which were connected to multiunit abutments (Fig 16a). A maxillary complete denture and a mandibular implant-supported prosthesis were placed and have functioned for a year without problem (Figs 16b and 16c).

Tissue engineered bone can enhance the calcification in bone healing

A vascularized fibular flap is often selected for mandibular reconstruction because it offers adequate length of bone and pedicle, constant geometry, and low donor site morbidity. However, to follow the mandibular arch, the fibula requires multiple osteotomies, which interrupt the medullary vessel and thereby vascular supply since the entire flap depends on the periosteum [43].

The fibular periosteum still supplies the external two thirds of the cortex after revascularization, while its internal third and the medulla have a reduced vascular supply [44].

Preservation of periosteal attachment is therefore considered a critical factor in DO, even if grafted fibular segments have healed and united. Several authors have reported on successful cases of vertical DO of the fibula grafted to reconstruct the mandible [43,45].

These cases were less complex than the present case, which included a patient with older age, a higher dose of irradiation,a larger transport segment, a longer distance of distraction, and damage to the labial periosteum resultant to simultaneous removal of osteosynthetic plates and screws. These conditions should reflect upon the partial resorption of the superior transport segment. Despite the reflection, the present case demonstrated new bone formation. Not only was the new bone formation less complicated on the labial side of the regenerate, it was also better quality inside, as observed radiographically and histologically, without a longer consolidation period. These favorable results might be attributed to the material injected into the distracted tissue.

In applying TEB to DO, they regarded the fibrous tissues in the distracted zone as the scaffold. Several animal studies have shown that the injections of cells with osteogenic potential into distraction gaps enhanced new bone formation with respect to volume and strength and that this enhancement led to shortening of the consolidation period [39-42].

The timing of the cell injections was further investigated; it appeared to have no effect on experimental outcome [41].

In this case the 15-mm distraction was considered relatively short, and the injection was administered at the end of the distraction because that is when the number of cells in the distraction gap with osteogenic potential is the lowest. The injected cells could work before their gradual recruitment via vessel.

Growth factors which alpha granules of the platelets secrete can activate cells, including MSCs and osteoblasts, through their membrane receptors [46].

Partial resorption of the transport segment, which left the gap between its neighboring bone, was recovered with the injectable bone. Its gel form allowed the contained cells to contact surface microarchitecture of implants placed simultaneously. For space making with a relatively large shield, a titanium mesh was considered superior to polytetrafluoroethylene membranes because they restrict new vascularity [47].

The lack of blood supply might limit bone regeneration with the TEB to a certain amount. DO has few limitations regarding distraction length but requires longer treatment time than grafting. These innovative methods in combination can allow more effective bone regeneration for adequate implant placement.

Discussion

Reconstruction of maxillofacial defects secondary to tumors and trauma relies on different sources of bone grafts with inherent morbidity. Stem-cell-based tissue engineering is a promising alternative for bone regeneration (Petite et al., 2000; Bianco et al., 2001; Rose and Oreffo, 2002). Oral & maxillofacial bone engineering is a fast-moving field with considerable potential clinical applications (Mao et al., 2006; Kaigler et al., 2006; Zhao et al., 2007).

The aim of this article is to summarize our current research on bone tissue engineering in Nagoya University Hospital and highlight important translational studies that has already been carried out on human subjects.

Unfortunately, only a very small proportion of the above clinical studies except our study make it to the bedside in the form of clinical trials or therapies. Because of practical and ethical reasons, it is sometimes impossible to have proper control groups and therein lies the difficulty of data interpretation. The clinical studies discussed here use a variety of approaches including bone marrow, MSC and scaffolds, and osteoinductive factors (PRPs) in treating a variety of conditions including implant, tumors defect, alveolar cleft. Our studies are small, observational phase 1-type studies with no control groups and they have short-term follows. Despite this, they do provide valuable information and we know that the clinical use of autologous bone marrow derived MSC is relatively safe and does not preclude the use of other techniques in the event of failure.

As described in this article, reconstruction of maxillomandibular defects using tissue-engineered bone has the potential to dramatically improve current methods that rely on sequential bone grafting followed by oral

surgeries. The abilities to eliminate donor-site morbidity related to autogenous bone-graft harvest, and to provide comprehensive oral rehabilitation therapies superior to current synthetic implant materials, would make a significant contribution to current dentistry.

The our case reports demonstrate the absence of the reaction of the transplant bed to the transplanted tissue-engineering construct. We observed rapid healing of the operation wound without mucosa ingrowth into the transplant. None patients had microbial inflammatory complications, exposure of the implanted material, or flap necrosis. X-ray examination after transplantation revealed ossified tissue similar to cortical lamina at the boundary of the transplants and oral mucosa. Histological examination of tissue samples from the center of the regenerate revealed the development of young low-mineralized bone tissue.

Thus, the results of our clinical study suggest that transplantation of tissue-engineering bone for bone defect reconstruction is a safe procedure allowing to solve some complex clinical problems of oral surgery.

Taken together, injectable tissue-engineered bone would provide a further option as a graft material for the bone reconstruction of other parts of the skeleton such as osteporotic fracture in the extremities, cranial bone regeneration after brain surgery, and osteoradiomyelitis. Future research will have to address the long-term rates, the stability of tissue-engineered bone, and the application of the therapy to less vascularized environments. We suggest that, based on the present findings, future clinical trials are warranted.

Acknowledgments

The author wishes to thank to Yoichi Yamada, Hideharu Hibi, and the members of the Department of Oral and Maxillofacial Surgery, Nagoya University Graduate School of Medicine for help, encouragement and contributions to the completion of this study. This work was partly supported by GCOE, the Ministry of Education, Culture, Sports, Science and Technology (MEXT) on Grant-in-Aid for young scientists (B) (15791163) and by Japan Society for the Promotion of Science (JSPS) on Grant-in-Aid for Scientific Research (B) (16390583).

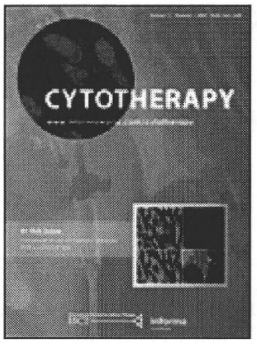
References

1. Laurie SW, Kaban LB, Mulliken JB, Murray JE. Donor-site morbidity after harvesting rib and iliac bone. Plast ReconstrSurg 1984;73:933–938.
2. Quarto R, Mastrogiacomo M, Cancedda R, Kutepov SM, Mukhachev V, Lavroukov A, Kon E, Marcacci M. Repair of large bone defects with the use of autologous bone marrow stromal cells. N Engl J Med 2001;344:385–386.

3. Stevenson S. The immune response to osteochondral allografts in dogs. J Bone Joint Surg Am 1987;69:573– 582.
4. Alman BA, De Bari A, Krajbich JI. Massive allografts in the treatment of osteosarcoma and Ewing sarcoma in children and adolescents. J Bone Joint Surg Am 1995;77:54–64.
5. Lord CF, Gebhardt MC, Tomford WW, Mankin HJ. Infection in bone allografts. Incidence, nature, and treatment. J Bone Joint Surg Am 1988;70:369–376.
6. Mankin HJ, Gebhardt MC, Tomford WW. The use of frozen cadaveric allografts in the management of patients with bone tumors of the extremities. Orthop Clin North Am 1987;18:275–289.
7. Langer R, Vacanti JP. Tissue engineering. Science 1993;260:920–926.
8. H.Hattori, M.Sato, K.Masuoka, et al: Cells Tissues Organs.,178, No.1 2-12, 2004
9. B.Fang, Y.Song, Q.Lin, et al., Pediatr Transplant., 11, No.7, 814-817, 2007
10. S. Lendeckel, A. Jodicke, P.Christophis, et al., J.Craniomaxillo.Surg., No.6, 370-373, 2004
11. K.Weinzierl, A.Hemprich, B Frerich, J.Craniomaxillofac. Surg., 34 No.8, 466-471, 2006
12. Pittenger MF, Mackay AM, Beck CB, et al. Multilineage potential of adult human mesenchymal stem cells. Science.1999;284:143-147.
13. Yamada Y, Boo JS, Ozawa R, et al. Bone regeneration following injection of mesenchymal stem cells and fibrin glue with a biodegradable scaffold. J Craniomaxillofac Surg. 2003;31:27-33.
14. Yamada Y, Ueda M, Naiki T, et al. Autogenous injectable bone for regeneration with mesenchymal stem cells and platelet-rich plasma: Tissue-engineered bone regeneration. Tissue Eng. 2004;10: 955-964.
15. Tatum H. Maxillary and sinus implant reconstructions. Dent Clin North Am. 1986;30:207-230.
16. Jensen J, Simonsen EK, Sindetpedersen S. Reconstruction of the severely resorbed maxilla with bone grafting and osseointegrated implants: A preliminary report. J Oral Maxillofac Surg. 1990; 48:27-32.
17. Marx RE. Clinical application of bone biology to mandibular and maxillary reconstruction. Clin Plast Surg. 1994;21: 377-392.
18. Raghoebar GM, Brouwer TJ, Reintsema H, et al. Augmentation of the maxillary sinus floor with autogenous bone for placement of endosseous implants: A preliminary report. J Oral Maxillofac Surg 1993;51:1198-1203.
19. Marx RE, Morales MJ. Morbidity from bone harvested in major jaw reconstruction. J Oral Maxillofac Surg. 1988;48: 196-203.
20. Smith JL. Osseous regeneration in preclinical models using bioabsorbable delivery technology for recombinant human bone morphogenetic protein 2(rhBMP-2). J Control Release. 1995;36: 183-195.
21. Gerhart TN, Kirker-Head CA, Kriz MJ, et al. Healing segmental femoral defects in sheep using recombinant human bone morphogenetic protein. Clin Orthop Relat Res. 1993;293:317-326.

22. Toriumi DM, Kotler HS, Luxenberg DP, et al. Mandibular reconstruction with a recombinant bone-inducing factor: Functional, histologic, and biomechanical evaluation. Arch Otolarnygol Head Neck Surg. 1991;117:1101-1112.
23. Sigurdsson TJ, Lee MB, Kubota K, et al. Periodontal repair in dogs: Recombinant human bone morphogenetic protein-2 significantly enhances periodontal regeneration. J Periodontol. 1995;66:131-138.
24. Boyne P, Marx RE, Nevins M, et al. A feasibility study evaluation rhBMP-2/absorbable collagen sponge for maxillary sinus floor augmentation. Int J Periodontics Restorative Dent. 1997;17:11-25.
25. Horswell BB, Henderson JM. Secondary osteoplasty of the alveolar cleft defect. J Oral Maxillofac Surg 2003: 61: 1082-1090
26. Zeitler D. Alveolar cleft grafts. In: Fonseca RJ, ed: Oral and Maxillofacial Surgery, Vol. 6 Cleft/ craniofacial/cosmetic surgery. Philadelphia: W.B. Saunders 2000: 75-86.
27. Fonseca RJ, Turvey TA, Wolford LM.Orthognathic surgery in the cleft patients.In: Fonseca RJ, ed: Oral and Maxillofacial Surgery, Vol. 6, Cleft/craniofacial/cosmetic surgery. Philadelphia: W.B.Saunders 2000: 87–146.
28. Boyne PJ, Nath R, Nakamura A.Human recombinant BMP-2 in osseous reconstruction of simulated cleft palate defects. Br J Oral Maxillofac Surg 1998:36: 84–90.
29. Kawamoto T, Motohashi N, Kitamura A, Baba Y, Suzuki S, Kuroda T. Experimental tooth movement into bone induced by recombinant human bone morphogenetic protein-2. Cleft Palate Craniofac J 2003: 40: 538–543.
30. Mayer M, Hollinger J, Ron E, Wozney J. Maxillary alveolar cleft repair in dogs using recombinant human bone morphogenetic protein-2 and a polymer carrier. Plast Reconstr Surg 1996: 98:247–259.
31. Hibi H, Yamada Y, Kagami H, Ueda M.Distraction osteogenesis assisted by tissue engineering in an irradiated mandible:a case report. Int J Oral Maxillofac Implants 2006: 21: 141–147.
32. Yamada Y. Ueda M., Hibi H, Nagasaka T, Translational research for injectable tissue-engineered bone regeneration using mesenchymal stem cells and platelet-rich plasma: from basic research to clinical case study. Cell Transplant 2004: 13: 343-355.
33. Van der Meij AJW, Baart JA, Prahl Andersen B, Valk J, Kostense PJ, Tuinzing DB. Bone volume after secondary bone grafting in unilateral and bilateral clefts determined by computed tomography scans. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2001: 92: 136-141.
34. Von Arx T, Hardt N, Wallkamm B. The TIME technique: a new method for localized alveolar ridge augmentation prior to placement of dental implants.Int J Oral Maxillofac Implants 1996:11: 387–394.
35. Schmelzeisen R, Schimming R, Sittinger M. Making bone: implant insertion into tissue-engineered bone for maxillary sinus floor augmentation – a preliminary report. J Cranio-Maxillofac Surg 2003:31: 34–39.
36. Schimming R, Schmelzeisen R. Tissueengineered bone for maxillary sinus augmentation. J Oral Maxillofac Surg 2004:62: 724–729.

37. Yen SL-K, Yamashita DD, Gross J, Meara JG, Yamazaki K, Kim TH, Reinisch J. Combining orthodontic tooth movement with distraction osteogenesis to close cleft spaces and improve maxillary arch form in cleft lip and palate patients. Am J Orthod Dentofacial Orthop 2005: 127: 224–232.
38. Swennen G, Dempf R, Schliephake H. Cranio-facial distraction osteogenesis: A review of the literature.Part 2:experimental studies. Int J Oral Maxillofac Surg 2002;31:123-135.
39. Takushima A, Kitano Y, Harii K.Osteogenic potential of cultured periosteal cells in a distracted gap in rabbits. J Surg Res 1998;78:68-77.
40. Tsubota S,Tsuchiya H,Shinokawa Y,Tomita K,Minato H.Transplantation of osteoblast-like cells to the distracted callus in rabbits.J Bone Joint Surg Br 1999;81-B:125-129.
41. Richards M, Huibregtse BA, Caplan AI, Goulet JA, Goldstein SA. Marrow-derived progenitor cell injections enhance new bone formation during distraction. J Orthop Res 1999;17:900-908.
42. Takamine Y, Tsuchiya H, Kitakoji T, et al. Distraction osteogensis enhanced by osteoblastlike cells and collagen gel. Clin Orthop 2002;399:240-246.
43. Nocini PF, Wangerin K, Albanese M, Kretschmer W, Cortelazz Vertical distraction of a free vascularized fibula flap in a reconstructed hemimandible:Case report. J Craniomaxillofac Sur2000;28:20-24.
44. Bähr W. Blood supply of small fibula segments:An experimetal study on human cadavers.J Craniomaxillofac Surg 1998;26:148-152.
45. Klesper B, Lazar F, Sießegger M, Hidding J, Zöller JE. Vertical distraction osteogenesis of fibula transplants for mandibular reconstruction: A preliminary study. J Craniomaxillofac Surg 2002;30:280-285.
46. Marx RE.Platelet-rich plasma:Evidence to support its use. J Oral Maxillofac Surg 2004;62:489-496.
47. von Arx T, Hardt N, Wallkamm B. The TIME technique:A new method for localized alveolar ridge augmentation prior to placement of dental implants. Int J Oral Maxillofac Implants1996;11:387-394.



Stem cells from human exfoliated deciduous teeth (SHED) enhance wound healing and possibility of novel cell therapy

Journal:	*Cytotherapy*
Manuscript ID:	CYTH-2010-0169
Manuscript Type:	Original Paper
Date Submitted by the Author:	12-Sep-2010
Complete List of Authors:	Nishino, Yudai Yamada, Yoichi Ebisawa, Katsumi Nakamura, Sayaka Okabe, Kazuto Umemura, Eri Hara, Kenji Ueda, Minoru
Keywords:	wound healing, stem cells from exfoliated deciduous teeth (SHED), human mesenchymal stem cells (hMSCs), cell therapy, hyaluronic acid (HA)

Stem cells from human exfoliated deciduous teeth (SHED) enhance wound healing and possibility of novel cell therapy

Yudai Nishino[1], Yoichi Yamada[2], Katsumi Ebisawa[3], Sayaka Nakamura[1], Kazuto Okabe[1], Eri Umemura[1], Kenji Hara[1], and Minoru Ueda[1]

Author's affiliations:

[1]Department of Oral and Maxillofacial Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan

[2]Center for Genetic and Regenerative Medicine, Nagoya University School of Medicine, Nagoya, Japan

[3]Department of Plastic and Reconstructive Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan

Address correspondence to:

Yoichi Yamada, D.D.S., Ph.D., Center for Genetic and Regenerative Medicine, Nagoya University School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8550, Japan.

Tel: +81-52-744-2348; Fax: +81-52-744-2352;

E-mail: yyamada@med.nagoya-u.ac.jp

The abbreviated title for the running head: Novel wound healing promotion therapy with SHED

ABSTRACT

Background

Stem cells from human exfoliated deciduous teeth (SHED) have been paid attention as a novel stem cell source with multipotent potentials in recent years. So we examined the effect on wound healing promotion with the unique stem cells from deciduous teeth as a medical waste.

Methods

It was used an excisional wound splinting model and evaluated the effect of wound healing among SHED, human mesenchymal stem cells (hMSCs), human fibroblasts (hFibro) and control (phosphate buffered saline: PBS) by macroscopic view, histological and enzyme-linked immunoadsorbent assay (ELISA) and investigated the expression of hyaluronan (HA), which was related to wound healing.

Results

SHED and hMSCs accelerated wound healing compared to hFibro and control group. There was statistically significant difference in wound healing area among hFibro, hMSCs, SHED compared to control after day 5. At day 7 and 14 after cell transplantation, the histological observation showed that transplanted PKH 26-positive cells were surrounded by human HA binding protein especially in hMSCs and SHED. HA expression volume values were 1558.41 ± 60.33 (control), 2092.75 ± 42.56 (hFibro),

2342.07 ± 188.10 (hMSCs) and 2314.85 ± 164.91 (SHED) ng/mg, respectively, and expressed significantly higher in hMSCs and SHED compared to hFibro and control at day 7 and 14 (P<0.05).

Discussion

Our results showed that SHED, hMSCs have similar effect of wound healing promotion compared to hFibro and control. This study implies that SHED might offer a unique stem cell resource and would keep possibility of novel cell therapies for wound healing in future.

Keywords

wound healing, stem cells from human exfoliated deciduous teeth (SHED), human mesenchymal stem cells (hMSCs), cell therapy, hyaluronic acid (HA).

Introduction

An intractable wound such as chronic wounds gives a patient great stress, and the treatment is very difficult [1,2]. The wound has been treated by using surgical operation and medical treatment so far. But there are various problems like an imperfect wound healing and scar. It is also not resulted in dramatic changes in wound closure or outcome [3]. Accordingly establishment of the treatment is essential. Now variety of the examination of the development of the ointment treatment, artificial skin and skin substitute as the treatment method of the wound is done. Additionally, cell therapy which is a low aggression, promotion of wound healing and scar less treatment has been paid to attention. There are several lines of evidences reported that fibroblasts and mesenchymal stem cells (MSCs) have been applied to accelerate wound healing through differentiation and paracrine effects [4-6]. Fibroblast injections are based on the hypothesis that the autologous fibroblasts would be capable of producing collagens for ongoing improvements without immune or allergic reactions. But clinical reports have recommended repeated injections in order to continue longer rhytide correction, and the reported results are less than optimal [7]. And fibroblast injections can reportedly increase inflammation and scar formation [7-10]. On the other hand, MSCs are referred to as stromal progenitor cells, selfrenewing and expandable stem cells and are able to

differentiate into osteoblasts, adipocytes, and chondrocytes and so on [11]. However bone marrow aspiration is an invasive procedure for the donor. In addition, the number, proliferation, and differentiation potential of MSC decline with increasing age [12]. In recent study, dental pulp also contains a population of multipotent stem cells with the capacity to differentiate into several different cells lineages in vitro and in vivo [13,14]. These studies facilitated experiments to address an important property of stem cells, the capacity of a given stem cell population. Human dental pulp cell also shows the property that resembled MSCs [15]. Therefore it is expected as new source of stem cell and is to be applied to many diseases same as MSC. And dental pulp cell has been already applied to cardiac infarction, Alzheimer's, Parkinson's disease or muscular dystrophy [16,17]. However there are few report as for application of dental pulp stem cells for wound healing.

Wound healing consists of inflammation, granulation tissue formation, re-epithelization and remodeling. The process of wound healing depends upon a variety of interactions between cells and the extracellular matrix. One of the extracellular matrix is hyaluronic acid (HA), which is well known that not only supports tissue architecture as a passive structural component of the matrix in various connective tissues but is also involved in dynamic cellular processes during wound healing. Thus, it

would be important to observe the HA in the process of wound healing.

In this research we examined the effect of stem cells from human exfoliated deciduous teeth (SHED) in wound healing with using wound healing skin defect model as described previously [18] by comparing to hMSCs, hFibro and control (phosphate buffered saline: PBS). It also would be evaluated in terms of HA expression which is thought to have important biological roles in skin wound healing. Taken together, this study would provide that SHED might offer unique stem cell resource with potentials of novel cell therapies for wound healing.

Methods

Animals

Seven-week-old KSN/Slc nude mice, which were obtained from the Chubu Kagaku Shizai Corporation (Nagoya, Japan), were used. The animal experiments were performed in accordance with the *Guide for Animal Experimentation of Nagoya University School of Medicine*.

Cell culture of hFibro, hMSCs and SHED

SHED were obtained from clinically extracted deciduous teeth at Regenerative Medicine Clinic and Department of Oral and Maxillofacial Surgery, Nagoya University Hospital, after obtaining informed consent from each patient. They were isolated and cultured as previously described [13,14]. Human MSCs and hFibro purchased from LONZA® Inc. (Walkersville, MD, USA). Cells cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing low glucose supplemented with 10% fetal bovine serum and 100 units of penicillin G, 100 μg of streptomycin, and 0.25 μg of amphotericin B (Sigma, St Louis, MO, USA), at 37°C in 95% humidified air and 5% CO_2.

Wound healing model and cell transplantation

The experimental protocol is summarized in Figure 1. Before cell transplantation, they labeled with a fluorescent cell linker PKH26 (Sigma-Aldrich, St Louis, MO, USA), according to the manufacturer's protocol. The animals were randomly divided into four groups. The groups were as follows: control group, phosphate-buffered saline (PBS); experimental groups, hFibro, hMSCs and SHED. The excisional wound splinting model was generated as described previously [18]. Briefly, two 8-mm fullthickness excisional skin wounds were created on each side of the midline. A donutshaped silicone splint was placed so that the wound was centered within the splint. An immediate-bonding adhesive (Krazy Glue Columbus) was used to fix the splint to the skin, followed by interrupted sutures to stabilize its position. Each wound received 4×10^6 cells in PBS injected intradermally around the wound at four injection sites and 1×10^6 cells in PBS applied onto the wound bed. And Tegaderm (3M, London, ON, Canada) was placed over the wounds.

Assessment of wound area

Digital photographs of wound were taken at days 0,1,2,3,4,5,6,7,8,9,10,11,12,13 and 14 after cell transplantation. Wound area was measured by using an image analysis

program (Scion Corporation, Frederick, MD, USA). The percentage of wound area was calculated as follows: Area of actual wound/Area of original wound ×100. Mice were sacrificed at day 7 and 14 after cell transplantation and evaluated by using an image analysis program.

Histologic examination and immunofluorescent staining

Extraction tissue were fixed in 4% paraformaldehyde and embedded in OCT compound (Tissue-Tek; Miles Inc., Elkhart, IN, USA). Sections were taken at 5-μm intervals and performed with hematoxylin-eosin staining. Immunofluorescent staining was used to confirm the presence of human hyaluronic acid (HA) generated by the injected cells (Rockland Immunochemicals Inc., Gilbertsville, PA). The HA were identified with a biotinylated hyaluronic acid binding protein (Seikagaku bio, Tokyo,Japan) diluted 1:100 in PBS for 2 hours at room temperature. After incubation, the slides were rinsed in PBS, and visualized with FITC-conjugated streptavidin (Beckman Coulter Inc., CA, USA) diluted 1:200 in PBS for 15 minutes at room temperature. The slides mounted with the mounting medium with DAPI (Vector Laboratories Inc.). Before reaction of hyaluronic acid binding protein tissue sections were preincubated with 1% bovine serum albumin for 1 hour to reduce autofluorescence. Endogenous biotin was blocked with streptavidin

biotin blocking kit (Vector Laboratories Inc., Burlingame, CA, USA) for 20 minutes at room temperature.

Enzyme-linked immunoadsorbent assay (ELISA) for hyaluronic acid (HA)

To determine the level of HA in each sample at 7 and 14 days, it was used hyaluronic acid quantitative test kit (Seikagaku bio, Tokyo, Japan) according to the manufacturer's protocol. The HA test kit is an enzyme-linked binding protein assay that uses a capture molecule known as hyaluronic acid binding protein (HABP). First, biotinylated-HABP solution is reacted. Next, HRP-conjugated streptavidin solution is added to secondary reaction. Stop solution road with them and a colored product was formed at a level proportional to the amount of HA in each sample that was measured at 450 nm. HA levels in the all samples are determined against a reference curve prepared from the reagent blank and the HA reference solutions provided with the kit.

Statistical analysis

Statistical differences among the volumes in different cells were evaluated by Tukey-Kramer test following one-way analysis of variance (ANOVA). A value of $P < 0.05$ was considered to be statistically significant.

Results

Macroscopic observation of wound healing area by effect of transplanted cells

Each wound samples collected from the dorsum of mice at day 7 and 14 *in vivo*. At day 7, healing was still incomplete all groups and especially in control group, the surface was still raw under macroscopic observation. And exudate was observed in control, hFibro, hMSCs groups. At day 14 wound contraction of hFibro, hMSCs and SEHD groups were almost completely closed in contrast to control group, which was not sufficiently (Figure 2). Digital image analysis showed that the percentage of wound area were 88.48 ± 3.49%, 42.64 ± 5.36% (control), 63.15 ± 4.93%, 19.33 ± 3.77% (hFibro), 46.41 ± 5.49%, 4.90 ± 2.36% (hMSCs), 47.77 ± 4.86%, 5.74 ± 2.85 (SHED) at 7 and 14 days, respectively.

Cell transplantation groups such as hFibro, hMSCs, and SHED demonstrated accelerated wound healing compared to control group There was statistically significant difference in wound area observed between hFibro, hMSCs, SHED groups and control group at all time points after day 5, and between hMSCs, SHED groups and hFibro group at all time points after day 6 ($P < 0.05$; Figure 3). However there was no statistically significant difference between hMSCs and SHED groups (Figure 3).

Histological observation and detection of human hyaluronic acid (HA) Produced by transplanted hFibro, hMSCs and SHED

At day 7 after cell transplantation, infiltrate of inflammatory cells were admitted in all groups. The wound epidermis was lack in control group especially. Cell transplantation groups, especially hMSCs, SHED groups were observed thick granulation tissue. And HABP detection was performed to confirm the presence of HA derived from transplanted cells in the each wound sides. The evaluation by fluorescence microscope showed that PKH 26 positive cells were surrounded by human HA in hFibro, hMSCs, and SHED groups (Figure 4). These results showed that the hFibro, hMSCs, and SHED groups, especially in hMSCs and SHED groups, produced human HA derived from the injected cells.

At day 14 after cell transplantation, the number of inflammatory cells in the wound area was reduced as compared to day 7. Cell transplantation groups were re-epithelialized with an epithelium (Figure 5). The evaluation by fluorescence microscope showed that PKH 26 positive cells (hFibro, hMSCs, SHED) and HA production also reduced (Figure 5).

Hyaluronic acid (HA) production measurement by enzyme-linked immunosorbent assay (ELISA)

HA volume was determined by measuring with Enzyme-Linked Immunosorbent Assay (ELISA) (Table 1). HA volume at day 7 and 14 were 1558.41 ± 60.33 ng/mg, 704.17 ± 40.26 ng/mg (control), 2092.75 ± 42.56 ng/mg, 1425.79 ± 56.31 ng/mg (hFibro), 2342.07 ± 188.10 ng/mg, 1653.10 ± 120.84 ng/mg (hMSC), 2314.85 ± 164.91 ng/mg, 1644.98 ± 120.70 ng/mg (SHED), respectively. Significantly increased amounts of HA in wounded tissues were observed in hMSCs, SHED groups and hFibro groups compared to control groups at day 7 and 14 ($P<0.05$). Moreover hMSCs, SHED groups showed significantly higher HA levels compared to hFibro groups at day 7 and 14 ($P<0.05$). There was no statistically significant difference in between hMSCs and SHED groups treated at day 7 and 14 ($P<0.05$; Table 1).