Pharmaceutical Research, Vol. 24, No. 8, August 2007 (© 2007)
DOI: 10.1007/s11095-007-9265-9

Research Paper

Preparation and Evaluation of Gene-transfected Cultured Skin as a Novel Drug Delivery System for Severely Burned Skin

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Received November 18, 2006; accepted February 7, 2007; published online March 22, 2007

Purpose. The purpose of this study is to prepare and evaluate gene-transfected cultured skin to establish a dermal patch consisting of cultured skin as a new and novel delivery system for severely burned skin.
Materials and Methods. Plasmid DNA encoding the green fluorescent protein (GFP) gene was used as a model gene and transfected to rat and human cultured dermis models (CDMs) using the hemagglutinating virus of Japan envelope vector (HVJ-E) to prepare gene-transfected CDM and evaluate GFP expression in the CDM. Two kinds of transfection methods were evaluated. In pre-transfection, the gene was first transfected into fibroblasts and then CDM was prepared using these gene-transfected cells. In post-transfection, the gene was transfected directly into CDM.
Results. GFP expression was observed in both the pre- and post-transfected CDMs. The post-transfection method showed higher GFP expression in the CDM than pre-transfection, although no statistically significant difference was observed. The cell viability of these transfected CDMs was also examined with MTT assay. Slight decrease in viability was observed in these transfected CDMs. These methods could be useful in preparing gene-transfected cultured skins with low cell damage.
Conclusion. Gene transfection to cultured skin may produce several dermal patches that release potent endogenous bioactive peptides.

KEY WORDS: cultured dermis model; gene delivery; gene-transfected cultured skin; green fluorescent protein; tissue engineering.

INTRODUCTION

A paradigm shift is now ongoing in therapeutic drugs and their research and development. This has resulted from the rapid development of biotechnology. Most therapeutic drugs of low molecular weight organic compounds have been mainly used since the mid twentieth century. However, bioactive peptides and proteins, and even genes, as a new category of therapeutic drugs, were utilized by the end of the last century. As a result, many innovative technologies have been developed for effective drug delivery, including transdermal patches, nanodevices, bioadhesive systems, implants, microfabricated systems, cell encapsulation devices, and novel nasal drug delivery systems (1). Moreover, regenerative medicine, to repair defective tissues or substitute biological functions of damaged organs by cell-based tissue regeneration, has become available by the recent development of tissue engineering as well as basic biology and medicine (2). A new therapeutic strategy with biological cells is an example that utilizes regenerative medicine, that is, skin substitutes, such as cultured epidermis, dermis, and whole skin, have been developed and some are already in use for treating severe burns (3–5). Biological cell-containing cultured skin substitutes based on the tissue engineering technique have various advantages, i.e., superior biocompatibility compared with classical acellular artificial skin, improved supply of skin grafts for transplantation, and biological cells in skin substitutes to promote tissue repair by releasing various cytokines and/or extracellular matrix proteins (6). Secondary infection by some bacteria, however, is a possible problem when implanting such cultured skin substitutes. Therefore, protecting wound skin using skin substitutes is the primary treatment, followed by drug therapies with antibacterial ointments (7,8). A greater therapeutic effect may be obtained by direct application of a cultured skin substitute containing antibiotics as a drug formulation on wound skin. Although several acellular wound dressings capable of releasing antimicrobial drugs have already been investigated (9–11), they are not expected to promote tissue repair by biological cells. In a previous study, a dermal patch consisting of a three-dimensional cultured human skin model (LSE-high, Toyobo Co. Ltd., Osaka, Japan) loaded with antibiotics was evaluated as a new drug delivery system (DDS) with two functions: first, to protect the wound skin surface; second, to prevent secondary infection via antibiotics (12). Analysis using diffusion equations suggested that the antibiotic concentration in the model wound skin was mostly over the minimum inhibitory concentration (MIC) against several bacteria when the dermal patch (antibiotic-loaded LSE-high) was applied on

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the model wound skin surface; however, several issues remain. Briefly, preliminary experiments using MTT assay of LSE-high showed that cell viability had a tendency to decrease by loading antibiotics. Therefore, dermal patches consisting of gene-transfected cultured skin, which secrete endogenous antimicrobial peptides such as human β-defensins instead of exogenous antibiotics, can be a new DDS for the treatment of severe burns without decrease in cell viability. Human β-defensin families are mainly expressed in phagocytes and epithelia of multiple organs, and the expression of human β-defensin-2 (HBD-2) has been reported in the skin, urogenital tract, trachea, and lung (13,14). HBD-2 was also originally isolated from the desquamated scales of psoriatic skin, suggesting that it may be involved in cutaneous defense and inflammation (13,15). A cultured skin substitute that introduced endogenous antimicrobial peptide genes, such as HBD-2, can be utilized as a dermal patch capable of preventing secondary infection.

In the present study, the preparation method of gene-transfected cultured skin expressing a model protein was investigated as the first approach to establish a dermal patch that secretes HBD-2. Plasmid DNA, pQBI25, encoding the green fluorescent protein (GFP) gene derived from *Aequorea victoria*, and a three-dimensional rat or human cultured dermis model (CDM) were used as a model gene and model cultured skin, respectively. Using two kinds of transfection methods, gene-transfected CDMs were prepared and then evaluated. Hemagglutinating virus of Japan envelope vector (HVJ-E) was used for gene transfection, which is expected to be a very useful tool for gene therapy or gene diagnosis (16,17).

MATERIALS AND METHODS

Cell Culture

Rat skin fibroblasts (FR cells, ATCC CRT 1213) and the primary cells of human dermal fibroblasts (HDFs) were purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan) and Toyobo Co., Ltd. (Osaka, Japan), respectively. These were cultured with Dulbecco's modified Eagle's medium (DMEM; Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal bovine serum (FBS; ICN Biomedicals, Inc., Aurora, OH, USA) and mixed antibiotics (Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA) of penicillin (50 U/ml) and streptomycin (50 μg/ml) at 37°C in a 5% CO_2 humidified incubator.

Preparation of Three-dimensional Cultured Dermis Model

Three-dimensional CDMs of rats and humans were constructed from cultured FR cells or HDFs and a Collagen Gel Kit (Toyobo Co., Ltd.) including bovine-derived acidic type I collagen solution, collagen-neutralizing solution, calf serum, and an adjunctive culture tray (Toyobo Co., Ltd.). Briefly, 16 m of neutralizing solution, 2 ml of calf serum, and 4 ml of fibroblast suspension (1.88×10^5 cells/ml medium) were added to 8 ml of collagen solution at 4°C, and each mixture was gently and immediately agitated after each step. Four milliliters of the final mixture was applied to the inside of each Transwell with a diameter of 24 mm in a culture tray; the outside of each Transwell was filled with 12 ml of medium. The culture tray was incubated at 37°C in a 5% CO_2 humidified incubator for 7 days (18). The medium used for CDM culture was the same as DMEM in the cell culture. Figure 1 shows the whole CDM in Transwell and microscopic observations of the HE-stained CDM cross-sections.

Plasmid DNA Preparation

Plasmid DNA, pQBI25, encoding the GFP (rsGFP) gene, was purchased from Takara Bio, Inc. (Shiga, Japan). pQBI25 was transformed into *Escherichia coli* HB101 competent cells (Takara Bio, Inc.) by the heat shock method. The transformed *E. coli* cells were spread over LB agar medium containing 50 μg/ml ampicillin (ampicillin sodium, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and incubated at 37°C for 12 h. Colonies were collected from LB agar and then grown in a shaker in LB medium containing 50 μg/ml ampicillin at 37°C. After large-scale amplification for 16 h, pQBI25 was collected and purified from the bacterial pellet using the QIAfilter Plasmid Maxi Kit (Qiagen K. K., Tokyo, Japan) according to the manufacturer's instructions. Purified pQBI25 was dissolved with TE buffer (10 mM Tris-HCl/1 mM EDTA, pH 8.0), and the concentration of 3 μg/μl was used in gene transfection.

Gene Transfection

A commercial product of HVJ-E, HVJ Envelope Vector kit, GenomONE®-Neo (Ishihara Sangyo Kaisha, Ltd., Osaka, Japan), was used to transfect pQBI25 into cells and the rat and human CDMs. The second method in the manufacturer's instructions supplied with the kit was used for fundamental procedures. The reaction mixture for gene transfection was prepared by two processes, entrapment and transduction.

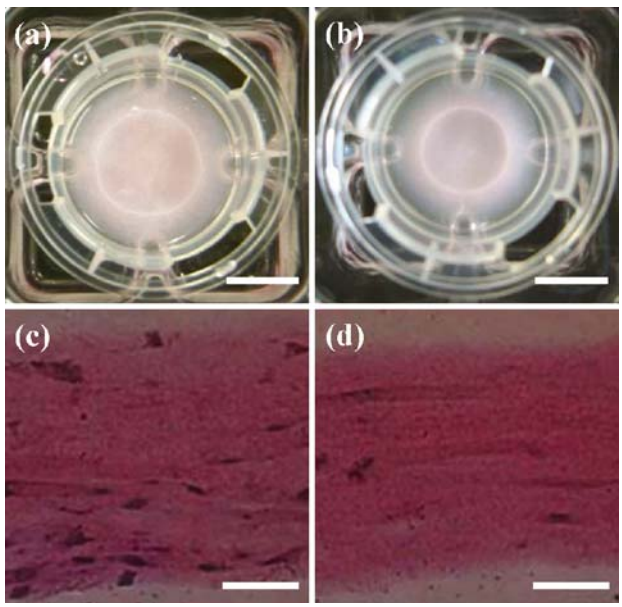

Fig. 1. Images of rat (**a**) and human (**b**) whole CDMs in Transwell and microscopic observation of cross-section of rat (**c**) and human (**d**) HE-stained CDMs. *Scale bars* are 10 mm (**a**, **b**) and 50 μm (**c**, **d**).

Table I. Reaction Mixture for the Second Method of GenomONE®-Neo

Step	Component	Volume (μl)	
		Per 60 mm Dish	Per CDM
Entrapment	HVJ-E (Neo)	80	40
	Buffer	20	10
	DNA/TE Solution (3μg/μl)	20	10
	Reagent B (entrapping reagent)	4	2
Transduction	Buffer	50	25
	Reagent C (transducing enhancer)	20	10
	Treatment Suspension	70	35

Briefly, pQBI25-entrapped HVJ-E was obtained by adding pQBI25 and the entrapping reagent into HVJ-E and mixing them gently, followed by centrifugation (10,000 × g at 4°C for 5 min), and then resuspended with buffer and the transducing reagent to obtain the reaction mixture (Table I).

In cell transfection, 1.0×10^5 FR cells or HDFs were pre-incubated in 4 ml of medium in plastic dishes (60 mm diameter) at 37°C in a 5% CO_2 humidified incubator overnight. These were replaced by the same volume of fresh medium and then 70 μl per dish of the reaction mixture was added. After incubation for 24 h under the same conditions, medium containing HVJ-E was removed from these cells and then 4 ml of fresh medium was added. The cells were additionally cultured for 24 h under the same conditions to obtain transfected FR cells or HDFs. These cells were used for observation with a fluorescence microscope (Olympus Corp., Tokyo, Japan) and fluorescence measurement (Fig. 2a).

To prepare and evaluate the gene-transfected CDM, two kinds of transfection methods, pre-transfection and post-transfection, were used. In pre-transfection, the gene was first transfected into FR cells or HDFs, and then the pre-transfected rat and human CDMs were prepared using these gene-transfected cells as mentioned above (Fig. 2b). In post-transfection, the rat and human CDMs were first prepared as mentioned above, and then the gene was directly transfected into the CDMs. Briefly, after culture for 7 days, the medium was removed from both inside and outside of the Transwell, and the CDMs were transferred to a 6-well plate which each well had a diameter of 3.5 cm and contained 3 ml of fresh medium. Two milliliters of fresh medium and then 35 μl of the reaction mixture for gene transfection were applied to the upper side of each CDM (inside each Transwell). After incubation for 24 h in a 5% CO_2 humidified incubator at 37°C, medium containing HVJ-E was removed from the upper side of the CDM and replaced with the same volume of fresh medium. The CDM was additionally cultured for 24 h under the same conditions to obtain post-transfected CDM (Fig. 2c). A subset of the pre- and post-transfected CDMs was used for confocal laser scanning microscopic observation of the cross-section, and the other CDM was used for fluorescence measurement and MTT assay.

Histology

Non-treated, pre-transfected, and post-transfected CDMs were placed in Tissue-Tek® OCT 4583 Compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and quickly frozen in isopentane at −20°C. The frozen tissue blocks were cut into 6–8 μm sections using a cryomicrotome (Leica CM3050S, Leica Microsystems, AG, Wetzlar, Germany) and collected on glass slides. FluoroGuard® Antifade Reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to prevent fluorescence degradation. Fluorescence was visualized using a confocal laser scanning fluorescence microscope (C1 plus, Nikon Corp., Tokyo, Japan).

Fluorescence Measurement

Non-treated or transfected fibroblasts were collected, and 300 μl of 0.1% Tween 20 solution was added. The cell suspension was then homogenized and subsequently freeze-thawed. These procedures were repeated two or three times to break cells. The cell homogenate was centrifuged (10,000 × g at room temperature for 10 min), and the total protein content of the supernatant was measured using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Inc.) based on the Coomassie Brilliant Blue (CBB) method. The GFP content was measured by fluorospectrophotometry at an excitation wavelength of 490 nm and fluorescent emission wavelength of 510 nm using standard GFP (Upstate Biotechnology, Inc., Lake Placid, NY, USA). The fluorescence intensity of a sample was standardized by dividing the obtained GFP by the total protein content (μg GFP/mg total protein).

A piece of non-treated or transfected CDM (1 cm × 1 cm) was minced and 300 μl of 0.1% Tween 20 solution was added. The tissue suspension was then homogenized and subsequently freeze-thawed. These procedures were repeated four or five

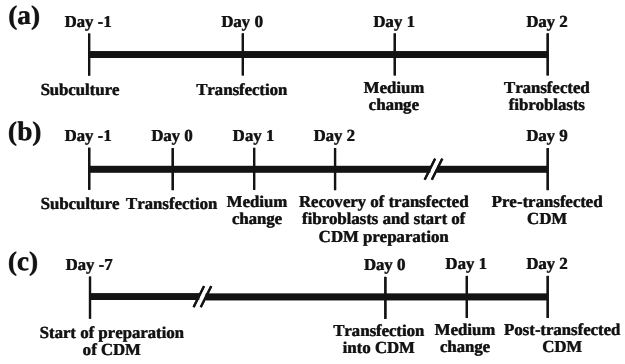

Fig. 2. Time course of preparation of transfected fibroblasts (**a**), pre-transfected CDM (**b**), and post-transfected CDM (**c**).

times. The tissue homogenate was centrifuged (10,000 × g at room temperature for 10 min), and the protein content of the supernatant was measured as mentioned above.

MTT Assay

The cell viability of CDM was evaluated by the calorimetric MTT conversion assay (19). The metabolic reduction of this soluble tetrazolium salt to a blue formazan precipitate is dependent on the presence of viable cells with intact mitochondrial function.

MTT solution (1.2 ml) at a concentration of 0.333 mg/ml in the medium was then applied to the underside of non-treated or transfected CDM. After reaction for 3 h at 37°C in a humidified atmosphere, the tissue sample was washed with pH 7.4 phosphate-buffered saline (PBS). A tissue disk of 8 mm diameter was made using a biopsy punch. The obtained tissue was transferred to a test tube and 1.0 ml of 0.04 N HCl in isopropanol was added to dissolve the formazan crystals. The absorbance of the solution was then spectrophotometrically measured at 570 nm.

Statistical Analysis

All experimental data are expressed as the mean ± S.E. Statistical analysis was performed using Student's *t* test or analysis of variance (ANOVA).

RESULTS

GFP Expression in Fibroblasts

pQBI25 was transfected into rat and human dermal fibroblasts (FR cells or HDFs) using GenomONE®-Neo. GFP expression was observed in transfected cells 48 h after transfection treatment by fluorescence microscopy (Fig. 3). At the same time, these transfected cells were collected and measured for the level of GFP expression. Fluorescence intensity of the transfected FR cells and HDFs was 1.9 ± 0.060 and 2.7 ± 0.33 μg GFP/mg total protein, respectively, significantly higher than that of the control cells (Fig. 4). Meanwhile, these GFP-expressing transfected cells were collected to prepare the pre-transfected human and rat CDMs.

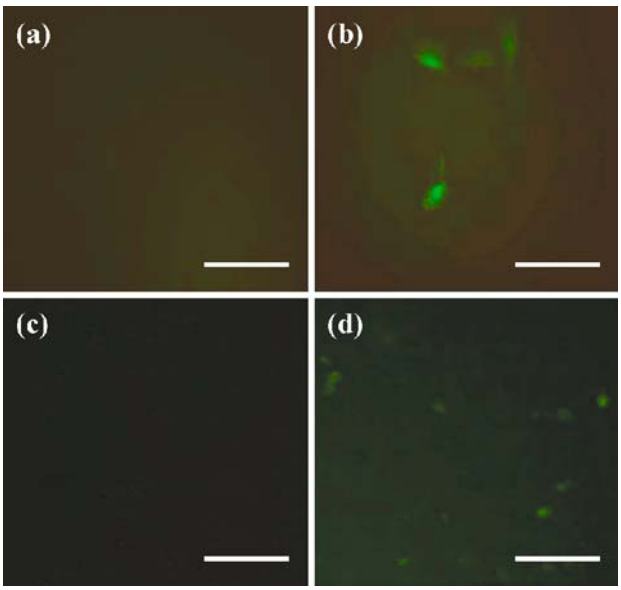

Fig. 3. Fluorescence microscopic observation of FR cell (**a**, **b**) and HDF (**c**, **d**). (**a**) and (**c**) are the controls and (**b**) and (**d**) are transfected fibroblasts. *Scale bars* are 20 μm.

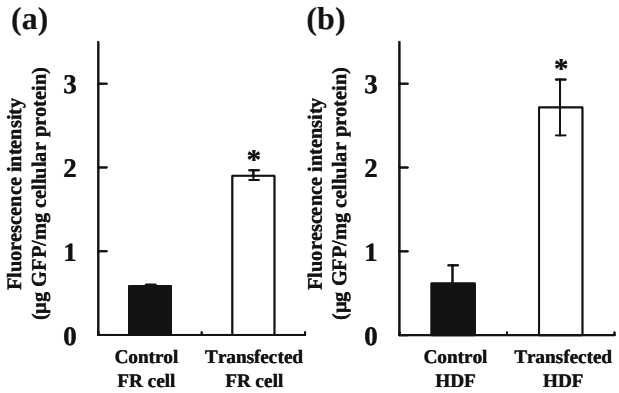

Fig. 4. Measurement of GFP expression in the control and transfected FR cell (**a**) and HDF (**b**) by fluorescence spectrophotometry. Data are shown as the mean ± SE ($n = 4$–6). * $p < 0.05$ vs control.

GFP Expression in CDM

Figure 5 shows the cross-sections of non-treated, pre-transfected, and post-transfected rat and human CDMs observed by confocal laser scanning fluorescence microscopy. GFP expression was detected in both the pre- and post-transfected CDMs, suggesting that the genes can be transfected into fibroblasts embedded in the three-dimensional cultured dermis using HVJ-E. No difference was observed between the rat and human CDMs. In addition, GFP expression was observed relatively uniformly throughout the collagen gel layer in the pre-transfected CDM, whereas GFP expression was localized in the collagen layer in the post-transfected CDM. These findings are assumed to be due to the different transfection method, i.e., the pre-transfection method probably allows gene-transfected cells to be dispersed uniformly into the collagen layer of CDM. However, in the post-transfection method, GFP expression was observed within the CDM, suggesting that genes can be transfected into cells in the CDM.

Figure 6 shows the fluorescence intensity in non-treated, pre-transfected, and post-transfected rat and human CDMs. Non-treated CDM 7 days after starting the CDM preparation was used as the control CDM, because the level of autofluorescence in the CDM was fairly constant after 5–10 days of the CDM preparation (data not shown). The fluorescence intensity in both the pre- and post-transfected CDMs was significantly higher than in the non-treated CDM. Although no significant difference was shown in the transfected CDM, the post-transfection method showed higher GFP expression in the CDM than pre-transfection. In addition, no significant difference was observed between the rat and human CDMs. HVJ-E can be a useful tool for gene transfection into three-dimensional cultured tissues,

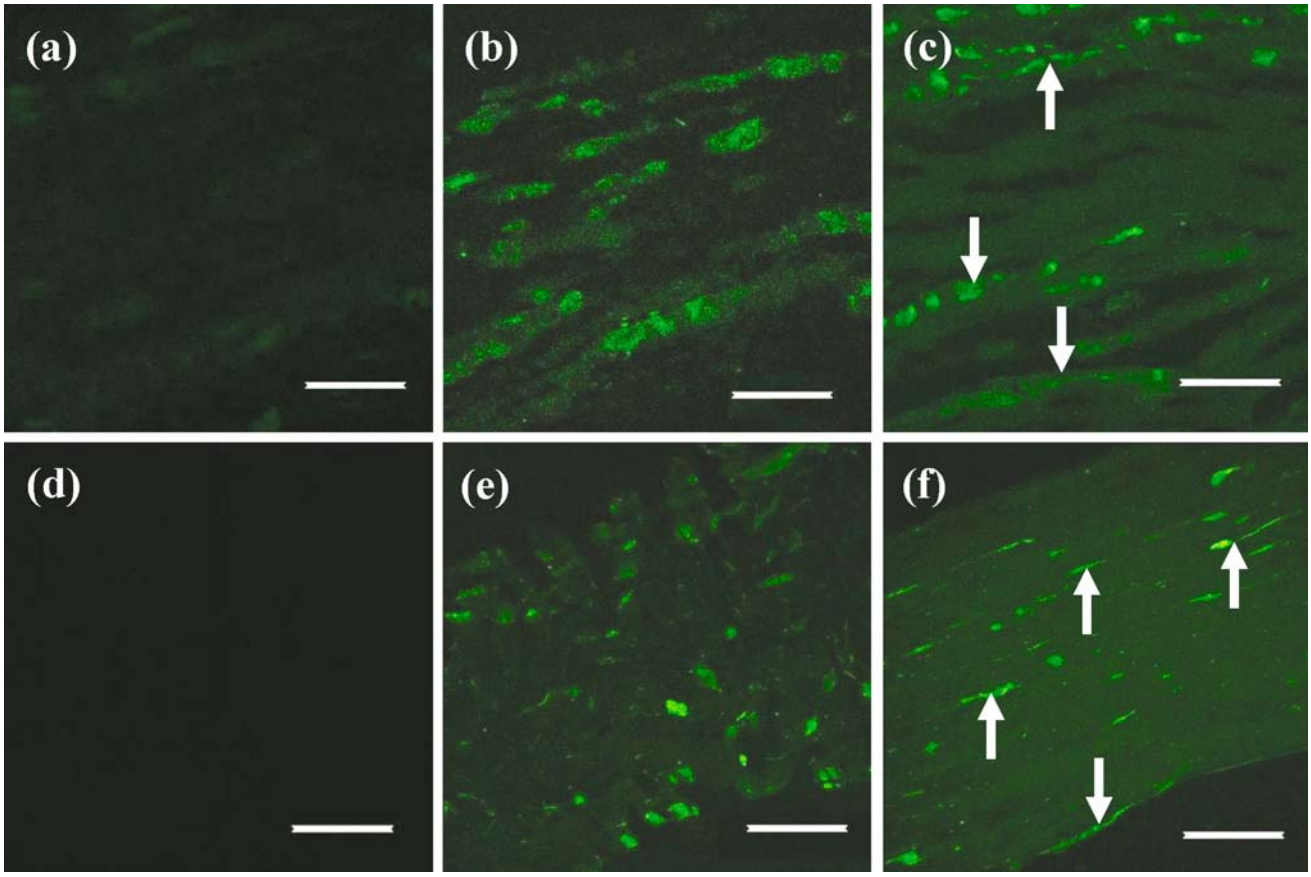

Fig. 5. Confocal laser scanning microscopic observation of CDM cross-section. (**a**), (**b**), and (**c**) are rat CDM and (**d**), (**e**), and (**f**) are human CDM. (**a**) and (**d**) are the control CDMs, (**b**) and (**e**) are pre-transfected CDM, and (**c**) and (**f**) are post-transfected CDM. *Arrows* show localized GFP along the collagen gel layer. *Scale bars* are 50 μm.

because HVJ-E has been reported in gene transfection not only into cells *in vitro* but also into animal tissues *in vivo* (20,21).

Cell Viability of CDM

The cell viability of these CDMs was measured by MTT assay to evaluate the effect of gene transfection on fibroblasts in the CDMs. Figure 7 shows the results of the MTT assay. Cell viability was expressed as a ratio of the value from a sample to that from a pure CDM 7 days after starting the CDM preparation. Similar results were obtained in the rat and human CDMs: slight decrease in viability was observed in both the pre- and post-transfected CDMs. Despite no significant difference, viability in the post-transfected CDM decreased slightly more than the pre-transfected CDM. This result may be due to the longer culture period of the CDM after transfection in the pre-transfection method. Briefly, because the pre-transfected CDM was cultured for 9 days after transfection into fibroblasts, viability may be recovered within this period; however, if a secretory protein-expressed

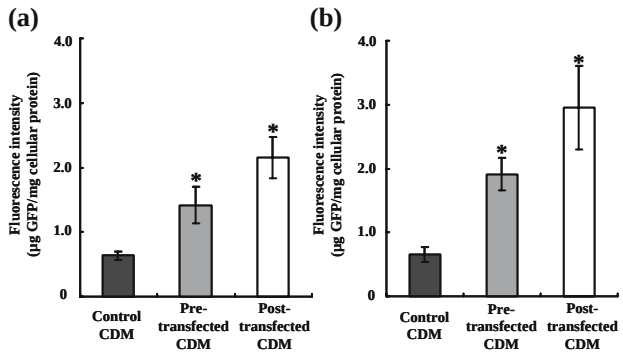

Fig. 6. Measurement of GFP expression in the control, pre-transfected, and post-transfected rat (**a**) and human (**b**) CDMs by fluorescence spectrophotometry. Data are shown as the mean ± SE ($n = 4$–6). * $p < 0.05$ vs control CDM.

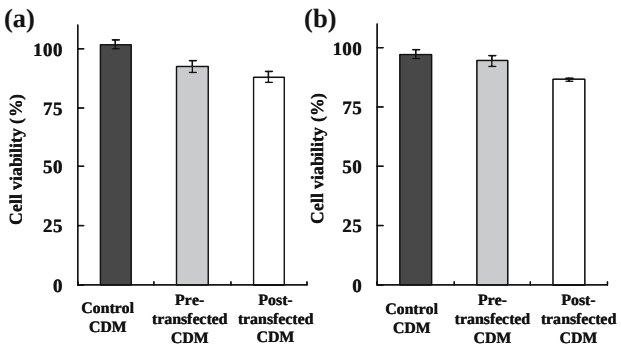

Fig. 7. Cell viability (%) of the control, pre-transfected, and post-transfected rat (**a**) and human (**b**) CDMs by MTT assay. Data are shown as the mean ± SE ($n = 3$–5).

gene is transfected into a CDM using the pre-transfection method, the produced protein may be released into culture medium during the culture period. Despite the difference between pre- and post-transfection methods, these results suggest that the present methods could be useful in preparing gene-transfected cultured skins with low cell damage.

DISCUSSION

Gene therapy is a tool to improve the treatment of acute wounds and accelerate healing of chronic wounds in skin. In the present study, the method for preparation a model protein, GFP, expressing gene-transfected cultured dermis, was investigated as the first approach to establish a dermal patch consisting of gene-transfected cultured skin, which secretes human endogenous antimicrobial peptides such as HBD-2. Two kinds of transfection methods, pre-transfection and post-transfection, were evaluated. GFP expressing the gene-transfected rat and human CDMs were successfully prepared using these two methods. In addition, slight decrease in viability was observed in these transfected CDMs. These results suggest that the present methods could be useful in preparing gene-transfected cultured skins with low cell damage.

However, in the present study, gene transfection or expression efficiency was not investigated in detail. Overexpression of transfected potent genes is required for a sufficient promoting effect on wound healing when such gene-transfected cultured skin is applied to wound skin (22,23). Gene overexpression can be achieved by improving gene transfection methods. For clinical application, transfection methods require both high transfection efficiency and no or low toxicity. HVJ-E has been developed as a non-viral transfection tool, consisting of an HVJ envelope with cell-fusion property and without viral genome RNA. Therefore, this tool can yield high gene expression efficiency, and is safer and easier than viral vectors. Meanwhile, gene transfection efficiency by HVJ-E may vary depending on the type of cell or gene and the features of the experimental system (24), suggesting that gene overexpression in gene-transfected cultured skin can be achieved by selecting an effective combination of cell, gene, and experimental system. The combination of HVJ-E and other non-viral transfection methods, i.e., electroporation and iontophoresis, can also be used to improve gene transfection efficiency. In addition, to establish useful gene-transfected cultured skins, it is important to regulate not only the level of gene expression and the secretion of peptides or proteins but also their period in the cultured skins. In the present study, gene expression was transient in the CDM; thus, further studies are necessary to improve the period of gene expression.

Although several issues remain, it is believed that genetically modified cultured skins are useful to improve wound healing. Recently, endogenous compounds and their relatives, such as bioactive peptides and oligonucleotides, have been used as new therapeutic drugs, in addition to low molecular weight organic compounds. Biological compounds and cells themselves may be the best therapeutic materials produced since the twentieth century until now. In addition, cultured skin substitutes containing biological cells are expected to be used as gene therapy vehicles not only for wound healing applications but also for the treatment of cutaneous or systemic genetic disorders (25). Since GFP was used as a model protein for preparation and evaluation of gene-transfected cultured skin in the present study, HBD-2 release from gene-transfected cells or cultured skin and the effect of HBD-2 or its gene on the cell viability of cultured skin should be evaluated. Further experiments are necessary to market cultured human tissues containing genes.

In conclusion, the present methods, both pre- and post-transfection, could become a useful tool in preparing gene-transfected cultured dermis with high cell viability. These methods can also be applied to other effective genes in wound healing. Gene transfection to cultured skin may produce several dermal patches that release potent endogenous bioactive peptides. This study is one of the first findings on DDS using biological tissues and genes. In the future, cells and tissues may be used as materials for new DDS. The present gene-transfected cultured skin for severely burned skin is an example.

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