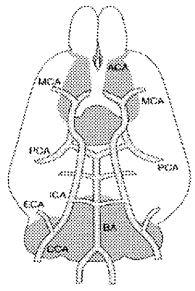
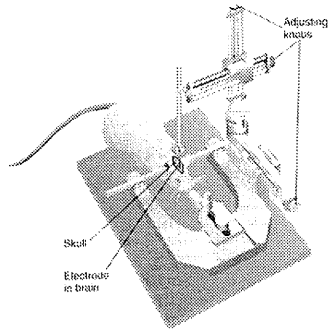
本研究で用いる乳歯歯髄幹細胞（SHED）は、名古屋大学医学部付属病院歯科口腔外科において、該当患者に書面および口頭で十分に説明し、本研究への理解と同意のもと提供された乳歯を使用した。同意書については、名古屋大学倫理委員会にて承認を受けたものを用いた。また、SHED は完全に匿名化されており、プライバシー保護のためドナー情報が不明な状態で移植実験に用いた。



(図 1)右総頸動脈永久焼灼術　麻酔下にて電気メスを用い、右総頸動脈(CCA)を永久焼灼する。

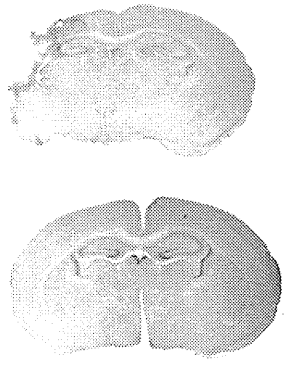


(図 2) Stereotaxic Injector　PVL モデルマウスを固定し、脳虚血部へ細胞移植を行う。

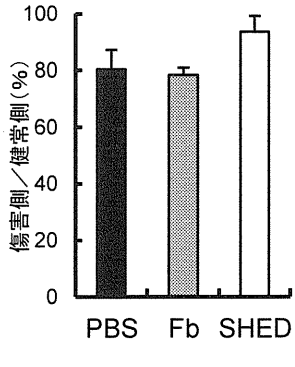
C. 研究結果

1）SHED 移植による PVL 治療効果の組織学的検討

はじめに、移植細胞が虚血部位に生着していることを、ヒト抗核抗体を用いて免疫染色を行い確認した。移植後 48 時間後に脳を取り出しパラホルムアルデヒド固定後、60 μm 切片を作製し、ヘマトキシリン・エオジン染色を用いて脳傷害の改善の程度を定量した(図 3)。その結果、hFb、PBS 移植群と比較して、SHED 移植群では有意に脳の萎縮、変形が少なかった(図 4)。



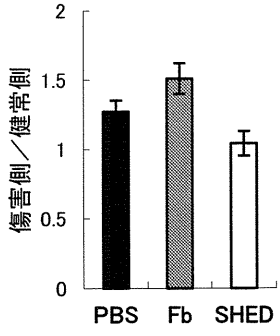
(図 3) ヘマトキシリン・エオジン染色　左：PBS 移植群／右：　SHED 移植群



(図 4) 健常側に対する傷害側の脳の大きさの割合を定量
SHED 移植群が有意に脳の萎縮や変形が少なかった

次に傷害脳における細胞死の割合を検討したところ、hFb、PBS 移植群と比較して、SHED 移植群では有意に細胞死が減少していることがわかった(図 5)。このことから、SHED は低酸素虚血傷害による細胞死に対し、神経保護的に作用することが示唆された。この細胞死が抑制された現象について詳細に解析するため、脳を皮質、脳梁、線条体、海馬の４つの部位に分け、さらにこの神経保護作用が脳

内に存在する神経系細胞のうち、どのポピュレーションに対して効果があるのか、細胞死マーカーである抗 Cleaved Caspase3 抗体と、神経細胞マーカーNeuN、オリゴデンドロサイトマーカーAPC、アストロサイトマーカーGFAP との二重染色を行い、解析を行った。その結果、SHED 移植群では皮質ではオリゴデンドロサイト、線条体と海馬では神経細胞の細胞死が特に減少していることがわかった。



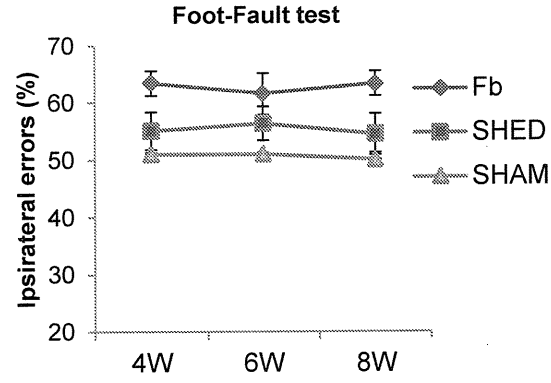
(図 5)健常側に対する傷害側の Caspase3 陽性細胞数の割合
SHED 移植群では有意に細胞死が減少していた

2）SHED 移植による安全性の検討

PVL モデルマウスへの SHED 移植 48 時間後、4 週間後、8 週間後に脳を取り出し、パラホルムアルデヒド固定後に 60 μm に薄切して切片を作製したのち、抗ヒト核抗体を用いて免疫染色を行い、移植細胞の局在を確認した。また、その周囲組織の腫瘍化、形態変化を検討した。その結果、移植細胞は移植部位に腫瘍化することなく生着しており、形態的にも著変は認めなかった。

3）SHED 移植による PVL モデルマウスの機能障害改善の評価

SHED、hFb を移植した PVL モデルマウスに対し、移植 4 週間後、6 週間後、8 週間後に Foot Fault-Test を行い、神経学的機能障害に対する治療効果の検討を行ったところ、SHED 移植群において有意に改善がみとめられた(図 6)。



(図 6)Foot Fault – Test　SHED 移植群は Fb 移植群に比較して、有意に神経学的機能障害の回復が認められた

平成２３年度研究報告

B. 研究方法

1）SHED 移植による PVL モデルマウスの治療効果の病理学的検討

SHED の細胞死抑制効果について、病理学的解析を行った。移植脳を HE 染色にて、前方、中央、後方の皮質、線条体、海馬を CA1、CA2、CA3、DG の８カ所に分類し、神経病理学スコアを用いて評価を行った。

2）SHED を移植した脳の炎症性および抗炎症性サイトカインの発現の定量

神経細胞死の抑制、構造の維持などの効果が得られた機序を調べるため、我々は炎症症状に注目し、PVL モデルマウスへ細胞移植を行ってから２４時間後の脳から mRAN を抽出し、炎症性および抗炎症性サイトカインの mRNA の発現を Real Time-PCR を用いて定量を行った。炎症性サイトカインとして、IL-1β、TNF-α について、抗炎症性サイトカイン

として、IL-4、IL-6、IL-10、IL-13 について、発現を解析した。

3）SHED のパラクライン効果の検討

細胞の培養上清（SHED-CM）を用いた実験を行った。コントロールとしてヒト線維芽細胞の培養上清（hFb-CM）、DMEM を用いた。細胞移植と同様の手法を用い，PVL モデルマウスを作製し、２４時間後に培養上清を脳内へ注入、その４８時間後に評価を行った。４８時間後にマウスを屠殺、灌流固定後、4%パラホルムアルデヒドを用いて固定した。脳を 60μmに薄切後、免疫染色および HE 染色を行った。HE 染色にて細胞の形態の評価を、神経細胞骨格を染色する MAP2 染色にて梗塞領域の評価を、Cleaved caspase-3 染色にてアポトーシスの評価を行った。

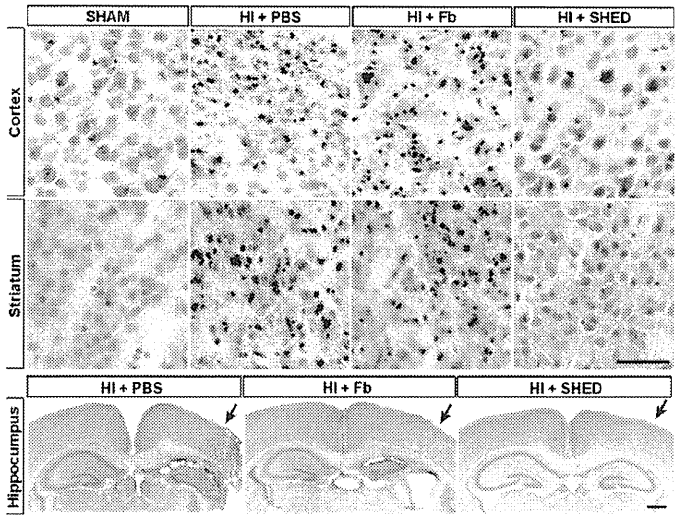
4）生存率および神経学的機能回復の評価

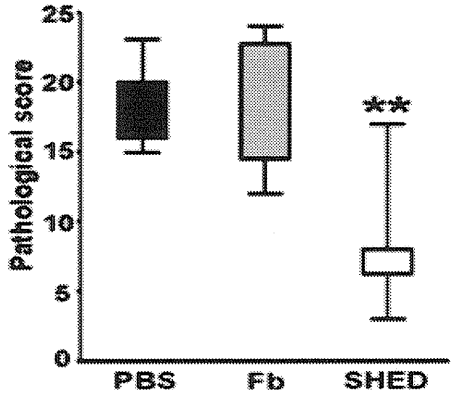
SHED-CM／hFb-CM／DMEM 投与後 4 週、6 週、8 週目に Foot Fault-Test を行い、神経学的機能の評価を行った。また、生存率についても Kaplan-Meier の生存曲線（Log-rank test）を用いて評価を行った。

C. 研究結果

1）SHED 移植による PVL モデルマウスの治療効果の病理学的検討

SHED 移植群においては、脳の組織構造が維持されており、病理学的にも PVL に対する治療効果を認めた。





（図 7）HE 染色像および神経病理学スコア

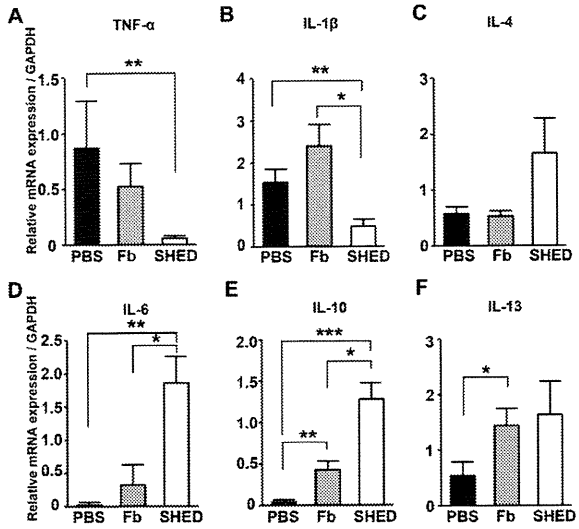
SHED 移植群では、PBS 群、hFb 移植群と比較して、有意に脳の神経細胞構造が保たれていた。

2）SHED を移植した脳の炎症性および抗炎症性サイトカインの発現の定量

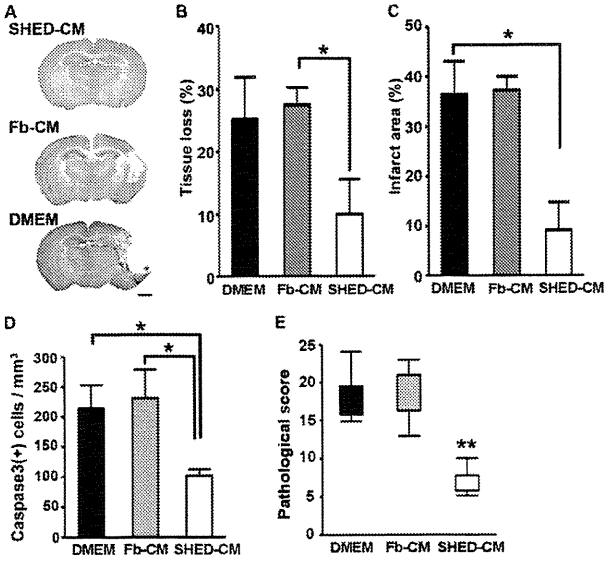
SHED 移植群では、炎症性サイトカインである TNF-α、IL-1β の発現が減少し、脳内では抗炎症性に作用するサイトカインの IL-4、IL-6、IL-10、IL-13 の発現が増加していた。SHED 移植群において、炎症性サイトカインの発現の抑制、抗炎症性サイトカインの発現

の増加を認めた。

（図８）Real Time-PCR 結果
A:TNF-α,B:IL-1β,C: IL-4, D:IL-6, E:IL-10, F:IL-13



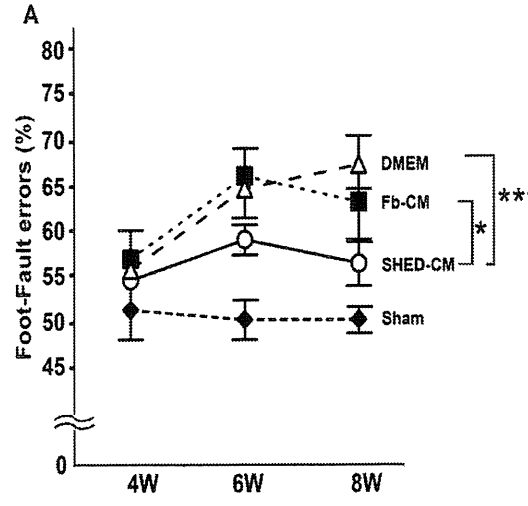
3）SHED のパラクライン効果の検討
SHED の培養上清（SHED-CM）を投与した群では、hFb-CM および、DMEM 群と比較して、細胞移植と同じ様に神経細胞死や脳の萎縮・変形の抑制、および梗塞領域の縮小を認めた。
また、病理学的評価においても、治療効果を認めた。



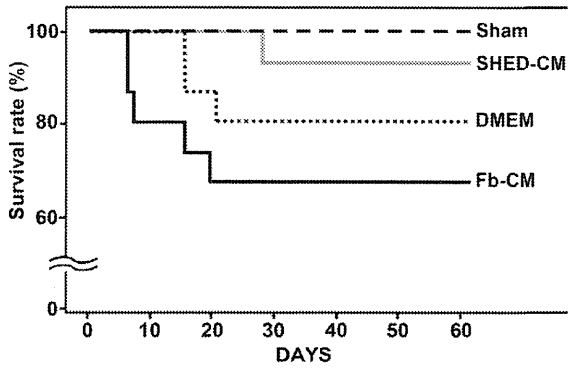
（図９）培養上清投与による PVL 治療効果
A: MAP2 染色像、B:組織損傷の定量、C:梗塞領域の定量、D:アポトーシス細胞の定量、E:神経病理学スコアによる病理学的評価
SHED-CM 投与群において、すべての項目で有意に治療効果を認めた。

4） SHED-CM 投与による PVL モデルマウスの機能障害改善の評価
SHED-CM、hFb-CM を投与した PVL モデルマウスに対し、移植 4 週間後、6 週間後、8 週間後に Foot Fault-Test を行い、神経学的機能障害に対する治療効果の検討を行ったところ、SHED-CM 投与群において有意に改善がみとめられた(図１０)。

（図１０）Foot Fault-Test



また、生存率においても SHED-CM 投与群は、hFb-CM 投与群に比べて改善傾向を認めた。



(図１１)　Kaplan-Meier Survival Curve

C.研究結果

以上の研究結果をまとめ、学会および論文に報告した。

D.考察

本研究において、PVL モデルにおける SHED 移植による治療効果を検討したところ、組織学的、機能的な解析において有益な結果を得ることができた。その機序について、炎症系について解析を行ったところ、　SHED 移植群では炎症性のサイトカインの発現が抑制され、抗炎症性サイトカインの発現が上昇していたことから、傷害脳の微小環境が炎症性のものから神経保護的なものへとシフトしたことが示唆され、これも抗アポトーシス作用の一端を担っていると考えられる。細胞移植後８週間経過した脳を解析したところ、SHED および hFb のニューロン前駆細胞、オリゴデンドロサイト前駆細胞、ミクログリア、アストロサイトなどの神経系譜細胞への分化を確認することができなかった。このことから細胞移植による上記の効果は、移植した SHED が失われた神経系細胞へ分化し、補填したのではないことを示唆している。そこで、これらの作用は移植した細胞から分泌される何らかの栄養因子によるものではないかと考え、細胞の培養上清のみを用いた実験を行ったところ、細胞移植と同様の治療効果を認めた。このことから、細胞から分泌される栄養因子に抗アポトーシス作用、抗炎症作用があることが示唆された。

E.結論

本研究では、医療廃棄物である乳歯より採取した SHED は、PVL モデルに移植することによって脳内の微小環境を神経保護的なものへと変化させることで、炎症やアポトーシスを抑え、神経細胞の維持、保護に作用することがわかった。また、その作用は SHED が失われた神経細胞へ分化し補填したためではなく、SHED から分泌される栄養因子によるものであることがわかった。さらにこのパラクライン効果は、細胞移植と同様の効果を発揮することがわかった。

SHED の培養上清のみで神経保護や機能改善に寄与することができたことから、細胞不要の再生医療への道が拓かれたと考える。これは、移植細胞の免疫拒絶反応や腫瘍化の問題が解決されたことを意味する。その栄養因子の成分について詳細な解析が、今後の研究課題である。

F.健康危険情報

本研究において国民の生命、健康に重大な影響を及ぼす事項は発生していない。

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Human dental pulp-derived stem cells protect against hypoxic-ischemic brain injury in naonatal mice

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◯Mari Yamagata

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Global COE Symposium on Neuro-Tumor Biology and Medicine

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H.知的財産権の出願・登録状況（予定を含む）

該当なし

研究成果の刊行に関する一覧表

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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 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; OR, original region.
Trauma mechanism and ABSI score.

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

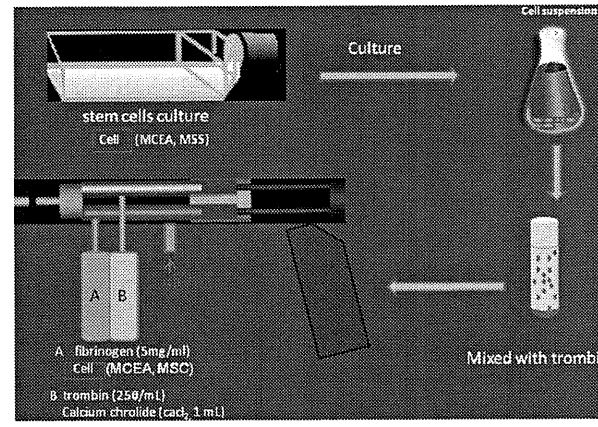

FIGURE 1. Prototype spray device for application of cell suspensions.

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

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.

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.

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

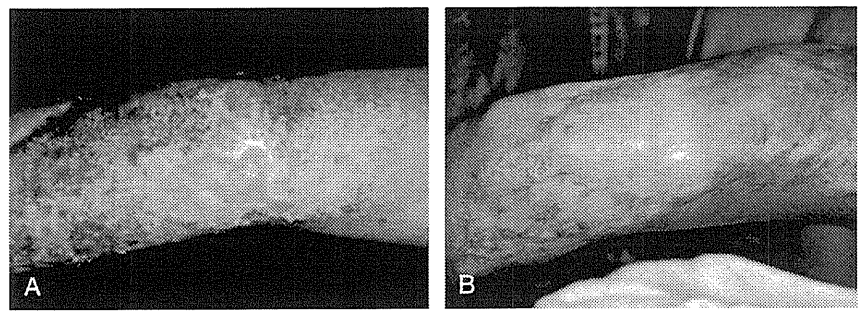

FIGURE 2. A 56-year-old male patient (patient 6, Table 1) presenting with deep dermal burns after flame injury (A). Results at follow-up after 12 months (B).

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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Cell-Based Cytokine Therapy for Skin Rejuvenation

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Abstract: The interaction between stem cells from human exfoliated deciduous teeth (SHEDs)–derived growth factors and human dermal fibroblast (HDF) 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 SHED-derived conditioned medium (SH-CM) can be used for the treatment of photoaging. Our results suggest that SHEDs and SH-CM should be constitutionally suited for photoaging treatment. Mainly with secreted growth factors or extracellular matrix 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.

Key Words: Dental pulp stem cell, cytokine, skin rejuvenation, photo aging

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Regenerative medicine using stem cells is a promising tool in a new clinical platform for a whole spectrum of intractable diseases. Various stem cells have been reported, including embryonic stem cells, induced pluripotent stem cells, and somatic stem cells. Because of ethical problems, immunorejection, and tumorigenesis, embryonic stem cells and induced pluripotent stem cells have many issues to overcome. Among them, somatic stem cells, especially mesenchymal stem cells (MSCs) isolated from various tissues including bone marrow (BMSCs), adipose tissue, skin, umbilical cord, and placenta,[1–4] have been used in clinical applications in skin regeneration. However, bone marrow aspiration is an invasive and painful procedure for the donor. In addition, the number, proliferation, and differentiation potential of BMSCs decline with increasing age.[5]

Dental pulp seems to be an alternative and more readily available source of stem cells. Stem cells from the pulp of permanent teeth (dental pulp stem cells [DPSCs]) and from exfoliated deciduous teeth (SHEDs) have been identified as a novel population of stem cells that have the capacity of self-renewal and multilineage differentiation and similar to BMSCs.[6,7]

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Moreover, DPSCs have been reported to have the potential for use in cell-based therapy for systemic disease, such as neurologic disease and cardiac disease, and to ameliorate ischemic disease.[8–10] The main advantage of using SHEDs is that it can be obtained noninvasively from deciduous teeth that are routinely extracted in childhood and generally discarded as medical waste without any ethical concerns. Despite extensive reports on DPSCs, the characteristics and possible applications of SHED-derived stem cell remain poorly understood.

Recent studies have shown that MSCs may contribute to skin repair. In addition, there have been extensive investigations into wound healing by the exogenous application of various growth factors. However, result of utilizing growth factors at a single dose, multiple doses, or the combined application of multiple factors, with the expectation of synergistic effects, have not yet been confirmed clinically. Mixed growth factors secreted from stem cells may have an ideal combination that can improve damaged skin condition.

On the other hand, it has become the focus of cosmeceuticals and dermatologists treating an aging population overexposed to the sun; various noninvasive treatments and topical cosmeceuticals have been used to treat some symptoms of photoaged skin, including wrinkles.[11–13] Despite numerous claims of the reversal of wrinkles, solid scientific evidence regarding this issue is limited. Aging can be divided into 2 categories: intrinsic and extrinsic. Extrinsic aging refers to components mediated by environmental factors, which include smoking, chemical exposure, and primarily UV-B exposure.[11,14,15] Extrinsic aging is characterized by fine and coarse wrinkling, roughness, dryness, laxity, and pigmentary lesion. This type of aging causes a decrease in epidermal thickness and atypia of keratinocytes. In the dermis, UV-B exposure has been shown to stimulate collagenase production by human dermal fibroblasts (HDFs) and to upregulate collagenase gene expression. This induces degeneration of collagen and deposition of altered elastic tissue, which is prominent as wrinkles and yellow discoloration of skin.[16,17] Lasers and several cosmeceuticals include derivatives of retinol, vitamin C, and topical growth factors, all of which are well known for inducing collagen synthesis from HDFs and have been used for the treating skin texture and wrinkling.[13]

In the previous studies, MSCs have been known to produce various cytokines such as vascular endothelial growth factor, hepatocyte growth factor, insulinlike growth factor, platelet-derived growth factor, and transforming growth factor β. Recently, the production and secretion of cytokines have been reported as an essential function of MSCs, and diverse pharmacological actions of MSCs have been demonstrated especially in skin biology.[7,18–20] For example, it was reported[21] that MSCs have cutaneous healing effect through the production of diverse growth factors. These growth factors activated HDFs, which increase the proliferation/migration of HDFs and mediate the secretion of collagen from HDFs. The antioxidant effect of MSCs also has been demonstrated by showing that the secretory factors of MSCs protect HDFs from oxidative stress. The application of topical growth factors stimulated the repair of facial photoaging resulting in new collagen synthesis, epidermal thickening, 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 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, Flextime1 (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

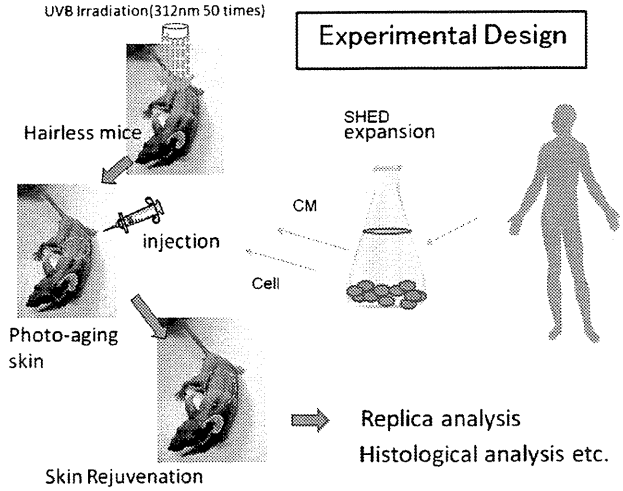

FIGURE 1. Experimental design in hairless mice. Wrinkle was induced by UV-B irradiation.

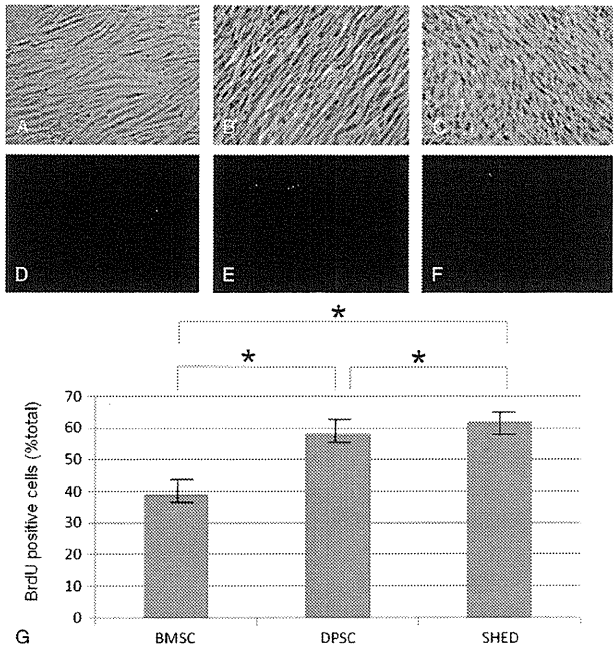

FIGURE 2. A–C, Cell morphology of (A) BMSCs, (B) DPSCs, and (C) SHEDs (original magnification ×40). D–F, Immunofluorescence staining of the stem cell marker STRO-1: (D) BMSCs, (E) DPSCs, and (F) SHEDs were positive for STRO-1 (green fluorescence). DAPI was used to visualize the nuclei (blue fluorescence). 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: $*P < 0.05$.

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.

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). Optical density 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

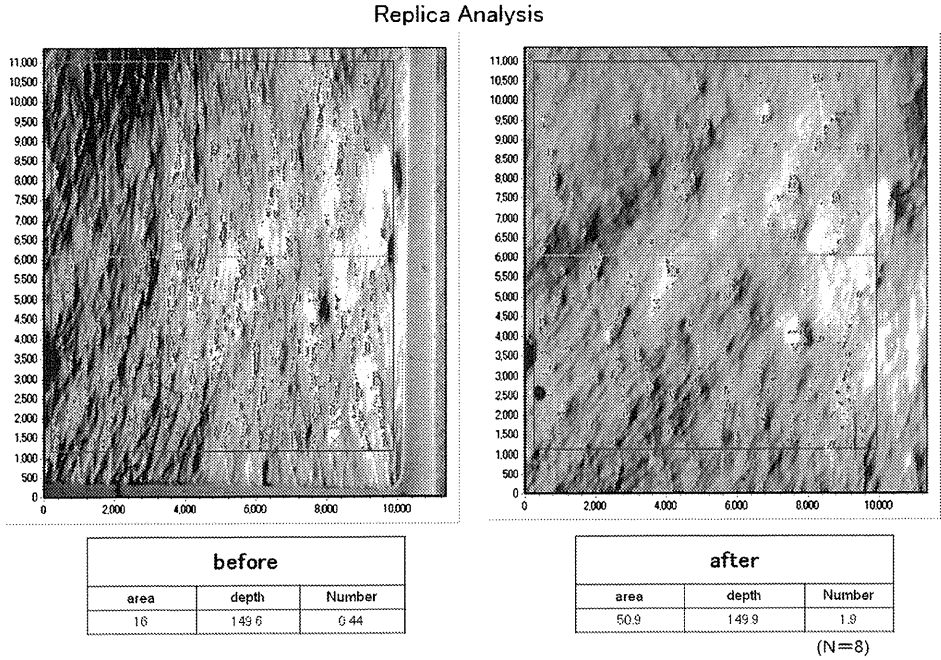

FIGURE 3. Evaluation of wrinkles by replica analysis after SH-CM injection 100% SH-CM treatment.

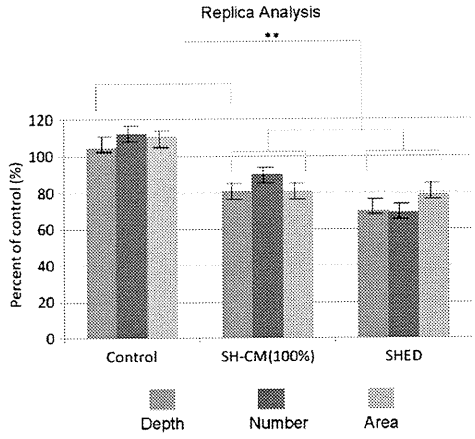

FIGURE 4. Improved parameters for wrinkles in natural level of SH-CM– and SHED-injected group.

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

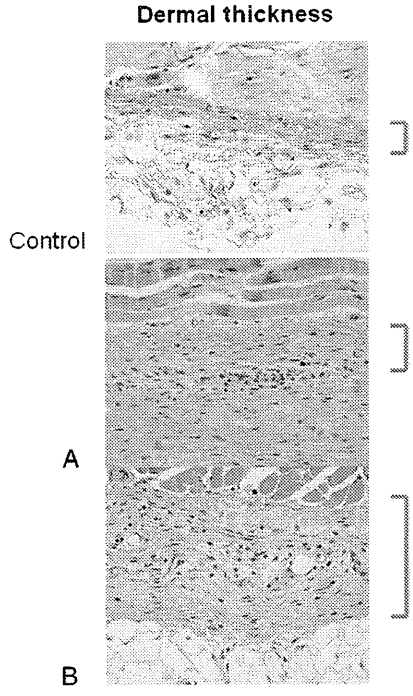

FIGURE 5. Hematoxylin-eosin staining. Collagen fibers were stained and were significantly increased in the SH-CM–treated group and SHED-injected group. B, SHED-injected group.

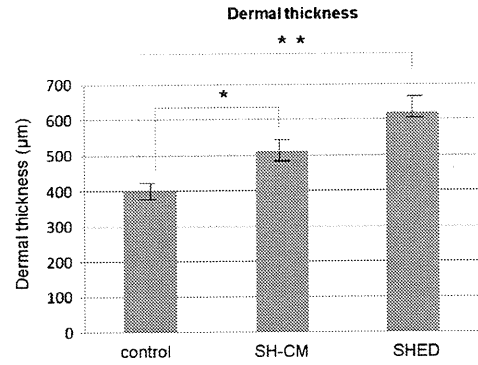

FIGURE 6. Hematoxylin-eosin staining, SH-CM–treated and SHED-injected group increased dermal thickness of hairless mice.

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 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).

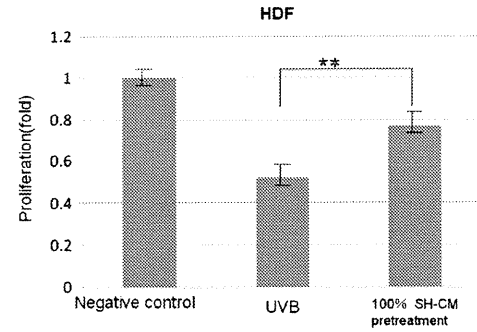

FIGURE 7. Effect of SH-CM on the proliferation of HDFs. UV-B irradiation significantly decreased the proliferation of HDFs, but pretreatment of SH-CM protected HDFs from UV-B.

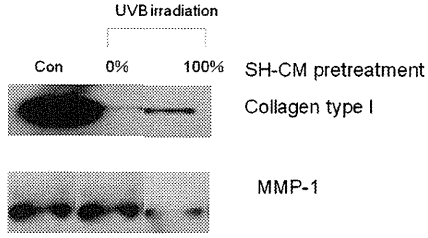

FIGURE 8. Effect of SH-CM on the expression of collagen type I and MMP-1.

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.

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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