

# Gene Transfer to Corneal Epithelium and Keratocytes Mediated by Ultrasound with Microbubbles

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**PURPOSE.** The cornea is an ideal organ for evaluating gene transfer because it can be treated noninvasively and monitored easily. The present study was performed to investigate the practical efficacy and safety of ultrasound (US) plus microbubble (MB)-mediated gene transfer to cornea.

**METHODS.** Cultured rabbit corneal epithelial (RC-1) cells were incubated in 24-well dishes with plasmid DNA having a green fluorescent protein (GFP) gene under a cytomegalovirus promoter. The cells were exposed to US under different intensities (1 MHz; power, 0.5~2 W/cm<sup>2</sup>; duration, 15-120 seconds; duty cycle, 20%-100%). The effect of simultaneous stimulation with MBs was also examined. Gene transfer was quantified by counting the number of GFP-positive cells under microscopy. Furthermore, *in vivo* gene transfer was examined by GFP plasmid injection into rabbit cornea and US exposure with MBs.

**RESULTS.** In the *in vitro* study, DNA exposure alone could not transfer gene into cultured RC-1 cells; US enhanced gene transfer slightly. Coexposure with MBs significantly increased gene transfer efficiency. In the *in vivo* study, DNA injection alone could transfer the gene to a limited degree, but plasmid injection plus US with MBs strongly increased gene transfer efficiency without apparent tissue damage, and gene transfer was achieved two dimensionally.

**CONCLUSIONS.** US with MBs greatly increases gene transfer to *in vivo* and *in vitro* corneal cells. This noninvasive gene transfer method may be a useful tool for clinical gene therapy. (*Invest Ophthalmol Vis Sci* 2006;47:558-564) DOI:10.1167/iovs.05-0889

A modality to efficiently deliver genes to living tissue is essential for gene therapy and genetic research. The basic technology of gene delivery can be divided into two categories,

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a virus vector-mediated method and a non-virus vector-mediated method.<sup>1-3</sup> The virus vector-mediated method can transfer the gene of interest with high efficiency, but concern about safety issues prevents clinical application for common diseases.<sup>3-5</sup> The non-virus vector-mediated method is comparatively safe, but gene transfer efficiency does not reach a satisfactory level.<sup>6-9</sup> Among these methods, the mechanical enhancing method is unique because it is free from a biochemical agent that has not been proven to be safe for humans; thus, clinical application might be more easily accepted. Electroporation can be used for this purpose but often results in severe cell damage.<sup>10,11</sup> In contrast, it recently became apparent that ultrasound (US) can enhance gene transfer to mammalian cells *in vitro* and *in vivo* without cell damage,<sup>12-16</sup> and US-mediated gene therapy has been reported in vessels and muscles of animal studies.<sup>17,18</sup> US is now widely used for clinical examinations and therapies, and its safety has been reliably established.

The cornea plays an important role in maintaining vision. Vision can be seriously impaired as a result of corneal cloudiness caused by insufficient wound healing or by the metabolic processes of cornea. Although corneal surgery is widely performed and may be performed even more often as more refractive surgery is performed, the cellular and molecular events that control wound healing within the corneal stroma are not well understood.<sup>19,20</sup> The cornea is an external tissue suitable for gene therapy because of its easy accessibility by surgical maneuvers, including US. In addition, gene transfer can be easily monitored by noninvasive methods such as microscopic observation.<sup>21</sup>

Microbubbles (MBs), which are gas bubbles measuring approximately 3  $\mu\text{m}$  in diameter, have been developed mainly as contrast agents to improve ultrasonographic images. They have shown promise in gene therapy for several reasons. MBs act as cavitation nuclei, effectively focusing ultrasound energy, and they can potentiate bioeffects. Evidence indicates that the ultrasound energy needed can be greatly reduced; therefore, the lower power used in diagnostic imaging systems may be sufficient to produce therapeutic effects.<sup>22-24</sup>

Simultaneous use of US and MBs has been found to increase gene transfer efficiency.<sup>25-32</sup> However, to our knowledge, few reports have been published of detailed analyses of US-mediated gene transfer with MBs. In this study, we performed a detailed analysis of gene transfer mediated by US with MBs using cornea, and we show two-dimensional gene transfer achieved by this method.

## METHODS

### In Vitro Study

**Cell Culture.** Rabbit corneal epithelial (RC-1) cells (JCRB0246) were obtained from Human Science Research Resource Bank (Tokyo, Japan) and incubated in modified Eagles medium (MEM; Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (FBS; Invitrogen-Gibco, Grand Island, NY) and streptomycin/penicillin (Wako, Osaka, Japan). All cells used in the studies were from passages 4 to 6.

**Plasmids.** An expression vector for the green fluorescent protein (GFP) gene, *pEGFP-N2*, a mammalian expression vector containing a cytomegalovirus immediate-early enhancer/promoter, was obtained from Clontech Co., Ltd. (Palo Alto, CA). Plasmids grown in *Escherichia coli* host strain XLI-blue were purified (Plasmid Kit; Qiagen, Valencia, CA) and suspended in TE buffer (pH 8.0) at a concentration of 1.0  $\mu\text{g}/\mu\text{L}$ . Plasmids were then suspended in phosphate-buffered saline (PBS; Invitrogen-Gibco), and the pH was adjusted to 7.35.

**Ultrasound Exposure.** RC-1 cells were collected with trypsin (Sigma-Aldrich), passed through a cell strainer (Becton Dickinson, Franklin Lakes, NJ), and put into a 48-well collagen type 1-coated glass bottom chamber (Asahi Technoglass, Chiba, Japan) filled with 400  $\mu\text{L}$  MEM with 10% FBS,  $4 \times 10^4$  cells/well. A plasmid solution 0.5  $\mu\text{L}$  was added to the medium. Immediately thereafter, US was exposed to the medium using a 6-mm probe generated by an US machine (Sonitron 2000; Richmar, Inola, OK). During exposure, the medium-containing cells were gently stirred by a magnetic stirrer (300 rpm). To induce MBs, perflutren protein type A microsphere (Optison; Amersham Health, Princeton, NJ)—a well-established, second-generation US medical diagnostic product with robust capability—was used. It is an albumin-shelled US contrast agent composed of approximately  $5$  to  $8 \times 10^8$  MBs per milliliter measuring between 2 and 4.5  $\mu\text{m}$  in diameter and filled with octafluoropropane. The indicated percentage was added to the plasmid solution (0.5, 0.25, or 0.1  $\mu\text{L}$ ), gently mixed, and left for 60 seconds. Immediately thereafter, the mixed solution was added to the dish and exposed to US as described.

**Gene Transfer Efficiency.** After gene transfer treatment, including plasmid alone or plasmid plus US exposure, the cells were incubated in a medium containing 10% FBS for 48 hours and then were observed by phase-contrast microscopy with or without a 515-nm filter (Olympus, Tokyo, Japan). A randomly selected field (4 fields/well with  $100 \times$  magnification) was photographed. The photographs were monitored by a NIH image analyzer, and the ratio of GFP-positive cells to all cells in each field was evaluated by masked observers. To determine the duration of GFP expression, GFP expression was evaluated on days 4, 8, 14, and 30.

**Cell Survival and Cell Damage.** Survival of cells was evaluated by counting living cells. Briefly, after 48 hours of incubation, the dishes were washed twice with PBS, four microscopic fields per well were photographed, and the living cells were counted. Cell damage was evaluated by lactate dehydrogenase (LDH)-releasing assay according to the manufacturer's instructions (Roche, Penzberg, Germany). Cells cultured on a 48-well plate were treated by each procedure, and cell damage was evaluated after 6 hours. The most severe cell damage was obtained by treatment with 1% Triton X (Sigma-Aldrich) and was used as a positive control. Cells with no treatment were applied as negative controls. Cell damage was expressed as: % LDH of sample – LDH of negative control/LDH of positive control – LDH of negative control.

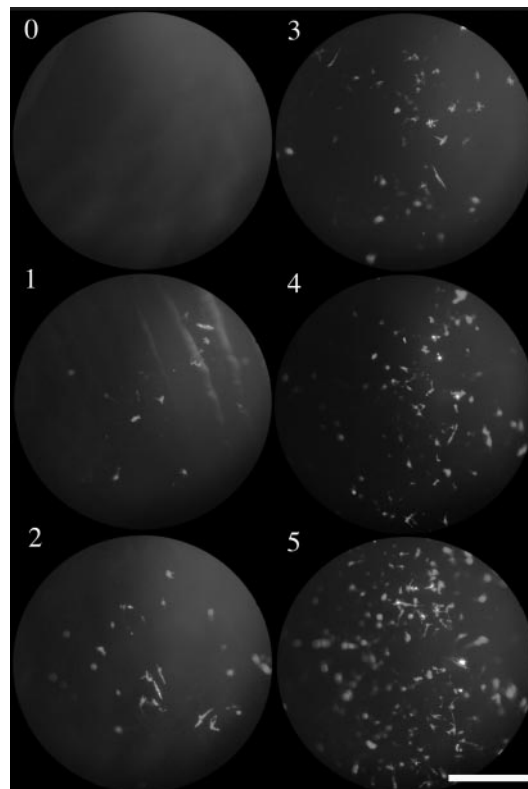
### In Vivo Study

After obtaining the approval of Kagoshima University ethics committee, all animals were used humanely in strict compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

New Zealand albino rabbits (male; age, 14 weeks; weight, 3000 g; KBT Oriental Co., Saga, Japan) were first anesthetized with intramuscular injection of ketamine hydrochloride (14 mg/kg) and xylazine hydrochloride (14 mg/kg). Plasmid 10  $\mu\text{L}$  mixed with PBS 2  $\mu\text{L}$  was injected under surgical microscopy into the center of each cornea using a syringe with a 30-gauge needle. Immediately thereafter, a 6-mm US probe was placed directly on the corneal surface, and US was generated. When MBs were used, plasmid 10  $\mu\text{L}$  mixed with perflutren protein (Optison; Amersham Health) 2  $\mu\text{L}$  was injected instead of a plasmid with PBS. The intensity of US was set at 1 MHz, 120 seconds, 50% duty cycle, and 3 US powers—1  $\text{W}/\text{cm}^2$ , 1.5  $\text{W}/\text{cm}^2$ , and 2  $\text{W}/\text{cm}^2$ —were examined.

**GFP Expression.** The presence of fluorescence signaling in the *in vivo* gene expression was determined using direct stereomicroscopy 72 hours after gene transfer (Olympus, Tokyo, Japan). The value of gene transfer was graded according to our scoring system by 3 masked observers. Only GFP expression scores agreed to by at least 2 of the observers were used in the analysis. Scoring criteria and representative photographs are shown in Figure 1. Transfection efficiency was expressed by a score of 0 to 5 (0 = not GFP positive; 5 = most intensely GFP positive).

**Histology.** All the animals were killed by overdose intravenous injection of pentobarbital. The eyes were enucleated 48 hours after treatment and were immediately frozen in liquid N<sub>2</sub>-cooled isopentane. Serial sections (6  $\mu\text{m}$ ) were sliced with a cryostat, placed on slides, and air dried. Regular fluorescent images and differential interference images were obtained by fluorescence microscopy (BX-FLA; Olympus, Tokyo, Japan) and were fitted using the IP Laboratory program (Scanalytics, Inc.; Fairfax, VA) on a personal computer. For light microscopy, the eyes were fixed with 3.7% formaldehyde in PBS, dehydrated with a graded alcohol series, and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin. For the electron microscopy, the tissue was fixed with 4% paraformaldehyde, and the specimens were then dehydrated in a series of graded ethanols and embedded in epoxy resin. Thin sections were cut on an ultramicrotome, stained with uranyl acetate-lead citrate, and observed with an electron microscope (JEM-100CX; JEOL, Tokyo, Japan), as described. All the specimens were then observed by 2 masked observers who received no information about the specimens.



**FIGURE 1.** Representative photographs of scoring criteria. Gene transfer efficiency was expressed as a score of 0 to 5. Scoring was performed by 3 masked observers according to the following criteria: 0: no positive cells; 1: 1 to 25 positive cells in each field; 2: 26 to 50 positive cells in each field; 3: 51 to 75 positive cells in each field; 4: 75 to 150 positive cells in each field; 5: 151 or more positive cells in each field. Bar, 400  $\mu\text{m}$ .

## Statistical Analysis

All values were expressed as mean  $\pm$  SEM. Analysis of variance with subsequent Scheffe test and Mann-Whitney *U* test was used to determine the significance of the difference in multiple comparison. Differences with a  $P < 0.05$  were considered significant.

## RESULTS

### In Vitro Study

**Gene Transfer by Ultrasound.** Numerous RC-1 cells were GFP positive after US exposure with the plasmid solution (Fig. 2A). Cells treated with the plasmid solution alone or the plasmid solution with MB perflutren protein (Optison; Amersham Health) alone did not show any fluorescein (Fig. 2B).

**Gene Transfer by US Plus MBs.** Under any of the following experimental US conditions—1 W/cm<sup>2</sup>, 60 seconds, duty cycle 50%; 1 W/cm<sup>2</sup>, 120 seconds, duty cycle 50%; 2 W/cm<sup>2</sup>, 60 seconds, duty cycle 50%; 2 W/cm<sup>2</sup>, 120 seconds, duty cycle 50%—the ratio of GFP-positive cells treated by US with MBs was significantly (two to four times) higher than that by US alone (Mann-Whitney *U* test,  $P < 0.01$  Fig. 2C). To identify the optimal conditions to transfer genes by US plus MBs, the following four parameters were examined.

**Duty Cycle.** According to our previous studies, three duty cycles (20%, 50%, or 100%) were examined under an intensity of 1 W/cm<sup>2</sup> and 60-second exposure with 20% MBs.<sup>29,30,32</sup> As a result, the GFP-positive cell ratios were 11.3% with a duty cycle of 20%, 18.0% with a duty cycle of 50%, and 35.6% with a duty cycle of 100%. The GFP-positive cell ratio of duty cycle 100% US plus MBs was significantly higher than that under the other three conditions (Scheffe test,  $P < 0.01$ ; Fig. 3A). On the other hand, the average number of survival cells was low with a duty cycle of 100%; for 79 cells per field, the duty cycle was 20%; for 171 cells per field, it was 50%; for 17.1 cells per field per, it was 100%. Cell damage was high in US, with a duty cycle of 20% or 100% in LDH assay (Fig. 3B). These results indicate that a duty cycle of 50% is preferable for obtaining high gene transfer efficiency with minimal cell damage.

**MBs.** Three concentrations of MBs—20%, 50%, and 100%—were examined under the US condition of 1 W/cm<sup>2</sup>, 60 seconds, and duty cycle 50%. The GFP-positive ratio was highest in cells treated by US with 20% MBs (Scheffe test,  $P < 0.01$ ;

Fig. 3C). Cell survival was lowest in the 100% MB group, and no significant difference was found between the 20% and the 50% MB groups (170.7 cells/field in 20% MBs, 163 cells/field in 50% MBs, 38 cells/field in 100% MBs). A similar tendency was found by LDH assay (data not shown). Thus, 20% MBs was preferable.

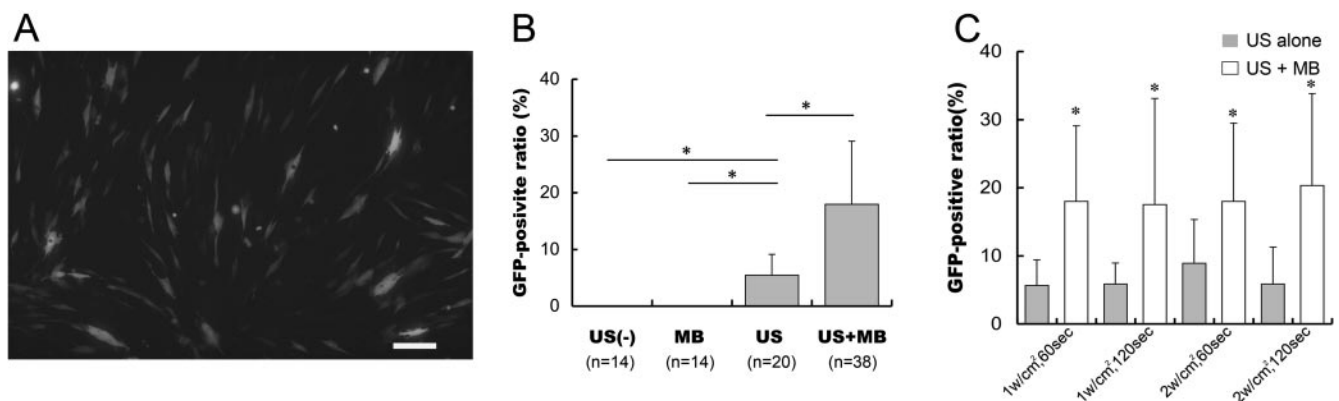
**Exposure Time.** According to our previous study, a US exposure duration exceeding 120 seconds significantly damaged cells<sup>29,32</sup>; thus, a US exposure time of 15 to 120 seconds was examined under the condition of 1 W/cm<sup>2</sup>, duty cycle 50%, with 20% MBs. As a result, the GFP-positive ratio was equally high for 60- and 120-second exposures (Fig. 3D). Cell damage was not apparent in any experimental group (Fig. 3E).

**US Intensity.** US intensities of 0.5, 1.0, 1.5, and 2.0 W/cm<sup>2</sup> were examined under the condition of 50% duty cycle and 60-second exposure with 20% MBs. A GFP-positive ratio of 0.5 W/cm<sup>2</sup> US group was significantly smaller than that in any of the other 3 groups (Scheffe test,  $P < 0.01$ ; Fig. 2F). LDH assay also indicated that cell damage was significantly high in the 1.5 and 2.0 W/cm<sup>2</sup> exposure groups ( $P < 0.01$ ; Fig. 3G), whereas little cell damage was observed in cells treated with 0.5 or 1.0 W/cm<sup>2</sup> US intensity. Representative images are shown in Figures 3H and 3I.

### In Vivo Study

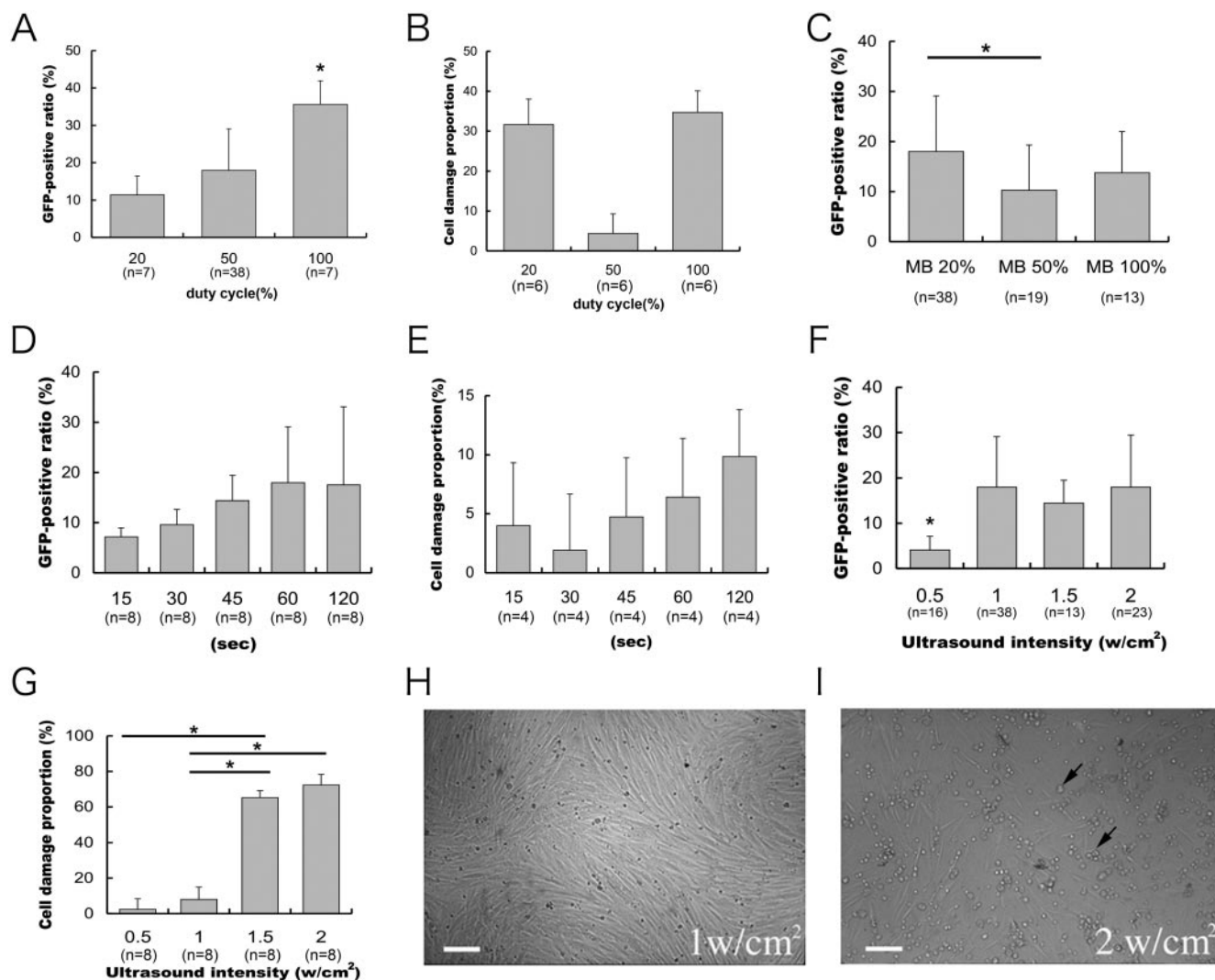
**Gene Transfer by US.** All GFP-positive cells in rabbit eyes underwent treatment. Eyes that received plasmid injection alone showed mild GFP-positive cells distributed within the injected area. GFP-positive cells were observed mainly in the corneal stroma. Average fluorescein score was 2.1 ( $n = 24$ ). On the other hand, eyes that received plasmid injection and US (1 W/cm<sup>2</sup>, 120 seconds, duty cycle 50%) had more GFP-positive cells (average score, 2.6;  $n = 14$ ) than those that received plasmid injection alone. However, the difference was not statistically significant (Fig. 4).

**Gene Transfer by US and MBs.** From the in vitro study, 20% MBs were chosen. The average score of GFP-positive cells in cornea was 3.5 for eyes treated by US of 1 W/cm<sup>2</sup>, 120 seconds, 50% duty cycle with MBs ( $n = 41$ ). A significantly higher score (4.5) was obtained with simultaneous treatment by US and MBs than by US alone (2.6) or MBs alone (2.0) (Scheffe test,  $P < 0.05$ ; Fig. 4), and it was achieved with US of 2 W/cm<sup>2</sup>, 120 seconds, and 50% duty cycle ( $n = 12$ ). GFP-



**FIGURE 2.** GFP-positive cells in gene-transferred rabbit corneal epithelial (RC-1) cells. (A) Fluorescence photograph of gene-transferred RC-1 cells. Many cells show a GFP-positive reaction in the whole cytoplasm. Bar, 100  $\mu$ m. (B) GFP-positive ratio by different methods. Numerous RC-1 cells were GFP positive after ultrasonic exposure with the plasmid solution. Cells treated with the plasmid solution alone or the plasmid solution with perflutren protein (Optison; Amersham Health) alone did not show any fluorescein (Mann-Whitney *U* test,  $*P < 0.01$ ). (C) GFP-positive ratio of cells treated by US exposure with MBs. GFP-positive ratio treated by US with MBs was significantly higher (2–4 times) than that by US alone (Mann-Whitney *U* test,  $*P < 0.01$ ). 1 W/cm<sup>2</sup>, 60 seconds, duty cycle 50%: US only,  $n = 14$ ; with MB,  $n = 38$ . 1 W/cm<sup>2</sup>, 120 seconds, duty cycle 50%: US only,  $n = 15$ ; with MB,  $n = 24$ . 2 W/cm<sup>2</sup>, 60 seconds, duty cycle 50%: US only,  $n = 15$ ; with MB,  $n = 23$ . 2 W/cm<sup>2</sup>, 120 seconds, duty cycle 50%: US only,  $n = 15$ ; with MB,  $n = 13$ .





**FIGURE 3.** Gene transfer by US and MBs under different conditions. (A) GFP-positive ratio at a different duty cycle. GFP-positive ratio of duty cycle 100% US plus MBs was significantly higher than under the other 3 conditions (Scheffe test,  $*P < 0.01$ ). (B) Cell damage at different duty cycle. LDH assay indicates that cell damage was high in US at duty cycle 20% or 100%. (C) GFP-positive ratio at different concentrations of MBs. GFP-positive ratio was highest in cells treated by US with 20% MBs (Scheffe test,  $*P < 0.01$ ). (D) GFP-positive ratio at different US exposure times. GFP-positive ratio was equally high at 60- and 120-second exposures. (E) Cell damage at different US exposure times. Cell damage was not apparent in any experimental group. (F) GFP-positive ratio at different US powers. GFP-positive ratio of 0.5 W/cm<sup>2</sup> US group was significantly smaller than that of any of the other 3 groups (Scheffe test,  $*P < 0.01$ ). (G) Cell damage at different US powers. LDH assay indicates that cell damage was significantly high in the 1.5 or 2.0 W/cm<sup>2</sup> exposure group ( $*P < 0.01$ ), whereas little cell damage was noticed in cells treated with 0.5 or 1.0 W/cm<sup>2</sup> US power. (H) Phase-contrast photograph of RC-1 cells immediately after US exposure. No apparent cell damage was observed. Bar, 100  $\mu$ m. (I) Phase-contrast photograph of RC-1 cells immediately after US exposure. Apparent cell damage was found in many cells (arrows). Bar, 100  $\mu$ m.

positive cells were observed exclusively where US was exposed (Fig. 5). US intensity greater than 3 W/cm<sup>2</sup> caused immediate stromal haziness, which resolved spontaneously with no treatment (data not shown).

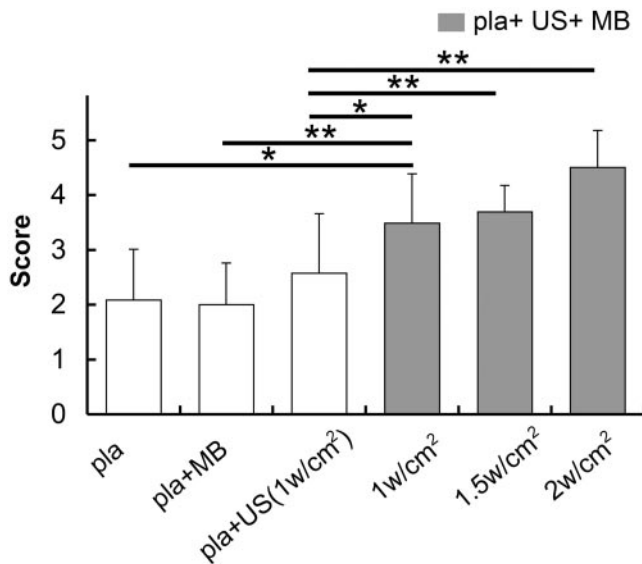
**Duration of GFP Expression.** The duration of GFP expression in cornea was evaluated in the eye treated with US and MBs under 20% MBs, 2 W/cm<sup>2</sup>, 120 seconds, duty cycle 50%. GFP-positive cells appeared on the following day and increased the strength and the number of GFP-positive cells for 8 days (Fig. 6). GFP-positive cells in cornea gradually decreased in number and strength over time, and the average GFP-positive score on day 14 was 2 ( $n = 13$ ) (Scheffe test  $P < 0.01$ ). On day 30, only a faint GFP-positive reaction was noticed ( $n = 4$ ).

**Histologic Findings.** Fluorescence microscopic examination showed that GFP was present in spindle-shaped cells in the targeted regions of the corneal stroma; thus, we speculated that keratocytes were also present (Fig. 7). Importantly GFP-

positive cells were limited to the US-exposed area. No GFP was detected in untreated cornea or other intraocular tissues, such as ciliary epithelial cells, trabecular meshwork, cells lining Schlemm canal, lens epithelial cells, or retina (data not shown). Light and electron microscopic examination 48 hours after treatment showed no corneal damage, such as opacity or persistent epithelial defects, after US plus MB treatment, even under the strongest power studied in this series (2 W/cm<sup>2</sup>, 120 seconds, duty cycle 100%; data not shown).

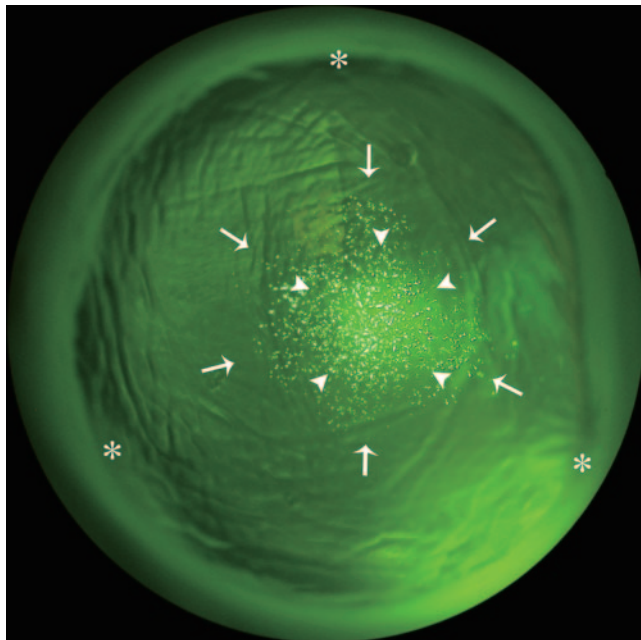
## DISCUSSION

US is broadly used for clinical imaging, and its safety has been reliably established. Only in the past few years have studies demonstrated US-enhanced gene delivery to mammalian cells in vitro and in vivo.<sup>12-18</sup> Moreover, the presence of MBs near

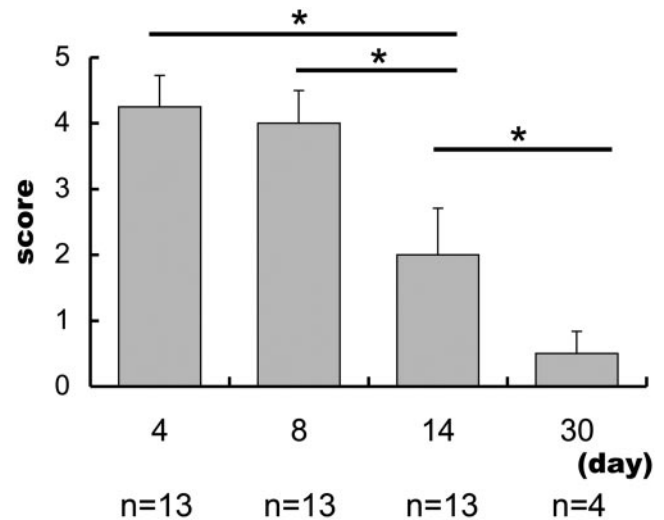


**FIGURE 4.** Gene transfer to rabbit cornea in vivo by US or MBs. Eyes that received plasmid injection alone or plasmid with MBs showed fluorescein-positive cells widely distributed within the injected area (plasmid alone,  $n = 24$ ; plasmid with MB,  $n = 8$ ). Eyes that received plasmid injection and US showed more numerous GFP-positive cells ( $n = 14$ ) than those that received plasmid injection alone. A significantly higher score was obtained by simultaneous treatment of US and MBs (1 W/cm<sup>2</sup>,  $n = 41$ ; 1.5 W/cm<sup>2</sup>,  $n = 13$ ; 2 W/cm<sup>2</sup>,  $n = 12$ ) than by US alone (2.6) or MBs alone (2.0) (Scheffe test, \* $P < 0.05$ , \*\* $P < 0.01$ ). Pla, plasmid DNA injection.

the cells further increases the gene transfection rate by lowering the acoustic pressure threshold needed to induce the microjets that penetrate the cellular membrane.<sup>25-32</sup> Sonography contrast-agent MBs with diameters ranging from 1 to 10

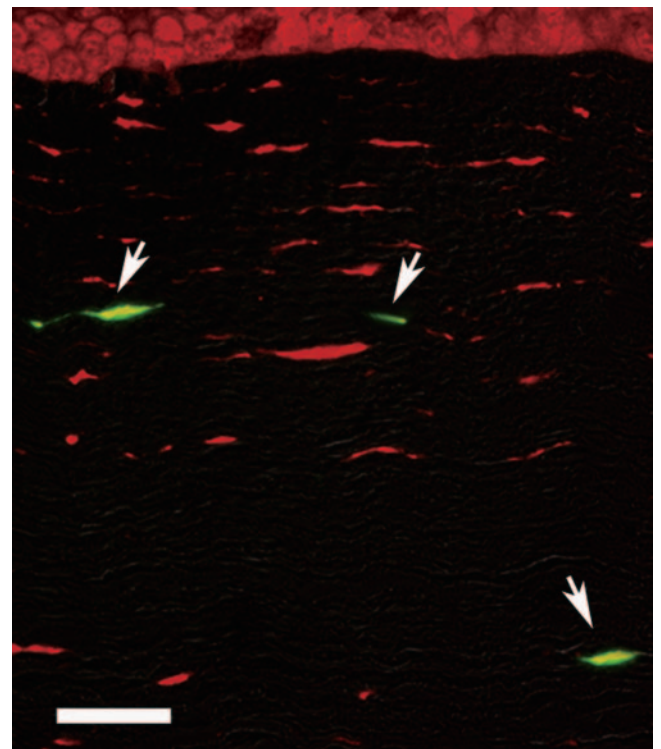


**FIGURE 5.** Fluorescence photograph of rabbit cornea 7 days after treatment. Twelve microliters plasmid with MBs was injected into the central cornea (arrows); this was followed by US exposure. Arrowheads indicate exactly where the US probe was placed. GFP-positive cells were specifically located where the US was exposed. A few GFP-positive cells are seen in the surrounding area. Asterisks indicate the corneal margin.



**FIGURE 6.** Duration of GFP expression in rabbit cornea was evaluated in the eye treated with US and MBs. GFP-positive cells appeared on the following day and increased the strength and number of GFP-positive cells for 8 days. GFP-positive cells in cornea gradually decreased in number and strength over time, and the average GFP-positive score on day 14 was 2 ( $n = 13$ ) (Scheffe test, \* $P < 0.01$ ). On day 30, only faint GFP-positive reaction was observed ( $n = 4$ ).

$\mu\text{m}$  are considered ideal for this purpose. To gain further insight into the practicability of sonoporation, the cornea was selected as the subject of the present study because it can be noninvasively treated and monitored with the use of standard ophthalmological equipment, allowing visualization the cornea and the surrounding tissues under high magnification and per-



**FIGURE 7.** Fluorescence microscope photograph of rabbit cornea after treatment with US and MBs (2 W/cm<sup>2</sup>, duty cycle 50%, 120 seconds). GFP is present mainly in spindle-shaped cells in the targeted regions of the corneal stroma (arrows). Bar, 10  $\mu\text{m}$ .

mitting easy determination of gene transfection and tissue damage.

To establish the optimal condition of US MB-mediated gene transfer to cornea, 98 different patterns of various US conditions were examined in vitro in preliminary studies because it is difficult in practice to study so many different patterns using rabbit eyes in vivo. First, the duty cycle of US was evaluated. Results clearly showed that a duty cycle of 100% is most effective to transfer genes. However, cell damage was too strong with a duty cycle of 100%. Therefore, a duty cycle of 50% was chosen for our purpose. Second, the amount of MBs perflutren protein (Optison; Amersham Health) was evaluated. Cytotoxicity was highest in 100% MBs, and gene transfer efficiency was highest in 20% MBs. Thus, 20% MBs was chosen. Third, the exposure time of US was evaluated. An exposure time longer than 120 seconds significantly damaged the cells, and an exposure time shorter than 60 seconds could not transfer the gene efficiently. Sixty- and 120-second exposure provided almost identical gene transfer efficiency and cell toxicity. Finally, US power was studied. It is understandable that high US power can transfer the gene to cells but also induce strong cell damage. Considering gene transfer efficiency and cellular damage, a US power of 1 or 2 W/cm<sup>2</sup> should be appropriate.

Contrary to in vitro experiments, US alone revealed no significant enhancement of gene transfer compared with controls. Because the cornea is composed of multiple cell layers and abundant extracellular matrix, it is postulated that higher US intensities are needed to produce sufficient microjets to damage cells or inject genes into cells. Adding MB with the plasmids, on the other hand, increased gene transfer efficiency by twofold to threefold. Optic examination showed that GFP was present mainly in keratocytes at the US-targeted regions of the corneal stroma. GFP was not detected in the untreated area of the cornea or other intraocular tissues. It is noteworthy that US induced no immediate corneal damage, such as opacity or defect of corneal/ciliary epithelial cells. Surrounding trabecular meshwork, cells lining Schlemm canal, lens epithelial cells, and retina seemed to be intact.

Previously, Wang et al.<sup>22</sup> reported perflutren protein (Optison; Amersham Health) with DNA could achieve effective gene transfer in muscle. However, effective gene transfer could not be performed in cornea in the present study. Because the cornea has more abundant extracellular materials than muscle, perflutren protein (Optison; Amersham Health) alone might not be effective for transferring DNA to cells.

Viral vector- or liposome-mediated gene transfer methods are effective for transferring genes to almost every cell that comes in contact with vectors<sup>33,34</sup> (e.g., the adenoviral vector). Because the long-term effects of gene transfer on recipient cells remain unclear, the cells to which genes are transferred should be strictly controlled. Especially when transferring genes to the cornea, the pupillary area should be avoided so as not to threaten vision. The present method greatly reduces this concern because of the precise targeting it makes possible.

In previous studies we reported that electroporation-mediated gene transfer can be achieved in rat cornea.<sup>35-37</sup> However, in larger animals, including rabbit, electroporation may be hazardous because the cardiovascular system is sometimes impaired by treatment (data not shown). Therefore, at present it might not be easy to apply electroporation for human gene therapy.

When the vehicle with the gene of interest is injected into tissue through a local gene delivery method such as liposome or viral vector injection, the vehicle spreads in every direction three dimensionally. Accordingly, gene transfer is achieved three dimensionally in a similar way. However, in the treatment of a surface organ such as skin or cornea, two-dimen-

sional gene transfer is sometimes preferable. Using the characteristics of ultrasound, the present method achieved two-dimensional gene transfer.

Thirty percent to 40% of the total corneal area was covered by a 12- $\mu$ L plasmid injection. Gene transfer was achieved in the area exposed to US. To expand the area of gene transfer, it was necessary to improve the injection method and the US probe. The present method can be applied to a variety of purposes, such as making it feasible to use highly fragile proteins.<sup>38,39</sup> With the present method, we were able to superficially deliver genes to a targeted tissue surface area two dimensionally. Thus, this sonoporation method could become a valuable modality for therapy and research that require surface-localized drug delivery or gene induction.<sup>40</sup>

Many questions remain before the present method can be applied clinically. The optimal US condition required for efficient gene transfer is highly dependent on the tissue, and the biologic structures responsible for the US effect vary greatly among species. These issues must be carefully explored. Of necessity, there is another limitation to the present study. We examined a rabbit corneal epithelial cell line in an the vitro study, but the gene-transferred cells were mainly keratocytes in the in vivo study. In our preliminary study, rabbit keratocytes were cultured; however, the morphology of these cells soon changed and differentiated into unidentifiable cells. Therefore, we used the rabbit corneal cell line RC-1.

To summarize, our studies show that using US in conjunction with commercially available MBs can enhance gene delivery to cells without damaging tissues. Although this modality was highly dependent on acoustic conditions and bubble concentration, its simplicity and noninvasiveness may provide a new avenue for microinjecting various substances into a wide range of living tissues.

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