IMMUNOHISTOLOGY

Cryosections from cell sheets were immunostained with monoclonal anti–keratin 3 antibodies (AE5, Progen Biotechnik), anti–β_1 integrin antibodies (P5D2, Santa Cruz Biotechnology), or anti-p63 antibodies (4A4, Santa Cruz Biotechnology) and fluorescein isothiocyanate–labeled or rhodamine-labeled secondary antibodies (Jackson ImmunoResearch Laboratories). Nuclei were costained with Hoechst 33342 (Molecular Probes) or propidium iodide (Sigma). Stained cells were observed using confocal laser scanning microscopy (LSM-510, Zeiss). The same concentration of corresponding normal nonspecific IgG provided negative controls, and native human corneal and limbal tissues were used as positive controls.

TRANSPLANTATION OF CELL SHEETS TO THE EYE

We removed the conjunctival and subconjunctival scar tissue from the cornea up to 3 mm outside the limbus to reexpose corneal stroma (Fig. 2, and a video clip in the Supplementary Appendix, available with the full text of this article at www.nejm.org). Subsequently, the harvested sheet of autologous oral mucosal epithelial cells was placed directly onto the exposed transparent stromal bed as described previously.[6,23] No sutures were required. The grafted corneal surface was then covered with a soft contact lens for protection during healing. After surgery, topical antibiotics (0.3 percent ofloxacin) and steroids (0.1 percent betamethasone) were initially applied four times a day and then tapered to three times a day. During the first week after surgery, betamethasone (1 mg per day) was administered orally to reduce postoperative inflammation. One month after surgery, the administration of topical corticosteroids was changed from 0.1 percent betamethasone to 0.1 percent fluorometholone. Because the patients had severe dry eye, proper wound healing could not be expected without tear supplementation. Preservative-free artificial tears were frequently used, and the puncta lacrimale of all the patients were occluded to increase tear retention.

RESULTS

CHARACTERIZATION OF TISSUE-ENGINEERED EPITHELIAL-CELL SHEETS

We compared cultured autologous oral mucosal-cell sheets with endogenous tissue both functionally and phenotypically. Oral mucosal epithelial cells cultured under these culture conditions resemble corneal epithelium, with three to five cell layers, small basal cells, flattened middle cells, and polygonal and flattened superficial cells (Fig. 1B), more than they resemble native oral mucosal epithelium (Fig. 1C), which is much thicker than corneal epithelium (Fig. 1D). The optical transparency of harvested cell sheets was equal to that of corneal epithelial-cell sheets originating from limbal stem cells (data not shown).[23]

Ultrastructural examination revealed an architecture of well-structured, compact, multilayered cell sheets with the expected microstructures of the native cells, including microvilli (Fig. 1E), tight junctions, desmosomes, and basement membrane. Such morphologic characteristics are similar to those of corneal epithelium in vivo. Native corneal epithelial cells and oral mucosal epithelial cells express keratin 3 as a characteristic phenotypic marker, and harvested epithelial-cell sheets also express keratin 3 (Fig. 1F).

The mean (±SE) colony-forming efficiency, calculated as the ratio of the number of stem or progenitor cells that can produce colonies to the total number of cells in the harvested tissue, was 2.1±0.9 percent for all four patients (with measurements performed in triplicate in each patient), confirming the presence of progenitor cells among the isolated oral mucosal epithelial cells. Correspondingly, β_1 integrin, reported to be an epithelial stem-cell and progenitor-cell marker[24] susceptible to digestion by trypsin, remained intact in the basal cells (Fig. 1G). The basal cells in the multilayered cell sheets also express p63 (Fig. 1H), a putative epithelial stem-cell marker.[25]

CLINICAL RESULTS OF TRANSPLANTATION OF THE CELL SHEET TO THE CORNEAL SURFACE

Attachment of the cell sheet to the stromal bed was spontaneous and uniform (Fig. 2, and video clip in the Supplementary Appendix). Within several minutes after placement without sutures, the grafted cell sheets remained intact and stably bound to the stromal surfaces, even after the extensive application of eyedrops. This observation is consistent with previous experiments with rabbit models, in which transplanted sheets of oral mucosal epithelial cells readily resisted outward displacement when the perimeters were pulled with forceps.

Immediately after surgery, the transplanted corneal surface was clear and smooth, without observable vascularization. Within one week, slit-lamp ex-

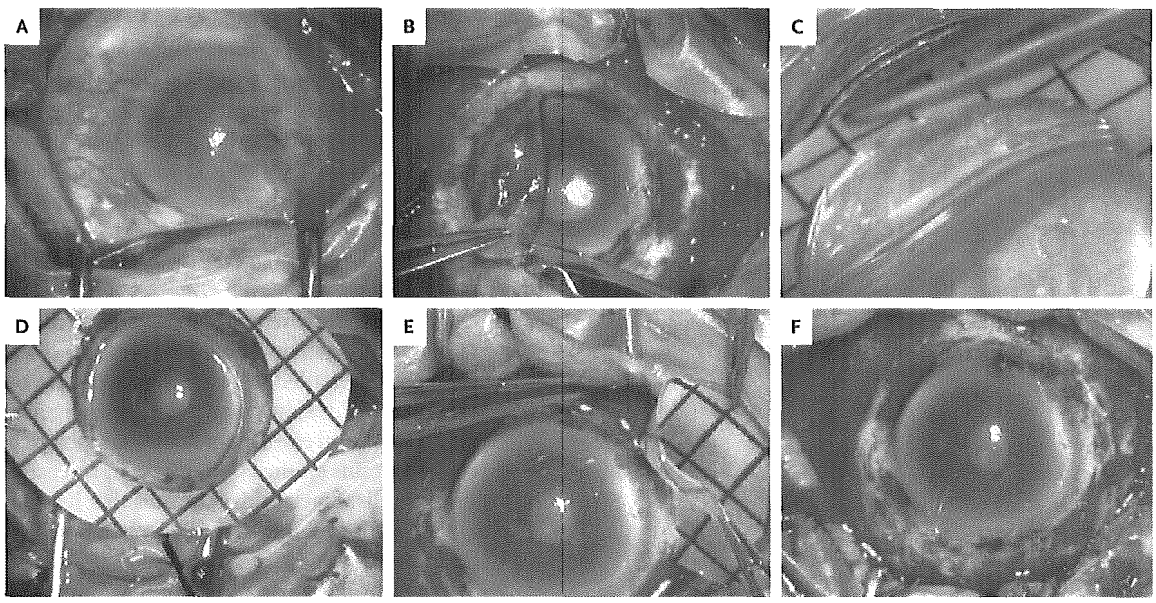

Figure 2. **Transplantation Procedures for Tissue-Engineered Autologous Epithelial-Cell Sheets.**

Preoperatively, the entire corneal surface was covered by conjunctival tissue with neovascularization (Panel A). In Panel B, conjunctival tissue over the cornea is surgically removed to reexpose transparent corneal stroma. Then, the sheet of tissue-engineered epithelial cells is harvested from a temperature-responsive culture insert with the use of a doughnut-shaped supporter ([black-and-white squares] Panel C) and placed on the stromal bed (Panel D). The sheet adheres to corneal stroma in a few minutes without sutures, and the supporter is removed (Panel E), leaving the cell sheet on the stroma (Panel F). A video clip can be viewed in the Supplementary Appendix, available with the full text of this article at www.nejm.org.

amination with fluorescein sodium staining showed complete reepithelialization of the corneal surface in all four eyes, revealing the tight junction–mediated barrier function. Corneal transparency was restored without any defects of the corneal epithelium. In all eyes, stromal vascularization gradually recurred in the peripheral cornea but not in the central zone. This vascularization was unlike subepithelial vascularization accompanied by conjunctival ingrowth, since it was localized to the deeper stroma and did not show the abnormally high fluorescein permeability characteristic of conjunctival epithelium.

During a mean follow-up period of 14 months, corneal transparency was maintained (Fig. 3 and Table 2). Maximally improved visual acuity was obtained 6, 2, 10, and 8 weeks after transplantation for Patients 1 through 4, respectively, and became stable thereafter. The length of time until visual acuity improved seemed to correspond to the length of time until the corneal stroma became less opaque. No complications were observed.

DISCUSSION

Our study shows that tissue-engineered cell sheets from autologous oral mucosal epithelium may serve as effective substitutes for allografts of limbal tissue in the reconstruction of the corneal and limbal surfaces. Four patients (four eyes) were consecutively treated with this approach, and corneal transparency was restored and postoperative visual acuity improved remarkably (Table 2). During the follow-up period, all corneal surfaces remained transparent, and there were no serious complications.

We developed this strategy on the basis of several observations from cell biology and medicine. First, in vivo oral mucosal epithelium expresses keratin 3, which is also expressed by the corneal epithelium but not by the epidermis.[1,27] Second, the excision of a small piece of oral mucosal tissue from the patient is straightforward, and the resulting wound heals within several days without incident or scarring. Third, transplantation of autologous

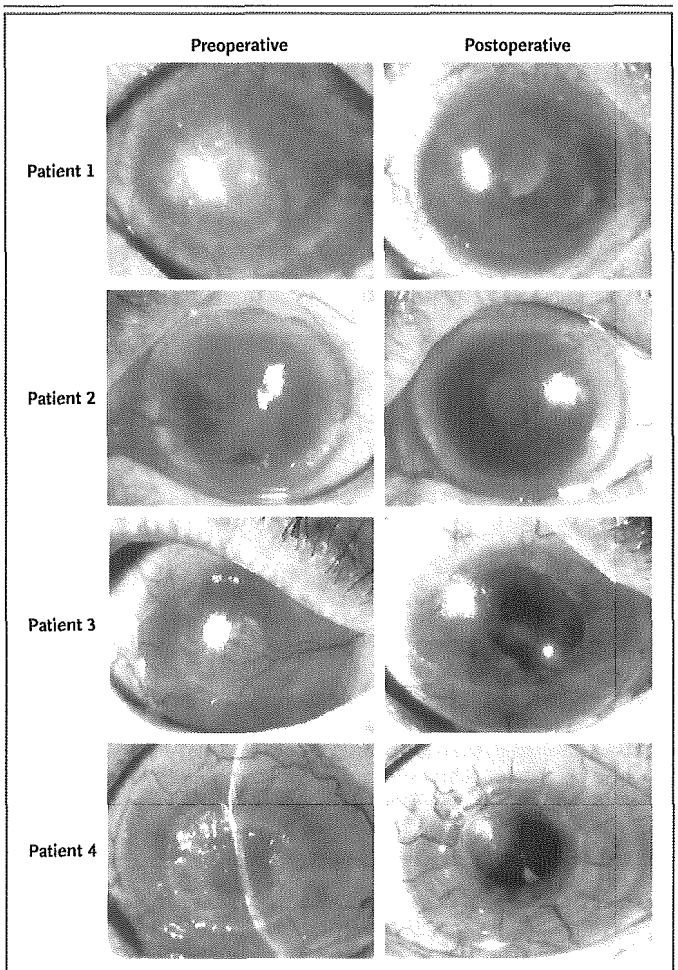

Figure 3. **Eyes of Patients before and after Transplantation of Sheets of Tissue-Engineered Autologous Epithelial Cells.**
These photographs were taken just before transplantation of the cell sheets and postoperatively at 13, 14, or 15 months.

buccal mucosal grafts directly onto ocular surfaces was previously reported in human patients[28] for the purposes of treating corneal ulcers, corneal perforations, and lid abnormalities (e.g., marginal entropion and trichiasis); these grafts are not useful for improving vision, since they contain opaque subepithelial fibrous tissue. In contrast, the transparency of carrier-free sheets of tissue-engineered epithelial cells fabricated from oral mucosal epithelial cells is similar to the transparency of corneal epithelial-cell sheets originating from limbal stem cells.[23]

Reconstruction with autologous oral mucosal epithelial cells offers substantial clinical advantages over allogeneic transplantation for treating severe diseases such as the Stevens–Johnson syndrome and ocular pemphigoid. It averts the risks of allogeneic immunorejection and immunosuppression. Severe tear-film and lid abnormalities often associated with these diseases continue to be a challenge, since immunologically driven inflammation of the ocular surface persists chronically in these patients.

Although decisive epithelial stem-cell markers that could provide evidence of the presence of these stem cells in grafted cell sheets have not yet been established,[29] results from colony-forming assays for oral mucosal epithelium show that excised oral tissue contains epithelial stem cells or at least progenitor cells. Since ocular surfaces that have been grafted with cell sheets retain their transparency for more than one year, and because the life spans of transient amplifying cells (cells committed to epithelial differentiation) are believed to be less than one year,[30] we conclude that progenitor cells with the potential to differentiate into new corneal epithelial phenotype are present in autografts of cell sheets.

Conjunctival epithelial cells invade the cornea after allogeneic transplantation because of the gradual depletion of allogeneic corneal epithelial cells due to epithelial rejection or stem-cell depletion.[31-33] It is unknown whether this also applies to autologous transplants. In the four eyes we studied, limited stromal vascularization occurred within a few months after transplantation of the cell sheet and reached a stable state within six months, with no appreciable growth thereafter. This stromal vascularization was observed only beneath cell sheets on peripheral corneas and should be distinguished from the subepithelial neovascularization accompanied by conjunctival ingrowth that results from the stem-cell loss associated with allografts, which occurs several months after transplantation. This finding suggests that grafted oral mucosal epithelial cells remained on the ocular surface.

It is possible that the reduction in host immunologic reactions associated with the grafting of autologous cells may minimize epithelial rejection, but further study is needed. The limited stromal neovascularization that we observed is probably caused by angiogenic factors secreted from tissue-engineered epithelial-cell sheets fabricated from oral mucosal epithelial cells originally located in

Table 2. **Surgical Outcome in Four Patients Who Received Transplants of Tissue-Engineered Autologous Oral Mucosal-Cell Sheets.**

Patient No.	Best Corrected Visual Acuity in Damaged Eye		Corneal Opacity (Grade)*			Complication	Months of Follow-up
	Preoperative†	Postoperative	Preoperative	1 Month after Surgery	At Last Observation		
1	Counting fingers	20/100	3	2	1	None	15
2	20/2000	20/25	3	1	1	None	14
3	Hand motion	20/300	3	1	1	None	14
4	20/2000	20/50	3	1	1	None	13

* The extent of corneal opacity was graded by three masked observers on the basis of the slit-lamp examination with a previously described system[26] and modifications for ocular-surface diseases. Grade 0 indicates clear or trace haze, grade 1 mild opacity, grade 2 moderately dense opacity partially obscuring details of the iris, and grade 3 severely dense opacity obscuring details of the intraocular structure. Grading is based on the opacity observed in all corneal layers, including epithelium, stroma, and endothelium.

† The visual acuity of patients who could not read a visual-acuity chart at a distance of 0.5 m was assessed by asking whether they could see the number of fingers held up by the examiner. If they could not, visual acuity was assessed by the patient's ability to see hand movement by the examiner.

vivo on the substantia propria, which is rich in vessels. However, the production of antiangiogenic factors such as thrombospondin by keratocytes[34] may limit vascularization to peripheral areas.

We observed that the transplanted cell sheets became more transparent and achieved smoother, integrated surfaces on the corneal stroma, further resembling normal corneal epithelium; a plateau was reached one to three months after transplantation. Originally, oral mucosal epithelium, located on substantia propria, is morphologically distinct from corneal epithelium in that it is much thicker and multilayered and has an irregular surface (Fig. 1C). The use of temperature-responsive harvesting allows the grafted carrier-free oral mucosal epithelial cells to interact immediately and directly with patients' corneal stromal keratocytes without interference from cell carriers such as fibrin gel and amniotic membranes.

Our transplantable epithelial-cell sheets used the common 3T3 feeder-layer method originally developed for the production of autologous epidermal-cell grafts[35] and used in the culture of other epithelial cells from various tissue sources, including the limbus.[16] This method has been clinically applied since the 1980s for the treatment of various skin conditions, including burns and giant nevi, although the Food and Drug Administration classifies these grafts as xenografts.

In summary, we have shown that sheets of tissue-engineered epithelial cells fabricated ex vivo from autologous oral mucosal epithelium are effective for reconstructing the ocular surface and restoring vision in patients with bilateral total stem-cell deficiencies. Long-term follow-up and experience with a large series of patients are needed to assess further the benefits and risks of this method, which offers the potential to treat severe ocular diseases that are resistant to standard approaches.

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TISSUE ENGINEERING
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Enhancement of Gap Junctional Intercellular Communication of Normal Human Dermal Fibroblasts Cultured on Polystyrene Dishes Grafted with Poly-*N*-isopropylacrylamide

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ABSTRACT

Technology developed to allow recovery of cells without enzyme treatment, involving a dish grafted with a thermoreactive polymer gel of poly-*N*-isopropylacrylamide (PIPAAm), was found to significantly enhance gap junctional intercellular communication (GJIC) in normal human dermal fibroblasts (NHDF cells). NHDF cells were cultured for 4 days on PIPAAm-grafted dishes irradiated with various doses of electron beams, and GJIC was assayed by the scrape-loading dye transfer method. The area of dye transfer was greater in the PIPAAm-grafted dishes than in the control culture dishes, indicating that the PIPAAm-grafted dishes enhanced the GJIC of NHDF cells. Connexin-43 (Cx43) expression was analyzed because Cx43 is considered to be a main component of the gap junctional channel. PIPAAm-grafted dishes irradiated with 100, 250, or 500 kGy of electron beams showed significantly enhanced expression of Cx43-NP, Cx43-P1, and especially Cx43-P2. Enhanced expression of Cx43-P2, a functional transmembrane protein, may be related to the promotion of GJIC. These results suggest that the PIPAAm-grafted dish not only enables the enzyme-free recovery of a cell monolayer for use in the construction of a three-dimensional artificial tissue, but also significantly contributes to the enhancement of GJIC, which may partly promote tissue strength on the surface of the PIPAAm-grafted dish.

INTRODUCTION

GAP JUNCTIONS exist on the cell membrane and work as intercellular channels that allow the exchange of substances with molecular masses up to 1 kDa, such as ions, sugars, and amino acids, by the function called gap junctional intercellular communication (GJIC).[1–3] Gap junctions are constructed from transmembrane proteins, called connexins,[4,5] that form a hemichannel, called a connexon. GJIC is suggested to be well correlated with passage of metabolites,[6] cell proliferation,[7] and cell differentiation[8]; thus, enhancement of the function of the gap junction is supposed to be important in the differentiation of engineered tissue products, such as those involving heart cells.[9–11] Poly-*N*-isopropylacrylamide (PIPAAm)-grafted dishes, which were originally developed as a thermosensitive scaffold for cell culture, are useful to maintain the GJIC of tissues cultured on them because they do not require enzyme treatment, which destroys connexins.[12–14]

PIPAAm is a thermoresponsive polymer that has a low critical solution temperature of 32°C: hydrated PIPAAm

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has an extended chain conformation below 32°C and dehydrated PIPAAm has a collapsed chain conformation above 32°C.[15–27] This property of PIPAAm has been exploited in intelligent materials for drug delivery systems and chromatography technology.[16–23] The PIPAAm-grafted dish has been found to enable the recovery of cell monolayers easily without enzyme treatment because cells cannot adhere to a hydrophilic surface below 32°C.[24–26] Cell monolayers are the basic units used to construct three-dimensional tissues *in vitro*. Because a cell monolayer recovered without enzyme treatment maintains normal adhesive and junctional proteins, it can easily adhere to the other tissues or cell sheets to construct a three-dimensional artificial tissue.[27–29] Thus, the PIPAAm-grafted dish has the potential to enable the development of new techniques in tissue engineering.

Although the PIPAAm-grafted dish has made a new era in tissue engineering possible, its effects on connexin-43 (Cx43) expression and GJIC have not been studied well. These effects are important because Cx43 plays an important role in cell proliferation and cell differentiation.

In this study, GJIC and expression of Cx43 molecules were examined by scrape-loading dye transfer (SLDT) assay[30] and Western blotting, respectively, using NHDF cells cultured on PIPAAm-grafted dishes irradiated with various doses of electron beams in order to clarify the safety and appropriateness of this material for the culture of artificial cultured tissues.

MATERIALS AND METHODS

Materials

N-isopropylacrylamide monomer (NIPAAm) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Isopropyl alcohol was obtained from Dojindo (Kumamoto, Japan), and Lucifer yellow dye was from Molecular Probes (Eugene, OR).

Cell culture

Normal human dermal fibroblasts (NHDF cells; Sanko Junyaku, Tokyo, Japan) were cultured in Dulbecco's modified Eagle's medium (GIBCO DMEM; Invitrogen, San Diego, CA), supplemented with 10% heat-inactivated fetal calf serum (FCS; Invitrogen) and antibiotics (penicillin [100 units/mL]-streptomycin [100 units/mL]) (Invitrogen) at 37°C. NHDF cells were maintained in a humidified atmosphere of 5% CO_2 and 95% air.

Preparation of PIPAAm-grafted culture dishes

One hundred microliters of 40% NIPAAm dissolved in isopropyl alcohol was added to 35-mm dishes and irradiated with various doses of electron beams (25, 100, 250, or 500 kGy), using an area electron beam-processing system (Nissin High Voltage, Kyoto, Japan). The PIPAAm-grafted dishes were then rinsed three times with ice-cold sterile water (2 ml) for 5 min, sealed, and dried under vacuum.

Cell morphology

NHDF cells were cultured on control and PIPAAm-grafted dishes. Confluent cells (after 4 days of culture) were fixed with formalin solution, stained with 3% Giemsa solution, and observed with an optical microscope.

Protein assay

The protein concentration of cells cultured on control and PIPAAm-grafted dishes was measured with a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL). Ten-microliter cell samples were

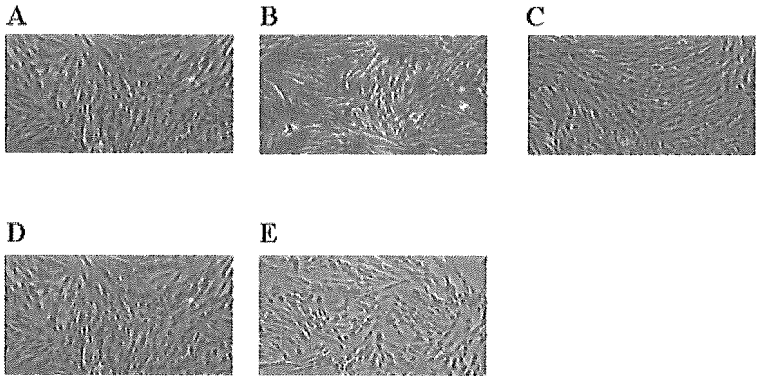

FIG. 1. Optical microscopy images of NHDF cells cultured on PIPAAm-grafted dishes. NHDF cells were cultured for 4 days on PIPAAm-grafted dishes prepared by irradiation with various doses of electron beams (0, 25, 100, 250, or 500 kGy). (**A**) Non-irradiated; (**B**) 25-kGy electron beam; (**C**) 100-kGy electron beam; (**D**) 250-kGy electron beam; (**E**) 500-kGy electron beam.

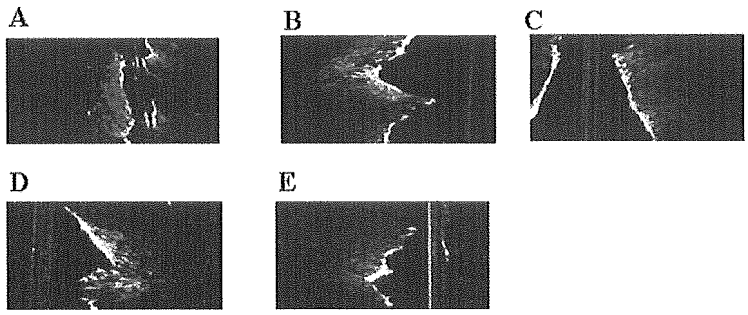

FIG. 2. Fluorescence of NHDF cells by SLDT assay. Transmission of Lucifer yellow into NHDF cells cultured on PIPAAm-grafted dishes irradiated with various doses of electron beams was detected 5 min after scrape-loading. (**A**) Nonirradiated; (**B**) 25-kGy electron beam; (**C**) 100-kGy electron beam; (**D**) 250-kGy electron beam; (**E**) 500-kGy electron beam.

added to 200 μL of the working solution and incubated at 37°C for 30 min in a 96-well plate. Absorbance was then measured at 562 nm in accordance with the manufacturer's protocols.

Scrape-loading dye transfer assay

NHDF cells were seeded on control and PIPAAm-grafted dishes at a density of 1×10^5 cells/mL and cultured for 4 days to form a confluent monolayer. Confluent NHDF cells were washed three times with phosphate-buffered saline containing Ca^{2+} and Mg^{2+} [PBS(+)], and the cell monolayer was scraped with a surgical blade. Fluorescent dye (Lucifer yellow; MW 457.2) at a concentration of 0.1% in PBS(+) was added.[30,31] Cells were exposed to the dye at 37°C for 5 min, and then the dye was discarded and the cells were washed four times with PBS(+). The distance that the dye had migrated was measured under a fluorescence microscope equipped with a type UFX-DXII CCD camera and super high-pressure mercury lamp power supply (Nikon, Tokyo, Japan). The dye migration was measured from the cut edge of the scrape to the edge of the dye front in the cells that were visually detectable.[30]

Western blotting

NHDF cells were cultured for 4 days. After being washed with ice-cold PBS(–) three times, the cells were lysed in 500 μL of lysis buffer (50 mM Tris-HCl [pH 6.8] containing 150 mM NaCl, 5 mM EDTA, 0.1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40) for 30 min on ice with shaking. The cell lysates were centrifuged (10,000 rpm) at 4°C for 20 min, and the supernatants were collected. The protein concentrations of the lysates were determined by BCA assay.

Equivalent amounts of protein sample were applied to 12% sodium dodecyl sulfate (SDS)–polyacrylamide gels and then transferred to a nitrocellulose membrane at 120 V for 60 min. The membrane was blocked with Block Ace (Yukijirusi, Tokyo, Japan) overnight at 4°C. After being washed for 30 min in PBS with 0.05% Tween 20, the membrane was incubated for 2 h with anti-Cx43 polyclonal antibody [diluted 1:1000 in PBS(–) with 0.05% Tween 20; Zymed Laboratories, South San Francisco, CA], followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (diluted 1:5000; Zymed Laboratories). The image was visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences/GE Healthcare, Little Chalfont, UK).

Statistical analysis

Significant differences between groups were evaluated by Student t test. Mean differences were considered significant when $p < 0.05$.

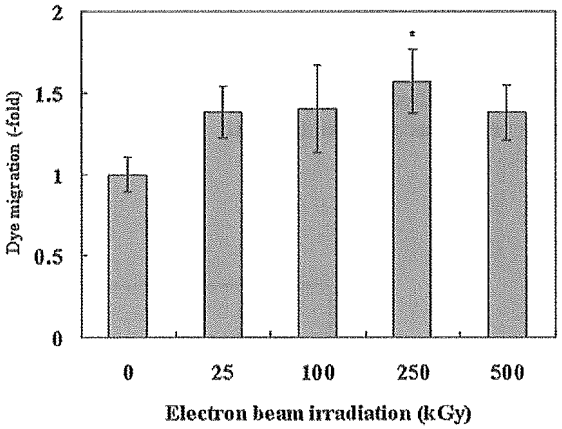

FIG. 3. Positive dye transfer in NHDF cells cultured on PIPAAm-grafted dishes. Transmission of Lucifer yellow was detected 5 min after scrape-loading in NHDF cells cultured on PIPAAm-grafted dishes irradiated with various electron beam doses (0, 25, 100, 250, or 500 kGy). Values represent means ± SD for three dishes. *Significant difference compared with control at $p < 0.05$ by t test.

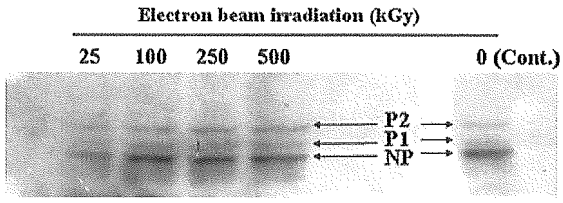

FIG. 4. Western blot of Cx43-NP, Cx43-P1, and Cx43-P2 expression; lysates of NHDF cells cultured on PIPAAm-grafted dishes irradiated with various doses of electron beams (0, 25, 100, 250, or 500 kGy) were applied to SDS–polyacrylamide gels. Fractionated proteins in the gels were transferred to nitrocellulose membrane and immunoblotted with anti-Cx43 polyclonal antibody as described in Material and Methods. Images of Cx43 on Western blot were captured with an Image scanner and analyzed with NIH Image software.

RESULTS

The appearance of NHDF cells grown on PIPAAm-grafted dishes irradiated with various doses of electron beams are shown in Fig. 1. No significant differences were observed by optical microscopy analysis between cells grown in dishes irradiated with various doses of electron beams. These results suggest that PIPAAm-grafted dishes are not toxic to NHDF cells.

The SLDT assay showed that dye migration in cells cultured on PIPAAm-grafted dishes irradiated with electron beams (25, 100, or 500 kGy) was enhanced by about 1.4-fold compared with that on control dishes. Interestingly, the dye migration in cells cultured on PIPAAm-grafted dishes irradiated with the 250-kGy electron beam was particularly enhanced, about 1.6 times higher than that on control dishes (Figs. 2 and 3). These results suggested that the GJIC of NHDF cells cultured on PIPAAm-grafted dishes was enhanced and that the GJIC on PIPAAm-grafted dishes irradiated with the 250-kGy electron beam was affected the most.

To further elucidate the effects of the PIPAAm grafting of culture dishes on GJIC, we analyzed the expression of Cx43, a transmembrane protein involved in GJIC. There are three forms of Cx43: Cx43-NP (nonphosphorylated Cx43), Cx43-P1 (monophosphorylated Cx43), and Cx43-P2 (another phosphorylated Cx43); Cx43-P2 is the most important and functional protein involved in GJIC. The results of Western blotting showed that the expression of Cx43-P1 and Cx43-P2 in NHDF cells cultured on PIPAAm-grafted dishes irradiated with 25, 100, 250, or 500 kGy of electron beams was considerably enhanced. Further, NHDF cells cultured on PIPAAm-grafted dishes irradiated with 100, 250, or 500 kGy of electron beams showed enhanced Cx43-NP expression (Figs. 4 and 5A). The Cx43-P2 expression of cells cultured on PIPAAm-grafted dishes irradiated with the 250-kGy electron beam dose showed the highest value, about 46% higher than that of control dishes. Cells cultured on PIPAAm-grafted dishes irradiated with electron beam doses of 25, 100, and 500 kGy were shown to have enhanced total Cx43 expression. Cells cultured on PIPAAm-grafted dishes irradiated with 100- and 250-kGy electron beam doses showed the highest total Cx43 expression, about 36.6% higher than that of control dish (Fig. 5B).

The Cx43-P2 expression of NHDF cells cultured on PIPAAm-grafted dishes irradiated with 25, 100, 250, and 500 kGy correlated well with GJIC ($R^2 = 0.9398$).

DISCUSSION

Thermoresponsive PIPAAm-grafted dishes irradiated with electron beams have been used to culture cell monolayers because the monolayers can be recovered without enzyme treatment, making PIPAAm a useful material for tissue engineering.

It has been reported that junctional proteins, cellular adherence proteins on the cell membrane, interact via

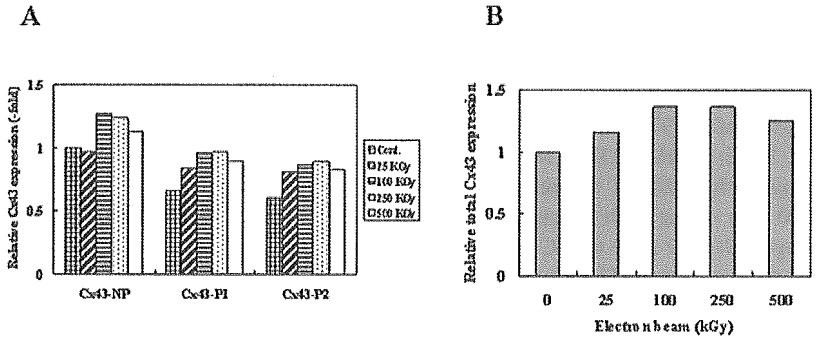

FIG. 5. Relative expression levels of Cx43-NP, Cx43-P1, and Cx43-P2 (**A**) and relative expression levels of total Cx43 (NP+P1+P2) (**B**) of NHDF cells cultured on PIPAAm-grafted dishes irradiated with various doses of electron beams (0, 25, 100, 250, or 500 kGy).

GJIC.[31] In this study, an SLDT assay demonstrated that dye migration in cultured NHDF cells was significantly enhanced in all PIPAAm-grafted dishes tested. Therefore, the chemical structure of the PIPAAm surface may stimulate junctional proteins on the cell membrane, and the stimulated junctional proteins may induce the enhancement of GJIC.

Cx43 expression of NHDF cells cultured on PIPAAm-grafted dishes irradiated with a 250-kGy electron beam changed significantly. Structural differences in PIPAAm triggered by the 250-kGy electron beam induced Cx43 protein expression by NHDF cells, probably by affecting the gene expression of NHDF cells. Further, total Cx43 expression was shown to be enhanced in cells cultured on PIPAAm-grafted dishes irradiated with various doses of electron beams (25, 100, 250, or 500 kGy). Differences due to the electron beam dose should be studied further.

Although the mechanism involved was not determined, it has been reported that basic fibroblast growth factor (bFGF) and keratinocyte growth factor (KGF) enhance GJIC activity and the expression of Cx43.[32–35] If bFGF and KGF in FCS are adsorbed onto the PIPAAm surface, cells can efficiently access these growth factors from the PIPAAm surface, and GJIC may be enhanced. It is also reported that bFGF activates protein kinase A (PKA),[36] an important regulator of Cx43, promoting the phosphorylation of Cx43 and enhancing GJIC.[37] Therefore, bFGF adsorbed onto the PIPAAm surface may bind its receptor and induce the activation of PKA, resulting in an enhancement of GJIC on NHDF cells caused by the increase in Cx43-P2 band protein.

In the process of posttranslational change, Cx43-P2 becomes insoluble in Triton X-100.[38] Thus, not all Cx43-P2 may be included in the lysate, and some Cx43-P2 may have been included in the pellet. More Cx43-P2 may have existed than was detected in the present results obtained by Western blotting.

In this study, it was shown that the use of PIPAAm-grafted dishes irradiated with various doses of electron beams enhanced GJIC and Cx43 expression in cultured NHDF cells. This suggests that PIPAAm-grafted dishes may promote efficient tissue regeneration, because GJIC plays an important role in increasing tissue strength.[39]

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