supplemented with 10% FBS following the method described previously. [21,22,23]. CD80- and CD86-transfected cells were kindly gifted by Dr. T. Yagita [24,25]. These two cells were cultured in RPMI 1640 medium (per liter: RPMI 1640, 10.4 g; $NaHCO_3$, 2.69 g; L-glutamine, 0.3 g) supplemented with 5% FBS. For all cells, medium was changed twice per week, and cell passage was performed when cells grew to confluency. Antibiotics such as 200 units/mL of penicillin and 0.2 mg/mL streptomycin (Sigma, MO, USA) were added to medium, and all the cell cultures were performed at 37°C with 5% CO_2 humidity. Medium for inoculation of *E. coli* is L-broth (per 1 L: polypepton, 10 g; NaCl, 10 g; Yeast extract, 5 g) or 2xYT (per 1 L: polypepton, 16 g; NaCl, 10 g; Yeast extract, 5 g).

Plasmid Construction and DNA Manipulation

Two plasmids, pEGFPN2 and pEGFPC1 comprising genes of enhanced green fluorescent protein (EGFP) and multiple cloning sites (MCS) at N terminal and C ter-

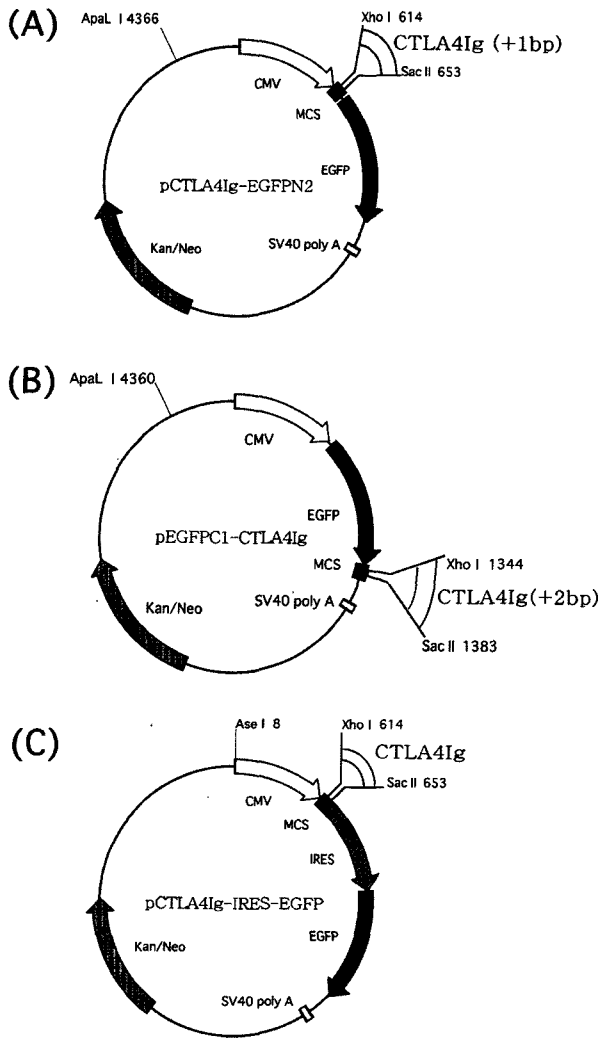

Fig. 2. Construction of three plasmids (A) pCTLA4Ig-EGFPN2, (B) pEGFPC1-CTLA4Ig and (C) pCTLA4Ig-IRES-EGFP by inserting genes of CTLA4Ig to plasmids pEGFPN2, pEGFPC1 and pIRES-EGFP, respectively. pCTLA4Ig-EGFPN2 was constructed by inserting the fragment of CTLA4Ig added with 1 base of A at the N-terminal of EGFP, pEGFPC1-CTLA4Ig was constructed by inserting a fragment of CTLA4Ig added with 2 bases of TT at the C-terminal of EGFP, and pCTLA4Ig-IRES-EGFP was constructed by inserting the fragment of CTLA4Ig ahead of IRES. The 1228 bp fragment of CTLA4Ig was inserted to the Xho1-SacII cutting site in MCS of each plasmid vector. All the three vectors contain CMV as promotor.

minal of EGFP, respectively, were used (Clontech Inc. CA, USA). Plasmid pIRES2-EGFP comprising a fragment encoding IRES (Internal Ribosome Entry Site) ahead of EGFP was also purchased from Clontech Inc. Plasmid CTLA4Ig/pMKITneo was kindly provided by Dr. Okumura [25], which is a vector of pMKIT comprising a neomycin marker and a full length of cDNA of the extracellular domain of CTLA4 linked to an immunoglobulin Ig Gγ2a chain, which enables this fused protein CTLA4-Ig to be secreted from cells. The construction of the three plasmids was shown in Fig. 2. Plasmids pCTLA4Ig-pEGFPN2 (Fig. 2A) and pEGFPC1-CTLA4Ig (Fig. 2B) were constructed by inserting the fragment of CTLA4Ig cut by XhoI-SacII from the CTLA4Ig/pMKITneo to the XhoI-SacII site in MCS of pEGFPN2 and pEGFPC1, respectively. For the DNA insertion to pEGFPN2 and pEGFPC1, one base of A and two bases of TT was PCR-added ahead of ATG starting codon of CTLA4Ig, respectively, which enables the open reading frame of CTLA4Ig to be translated correctly. Plasmid pCTLA4Ig-IRES-EGFP (Fig. 2C) was constructed by inserting the Xho1-SacII cutting fragment of CTLA4Ig to the XhoI-SacII site in MCS of pIRES2-EGFP. All of the DNA insertion and ligation were performed using T4 ligase (Takada, Japan). The DNA amplification of the constructed plasmids was achieved by transforming the plasmid DNA to *E. coli* JM109, and the transformants were grown in L-broth or 2x YT medium containing kanamycin at a concentration of 30 μg/mL. All the DNA manipulations and purification of plasmids were carried out using standard methods described elsewhere [26].

Electroporation and Limiting Dilution

500 μg each of the three constructed plasmids pEGFP-CTLA4Ig, pCTLA4Ig-EGFP and pCTLA4Ig-IRES-EGFP were dissolved in PBS buffer containing 11 mM glucose, and transfected to glomerular epithelial cells by electroporation at 960 μF, 250 volt using a Gene Pulser (Bio Rad, CA, USA). The transfected cells were cultured in a selective medium containing 0.7 mg/mL neomycin to exclude non-transfected cells. Fresh medium was changed every 3 days until colonies of the viable cells appeared. After trypsin treatment, cells were washed and subjected to limiting dilution in 96-well microplate (Iwaki, Japan). Briefly, the cell suspension was diluted to a concentration of 5 cells per mL, and then seeded into each well with 200 μL. On the next day, all wells were examined and only those containing a single cell were selected for growth of clones for another one month until confluent. When cells became confluent, they were transferred to a 48-well plate, and then 24-well, 12-well, 6 well and finally 100-mm dish. Cells in each 100-mm dish could grew to 10^8 cells. Through these processes, positive clones derived from single cell were selected, and all clones were stored in liquid nitrogen until use.

Microscopic Observation

In order to investigate GFP expression, the neomycin-selected cells derived from each single cell were seeded on 10 mm^2 cover glass and then observed by Leica DMR microscope (Model: RXA-6; Green filter: VG9) under visible light and fluorescent light every 2 or 3 days for one week.

Immunofluorescence Assay

In order to investigate the expression and secretion of CTLA4Ig from the clone cells selected by limiting dilu-tion, the supernatant of cell culture was collected, and added to each coverglass on which CD80- and CD86-cells were cultured, respectively. Cell culture medium without cells was used as negative control, and the protein of CTLA4Ig purified by our laboratory [18] was used as positive control. Cells were grown to confluency and then washed with phosphate-buffered saline (PBS) for four times, subsequent the addition of the first antibody at 37°C for 45 min. After four washes with PBS, the secondary antibody was added and cells to the fluorescent were cultured for another 45 min. Finally, cells were washed for four times and subjected to microscope. The first monoclonal antibodies (mAb) of CD80 and CD86 were anti-mouse CD80 mAb and anti-mouse CD86 mAb (DaiNippon Seiyaku, Japan), respectively. Both were added to the cell medium at 100 folds of dilution. The second antibody for both cells was FITC-labeled goat-anti-mouse IgG and was added to the cell medium at 1000 fold of dilution.

Cell Transplantation *In Vivo*

10^9 cells were collected and washed with PBS for three times, then suspended in PBS. The prepared cell suspension (pH 7.2) was then injected to a group of 30 female BALB/c mice (SLC, Japan) at an age of 6 months with autoimmune disease. The peritoneal injection was at a dose of 10^9 cells/mL per mouse and was carried out twice at the beginning of two weeks. Mice treated with cell injection were bred in cages and their survivability was estimated every month until all of the negative control mice died. For comparison, 30 female mice at the same age served as positive control, which were injected with purified proteins of supernatant collected from cell culture containing 10^9 cells. Proteins were purified by subjecting the cell supernatant to protein A column and the purified protein was confirmed to be CTLA4Ig with molecular weight of 120 kd by SDS-PAGE and Western blot (data not shown). The anti-CTLA4 and anti-IgG2a antibodies for Western blot were kindly gifted by Dr. Yagita in Department of Immunology, Juntendo University, Japan, and the site of CTLA4Ig on the blotted membrane was visualized by the reaction of ECL kit solution [18]. 0.1 mL of the purified CTLA4-Ig was peritoneal injected to the mice at a dose of 75 μg/mL and was carried out twice per week and lasted for 6 months. Another 30 mice treated with neither cells nor protein served as negative control. All the experiments were performed to each groups in triplicate. The survivability of each group of mice was measured and statistical analysis was performed by two-way analysis of student's t-test. Results are expressed as means ± SE and the significance level is expressed as the probability (p).

RESULTS AND DISCUSSION

Electroporation and Microscopy of the GFP Expression

CR1-expressing cells constructed previously [1] were transfected with three plasmids pCTLA4Ig-EGFPN2, pEGFP-CTLA4Ig and pCTLA4Ig-IRES-EGFP by electroporation, respectively. Cells were cultured in selective medium for 1-2 weeks. The electroporation caused 20% of the 10^7 cells inviable, and the subsequent selective medium excluded the non-transfected cells. Finally 5×10^3 cells remained viable. Fig. 3 shows the microscopic results of EGFP expression on cells. Cells without transfection were used as control which were cultured in medium without neomycin. Microscopic observation shows that the control cells and cells transfected with pCTLA4Ig-IRES-EGFP grew to confluent (Figs. 3A and 3D), while cells transfectd with pCTLA4Ig-EGFPN2 and pEGFPC1-CTLA4Ig grew slowly and did not reach confluent (Fig. 3C and 3D). Fluorescent microscopy confirmed that no fluorescence was detected on control cells (Fig. 3E), and neither were those transfected with pCTLA4Ig-EGFPN2 (Fig. 3F). However, fluorescence was detected in cells transfected with pEGFP-CTLA4Ig although the cell number was few (Fig. 3G). In contrast, cells transfected with pCTLA4Ig-IRES-EGFP showed significant fluorescence in several cell colonies (Fig. 3H). These results suggest that pCTLA4Ig-IRES-EGFP is the best construction among the three to express EGFP. Moreover, the appearance of fluorescent colonies in Fig. 3H may suggest that EGFP gene was maintained stably during cell division. It is notable that IRES, an internal ribosome entry site, can promote translation initiation independently from the classical cap-dependent mechanism and serves as an individual promoter in addition to CMV [27-29]. For pCTLA4Ig-IRES-EGFP transfected cells, fluorescent cells were observed (Fig. 3H). Although the various fluorescent results may be influenced by EGFP's form, it is most likely that IRES, instead of CMV, initiated the translation of the proximate gene of EGFP because it was found that cells transfected with pCTLA4Ig-EGFP lacking IRES could not express EGFP at all (Fig. 3F). On the other hand, promoter CMV in pEGFP-CTLA4Ig slightly initiated the translation of the proximate gene of EGFP (Fig. 3G). This fact implies that CMV is not sufficient to initiate the translation for both genes. In other word, CMV may be able to initiate the translation of proximate gene but not enough for distant gene. This mechanism may interpret the result of Fig. 3F in which EGFP is a distant gene which was not expressed at all. Taken together, to express two genes effectively requires two promoters, the first promoter such as CMV is for the proximate gene and the second promoter such as IRES for the distant gene. In the case of pCTLA4Ig-IRES-EGFP, CMV served the expression of CTLA4Ig and IRES the expression of EGFP. Thus, cells under this kind of construction were chosen for further limiting dilution.

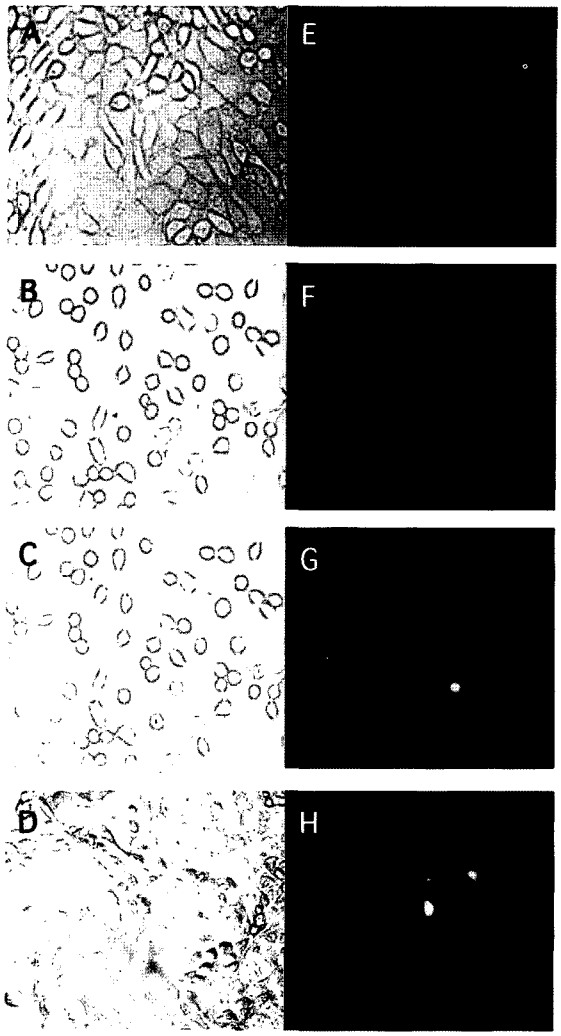

Fig. 3. Cells transfected with each type of plasmid were observed by microscope under visible light (A, B, C, D) and fluorescent light (E, F, G, H). Cells without any transfection of plasmid was used as control (A), and no fluorescent cells could be detected (E). Cells transfected with pCTLA4Ig-EGFPN2 (B) showed no expression of GFP fluorescence (F). On the other hand, Cells transfected with pEGFPC1-CTLA4Ig (C) showed GFP fluorescence on one cell (G), whereas cells transfected with pCTLA4Ig-IRES-EGFP (D) revealed GFP fluorescence on several colonies (H).

Clones Selected by Limiting Dilution

Clones selected by limiting dilution excluded the possibility of the contamination of non-expressing cells. Of 300 cells plated, 60 clones (20%) were obtained. This efficiency of cell recovery is higher than those reported previously [20,30-32]. Fig. 4 shows the microscopy of one clone randomly selected from the 30 clones after continuous cell passage for 6 months. Several domes were observed in the monolayer cell culture (Fig. 4A). Since such kind of domes formation was also found in

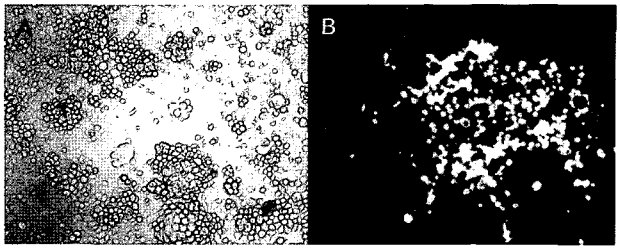

Fig. 4. The clone selected by limiting dilution was observed by microscope under visible light (A) and fluorescent light (B). Cells derived from the selected clone not only proliferated but also expressed high GFP fluorescence after cell division.

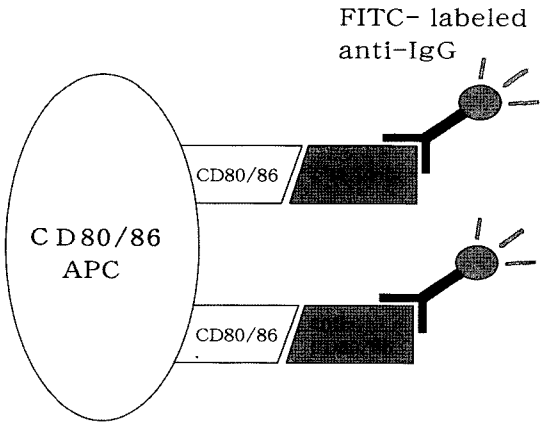

Fig. 5. The method of immunofluorescence assay. CD80/86 on the cell surface of APC are capable of conjugating CTLA4Ig and/or the first monoclonal antibody of anti-CD80/86 antibody. When the second antibody, fluorescent isothiocyanate (FITC)-labeled anti-IgG antibody, conjugates CTLA4Ig and/or CD80/86, the cells show immunofluoresence and can be observed by fluorescent microscope. In contrast, CD86-expressing cells incubated with anti-CD80 antibody, or CD80-expressing cells incubated with anti-CD86 antibody shows no immunofluorescence after interacting with FITC-labeled second antibody. This method is useful to detect the existence of CTLA4Ig in the cell culture.

the confluent cell culture [22,23], and was clarified to be a simple cuboidal epithelium with a little increment of C24 fatty acid of ganglioside GM [21], the morphology of the transfected cells might be considered as normal as those cells of monolayer. The fluorescent microscopy showed that 80% of the cells revealed high expression of EGFP indicating that the function of the plasmid was maintained stably during cell division.

Immunofluorescence Assay

In addition to EGFP expression, the expression and secretion of CTLA4Ig were also determined by immunofluorescence assay. Fig. 5 shows the strategy to determine CTLA4Ig expression. Since CD80/86 are capable of conjugating CTLA4Ig and/or the first monoclonal

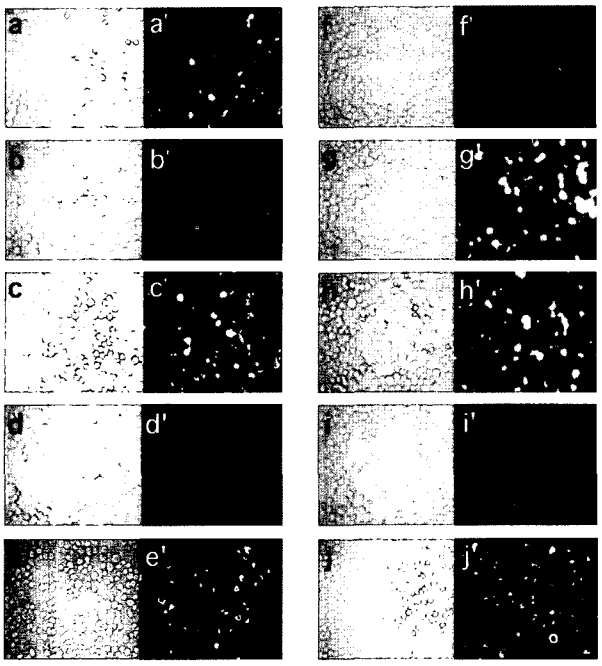

Fig. 6. Results of immunofluorescence assay of CD80- and CD86-expressing cells incubated with anti-CD86 antibody, anti-CD80 antibody, purified CTLA4Ig, supernatant of control cell culture and supernatant of cell culture from pCTLA4Ig-IRES-EGFP transfected cells. The left panel shows the microscopic observation of CD-86 expressing cells under visible and fluorescent light when incubated with anti-CD86 antibody (a, a'), anti-CD80 antibody (b, b'), purified CTLA4Ig (c, c'), supernatant of control cell culture where cells were not transfected with pCTLA4Ig-IRES-EGFP (d, d'), and supernatant of pCTLA4Ig-IRES-EGFP-tranfected-cell culture (e, e'). The right panel shows the microscopic observation of CD-80 expressing cells under visible and fluorescent light when incubated incubated with anti-CD86 antibody (f, f'), anti-CD80 antibody (g, g'), purified CTLA4Ig (h, h') supernatant of control cell culture (i, i') and supernatant of pCTLA4Ig-IRES-EGFP -tranfected-cells culture (j, j').

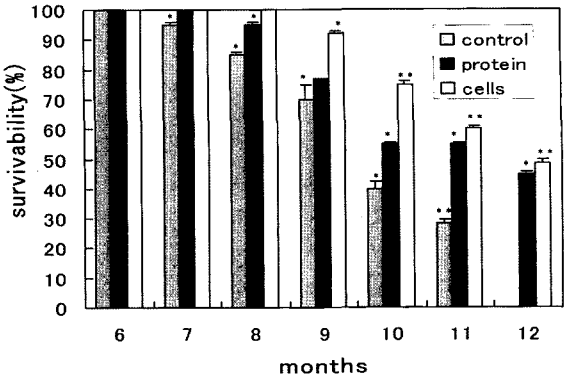

Fig. 7. Survivability of three groups of mice with autoimmune disease at time intervals from age of 6 months to 12 months. ▨: negative control mice treated with neither proteins nor cells; ■: protein from supernatant of cell culture twice every week; □: test mice treated with 10^9 cells/mL/mouse twice at the beginning of two weeks. Each group contains 30 mice, and each experiment was performed in triplicate. Survivability is expressed as means ±SE, and SE is expressed as bar. *, $p < 0.05$, **, $p < 0.01$.

antibody of anti-CD80/86, only the supernatant containing CTLA4Ig in the clone cell culture and/or anti-CD80 mAb, anti-CD86 mAb can highlight cells when the FITC-labeled second antibody is incubated together. In contrast, CD86-expressing cells incubated with anti-CD80 mAb, or CD80-expressing cells incubated with anti-CD86 mAb, yielded no fluorescence after the addition of the second antibody. The clone cells used for immunofluorescence assay were those selected by limiting dilution after continuous passage for 6 months. Fig. 6 shows the results of immunofluorescence assay. CD80-expressing cells reacted with anti-CD-80 mAb serves as positive control (Fig. 6a). It is not surprising to detect the fluorescence of these cells because of the specific conjugation of antigen to antibody (Fig. 6a'). Negative control was shown in Fig. 6b, in which anti-CD86 mAb did not conjugate CD80, and thus no fluorescence was detected in Fig. 6b'. Meanwhile, the purified CTLA4Ig protein [23] also serves as a positive control shown in Fig. 6c, while supernatant of cell culture without gene transfection serves as another negative control shown in Fig. 6d. Fluorescence was easily detected in positive control (Fig. 6c') whereas no fluorescence in negative control (Fig. 6d'). The supernatant collected from the clone cell indicated the same result (Fig. 6e') as those observed in positive control (Figs. 6a' and 6c'). This suggests that CTLA4Ig was well expressed and secreted by the clone cells. The same results were also detected in CD86-expressing cells (Fig. 6h' and 6i') except that anti-CD80 mAb was not specific to CD-86 and thus no conjugation appeared (Fig. 6f'). However, anti-CD86 mAb showed significant conjugation with CD86 (Fig. 6g'), and thus the supernatant of clone cells showing the same fluorescence (Fig. 6j') indicated the existence of CTLA4Ig in the clone cell culture. These results proved that the selected clone could express CTLA4Ig as long as EGFP expressed.

Taken together, using vector pIRES-EGFP to construct desired genes and using limiting dilution to select highlight clones provide an easy way to detect the expression of desired gene simply by watching the reporter gene EGFP through fluorescent microscope. Such a method is expected to be useful for the investigation of expression of many other genes. Since the above clones already contained CR1 gene which was transfected to cells previously, CR1 expression was also confirmed (data not shown) but the details will not be discussed. Further application of cell therapy using the selected clones were performed to investigate whether the immune tolerance protein CTLA4Ig as well as CR1 work together to prolong the animal survivability .

Cell Trasplantation *In Vivo*

Fig. 7 shows the results. For negative control group, all the mice with autoimmune disease died at the age of 12 months. The positive control group, in which proteins purified from the supernatant of 10^9 cells culture were administrated twice per week for half a year, showed 45% of mice survived at the age of 12 months. It is interesting to find that mice treated with 10^9 cells twice at the beginning of two weeks revealed the same survivability as those treated with purified proteins. Although it is not clear which one, CR1 or CTLA4Ig, plays more important role, or both of them play the same important role, in prolonging the animal life. The fact suggests that cell transplantation provided an effective and convenient way for cell therapy of autoimmune disease at lower cost. It should also be noted that the purification of proteins from the cell culture requires expensive column, labor and time. On the other hand, cell therapy only requires cell preparation without complicated operation. Treatment of murine lupus with CTLA4Ig protein was reported earlier [14], and cell transplantation of virally transduced cells into dermis of SCID mice had also been discussed elsewhere [4], but treatment of autoimmune disease with cells capable of removing immune complex and inducing immune tolerance simultaneously has not been mentioned yet.

In conclusion, our experimental results provide a simple and easy detection of the expression of desired gene through the fluorescent reporter gene. We also suggest a concept for cell therapy of autoimmune disease by cell transplantation. Further investigation on the mechanism to distinguish the role of expressed genes *in vivo* should be undertaken and improvement of practical technique for cell transplantation is required.

Acknowledgments Mr. Ohga and Ms. Ogura are of equal contribution to this study. This study was partly supported by a Grant-In-Aid for Scientific Research C (13680294) from the Ministry of Science, Education and Culture of Japan.

REFERENCES

[1] Wang, P. C., K. Deng, M. S. Horisawa, M. Matsumura, and Deng (1999) Construction of glomerular epithelial cells in vitro for removal of immune complex. *Artificial Organs* 2: 170-175.

[2] Gorodetsky, R., R. A. Clark, J. An, J. Gailit, L. Levdansky, A. Vexler, E. Berman, and G. Marx (1999) Fibrin microbeads (FMB) as biodegradable carriers for culturing cells and for accelerating wound healing. *J. Invest. Dermatol.* 112: 866-872.

[3] Breitbart, A. S., J. M. Mason, C. Urmacher, M. Barcia, R. T. Grant, R. G. Pergolizzi, and D. A. Grande (1999) Gene-enhanced tissue engineering: applications for wound healing using cultured dermal fibroblasts transduced retrovirally with the PDGF-B gene. *Ann. Plast. Surg.* 43: 632-639.

[4] Radfar, A. J., P. D. Robbins, J. Huard, F. R. Rosas, J. E. Dohar, and P. A. Hebda (2000) Transplantation of virally transduced cells into the dermis of immunocompetent and immunodeficient (SCID) mice to determine gene expression profile and differential donor cell survival. *Wound Repair Regen.* 8: 503-510.

[5] Kino, T., H. Hatanaka, M. Hashimoto, M. Nishiyama, T. Goto, M. Okuhara, M. Koisaka, H. Aoki, and H. Imanaka (1987) FK-506, a novel immunosupressant isolated from a Streptomyces I. Fermentation, isolation, and physicochemical and biological characteristics. *J. Antibiot.* 11: 1249-1255.

[6] Kino, T., H. Hatanaka, S. Miyata, N. Imamura, M. Nishiyama, T. Yagima, T. Goto, M. Okuhara, M. Kohsaka, H. Aoki, and T. Ochiai (1987) A novel immunosupressant isolated from a Streptomyces II. Immunosuppressive effect of FK-506 *in vitro*. *J. Antibiot.* 11: 1256-1265.

[7] Spencer, C. M., K. L. Goa, and J. C. Gills (1997) Tacrolimus an update of its pharmacology and clinical efficiency in the management of organ transplantation. *Drugs* 54: 925-975.

[8] Fung, J. J., K. Abu-Elmagd, S. Todo, R. Shapiro, A. Tzakis, M. Jordan, J. Armitage, A. Jain, M. Alessiani, and M. Martin (1990) Overview of FK506 in transplantaiton. *Clin. Transplant.* 115-121.

[9] Kazatchkine, M. D., D. T. Fearon, M. D. Appay, C. Mandat, and J. Bariety (1982) Immunohistochemical study on the human glomerular C3b receptor in normal kidney and in seventy-five cases of renal diseases. *J. Clin. Invest.* 69: 900-912.

[10] Freeman, G. J., J. G. Gribben, V. A. Boussiotis, J. W. Ng, V. A. Restivo Jr, L. A. Lombard, G. S. Gray, and L. M. Nadler (1993) Cloning of B7-2: A CTLA-4 counter-receptor that costimulates human T cell proliferation. *Science* 262: 909-911.

[11] Linsley, P. S., W. Brady, M. Urnes, L. S. Grosmaire, N. K. Damle, and J. A. Ledbetter (1991) CTLA-4 is a second receptor for the B cell activation antigen B7. *J. Exp. Med.* 174: 561-569.

[12] Nakajima, A. and M. Azuma (1997) Costimulatory molecules in autoimmunity: Role of CD28/CTLA4-CD80/CD86. *Nippon Rinsho.* 55: 1419-1424.

[13] Azuma, M., D. Ito, H. Yagita, K. Okumura, J. H. Phillips, L. L. Lanier, and C. Somoza (1993) B70 antigen is a second ligand for CTLA-4 and CD28. *Nature* 366: 76-79.

[14] Finck, B. K., P. S. Linsley, and D. Wofsy (1994) Treatment of Murine Lupus with CTLA4Ig. *Science* 265: 1225-1227.

[15] Judge, T. A., Z. Wu, X. G. Zheng, A. H. Sharpe, M. H. Sayegh, and L. A. Turka (1999) The role of CD80, CD86, and CTLA4 in alloimmune responses and the induction of long-term allograft survival. *J. Immunol.* 162: 1947-1951.

[16] Lu, P., Y. L. Wang, and P. S. Linsley (1997) Regulation of self-tolerance by CD80/CD86 interactions. *Curr. Opinion Immunol.* 9: 858-862.

[17] Lane, P. (1997) Regulation of T and B cell responses by modulating interactions between CD28/CTLA4 and their ligands, CD80 and CD86. *Ann. N.Y. Acad. Sci.* 5: 392-400.

[18] Gao H. Y., P. C. Wang, and K. Takagi, O. Shimozato, H. Yagita, T. Okigaki, and M. Matsumura (1999) Expression of a soluble form of CTLA4 on macrophage and its biological activity. *Cell Res.* 9: 189-199.

[19] Zhang, G., V. Gurtu, and S. R. Kain (1996) An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells. *Biochem. Biophys. Res. Comm.* 227: 707-711.

[20] Itoh, T. (1983) Establishment of a myoid cell clone from rat thymus. *Cell Tiss. Res.* 231: 39-47.

[21] Tomono, Y., C. Moritoh, T. Yasuda, and T. Okigaki (1995) Fatty acid composition of ganglioside GM3 of renal glomerular epithelial SGE1 cells during spontaneous dome formation *in vitro*. *Cell Struc. Func.* 20: 269-274.

[22] Yamada, M., C. Moritoh, M. Kawaguchi, and T. Okigaki (1989) Growth, morphology, function, and morphogenetic properties of rat renal glomerular epithelial cells in vitro: effects of retinyl acetate. *Eur. J. Cell Biol.* 49: 252-258.

[23] Yamada, M., M. Kawaguchi, H. Takamiya, H. Wada, and T. Okigaki (1988) Establishment and characterization of an epithelial cell line, SGE1, from isolated rat renal glomeruli. *Cell Struc. Func.* 13: 495-513.

[24] Kato, K., K. Okumura, and H. Yagita (1997) Immunoregulation by B7 and IL-12 gene transfer. *Leukemia* 11: 572-576.

[25] Enomoto. A., K. Kato, H. Yagita, and K. Okumura (1997) Adoptive transfer of cytotoxic T lymphocytes induced by CD86-transfected tumor cells suppresses multi-organ metastases of C1300 neuroblastoma in mice. *Cancer Immunol. Immunother.* 44: 204-210.

[26] Maiatis, T., E. F. Fritsch, and J. Sambrook (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA.

[27] Creancier, L., P. Mercier, A-C. Prats, and D. Morello (2001) c-myc Internal ribosome entry site activity is developmentally controlled and subjected to a strong translational repression in adult transgenic mice. *Mol. Cell. Biol.* 21: 1833-1840.

[28] Johansen, L. K. and C. D. Morrow (2000) Inherent instability of poliovirus genomes containing two internal ribosome entry site (IRES) elements supports a role for the IRES in encapsidation. *J. Virol.* 74: 8335-8342.

[29] Jang, S. K., H-G. Krausslich, M. J. H. Nicklin, G. M. Duke, A. C. Palmenberg, and E. Wimmer (1988) A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during *in vitro* translation. *J. Virol.* 62: 2636-2643.

[30] Dong, Q. G., S. Bernasconi, S. Lostaglio, R. W. De Calmanovici, I. Martin-Padura, F. Breviario, C. Garlanda, S. Ramponi, A. Mantovani, and A. Vecchi (1997) A general strategy for isolation of endothelial cells from murine tissues: Characterization of two endothelial cell lines from the murine lung and subcutaneous sponge implants. *Arterioscler. Thromb. Vasc. Biol.* 17: 1599-1604.

[31] Kettman, J. and R. W. Dutton (1975) The role of antigen in the immune response: Analysis by limiting dilution methods. *Cell. Immunol.* 17: 228-239.

[32] Wang, P. C., S. Kimura, K. Todokoro, T. Okigaki, Y. H. Kao, and M. Matsumura (1966) Stable expression of human complement receptor CR1 gene on rat renal glomerular epithelial cell SGE1. *Cell. Eng.* 1: 179-182.

[Received April 8, 2002; accepted August 2, 2002]