and the clinical appearance of smoother skin with less visible wrinkling.[22,23] As several growth factors involving skin regeneration are secreted from MSCs, it is hypothesized in this study that SHEDs and SHED-derived growth factors may improve UV-B–induced photodamage. Therefore, wrinkles were induced in hairless mice after an 8-week regimen of UV-B irradiation, and the antiwrinkle effect was investigated by the subcutaneous injection of SHEDs and its conditioned medium. In addition, mechanisms for improving wrinkling via paracrine routes were investigated further using SHED-derived conditioned medium (SH-CM) in cultured HDFs.

MATERIALS AND METHODS

Subjects and Cell Cultures

Human dental pulp tissues were obtained from clinically healthy extracted deciduous teeth and permanent teeth from 8 patients. The ethics committee of Nagoya University approved our experimental protocols. Stem cells from human exfoliated deciduous teeth and pulp of permanent teeth were isolated and cultured as previously described.[6,7] Briefly, the pulp was gently removed and digested in a solution of 3 mg/mL collagenase type I and 4 mg/mL dispase for 1 hour at 37°C. After filtration using 70-mm cell strainers (Falcon; BD Labware, Franklin Lakes, NJ), cells were cultured in Dulbecco's modified Eagle medium (DMEM; GIBCO, Rockville, MD) containing 20% mesenchymal cell growth supplement (Lonza Inc, Walkersville, MD) and antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin, and 0.25 mg/mL amphotericin B; GIBCO) at 37°C under 5% CO_2. After primary culture, the cells were subcultured at about 1 × 104 cells/cm^2. From 1 to 3 passages, cells were used in the experiments. Human BMSCs were purchased from Lonza Inc and cultured according to the manufacturer's instructions.

Analysis of Cell Proliferation

The proliferation rates of SHEDs, DPSCs, and BMSCs were assessed by bromodeoxyuridine (BrdU) incorporation for 12 hours using a BrdU staining kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA) (n = 3 for each group). The experiment was repeated 5 times. Statistical differences were evaluated by the Tukey-Kramer test following 1-way analysis of variance.

Immunofluorescence for STRO-1 SHEDs, DPSCs, and BMSCs was fixed with 3% paraformaldehyde and then rinsed twice with phosphate-buffered saline (PBS) and treated with 100 mM glycine for 20 minutes. Cells were then permeabilized with 0.2% Triton-X (Sigma-Aldrich, St Louis, MO) for 30 minutes and subsequently incubated in a mixture of 5% donkey serum and 0.5% bovine serum albumin for 20 minutes. Next, the cells were incubated with primary antibody mouse anti–human STRO-1 antibody (1:100; R&D, Minneapolis, MN) for 1 hour, incubated for 30 minutes with a secondary antibody goat anti–mouse immunoglobulin M–fluorescein isothiocyanate antibody (1:500; Southern Biotech, Birmingham, AL), and mounted using Vectashield with DAPI (Vector Laboratories Inc, Burlingame, CA).

Animal Experiment

Five-week-old female hairless mice (Hos: HR-1) were provided from SLC Inc (Shizuoka, Japan). All mice were housed in climate-controlled quarters (22°C ± 1°C at 50% humidity) with a 12-hour/12-hour light-dark cycle. Animals were allowed free access to water and a chow diet and were observed daily. The mice were irradiated dorsally using the UV-B–emitting system RMX-3W (Handok Biotech, Seoul, Korea) for 8 weeks, 5 times a week. A bank of 10 Toshiba SE lamps was used without any filtering for UV-B (peak of emission near 312 nm, the irradiance between 290 and 320 nm corresponding to 55% of the total amount of UV-B). The distance from the lamps to the animals' backs was 89 cm. During exposure, the animals could move around freely in their cages. The irradiation dose was 1 MED (minimal erythemal dose; 60 mJ/cm^2) in the first 2 weeks, 2 MED (120 mJ/cm^2) in the third week, 3 MED in the forth week (180 mJ/cm^2), and 4 MED (240 mJ/cm^2) in the fifth through eighth weeks. The total UV-B dose was approximately

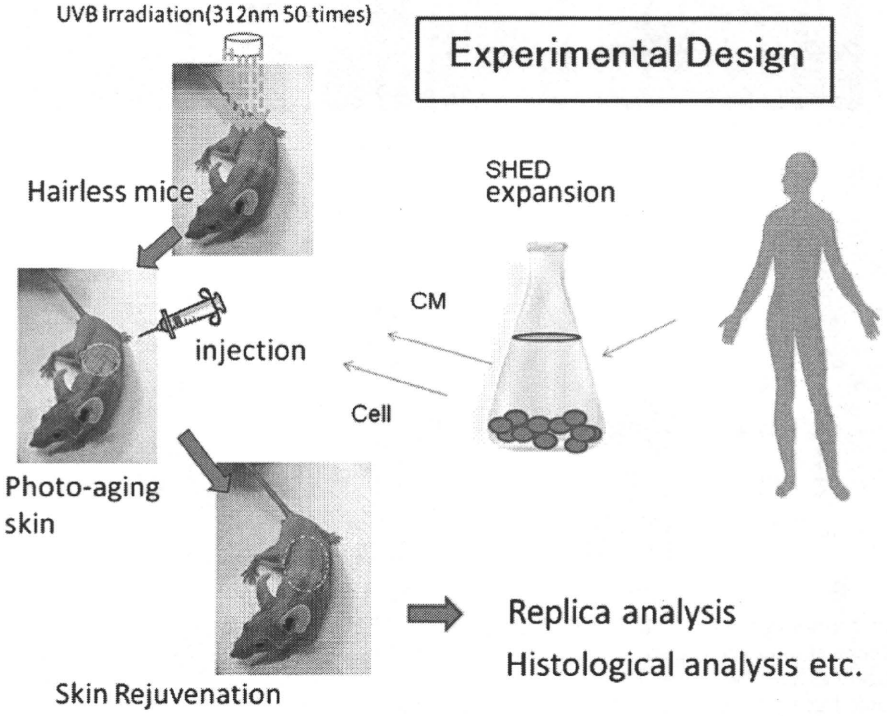

FIGURE 1. Experimental design in hairless mice. Wrinkle was induced by UV-B irradiation.

115 MED (6.9 J/cm^2). Five weeks after wrinkle induction, SH-CM (100%) was subcutaneously injected into the restricted area of the mice. As a positive control, PBS-suspended SHEDs (4×10^5) were injected directly into the dermis, and as a negative control, the dermis was treated by PBS only (Fig. 1).

Preparation of SH-CM

Stem cells from human exfoliated deciduous teeth (4×10^5 cells) were cultured in DMEM/F12 (Invitrogen–Gibco BRL, Grand Island, NY) serum-free medium. Conditioned medium of SHEDs was collected after 72 hours of culture, centrifuged at 300*g* for 5 minutes, and filtered using a 0.22-mm syringe filter.

Skin Replica and Image Analysis

At the time of wrinkle induction and 1 week after the injection, negative replicas of the dorsal skin surface were taken by using a silicon-based impression material, Flextimel (Heraeus Kulzer, New York, NY). To obtain replicas of the wrinkles from the same skin area, the skin was marked using an oil-based marker pen. Five weeks after final injection of SH-CM and SHED skin, impressions were taken from the marked area. For ease of measurement, all replicas were cut into square pieces of 1 cm, and the back of each replica was processed into a flat plane using the same impression material. Light was directed at a 208 angle, and images were incorporated from replica using a CCD. The image of the negative replicas was observed using a wrinkle analysis system skin visiometer SV 600 (Courage & Khazaka, Cologne, Germany). The parameters used in the assessment of the skin wrinkles are number, depth, and area.

Histology

Dorsal skins (1 × 1 cm) were fixed with a 10% formalin neutral buffered solution, embedded in polyester wax, and sectioned at 6 mm. The sections were subjected to hematoxylin-eosin and Masson trichrome staining.

HDF Culture and UV-B Irradiation Dose

Human dermal fibroblasts were cultured in a DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin in 5% CO_2 at 37°C. After starvation with serum-free medium for 24 hours, cells were washed with PBS and exposed to UV-B with 3 to 4 drops of PBS. UV-B irradiation was carried out using a UV lighter (Waldmann, Schwenningen, Germany). Immediately after irradiation, PBS was aspirated and replaced with complete medium. UV-B irradiation doses were tested in 50 to 250 mJ/cm^2 and finally fixed to be 70 mJ/cm^2 for further experiment.

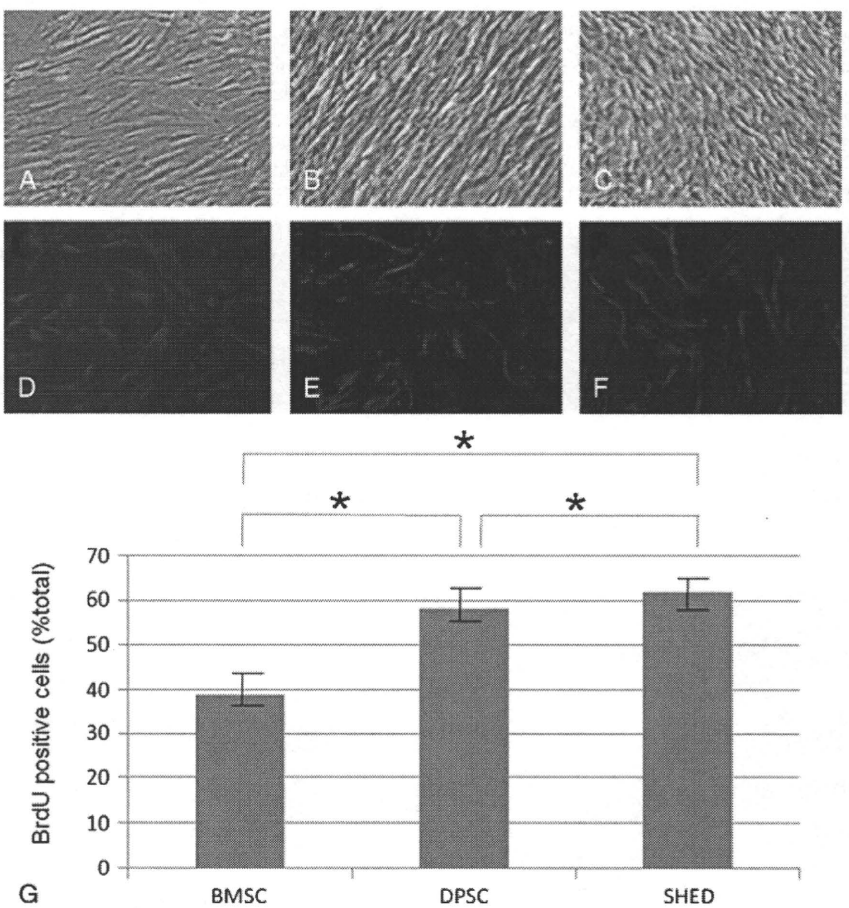

FIGURE 2. A–C, Cell morphology of (A) BMSCs, (B) DPSCs, and (C) SHEDs (original magnification ×40). D–F, Immunofluorescence staining of the stem cell marker STRO-1: (D) BMSCs, (E) DPSCs, and (F) SHEDs were positive for STRO-1 (green fluorescence). DAPI was used to visualize the nuclei (blue fluorescence). G, The proliferation rates of SHED, DPSCs, and BMSCs were assessed using BrdU; SHEDs have a significantly higher proliferation rate than DPSCs and BMSCs. Bar: SD. Significance: *$P < 0.05$.

Cell Proliferation Assay

Human dermal fibroblasts were plated at a density of 5×10^3 cells per well in 96-well plates, and the proliferation of HDFs was measured using a CCK-8 Kit (Dojindo, Gaithersburg, MD). After starvation with serum-free medium for 24 hours, the cells were continuously cultured for 24 hours with or without SH-CM and exposed to UV-B (70 mJ/cm^2) for 90 seconds. Then, UV-B–irradiated cells were cultured in complete medium for 24 hours and harvested. Human dermal fibroblasts were added to 10 mL of the CCK-8 solution and incubated for 3 hours.

The absorbance was measured at 450 nm using a microplate reader (TECAN, Grödig, Austria). OD values of each well were calculated to their relative cell numbers with comparable standard curves.

Western Blot Analysis

Human dermal fibroblasts (2×10^4 cells/well) were seeded in 24-well plates and pretreated as described above. Then, the cells were lysed in a RIPA buffer (50 mM Tris-HCl, 0.15 M NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium dodecyl sulfate, 50 mM NaF, 1 mM Na_3VO_4, 5mM dithiothreitol, 1 mg/mL leupeptin, and 20 mg/mL phenylmethanesulfonyl fluoride, pH 7.4). Fifty micrograms of proteins was separated on an 8% sodium dodecyl sulfate–polyacrylamide gel by electrophoresis. The proteins were transferred to polyvinylidene fluoride membranes. The membranes were incubated with antibodies of collagen type I (Santa Cruz, Saint Louis, MO) and matrix metalloproteinase 1 (MMP-1) (Calbiochem, Darmstadt, Germany). Then, the membranes were washed and incubated with horseradish peroxidase–conjugated anti–goat immunoglobulin G antibody (1:10,000; Santa Cruz). The blots were reacted with Immobilon Western reagent and exposed to x-ray film.

RESULTS

Characterization of SHEDs, DPSCs, and BMSCs

Stem cells from human exfoliated deciduous teeth and pulp of permanent teeth displayed a fibroblastic morphology resembling BMSCs (Figs. 2A–C). Immunofluorescence analysis indicated that SHEDs, DPSCs, and BMSCs contained STRO-1–positive cells (Figs. 2D–F). The proliferation rate of SHEDs was significantly higher than that of DPSCs and BMSCs (Fig. 2G).

SH-CM Reduced UV-Induced Wrinkles

During the period of UV exposure, the mice were observed for fine wrinkling of the skin. However, the SH-CM–treated group and SHED-injected group appeared to have fewer wrinkles than the PBS group during the treatment (n = 8 for each group). In a replica analysis, Figure 3, Figure 4 shows that repeated SH-CM treatment improved the fine wrinkles induced by UV-B irradiation. The SHED-injected group showed the same tendency as SH-CH group. When our group measured the parameters for the wrinkles of replicas with the skin visiometer SV 600, injection of natural level (100%) of

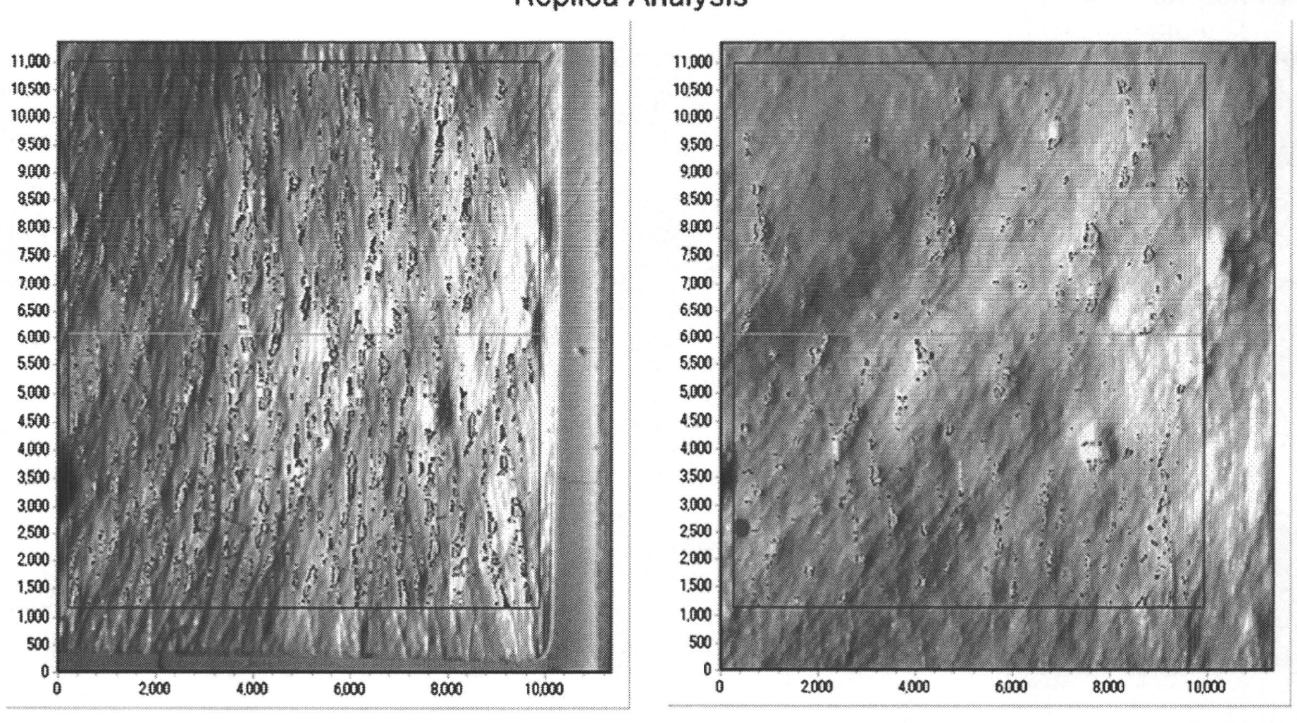

FIGURE 3. Evaluation of wrinkles by replica analysis after SH-CM injection (A) treated, (B) 100%, SH-CM–treated group.

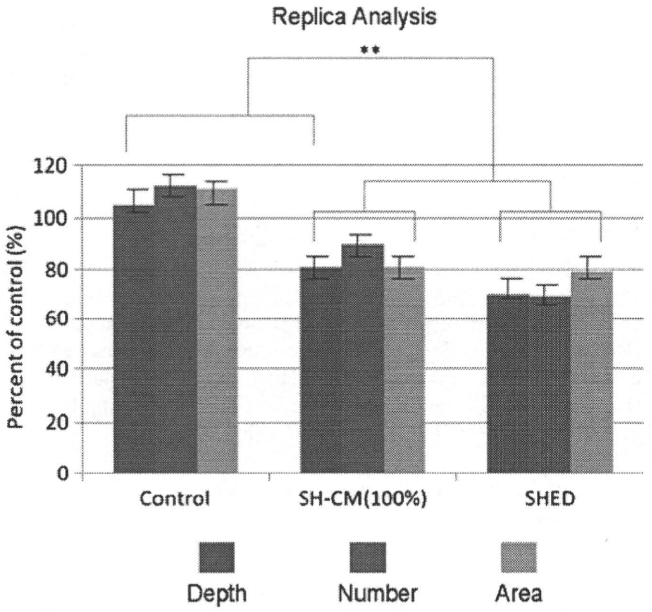

FIGURE 4. Improved parameters for wrinkles in natural level of SH-CM– and SHED-injected group.

SH-CM significantly reduced all parameters for wrinkles. However, SHED was shown to be more effective than SH-CM.

Histological Observation

UV-B–irradiated hairless mice showed great changes in skin appendages, and the effect of SH-CM on dermal thickness in UV-B–irradiated hairless mice was investigated. Figure 5 shows the histologic measurements of the dermal thickness of the hairless mouse skin by hematoxylin-eosin staining. Measurement of the dermal thickness showed significant increases in SHED-injected group and SH-CM–treated group (Fig. 6), and a marked increase in collagen bundles was observed in both groups, but not in the control group (Fig. 5). F5 F6

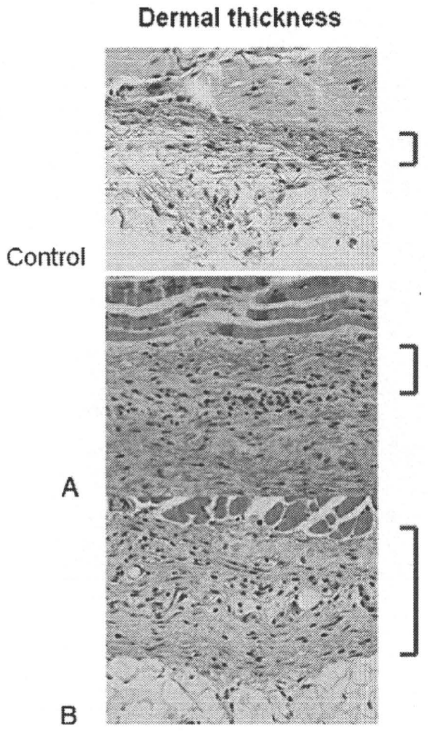

FIGURE 5. Hematoxylin-eosin staining. Collagen fibers were stained and were significantly increased in the SH-CM–treated group and SHED-injected group. B, SHED-injected group.

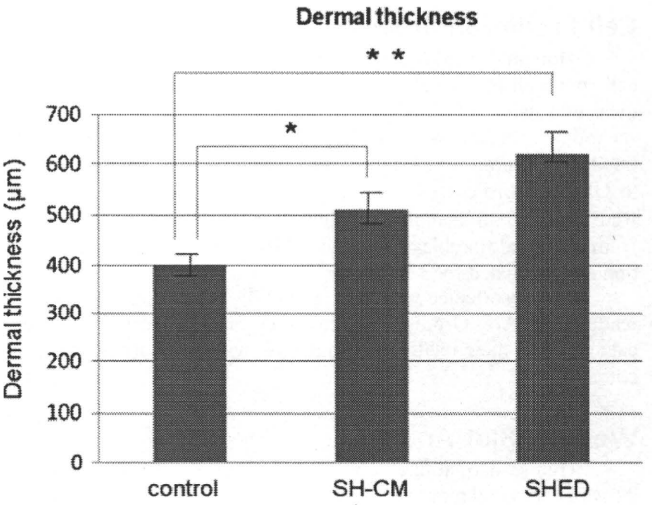

FIGURE 6. Hematoxylin-eosin staining, SH-CM–treated and SHED-injected group increased dermal thickness of hairless mice.

SH-CM Increased the Proliferation of HDFs

To further study the paracrine mechanism regarding the improvement of skin wrinkles with SHEDs, a cell proliferation assay was performed in primarily cultured HDFs with SH-CM. UV-B irradiation significantly decreased the proliferation of HDFs, but pretreatment of SH-CM showed a protective effect on HDFs (Fig. 7). As the SH-CM contains diverse growth factors and a unique characteristic of growth factors is their ability to initiate mitosis of quiescent cells, enhanced proliferation by SH-CM in this experiment might be mediated by growth factors secreted from SHEDs. F7

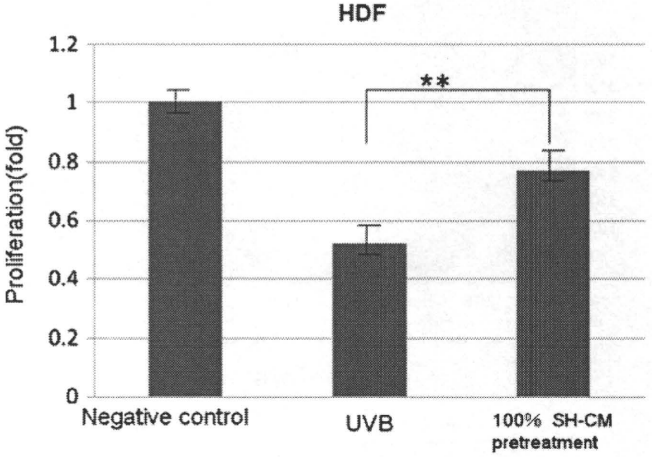

FIGURE 7. Effect of SH-CM on the proliferation of HDFs. UV-B irradiation significantly decreased the proliferation of HDFs, but pretreatment of SH-CM protected HDFs from UV-B.

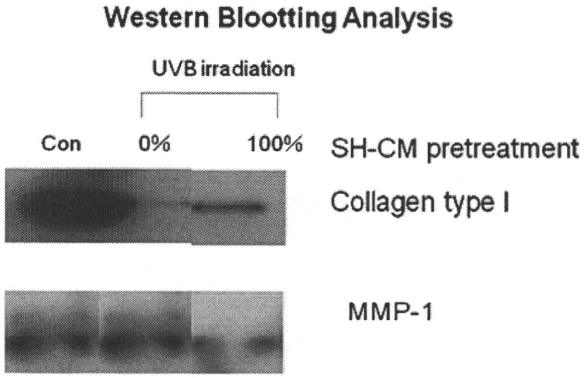

FIGURE 8. Effect of SH-CM on the expression of collagen type I and MMP-1.

Expression of Collagen Type I and MMP-1

Because collagen contents in the dermis were significantly increased in SH-CM–treated hairless mice, protein expressions of collagen type I and MMP-1 were examined in HDFs after SH-CM treatment (Fig. 8). UV-B irradiation clearly reduced the expression of collagen type I and induced that of MMP-1. However, expression of collagen type I was significantly increased after SH-CM pretreatment, whereas that of MMP-1 was decreased after SH-CM pretreatment. These results indicate that increased collagen contents in the dermis of SH-CM–treated hairless mice were mediated by the stimulation of collagen synthesis and the inhibition of collagen degradation in dermal fibroblasts.

DISCUSSION

In this study, we investigated the characteristics of SHEDs compared with DPSCs and BMSCs, which have been considered the standard for stem cell sources in tissue engineering and regenerative medicine. The results indicated that SHEDs possessed high proliferation ability and were enriched with extracellular matrix (ECM), suggesting that they are a useful source for stem cell–based therapy. STRO-1–positive cells were found in SHEDs, DPSCs, and BMSCs. STRO-1 recognizes a trypsin-resistant cell-surface antigen present on a subpopulation of bone marrow cells, including a predominant proportion of the high growth and differentiation potential skeletal stem cell and colony-forming unit–fibroblastic populations.[7,22] High proliferative capacity is one of the most critical characteristics of postnatal somatic stem cells.[23] Proliferation studies using BrdU revealed that SHEDs showed the highest population rate among SHEDs, DPSCs, and BMSCs. Previously, our group[24] has reported that SHEDs expressed several growth factors such as fibroblast growth factor (FGF), transforming growth factor β, connective-tissue growth factor, nerve growth factor, and bone morphogenetic protein, which were associated with this pathway at higher levels by micro array analysis. FGF-2 has been reported as a cytokine that acts to promote the proliferation of numerous kinds of cells and control ECM generation during tissue generation and wound healing.

Studies in recent years have inspired us to realize the paracrine in factors, such as vascular endothelial growth factor, keratinocyte growth factor, and FGF, to the skin regeneration, and this suggests that stem cell transplantation is also "cell-based" cytokine therapy. "Of importance, in this study, we used conditioned media containing growth factors to avoid negative effects of UV-B to HDFs. The concept of paracrine effects mediating at least part of the effects of stem cell therapy is not inconsistent with previous data. The overall benefits of cell-based cytokine therapy in wound healing will require further investigation. The contribution of keratinocyte differentiation of SHED-derived growth factors to reepithelialization in wound closure needs to be determined. Also, the benefits of SHED-derived growth factors in wound healing, tissue remodeling, and skin graft genesis await further investigation. We believe that a better understanding through these investigations will help us understand skin rejuvenation and the wound-healing process and develop novel therapies for skin defects and chronic wounds in the future.

Photoaging is a complex process having pathologic similarities to skin wounds. Mesenchymal stem cells play key roles in these processes as they interact with keratinocytes, fat cells, and mast cells. They also are the source of ECM proteins; fibrillar types I and III collagens are significantly reduced in the papillary dermis, and their reduction has been shown to correlate well with the clinical severity of photoaging. This reduction results from a combination of reduced procollagen biosynthesis and increased enzymatic breakdown via the actions of MMPs. Fisher et al[25] showed that UV irradiation induced the synthesis of MMPs in human skin in vivo.[26] Among the MMP family, MMP-1, MMP-13, and membrane-type MMP-14 display collagenolytic activity, and MMP-2 and MMP-9 have been described as true elastases. Matrix metalloproteinase–mediated collagen and elastin destruction accounts for a large part of the connective tissue damage that occurs in photodamaged skin.[16,25,27] In this study, our group found that SH-CM not only inhibited a UV-B–induced decrease in type I collagen but also attenuated UV-B–induced MMP-1 expression in HDFs. Wound healing and skin rejuvenation from photodamage are a complex but orderly process and are orchestrated via cytokines and growth factors. Therefore, these data combine with those of the current study imply that local cytokine release may be an important factor mediating the beneficial SHED rejuvenation effects seen after delivery of SH-CM. Local delivery of SHEDs may also cause circulating stem progenitor cells to home to the region of injury and contribute to healing.

In conclusion, the interaction between SHED-derived growth factors and HDFs has been investigated for the first time, as the application of SHEDs for dermal wound healing remains speculative. Stem cells from human exfoliated deciduous teeth have effects on HDFs by increasing collagen synthesis and by activating proliferation and migration activity of HDFs, suggesting that SHEDs or SH-CM can be used for the treatment of photoaging and wound healing. Our results also suggested that SHEDs should be constitutionally suited for dermal wound healing compared with MSCs. Mainly with secreted growth factors or ECM proteins, SHEDs contribute to enhanced wound-healing potential of HDFs. Further mechanism studies using neutralizing antibodies against each growth factor may clarify the role of soluble factors of SHEDs in wound-healing process.

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AQ1 = "simller" was changed to "similar." Please check if correct.

AQ2 = Is "208 angle" correct as meant?

AQ3 = Please spell out "OD."

AQ4 = Please check Fig 3 caption if correct. Should this be "Evaluation of wrinkles by replica analysis before (A) and after (B) 100% SH-CM treatment"?

AQ5 = Please indicate which data pertain to A ("SH-CM–treated group"?).

AQ6 = "They also are the source of ECM proteins of fibrillar types I and III collagens are significantly reduced in the papillary dermis" was changed to "They also are the source of ECM proteins; fibrillar types I and III collagens are significantly reduced in the papillary dermis." Please check if correct.

AQ7 = "SHED juvenation effects" was changed to "SHED rejuvenation effects." Please check if correct.

AQ8 = Refs 7 and 19 are the same. Thus, Ref 19 citation in the text was changed to Ref 7; former Ref 19 was deleted from the reference list, and references were renumbered accordingly. Please check.

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Sprayed Cultured Mucosal Epithelial Cell for Deep Dermal Burns

Minoru Ueda, DDS, PhD

Abstract: Mucosal epithelial cells have various advantages compared with epidermal cells, such as their high proliferation ability and long biologic activity. The objective of this study was to assess the clinical results after sprayed application of cultured mucosal epithelial autograft (CMEA) suspensions onto deep dermal burn wounds.

Ten patients with deep dermal burns were included in a prospective study. The average total-body-surface-area burn was 17.7% (8%–45%). The average Abbreviated Burn Severity Index was 6.3 points (4–9 points). The application of sprayed CMEA suspension was performed onto an average body surface area of 2.05% (0.5%–5%; median, 2%). Eight patients were recruited for clinical follow-up after an average of 10 months (3–18 months).

The average Vancouver Scar Scale score at follow-up was 1.5 points (range, 0–5 points). The average period of epithelialization in wound surface was 12.5 days. Our data show that enzymatic and careful surgical debridement and consecutive application of CMEA suspensions using a spray technique result in excellent cosmetic outcomes compared with any other methods.

Key Words: Deep dermal burns, cultured mucosal epithelial, sprayed cell transplantation

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The application of cultured epithelial cell autografts enables reconstruction of skin even in burn victims with large total-body-surface burns since the late 1970s and early 1980s of the last century.[1–3] A skin biopsy is sent to a specialized laboratory within few hours after burn injury in the ideal case. This procedure facilitates early in vitro skin cell expansion. Simultaneously, the patients undergo intensive care therapy for burn shock, burn wound excision, and wound preparation for cultured epithelial cell autograft delivery. In practice, cultured epithelial cell autografts are available after 10 to 14 days, depending on the cell expansion behavior in the laboratory and the body surface to be covered.

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On the other hand, it has been known that the proliferation rate of mucosal cells was higher than that of epidermal cells. According to our previous study,[4] at culture day 15, the epidermal cell was slightly decreased, whereas that of the mucosal cell culture remained unchanged. Total cell counts showed that mucosal cells possessed greater proliferating ability than epidermal cells. The results of 3(4,5-dlmethyel-thlazoyl-2-yl)2,5 diphenyltetrazolium bromide assay confirmed this observation and also suggested that the mucosal cells maintained biologic activity longer than did the epidermal cells. The most important morphologic characteristic of mucosal cells in culture was their low grade of differentiation. Interestingly, the epidermal cells showed enucleation and keratinization progressively during culture, whereas the mucosal cells showed no obvious enucleation when examined by light microscopy. Transmission electron microscopy showed a smaller number of desmosomes in cultured mucosal cells than epidermal cells. The results of several studies including ours revealed cultured mucosal epithelial cells to be a possible material for grafting for burned patients.

The cultured epithelial cell autografts were delivered traditionally as a sheetlike material on collagen carrier sheets. Because the cultured epithelium is very weak mechanically, care must be taken not to break the cultured epithelium in grafting. New methods to produce cultured epithelial cell autografts suspensions have been introduced recently.[4] These suspensions can be sprayed onto burn wounds using special spray devices and spray nozzles, facilitating an even distribution of cultured epithelial cell autografts over the wounds in a very short time.[5–7] We used cultured mucosal epithelial cell for spraying because of its advantageous character such as high proliferation ability and long biologic activities.

The objective of this study was to assess the clinical results after sprayed application of cultured mucosal epithelial autograft (CMEA) suspensions onto deep dermal burn wounds with special reference to the period until re-epithelialization and scar formation in the bed.

PATIENTS AND METHODS

Patients

Ten patients at the ages of 15 to 58 years (mean, 46.1 years) were included in a prospective study between January 2004 and December 2005. Six patients were male, and 2 were female. The average total-body-surface-area burn was 15.1% (8%–45%). The average Abbreviated Burn Severity Index was 6.7 points (4–12 points). The application of sprayed CMEA suspension was performed onto an average body surface of 2.05% (0.5%–5%; median, 2%).

The burn depth of the CMEA-treated regions was deep dermal in all cases. The data for each patient are shown in Table 1. The T1 average interval between trauma and CMEA suspensions application was 10 days (8–12 days) after injury. The burn wounds were prepared by enzymatic debridement using collagenase-consisting cream during the days before CMEA suspension delivery and by

TABLE 1. Data From CMEA Spray Application With Respect to Patient Data, Treated Region and Body Surface Area (% BSA CMEA), Total-Body-Surface-Area (TBSA) Burn, and Body Surface Area Treated Surgically (% OR)

Patient	Sex	Age, y	Treated Region	BSA % CMEA	TBSA, %	% OR	Trauma	ABSI
1	M	58	Leg	5	45	46	Explosion	9
2	M	40	Leg	2	28	28	Explosion	7
3	M	54	Leg, hand	5	8	7	Flame burn	5
4	M	38	Leg	2	16	14	Explosion	6
5	W	47	Hand	1.5	10	10	Flame burn	6
6	W	53	Hand	0.5	13	13	Scald	7
7	M	52	Hand	2	11	8	Flame burn	5
8	M	55	Leg	0.5	16	16	Explosion	7
9	M	49	Leg, hand	0.5	16	12	Explosion	7
10	M	15	Chest	1.5	14	8	Flame burn	4
	Average	46.1		2.1	17.7	16.2		6.3

ABSI indicates Abbreviated Burn Severity Index.
Trauma mechanism and ABSI score.

AQ1

sharp surgical spoons briefly before spray application at the day of surgery. Eight patients were recruited for clinical follow-up after an average of 10 months (3–18 months). The Vancouver Scar Scale (VSS) was used,[7] which is known as the burn scar index. The VSS evaluates vascularity (redness), height (hypertrophy), pliability (contracture and elastic texture), and pigmentation. The VSS has been validated on defined scar areas of 4 cm^2 used for burn scar assessment at follow-up.

MATERIALS AND METHODS

Production of Epithelial Cell Suspension

Oral mucosa was obtained from the patients, and subcutaneous and submucosal tissues were removed with scissors. The resulting samples were cut into small pieces, which were immersed twice in phosphate-buffered saline solution containing antibiotics (penicillin G 1000 U/mL, kanamycin 1 mg/mL, amphotericin [Fungizone] 2.5 μg/mL) for 30 minutes at 37°C. Afterward, those tissues were immersed in Dulbecco's modified Eagle medium (DMEM) containing 1000 protein units of dispase per milliliter for 16 hours at 4°C. They were then treated with 0.25% trypsin solution for 30 minutes at room temperature to separate the cells. The enzyme activity was eliminated by washing with DMEM containing 10% fetal calf serum (FCS). Then the specimens were stirred in DMEM containing 5% FCS for 30 minutes. The suspension was filtered through nylon gauze (50 μm) to remove unsatisfactory segments, and a suspension of purified epidermal and mucosal cells was obtained. It was centrifuged twice for 5 minutes at 1500 revolutions per minute, and the cell pellet was resuspended in culture medium.

3T3-J2 cells were used as the feeder layer. They were kindly provided by Dr. Howard Geen (Department of Cellular and Molecular Physiology, Harvard Medical School, Boston, MA) and treated with 4 μg of mitomycin C per milliliter in DMEM without FCS for 2 hours before epithelial cell inoculation. The 3T3-J2 cells were rinsed with phosphate-buffered saline (−) to remove mitomycin C and cultured for 24 hours in DMEM.

A 3:1 mixture of DMEM and Ham F12 medium supplement was made with the following: FCS 5%, cholera toxin 10 ng/mL, hydrocortisone 0.5 g/mL, transferring 5 μg/mL, triiodothyronine 2×10^{-9} M, insulin 5 μg/mL, penicillin 100 U/mL, kanamycin 0.1 μg/mL, and amphotericin 0.25 μg/mL. Human recombinant epidermal growth factor was added at 10 ng/mL when cell adhesion was complete. 3T3-J2 cells treated with mitomycin C were inoculated into a Petri dish (35 mm in diameter) at a density of 2×10^4. Then epithelial cells were inoculated on the surface of the feeler layer (1×10^4 cells/cm^2). The Petri dishes were kept in an atmosphere of 5% CO_2 in room air. The medium was changed every 2 days. These cultured cells were dispersed with 0.1% trypsin and suspended. After the cells reached 70% to 80% confluence (end of passage 0), keratinocytes were detached with 0.25% trypsin/0.02% EDTA solution and subcultivated in serum-free medium (passage 1) until passage 3. The cells were counted before and after passaging using an improved Neubauer hemocytometer (Merck, Darmstadt, Germany). The CMEA can be sprayed onto burn wounds as a suspension in culture medium at the end of each passage using our special spraying device.

An average of 3.9 (SD, 4.8) million cells (range, 0.35–14 million cells) was applied onto the wounds of the patients of the study population. Ten patients received the cells after passages 1 and 3 after passage 2.

Cell Spray Device

The fibrin delivery method made use of the Tisseel VH fibrin sealant system (Baxter, Glendale, CA). This preparation contains human fibrinogen and thrombin. The following protocol was used to make a fibrin gel with final concentrations of 5 mg/mL fibrinogen and 25 U/mL thrombin, utilizing a 1-mL Tisseel VH kit. This kit used 2 liquid phases that can be either extruded through a dual-chamber applicator or sprayed through the applicator with an inert gas carrier. To make the thrombin component, 1 mL of calcium chloride ($CaCl_2$) mixture from the kit was added using a sterile syringe to the 1-mL bottle of thrombin, and the mixture was allowed to dissolve. One part of this solution was then added to 9 parts of sterile 30 mM $CaCl_2$ in normal saline (0.9% sodium chloride). The fibrinogen/sealer protein component was made by adding 1 mL of sterile normal saline to the sealer protein bottle. One part of this solution was then added to 9 parts of sterile normal saline. The protease inhibitor aprotinin included with the kit was not used. All solutions were used within 4 hours. The total volume of fibrin gel was predetermined by the size of the wound to be covered. At the time of application to each wound, a small amount of the fibrin gel was placed on a tissue culture plate, covered in media, and incubated under standard conditions to verify and confirm cell viability and migration of the cells from the fibrin.

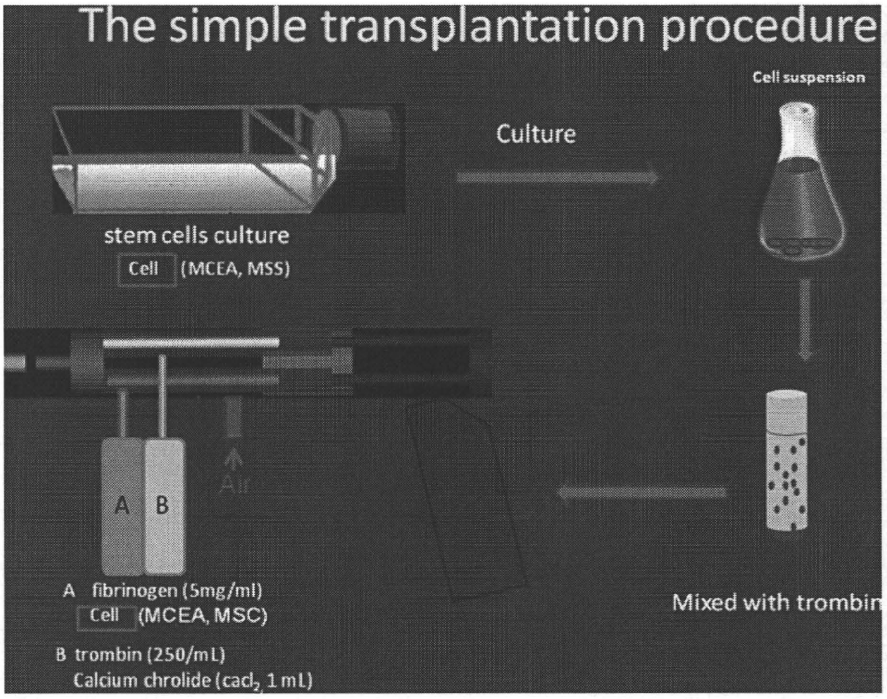

FIGURE 1. Prototype spray device for application of cell suspensions.

The spray device was originally fabricated in the United States. It consists of a base unit and a handgrip with spray nozzle (Fig. 1). A 1- or 2-mL syringe is fixed onto the slide of the handgrip to adjust the fluid flow (in milliliters per minute) of the cell suspension. An additional pump inside the base unit is connected via a pressure tube with the handgrip. The airflow (in liters per minute) generated by the pump is compacted by the spray nozzle. An airflow of 3.7 L/min and a fluid flow of 4.2 mL/min were used for all clinical applications. Preliminary data from our laboratory demonstrated that this adjustment results in a spray pressure of 8.2 mm Hg.

RESULTS

No adverse events were observed after CMEA suspension application. The 8 patients with follow-up presented with an average VSS score of 2.4 ± 2.2 points (range, 0–8 points).The average period of epithelialization in wound surface was 12.5 days (Table 2).

DISCUSSION

Deep dermal burns of the body surface are traditionally treated by tangential excision and full- or split-thickness skin grafting. Both usually lead to unsightly scar formation, and this is also associated with a new defect in the donor site. Waiting for spontaneous wound healing represents another alternative. However, in those patients with deep burns that require prolonged periods for spontaneous wound healing, tangential excision of the wound and resurfacing with skin grafts seem to give better cosmetic results than conservative management, with no greater incidence of functional complications.[9] Both skin grafting and spontaneous wound healing of deep dermal burns lead to scarring and disfigurement. Burn injuries often have a deleterious effect on the psychologic and functional well-being of the patient. Even when no skin grafting is performed, there seems to be significant of the physical and emotional function of adults after they have incurred partial-thickness burns[10] (Fig. 2).

The follow-up is still short, and the number of the patients is small, in our study. Taking into consideration that burn scars generally improve over time, it can be expected that the longer-term results will be even better. Our data are still of preliminary character because the study was performed without a control group. The method of CMEA spray application has become our standard of care for these indications. The faster wound closure, the promotion of spontaneous wound healing by keratinocyte application, and the preservation of donor sites are further advantages of the method.[11,12]

In general, mucosal cells that were used in this study proliferated faster than did epidermal cells. This finding seems to reflect the characteristics of mucosal and epidermal cells in vivo; the turnover time of mucosal cells was 11 days, whereas that of epidermal cells was 27 days. As a result, the period for confluent mucosal cell was 10 days, and for epidermal cell, 14 days. This difference was not significant. However, it suggests that the mucosal cell possesses a little greater proliferating ability and indicates the usefulness of mucosal cells to cultured epithelial suspension.

TABLE 2. VSS Scores and Period in Epithelialization

Patient	VSS	Period of Epithelialization	Follow-up, mo
1	0	7	18
2	1	10	18
3	1	12	12
4	1	11	10
5	2	8	13
6	5	14	4
7	1	11	11
9	1	13	10
Average	1.5	10.8	12

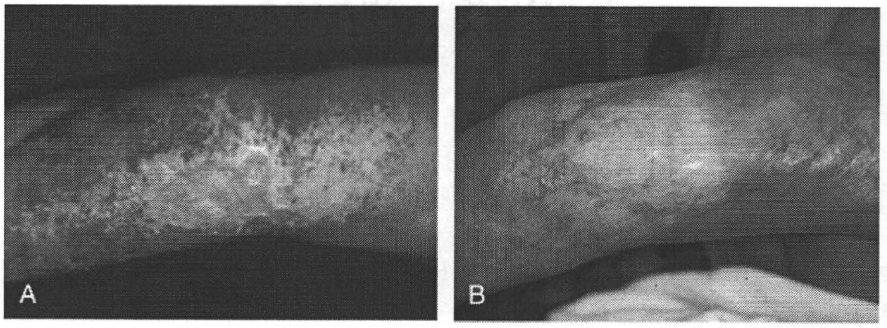

FIGURE 2. A 56-year-old male patient (patient 6, Table 1) presenting with deep dermal burns after flame injury (A). Results at follow-up after 12 months (B).

One of the difficult problems in culturing epithelial cell is the reduction of its viability during culture. It is necessary to maintain the cells' ability to proliferate until the cell is transplanted. The reduction of the viability seems to depend on the differentiation of epithelial cells. The differentiated epithelial cells, especially epidermal cells, showed enucleation and keratinization on the surface contacting the medium. The enucleated cells might have had reduced viability and prevented medium transmission or transportation to basally located cells. On the other hand, mucosal cells seldom showed enucleation and keratinization on the surface and the base. This property of mucosal cell may be an advantage for maintaining viability. From this clinic study, mucosal cells were found to possess sufficient potential for spray grafting, as was the epidermal cell. If the clinical aspects of cultured mucosal cell spray were investigated in detail, mucosal cells would be an appropriate source of grafting for burned patients.

In conclusion, our data show that enzymatic and careful surgical debridement and consecutive application of mucosal epithelial cell suspensions using a spray technique result in excellent cosmetic outcomes compared with any other method.

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3. Injectable tissue engineered bone -biological requirement and clinical relevant-

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Introduction

The tremendous need for bone tissue in numerous clinical situations and the limited availability of suitable bone grafts are driving the development of new approaches to bone repair. In the past the "gold standard" bone graft materials is autologous bone graft and this is limited in supply and its harvesting is associated with significant morbidity [1,2]. Approximately 8% of iliac grafts result in major complications such as infection, blood loss, nerve injury, short- and long-term pain, and functional deficit.

The use of allografts avoids donor site issues but these grafts are associated with risks of infection and possible immune response of the most tissue [3], which can lead to high rates of complications [4-6]. Thus, there is a trend toward tissue engineering as an alternative to the traditional techniques in bone repair. Langer and Vacanti defined tissue engineering as "an interdisciplinary

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field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ [7]".

Regeneration of the bone tissue is the most studied field in tissue engineering. According to the concept, equivalents of the bone tissue can be obtained by targeted osteogenic differentiation of multipotent mesenchymal stem cells (MSC) of the bone marrow (BM). MSC predifferentiated towards osteogenic lineage are applied on biocompatible materials maintaining osteoinduction and possessing sufficient osteoconductive properties [8] transplanted into the bone defect area.

Creation of bone equivalents is now beyond the scope of experimental numerous experimental study the possibility of effective reconstructed the bone tissue using various biodegradable material and MSC [9-11].

In tissue engineered bone carrying MSC from the BM tissue was performed at the department of Oral Surgery in Nagoya University Hospital in accordance with the research protocol approved by the Nagoya University Ethics committee (Permission No. 172) and in compliance with Helsinki Declaration (2000). Here we present the results of the study.

Tissue engineered bone

Cell preparation

Mesenchymal stem cells (MSC) were isolated from the patient's iliac crest marrow aspirates (10 mL) according to the reported method. Briefly [12], the basal medium, low-glucose Dulbecco's Modified Eagle's Medium, and growth supplements (50 mL of serum, 10 mL of 200 mM L-glutamine, and 0.5 mL of penicillin–streptomycin mixture containing 25 units of penicillin and 25 g of streptomycin) were purchased from Cambrex Inc. (Walkersville, MD). Three supplements, dexamethasone, sodium glycerophosphate, and L-ascorbic acid 2-phosphate, for inducing osteogenesis were purchased from Sigma Chemical Co. (St. Louis, MO). The cells were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. The MSCs were replated at densities of 3.1×10^3 cells/cm^2 in 0.2 mL/cm^2 of control medium. The differentiated MSCs were confirmed by detecting alkaline phosphatase activity using p-nitrophenylphosphatase as a substrate.

In culture, MSCs were trypsinized and used for implanting. For the safety of cultured cell, the culture media were examined for contaminations of bacterium, fungus, and mycoplasma before transplantation.

Platelet-rich plasma preparation

Preoperative hematological assessments included a complete blood count with platelet levels. The resulting pellet of platelets (PRP) was extracted 1 day before surgery. The PRP was isolated in a 200-mL collection bag containing the anticoagulant citrate under a sterilized condition at the blood transfusion service department of Nagoya University Hospital, Japan. Briefly, the blood was first centrifuged for 10 minutes at 350g. Subsequently, the yellow plasma containing the buffy coat, which contained the platelets and leukocytes, was removed. A second centrifugation at 3500g for 10 minutes was performed to combine the platelets into a single pellet and the plasma supernatant, which was platelet-poor plasma and contained relatively few cells, was removed. The buffy coat/ plasma fraction (PRP) was resuspended in 20 mL of residual plasma and used in the platelet gel.

Tissue engineered bone preparation

The PRP was stored at 22°C in a conventional shaker until used. Human thrombin in a powder form (5000 units) was dissolved in 5 mL of 10% calcium chloride in a separate sterile cup. Next, 3.5 mL of PRP, MSCs (1.0×10^7 cell/mL), and air were aspirated into a 5-mL sterile syringe. In a second 2.5 mL syringe, 500 μL of the thrombin/calcium chloride mixture was aspirated. The cells were resuspended directly into the PRP. The 2 syringes were connected with a T connector and the plungers of the syringes were alternatively pushed and pulled allowing the air bubble to transverse the 2 syringes. Within 5 to 30 seconds, the contents assumed a gel-like consistency as the thrombin affected the polymerization of the fibrin to produce an insoluble gel (Fig. 1).

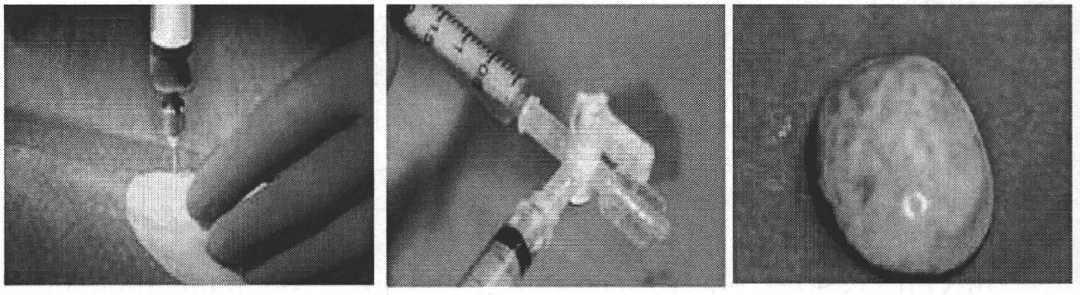

Figure 1

Application for ridge augmentation and dental implant placement

In the field of implant surgery, bone availability is the key to successful placement of endosseous implants in the posterior maxilla and mandible. When the thickness of the bone between the sinus and alveolar crest is less than 5 mm, increasing the thickness of the alveolar sinus floor through grafting is necessary to support the required length of implants. On the other hand, the distance from the mandibular canal is a critical condition to avoid serious nerve injury during implant installation. In a case with insufficient alveolar bone, vertical ridge augmentation through onlay grafting is needed to increase the alveolar bone height.

Because of these circumstances, we attempted to regenerate bone in a significant osseous defect with minimal invasiveness and good plasticity, and to provide a clinical alternative to the previous graft materials. The new technology 'Tissue Engineered Bone (TEB)' that we developed is so called "injectable bone,"[13,14] and involves the morphogenesis of new tissue using constructs formed from isolated cells with biocompatible scaffolds and growth factors.

We evaluate the clinical results, after functional loading, peri-implant tissues of titanium fixtures that had been placed in regions augmented using the injectable bone.

Patient selection

There were 14 cases aged from 44 to 74 years (mean age 54.6 years). Six patients with partially or totally edentulous ridges were scheduled for sinus floor grafting and 8 patients underwent concurrent onlay plasty. All patients had conventional denture retention problems because of severe anterior or posterior alveolar ridge atrophy. In cases of the maxilla, patients had a residual sinus floor of less than 5 mm in height, to such an extent that the sinus graft and implant would have resolved the problem (Table 1); in the other patients, a large part of the residual alveolar arch was atrophied in the horizontal and sagittal directions (Table 1).

After routine oral and physical examinations, patients were selected and TEB grafting was planned because the patients preferred not to undergo any surgery for harvesting of the autogenous bone. In all cases, the reconstruction included sinus floor grafting and onlay plasty in the anterior or part of the posterior maxilla and mandible with simultaneous implant replacement. All patients were healthy and free from any disease that may have influenced the treatment outcome (e.g., diabetes, immunosuppressive chemotherapy, chronic sinus inflammation, rheumatoid arthritis). The patients were informed

extensively about the procedures, including the surgery, graft material, implants, and uncertainties of using a new bone-regenerative method. They were asked for their cooperation during treatment, and the research protocol was approved by the university ethics committee (Fig. 2).

Table 1

Patient data

	age	sex	location	operation	No. of implants
1	51	F	7 6 | 6 7	Maxillary sinus lift	6
2	60	F	| 5 6 7	Maxillary sinus lift	3
3	44	F	7 6 |	Maxillary sinus lift	2
4	54	F	7 6 5 | 5 6 7	Maxillary sinus lift	6
5	50	F	6 5 4 |	Maxillary sinus lift	3
6	56	F	| 5 6 7	Maxillary sinus lift	3
7	52	F	7 6 |	Onlay graft	3
8	74	M	7 6 5 4 |	Onlay graft	4
9	54	F	7 6 |	Onlay graft	3
10	54	M	2 3 |	Onlay graft	2
11	54	F	2 3 |	Onlay graft	2
12	58	F	7 6 5 4 |	Onlay graft	4
13	52	F	5 - 2 | 2 - 5	Onlay graft	8
14	52	F	5 - 1 | 1 - 5	Onlay graft	8

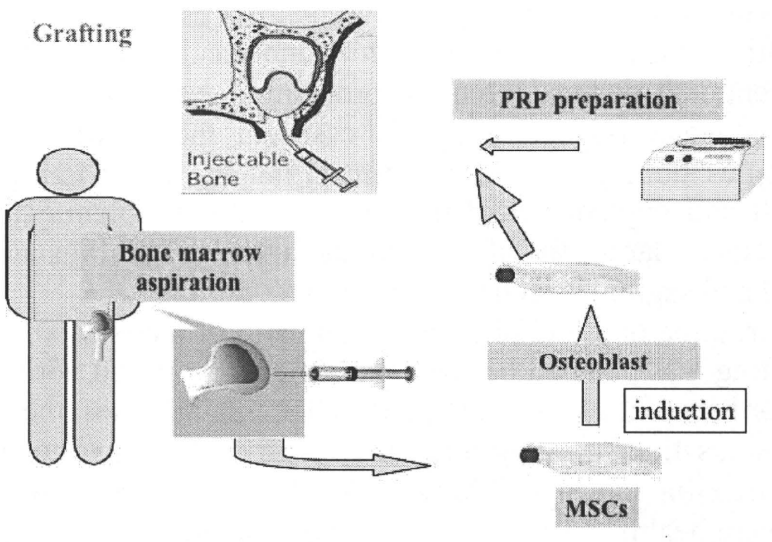

Figure 2