Application of Mesenchymal Stem Cells (MSC) to Regenerative Dentistry and Identification of Molecular Markers for MSC

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ABSTRACT

We characterized human bone marrow-derived mesenchymal stem cells (MSC) by identification of molecular markers and used these markers in clinical studies for treatment of periodontal disease: Auto-transplantation of MSC into periodontal defects enhanced regeneration of cementum, periodontal ligament and alveolar bone. Stem cell therapy could be a new frontier in dentistry.

Stem Cell Therapy May Induce Revolution of Dentistry

A newt has abundant stem cells, and its tail regenerates easily, but human tissues regenerate poorly, perhaps because human beings have fewer stem cells. If this is indeed the case, transplant of stem cells expanded ex vivo could induce regeneration of human tissues: This cell therapy would mean a broad new option for dentistry and medicine. With this optimistic view, we started studies on stem cells in 2000. At that time, the Japanese government encouraged university professors to set up venture companies on campus. Since regenerative dentistry seemed to be a promising field, I set up a venture company - Two Cells Inc. - in 2003. But which stem cells would be the most promising transplantable cells? There are many candidate stem cells, including embryonic stem cells (ES cells), hematopoietic stem cells, and mesenchymal stem cells (MSC).

Comparison of MSC and ES Cells

The human body contains 75 trillion (7.5×10^{13}) cells of ~ 200 different lineages. ES cells can differentiate into all these cells, and proliferate infinitely in vitro, so many researchers are using ES cells to explore principles of the stem-cell system and to develop stem-cell therapy. We decided to use bone marrow-derived MSC.

Bone marrow MSC can differentiate into bone, cartilage, muscle, blood vessels and nerves in culture, or by transplantation into tissue: They may be useful for treatment of periodontal diseases, osteoporosis, bone fracture, osteoarthritis, myocardiac infarction, brain infarction, and degenerative nerve diseases. In addition, MSC can differentiate into hepatocytes, which shows their remarkable plasticity (Sato et al., 2005). Furthermore, MSC support hematopoiesis, and suppress graft versus host disease. In contrast, ES cells induce graft versus host disease, and may form teratomas after transplantation. The isolation of ES cells from patients is impossible because ES cells are absent in the adult body, whereas MSC can easily be isolated from patients' bone marrow. Unlike ES cells, MSC do not present ethical dilemmas, and there is no immunological rejection. Accordingly, transplantation of MSC - but not ES cells - has already been used for treatment of periodontal disease, osteoarthritis, bone diseases, skin ulcer, and myocardial infarction, etc., in Japan and other countries.

Super-expansion Method for MSC

At least 10^7 to 10^9 MSC are required for cell therapy, but adult bone marrow contains only a small number of MSC (<0.01%). The expansion of MSC in culture is therefore prerequisite for regenerative medicine, but proliferation activity of MSC has proved poor in conventional cultures. To combat this problem, we used fibroblast growth factor-2 (FGF-2) or extracellular matrix (ECM)-coated dishes.

In conventional cultures, bone marrow cells were seeded on plastic tissue culture dishes, and adherent cells were incubated with DMEM medium containing 10% fetal bovine serum. These cells underwent sequential passages, and then the cells were incubated with osteogenic-induction medium, chondrogenic-induction medium or adipogenic induction medium for 21-28 days. However, these MSC soon lost proliferation and differentiation potentials in vitro.

With our method, FGF-2 was included in the culture medium, or MSC were seeded on basement membrane-like ECM-coated dishes. FGF-2 or ECM stimulated proliferation of MSC and maintained the differentiation potential throughout many mitotic divisions (Tsutsumi et

al., 2000; Matsubara et al., 2004): After incubation with the differentiation-induction medium, almost all cells grown with FGF-2 or on ECM-coated dishes developed into osteoblasts, chondrocytes or adipocytes (Fig. 1). Using this super-expansion method, the large number of MSC required for cell therapy can thus easily be prepared from a small volume of marrow aspirates (0.5-2 ml).

In vivo stem cells bind to certain extracellular matrices or special cells - "Niche" - that stabilize their undifferentiated state and their self-renewal capacity. In addition, "Niche" may protect stem cells from apoptosis during microenvironmental changes, since stem cells need to regenerate injured tissues that have survived. Basement membrane ECM is required for proliferation and maintenance of the undifferentiated state of some stem cells, such as keratinocyte stem cells and muscle satellite cells in vivo. We found that basement membrane ECM is also useful for maintenance of self-renewal capability and the multi-lineage differentiation potential of MSC in vitro (Matsubara et al., 2004). When tissue is injured, ECM is also injured, and injured EMC often do not support self-renewal of stem cells. In such case, FGF-2 and related growth factors are released from heparan-sulfate proteoglycan of ECM and stimulate proliferation of stem cells. Either ECM or FGF is used for maintenance of stem cells, depending upon, respectively, the absence or presence of injury/inflammation. Accordingly, FGF-2 did not further increase the effect of basement membrane ECM on the proliferation of MSC (Matsubara et al., 2004). In any case, the super-expansion method is a reasonable, powerful and reliable method for the expansion of MSC, and FGF or ECM may be essential for proliferation of MSC both in vitro and in vivo.

The effect of exogenous FGF-2 on MSC proliferation was weaker with medium containing fetal bovine serum than with medium containing human serum, perhaps because fetal bovine serum contains FGF-like growth factors at higher concentrations. In medium with 10% human serum, MSC underwent proliferation for a few generations, but addition of FGF-2 markedly extended the life-span of MSC even in medium containing human serum (Matsubara et al., 2004). This is a considerable merit, since doctors prefer to use auto-serum isolated from patients.

Cell Therapy for Periodontal Disease

We examined whether auto-transplant of MSC could promote regeneration of periodontal tissues in a dog model. Bone marrow MSC were isolated from beagle dogs and expanded in vitro. The expanded MSC were mixed with 2% type I collagen at various cell concentrations and transplanted into Class III defects. Collagen gel alone was implanted into the defects as a control. After transplantation, the gums were sutured (Kawaguchi et al., 2004, 2005).

In the control group, without cells, regeneration was poor. However, in the MSC group, new formation of cementum and bone, and adequate width of periodontal ligament were observed. Furthermore, the new cementum contained Sharpey' s fibers. Using GFP-labeled MSC, we confirmed that the newly formed bone and cementum, as well as the ligament, were derived from transplanted MSC (unpublished data). This transplantation of MSC revealed that differentiation into cementoblasts occurs in an earlier stage than does differentiation into osteoblasts: Perhaps adhesion of MSC on the surface of denuded dentine enhanced cementoblast differentiation, and the absence of a calcium-phosphate scaffold (adherent surface) in the future alveolar bone area delayed osteoblast differentiation.

On the basis of these animal studies, we commenced MSC therapy for periodontal diseases at Hiroshima University Hospital in 2004. We used individual

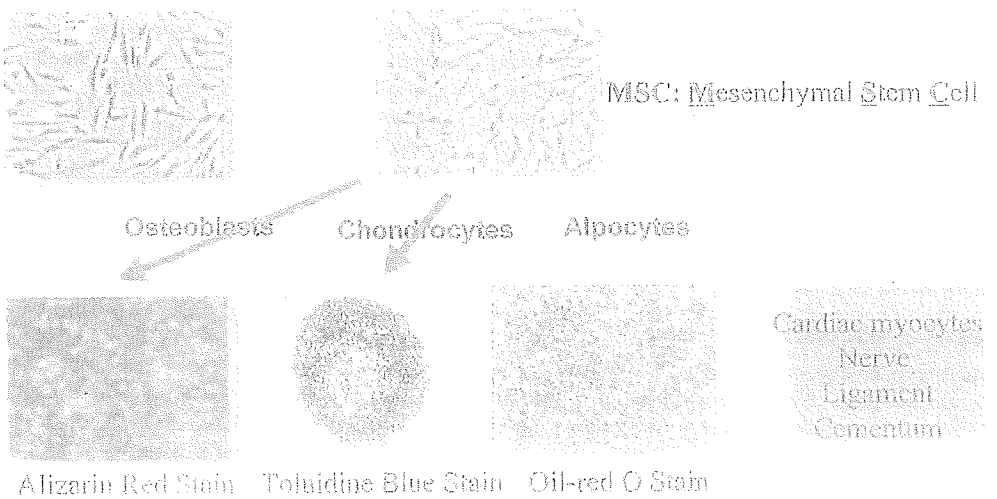

Fig. 1 Multi-lineage differentiation potentials of MSC expanded using the super-expansion method. FGF-2 was added to MSC cultures, or MSC were seeded and maintained on ECM-coated dishes. These MSC retained osteogenic, adipogenic and chondrogenic potentials throughout many mitotic divisions.

patients' serum to expand MSC in culture, because bovine serum may contain BSE prion and unknown pathogens. To isolate human serum from patients safely, we - together with stuffs of JMS Inc and Two Cells Inc - designed special bags. Two hundred ml blood was collected using a pump at a rate of 70 ml/min; the bags were centrifuged, and the serum was separated from the clot. On another day, we aspirated bone marrow from iliac bone, and the bone marrow aspirates were mixed with culture medium and patient' s own serum, and cells were seeded on tissue culture dishes. These cells were incubated in a CO_2 incubator, and we then confirmed the absence of bacteria, fungi, endotoxin and mycoplasma in the medium and the MSC layer in culture by clinical tests. MSC obtained from cultures at passage 3 were harvested and mixed with collagen gel. The gel containing MSC was transplanted to periodontal tissue defects. Comparison of bone density with X-ray 6 months before and after the MSC transplantation showed increased bone density around the tooth. We will report details of these studies in the near future.

Jaw/alveolar bone-derived MSC

Usually MSC are isolated from the ilium, but this causes considerable pain because iliac bone is covered by thick skin and muscle. To reduce pain, we isolated and expanded MSC - at a high success rate (70%) - from alveolar/jaw bone, which is covered by thin mucosa alone: These MSC had potent osteogenic potential in vitro and in vivo, although their chondrogenic and adipogenic potential was less than that of iliac MSC (Matsubara et al., 2005). Jaw MSC may be useful for treatment of oral diseases including periodontal disease, since they can easily be obtained during tooth extraction. It will be interesting to examine whether jaw MSC have greater potential for periodontal regeneration than do iliac MSC.

Adhesion of MSC to Scaffolds

MSC adhere poorly to some scaffolds, depending upon the scaffold material, and are often damaged by proteases or mechanical stimuli at site of transplantation. We found, however, that MSC - along with some other cells - that were exposed to PHA-E or ConA increased their adhesion capacity on plastic tissue culture dishes and on plates of hydroxyapatite, titanium and poly-DL-lactic-co-glycolic acid (PLGA). These cells, moreover, built up resistance to proteases and/or mechanical stimuli (Nishimura-H, et al., 2004). Thus, the lectins may have great potential in tissue engineering and cell therapy.

Molecular Markers for MSC

MSC and fibroblasts are indistinguishable in appearance: Neither marker genes nor cell surface antigens specific for MSC have been identified, so no one knows the real face of MSC. To characterize MSC at a molecular level, we compared gene expression profiles in MSC, fibroblasts, osteoblasts, and adipocytes using GeneChip: The DNA microarrays contain 54000 locations and millions of DNA strands built up in these locations. MSC were isolated from three young volunteers and expanded in vitro. These cells were not incubated, or incubated in osteogenic-induction medium, chondrogenic-induction medium or adipogenic-induction medium for 28 days.

We isolated total RNA from MSC, fibroblasts, MSC-derived osteoblasts, MSC-derived chondrocytes, and MSC-derived adipocytes. No degradation of the RNA samples was found, and cDNA was synthesized with T7 oligo-dT primers. Biotin-labeled cRNA was synthesized by in vitro transcription. Fragmented, biotin-labeled cRNA was then hybridized overnight with DNA strands for 54000 probes (48000 genes) on Affimetrix DNA chip (Human Genome, U133, +2.0). After incubation with fluorescent Strepto-avidine, the image was scanned, and the data were analyzed using a computer soft - GeneSpring. The expression level of each gene was standardized according to the GeneSpring global median normalization method: This is the method commonly used for standardization of gene expression levels.

Osteoblasts, chondrocytes and adipocytes can revert to MSC under some culture conditions, whereas fibroblasts cannot revert to MSC-like cells, and unlike MSC, fibroblasts do not have any differentiation potential. Thus, some genes expressed selectively in MSC, osteoblasts, chondrocytes and/or adipocytes - but not in fibroblasts - must be linked with the differentiation program. Using this premise, MSC-specific genes were next selected by gene filtering using GeneSpring: The criteria were more than an increase 4-times greater in MSC than in adipocytes, chondrocytes, fibroblasts, and osteoblasts. This analysis identified 88 and 127 MSC-specific gene markers, up-regulated and down-regulated, respectively.

We also identified 28 genes expressed selectively in MSC and osteoblasts (Group B), and 49 genes expressed in MSC and chondrocytes (Group C), etc. MSC-specific genes were 0.16% of total genes, and MSC/chondrocyte-specific genes were 0.09%. The number of MSC-specific genes was smaller than that of chondrocyte-sepcific genes, suggesting a low degree of transcription regulation in MSC. We also identified osteoblast-, chondrocyte-, or adipocyte-characteristic genes, as well as overlap genes expressed both in MSC and one of the differentiated cells.

Clinical Use of MSC Marker Genes

Tissue factor pathway inhibitor-2 (TFPI-2) was expressed in MSC alone, and leukemia inhibitory factor (LIF) was expressed in MSC and chondrocytes, but not in the other examined cells (data not shown). LIF and TFPI-2 were expressed in standard MSC lines and in four patients' MSC (P1-P4), but not in standard fibroblast lines (Fig. 2). In addition, we found that MMP1 and collagen type XV were expressed in fibroblasts, but not MSC. Accordingly, MMP1 and collagen type XV were not expressed in the patients' MSC (plastic adherent cells) (Fig. 3), indicating that fibroblasts were not present in the patients' MSC before transplantation (Ishii et al., 2005). As a result of this quality examination, we can confidently transplant MSC to patients' defects.

CONCLUSION

Bad habits - such as poor tooth-brushing, lack of exercise, and imbalances of calcium, sugar and fat intake - are causes of periodontal disease, osteoporosis, diabetes and

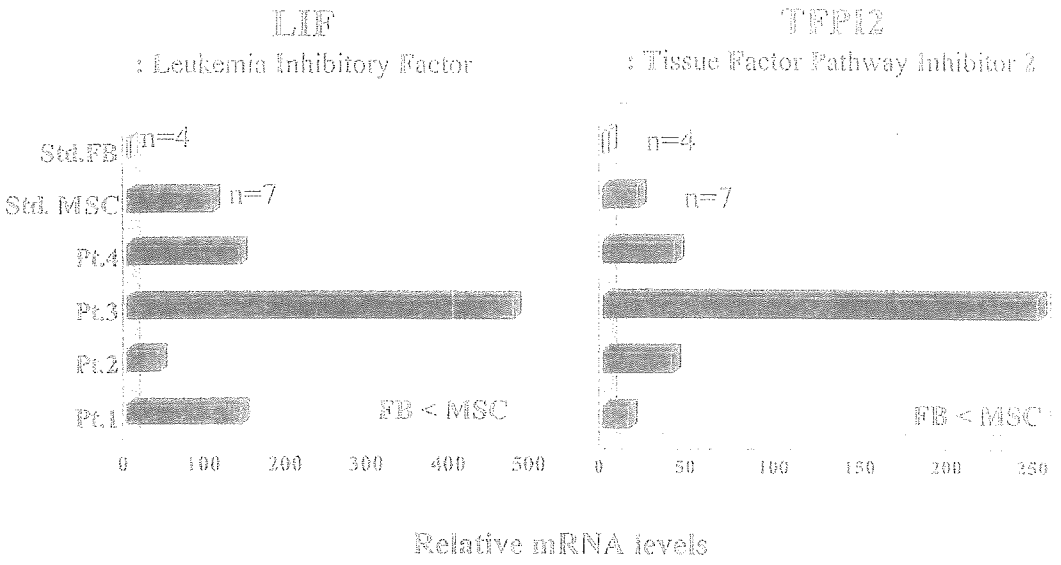

Fig. 2 The use of MSC marker genes in clinical studies. The expression levels of the genes in transplantable MSC from four patients were similar to those in standard MSC.

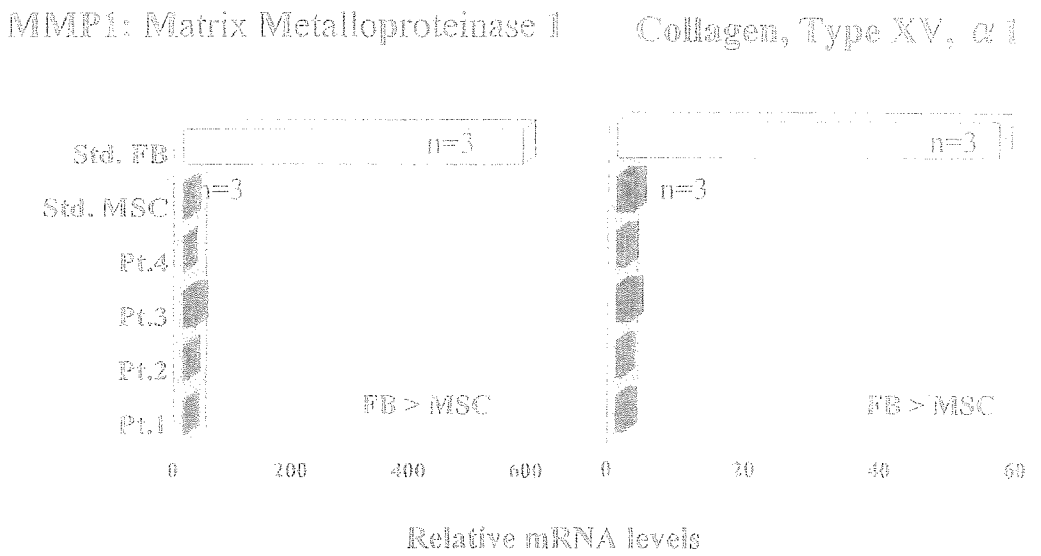

Fig. 3 MSC marker genes were used in clinical studies to confirm the absence of fibroblasts in transplantable cells.

myocardiac infarction, etc. However, age-dependent decreases in available stem cells may also have a great impact on these diseases. If this is true, the regeneration-failure syndrome should be treated with a sufficient number of stem cells. Two Cells Inc supports this business, and the application of MSC will brighten the future both dentistry and medicine.

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Basic and Clinical Studies of Periodontal Tissue Regeneration by Transplantation of Own Bone Marrow Mesenchymal Stem Cells

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ABSTRACT

Use of suitable cells seeded into periodontal defects would be a powerful strategy to promote regeneration of periodontal tissue. Recent interest has focused on mesenchymal stem cells (MSCs) isolated from bone marrow, which have the potential for multilineage differentiation. Transplantation of bone marrow-derived MSCs into periodontal osseous defects would be a useful option for periodontal tissue regeneration. We have been investigating a possibility of MSCs therapy for periodontal diseases.

Our animal studies indicated that transplanted MSCs into experimentally periodontal defects survive and differentiate into appropriate periodontal cells, resulting in enhancement of periodontal tissue regeneration. Based on these scientific evidences, clinical experience entitled "periodontal tissue regeneration by transplantation of own bone marrow MSCs" has been started. Human bone marrow cells are obtained from the iliac crest and expanded in vitro at Cell and Tissue Engineering Center in Hiroshima University Hospital. MSCs are, then, isolated and mixed with Atelocollagen at final concentration of $2x10^7$ cells/ml. These MSCs in Atelocollagen are transplanted into periodontal osseous defects at the periodontal surgery.

This review article summarizes our animal studies and initial clinical experience of periodontal tissue regeneration by transplantation of own bone marrow MSCs.

Key Words: periodontal tissue regeneration, mesenchymal stem cells, transplantation, regenerative therapy, periodontal diseases

Periodontal Tissue Regeneration and MSCs

A major goal of periodontal therapy is to reconstruct healthy periodontium destroyed by periodontal diseases. Previous studies have shown that conventional regenerative therapies such as guided tissue regeneration, topical application of enamel matrix derivative or various polypeptide growth factors are useful for periodontal tissue regeneration (Cortellini *et al.*, 2005; Wang *et al.*, 2005). Since the origin of regenerative cells in these current regenerative therapies is residual periodontal tissues, the type of periodontal osseous defect is an important factor for periodontal tissue regeneration. Indications for these therapies are, therefore, limited to cases of two or three walls bony defects or class II furcation lesions. In this context, use of suitable cells seeded into periodontal defects might be a powerful strategy to promote regeneration of periodontal tissue. The cells should be nonimmunogenic, highly proliferative and easy to harvest, and have the ability to differentiate into various types of cells composing periodontal tissue.

Recent interest has focused on mesenchymal stem cells (MSCs) isolated from bone marrow, which have potential for multilineage differentiation. Bone marrow MSCs can easily be obtained repeatedly and differentiate into osteoblasts, chondrocytes, tenocytes, adipocytes, muscle cells or nerve cells *in vitro* and *in vivo* (Baksh *et al.*, 2004). Thus, transplantation of bone marrow MSCs may provide a new method for treatment of osteoporosis, arthritis, cardiac diseases, and degenerative nerve diseases. Taking all these findings into consideration, it is conceivable that MSCs might be useful for periodontal tissue regeneration. We have been investigating a possibility of MSCs therapy for periodontal diseases. Our preclinical and clinical studies of periodontal tissue regeneration with MSCs are summarized in the following text.

Animal Studies

To determine the effect of periodontal tissue regeneration with MSCs, autologous bone marrow MSCs were transplanted into periodontal defects in dogs (Kawaguchi *et al.*, 2004). Bone marrow aspirates of 1 ml were taken from the iliac crest of each animal. Cell culture was performed in accordance with the technique described by Tsutsumi et al. (Tsutsumi *et al.*, 2001). The MSCs, having been cultured for 2 weeks were suspended, mixed with Atelocollagen (2% type I collagen extracted from bovine calf skin by pepsin digestion, Koken Co., Ltd., Tokyo,

Japan) at final concentration of $2x10^6$, $5x10^6$, $1x10^7$ or $2x10^7$ cells/ml, and auto-transplanted into experimental class III defects. Atelocollagen alone was implanted into the defects as a control. In Atelocollagen, the antigenic telopeptide region has been removed by pepsin digestion. This immunogenic advantage has enabled Atelocollagen to be used in various clinical settings. Furthermore, autologous chondrocytes embedded in Atelocollagen transplanted into cartilage defects were reported to promote repair (Ochi *et al.* 2001). Accordingly, Atelocollagen was chosen as a biomaterial scaffold to hold MSCs in a temporary matrix, and to keep them suspended during transplantation and early period of regeneration. One month after transplantation, histological findings showed that the defects were regenerated with cementum, periodontal ligament and alveolar bone in MSCs-Atelocollagen transplanted groups (Figure 1). Less periodontal tissue regeneration was observed in the control group than in the MSCs-Atelocollagen groups. Morphometric analysis revealed that the percentage of new cementum length in the $5x10^6$ and $2x10^7$ cells/ml groups and the percentage of new bone area in the $2x10^7$ cells/ml group were significantly higher than that in the control group ($p<0.01$). These results revealed that transplantation of bone marrow MSCs enhanced periodontal tissue regeneration.

Next animal study was undertaken to elucidate the performance of MSCs after transplantation. Green fluorescent protein (GFP)-transduced MSCs were cultured and mixed with Atelocollagen at final concentration of $2x10^7$ cells/ml and were auto-transplanted into experimental class III defects in the dogs. One month after GFP-transduced MSCs transplantation, immunohistochemical results showed that GFP-positive cells were present in the whole area of the defects. Cementoblasts arranged along the denuded surface, osteoblasts and osteocytes of regenerated bone, and fibroblasts of the regenerated periodontal ligament were GFP-positive. These findings suggested that some transplanted MSCs survive, differentiate into periodontal tissue cells and release various kind of cytokines, all of which promote periodontal tissue regeneration.

These animal studies demonstrated the therapeutic potential of bone marrow MSCs transplantation for periodontal tissue regeneration.

Clinical Experience

Based on the results of the animal studies, since 2004, clinical study entitled "Periodontal Tissue Regeneration by Transplantation of Own Bone Marrow MSCs" has been started at Hiroshima University Hospital. A brief flow chart of this study was shown in Figure 2. By November 2005, 7 patients with periodontal osseous defects caused by periodontitis had been treated with this procedure.

Patients

The clinical study protocol and the consent form were approved by local ethics committees at both Hiroshima University School of Dentistry and Hiroshima University Hospital. We obtained written informed consent from all patients.

Patients between 20 and 65 years old were eligible for the study if they had the following characteristics: (1) they had received initial preparation of periodontal treatment and maintained good oral environment; (2) there were intrabony osseous defects caused by periodontitis. We excluded patients who met one of the following criteria: malignant diseases, severe diabetes, hepatic dysfunction, severe morbidity, or unwillingness to participate.

Bone marrow aspiration and transplantation surgery were performed at Clinic for Cell and Tissue Transplantation Therapy, Hiroshima University

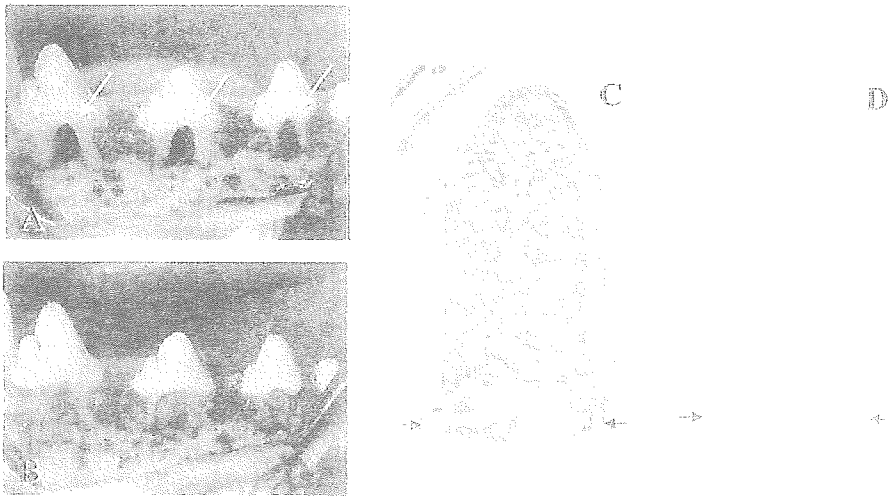

Fig.1 Periodontal regeneration by MSCs transplantation in dogs
Surgical view of the mandibular premolar area after defect (arrows) preparation (A); after transplantation of various concentration of MSCs in Atelocollagen (B). Light micrographs of 1 month after transplantation of MSCs ($2x10^7$ cells/ml) in Atelocollagen (C) and Atelocollagen alone (D). Arrows indicate apical border of denuded root surface.

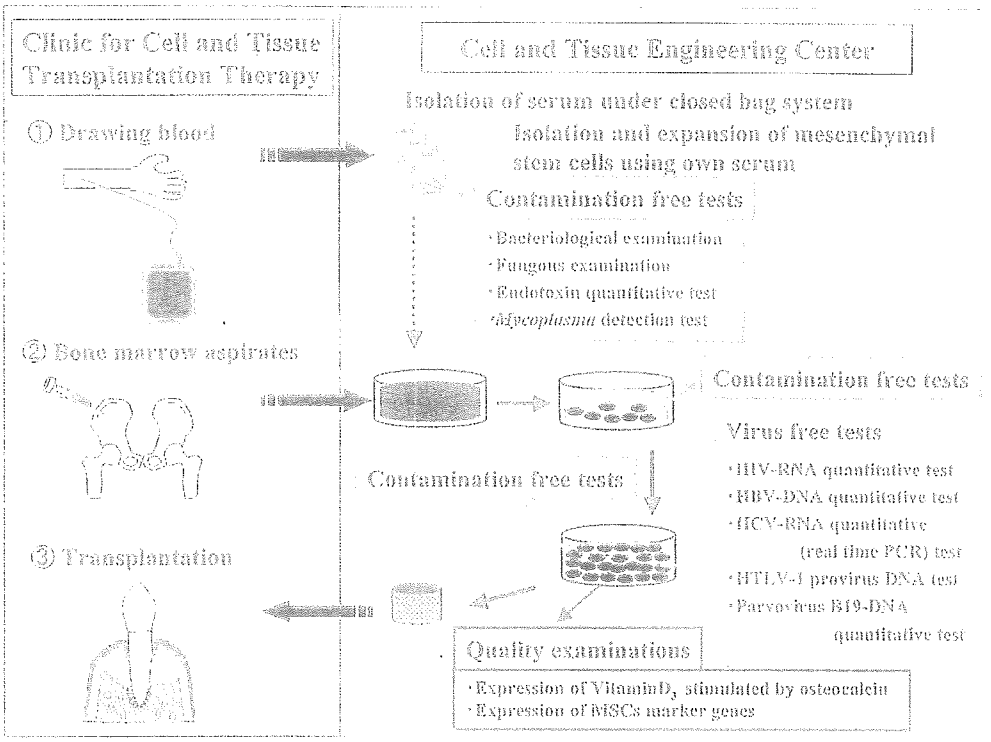

Fig.2 Flow chart of clinical study

Hospital, and isolation of MSCs and cell culture were performed at Cell and Tissue Engineering Center (CTEC), Hiroshima University Hospital.

Autologous serum for isolation and expansion of MSCs

In most previous experimental (Pittenger *et al.*, 1999, Tsutsumi *et al.*, 2001) and clinical (Vacanti *et al.*, 2001, Bang *et al.*, 2005) transplantation studies, fetal calf serum (FCS) has been used for the *in vitro* expansion of MSCs. FCS may include viral or bacterial infections and prion (bovine spongiform encephalopathy), which can cause variant Creutfeldt-Jakob disease. Other potential problems of FCS are immune or local inflammatory reactions due to contamination with bovine protein, which cannot be excluded even by several washings after expansion (Spees *et al.*, 2004). In this context, MSCs should be cultured in xeno-free environment for clinical application, suggesting that it is more appropriate to culture MSCs with autologous serum than with FCS.

We developed completely closed bag system separating autologous serum. Venous whole blood (200 ml) was drawn into special bags (JMS Co., Inc., Hiroshima, Japan). These bags were carried to CTEC and centrifuged at 2,500 *g* for 10 minutes. Approximately 90 ml of serum could be separated from the clot, corrected into other bags under closed environment, and then, stored at -20℃ until ready for use. Sixteen ml of the serum was drawn out to check the "contamination" : bacteriological and fungous examinations, endotoxin quantitative test and *mycoplasma* detection test.

Bone marrow aspiration, isolation of MSCs, and cell culture

We aspirated 15 ml of fresh marrow cells, under local anesthesia, from the posterior iliac crest of patients, and corrected to disposable syringes containing heparinized phosphate buffered saline (PBS). These syringes were carried to CTEC, centrifuged at 200 *g* for 5 minutes, and the supernatant of plasma with the fat layer was discarded. The residue, including buffy coat and red blood cells, was mixed with Dulbecco modified eagles' medium (DMEM) containing 10% of autologous serum from the patient, 50 μg/ml gentamycin and 100 μg/ml streptomycin, and seeded on 100-mm tissue culture dishes. The cells were cultured in a humidified atmosphere of 95% air with 5% CO_2 at 37℃. After 3 days of culture, non-adherent cells were removed by replacing the medium. The attached cells formed colonies within 5 to 7 days, thereafter, fibroblast growth factor-2 (3 ng/ml) was added into the culture with fresh medium. The medium change was done every 3 days. Passages were performed when cells were subconfluent.

Cells were cultured for 3 weeks, then checked for "contamination" mentioned above and "virus free tests" on cultured cell samples: human immunodeficiency virus (HIV)-RNA quantitative test, hepatitis B virus (HBV)-DNA quantitative test, hepatitis C virus (HCV)-RNA quantitative (real time PCR) test, human T-cell leukemia virus (HTLV)-I-provirus DNA test, and Parvovirus B19-DNA quantitative test. Characteristics of cultured cells were also checked (Ishii *et al.*, 2002).

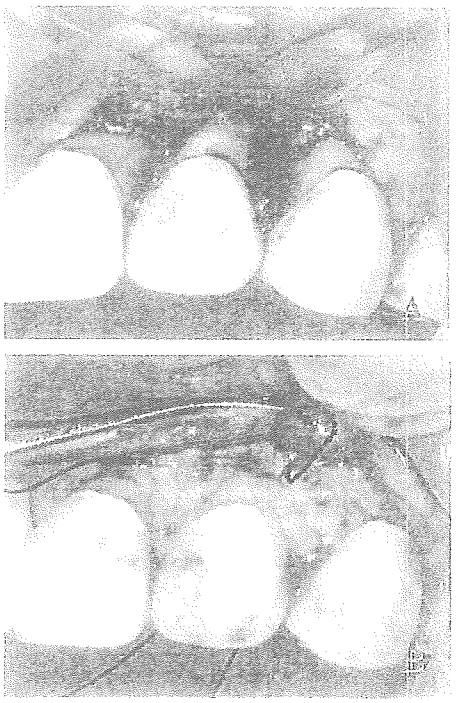

Fig.3 Clinical appearances of periodontal surgery before (A) and after (B) transplantation of MSCs in Atelocollagen

Cell preparation for transplantation

On the day of transplantation, MSCs in cultures of the 3rd passages were harvested using trypsin- EDTA, washed with PBS prior to being embedded in 3% Atelocollagen. The final cell density was adjusted to 2×10^7 cell/ml. The MSCs-Atelocollagen composites were further incubated at 37°C for 30 minutes, carried to Clinic for Cell and Tissue Transplantation Therapy and then, transplanted into periodontal osseous defects at the periodontal surgery (Figure 3).

Clinical results

The results in 7 patients who were followed up for at least 6 months have shown good clinical course. None of the patients exhibited any adverse event or side effect. In a case followed up 1 year, X-ray evaluation showed complete radiographic resolution of the intrabony component of the defect. However, more prolonged follow-up and a larger number of patients are necessary to confirm the effectiveness of MSCs therapy.

Future Perspectives

Although our animal studies indicated that transplanted MSCs into periodontal defects survive and differentiate into appropriate periodontal cells, resulting in enhancement of periodontal tissue regeneration, the precise mechanism whereby MSCs differentiate into cementoblasts, osteoblasts and periodontal ligament fibroblasts is still unknown. Further basic studies (e.g., the mechanisms of MSCs differentiation in vivo and appropriate scaffold in case of large periodontal osseous defects) and the continuous clinical experience are needed to establish the magnitude of the benefit and the effects on periodontal tissue regeneration after MSCs therapy.

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Serum-Free Spheroid Culture of Mouse Corneal Keratocytes

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Purpose. To develop a serum-free mass culture system for mouse keratocytes.

Methods. Corneas of C57BL6/J mice were enzyme digested after the epithelium and endothelium were removed. Stromal cells were cultured in serum-free DMEM/F12 (1:1) containing epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), and B27 supplement. Primary spheres were dissociated by trypsin and subcultured as suspended secondary spheres. Cells from postnatal day (P)6 to P10 spheres were subcultured onto plastic dishes or type I collagen gels for phenotype analysis. The expression of the keratocyte markers keratocan, aldehyde dehydrogenase (Aldh), and CD34, were analyzed by RT-PCR, and vimentin and α-smooth muscle actin (α-SMA) were examined by immunocytochemistry.

Results. Primary keratocytes formed spheres, which were cultured for over 12 passages. Suspended sphere cells expressed vimentin, keratocan, CD34, and lumican, but were negative for cytokeratin K12 (K12) and Pax6. Sphere cells subcultured on plastic exhibited a dendritic morphology characteristic of keratocytes, and maintained keratocan, Aldh, and CD34 expression in serum-free medium. Sphere cells subcultured with 10% serum became fibroblastic, and expressed α-SMA when stimulated by transforming growth factor (TGF)-β. α-SMA-positive cells demonstrated contractile properties on collagen gels, compatible with the myofibroblast phenotype.

Conclusions. The phenotype of mouse keratocytes can be maintained in vitro for more than 12 passages by the serum-free sphere culturing technique. (*Invest Ophthalmol Vis Sci.* 2005;46:1653–1658) DOI:10.1167/iovs.04-1405

The corneal stroma is characterized by a well-organized extracellular matrix consisting of a dense network of collagen fibrils and proteoglycans that are produced by keratocytes, the principal stromal mesenchymal cell of cranial neural crest origin.[1,2] In adult tissue, keratocytes are mitotically quiescent cells with a flat, dendritic morphology. Keratocytes form a three-dimensional network of cells through their extensive dendritic processes, linked via gap junctions,[3–10] and secrete collagens and keratan sulfate proteoglycans such as lumican, mimecan, and keratocan.[11–15] The corneal stroma is rich in total keratan sulfate proteoglycan content,[16] but contain relatively small amounts of dermatan sulfate proteoglycans.[17]

During corneal wound healing, the quiescent keratocytes are activated and transform into fibroblasts and/or myofibroblasts, losing their characteristic dendritic morphology. Keratan sulfate proteoglycans are downregulated,[11,18] whereas keratocytes proliferate and migrate to the site of injury, causing scar formation.[19–22] The conversion to myofibroblasts, characterized by intense expression of the contractile protein α-smooth muscle actin (α-SMA),[21,23,24] is induced by endogenous and exogenous transforming growth factor (TGF)-β.[25–27]

Ex vivo expansion of keratocytes is often performed to investigate keratocytes in vitro, and various culture techniques have been reported involving the use of plastic substrates. However, when cultured in serum-containing medium, collagenase-isolated keratocytes from bovine[28] and rabbit[27,29] corneas readily lose their in vivo quiescent phenotype and acquire a fibroblastic phenotype with altered physiological properties.[28,30,31] In the presence of 2% to 10% serum, keratan sulfate proteoglycan production is greatly reduced or absent in keratocyte-derived fibroblasts,[28,32,33] whereas production of dermatan sulfate proteoglycans is upregulated. Furthermore, TGF-β stimulation or culture at low densities[30] causes corneal fibroblasts to differentiate further into myofibroblasts with a more spread-out morphology.[26,29,32,34] Serum-free cultures have been reported to be effective in the maintenance of the dendritic morphology of keratocytes and the production of keratan sulfate proteoglycans.[27,28,30–33,35,36] However, the cultivation of a large quantity of cells by subculturing has been difficult.

In this report, we introduce our method for subculturing mouse corneal keratocytes in large quantities, using a modified version of a suspension culture method originally described for neural stem cells.[37–39] In our study, the sphere culture of keratocytes did not require serum, and the dendritic keratocyte phenotype was restored when subcultured on plastic substrate in serum-free medium.

Materials and Methods

Cell Culture

All animals were handled in full accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Stromal cells were dissociated from adult C57BL6/J mice (7–8 weeks old) and then cultured as described previously[40] with modifications. In brief, cornea tissue was excised in Hanks' balanced salt solution (HBSS) supplemented with 10% fetal bovine serum (FBS) by circular incision outside the limbus. The iris, ciliary body, and Descemet's membrane including the endothelium were bluntly dissected from the cornea. The remaining stroma with epithelium was incubated in 5 mg/mL of Dispase II (Roche Diagnostics, Indianapolis, IN) at 4°C overnight. Loose epithelial sheets were then removed, and corneal stromal discs were cut into small segments and digested in 0.05% trypsin (Sigma-Aldrich, St. Louis, MO) for 30 minutes at 37°C, followed by 78 U/mL

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TABLE 1. PCR Primers

Gene	Primer Sequence (5′–3′)	Product Size (bp)	GenBank Accession ID
Cytokeratin K12	Forward: TCCTCCTGCAGATTGACAACG Reverse: TTCCAGGGACGACTTCATGG	511	NM_010661
Pax6	Forward: AGTTCTTCGCAACCTGGCTA Reverse: TGAAGCTGCTGCTGATAGGA	500	NM_013627
Keratocan	Forward: AGGATGCCTTCATTCACGGAC Reverse: GCTCATTGTGGTGCTTATGGGG	491	NM_008438
Lumican	Forward: TGCTGTCTCGGCTTCTCTGAAAG Reverse: AACATCCCCCACATTCCCAACC	567	NM_008524
CD34	Forward: CCTTATTACACGGAGAATGGTGGAG Reverse: AAGAGGCGAGAGAGGAGAAATGGG	477	NM_133654
Vimentin	Forward: GAACGGAAAGTGGAATCCTTGC Reverse: GGTTGGCAGAGGCAGAGAAATC	591	NM_011701
Aldh	Forward: CTTCCAGCGGGTCATAAATCTG Reverse: AGCCAGCAAAACAAGTGTCAGG	528	NM_007436
Gapd	Forward: GACCACAGTCCATGCCATCAC Reverse: TCCACCACCCTGTTGCTGTAG	453	NM_008084

collagenase (Sigma-Aldrich) and 38 U/mL hyaluronidase (Sigma-Aldrich) for 30 minutes at 37°C.

Stromal cells were mechanically dissociated into single cells, and cultured in DMEM/F12 (1:1) supplemented with 20 ng/mL epidermal growth factor (EGF; Sigma-Aldrich), 10 ng/mL of fibroblast growth factor 2 (FGF2, Sigma-Aldrich), B27 supplement (Invitrogen, Carlsbad, CA), and 10^3 U/mL leukemia inhibitory factor (LIF; Chemicon International Inc., Temecula, CA) at a density of 5×10^5 cells/mL in a 37°C 5% CO_2 incubator. Initial culture was performed in 24-well plates or 35-mm dishes and then subcultured to 25-cm^2 culture flasks. The spheres were then further subcultured in 75 cm^2 culture flasks after 7 to 14 days, which was repeated every 7 to 14 days. Medium was changed every 5 to 7 days. All dishes and flasks used for sphere culture were polystyrene, noncoated vessels obtained from Asahi Techno Glass (Tokyo, Japan). Stromal sphere cells were examined by immunocytochemistry and RT-PCR. To allow cells to differentiate, cells dissociated from corneal spheres were cultured in serum-free or DMEM/F12 medium (10% FBS) supplemented with or without 2 ng/mL TGF-β (Sigma-Aldrich) for 4 days. Subcultured cells were stained by calcein-AM (Dojindo Laboratories, Tokyo, Japan), as described,[41] to visualize cell morphology. Primary stromal discs of mouse cornea were cultured in keratinocyte-serum free medium (K-SFM; Invitrogen) or DMEM/F12 with 10% FBS for 10 days (37°C, 5% CO_2), to identify any contamination by epithelial cells.

Immunocytochemistry

Immunocytochemistry was performed as described previously.[42] In brief, mouse corneal sphere cells and cells freshly isolated from mouse cornea were attached to glass slides by cytospin preparation (Auto Smear CF-120; Sakura, Tokyo, Japan) and then fixed in 4% paraformaldehyde for 15 minutes at 4°C. Cells were incubated in blocking serum for 30 minutes and then incubated with primary antibodies for 60 minutes. Primary antibodies used were anti-cytokeratin K12 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Pax6 (1:500, Chemicon International, Inc.), anti-vimentin (1:100, Santa Cruz Biotechnology), and anti-αSMA (1:200, Laboratory Vision, Fremont, CA). Immunoreactivity of primary antibodies was visualized with secondary antibodies conjugated with Cy3 or FITC (Jackson ImmunoResearch Laboratories, West Grove, PA).

Reverse Transcription–Polymerase Chain Reaction

Sphere cells and freshly dissociated corneal cells were collected and immediately frozen in liquid N_2. cDNAs were synthesized with a cDNA synthesis kit (Life Sciences, Inc., St. Petersburg, FL) from total RNA also prepared with a kit (RNeasy; Qiagen, Hilden, Germany). Gene-specific primers used for cytokeratin K12 (K12), Pax6, vimentin, keratocan, lumican, CD34, aldehyde dehydrogenase (Aldh), and Gapd are shown in Table 1. PCR was then performed (GeneAmp 9700; Applied Biosystems, Foster City, CA). The PCR products were analyzed by agarose gel electrophoresis.

Collagen Gel Contraction Assay

Collagen gel contraction assay was performed as described previously,[43–47] with some modifications. Collagen gels were prepared according to instructions provided by the manufacturer (Cellmaterix Type I-A; Nitta Gelatin, Osaka, Japan). In brief, collagen was mixed with 10-fold concentrated DMEM/F12 medium and 50 mM NaOH containing 260 mM $NaHCO_3$ and 200 mM HEPES at a proportion of 8:1:1 (vol/vol/vol) at 4°C. Then a 0.2-mL aliquot of the solution was placed in the center of each well of a six-well cell culture cluster (Corning Inc., Corning, NY) and allowed to polymerize at 37°C for 30 minutes in a cloning ring 10 mm in diameter (Asahi Techno Glass). Cells cultured in medium containing 10% FBS were harvested and suspended at 2×10^5 cells /mL. Eighty-five micrometers of the cell suspension was applied to a polymerized collagen gel and incubated overnight in a 37°C 5% CO_2 incubator. On day 1, the cloning ring was removed, and 2.5 mL of 10% FBS-containing medium was added to each well to submerge the cells. To examine TGF-β-dependent collagen gel contraction, TGF-β was added at a 0.1- or 1-ng/mL final concentration. As an inhibitor, an anti-TGF-β antibody (0.1 ng/mL) was also added in the medium for selected dishes. FBS-containing media with or without TGF-β and/or TGF-β antibody were changed on day 3. Gel thickness was measured on day 5 with an inverted phase-contrast microscope, by adjusting the plane of focus from the bottom to the top of the gel and recording the distance that the stage had been moved.

Data are expressed as the mean ± SD. Post hoc comparisons between groups was performed with the Tukey procedure. Differences were considered significant at $P < 0.01$.

RESULTS

Sphere Formation from Stromal Cells

More than five mice were used to prepare corneal stromal cells in each experiment. From 10 corneas, $1.32 \pm 0.16 \times 10^4$ cells ($n = 3$) were isolated, and subcultured cells proliferated into spheres, to yield an average of $7.97 \pm 0.35 \times 10^7$ cells per 75 cm^2 flask ($n = 6$) after four passages (P4). Sphere cells were propagated for >12 passages through 5 months without loss of viability. To avoid contamination of epithelial and endothelial cells, stromal discs were carefully prepared as described in the Materials and Methods sections. Dissociated cells from mouse stromal discs formed spheres when cultured in serum-free

medium containing EGF and FGF2 (Fig. 1A, left). To exclude the possibility that spheres may have originated from contaminating epithelial cells, we first performed primary cultures of mouse corneal discs, with or without dispase treatment, followed by epithelium separation. K-SFM with low Ca^{2+} was used to examine epithelial expansion.[48,49] When untreated discs were cultured, migration of epithelial and stromal cells was observed in K-SFM and in DMEM/F12 containing 10% FBS, respectively (Fig. 1B, left). There were no epithelial cells migrating from dispase-treated discs in both media, whereas fibroblasts migrated from the discs in DMEM/F12 with serum (Fig. 1B, right). We further cultured dissociated epithelial cells under conditions that allowed stromal spheres to form by 14 days. As a result, no spheres were observed in the epithelial cell culture (Fig. 1A, right). To demonstrate whether the spheres were hollow or solid, confocal microscopy of 4′,6′-diamino-2-phenylindole (DAPI)-stained spheres was performed. Imaging in different focal planes showed that the inside of spheres was filled with cells, not hollow (Fig. 1C).

We then examined the expression of epithelial and stromal markers in primary and subcultured spheres (P10) by RT-PCR. Stromal markers examined were the proteoglycans, keratocan, and lumican,[13,14,50,51] as well as CD34, which was recently reported to be expressed in keratocytes.[52–54] As shown in

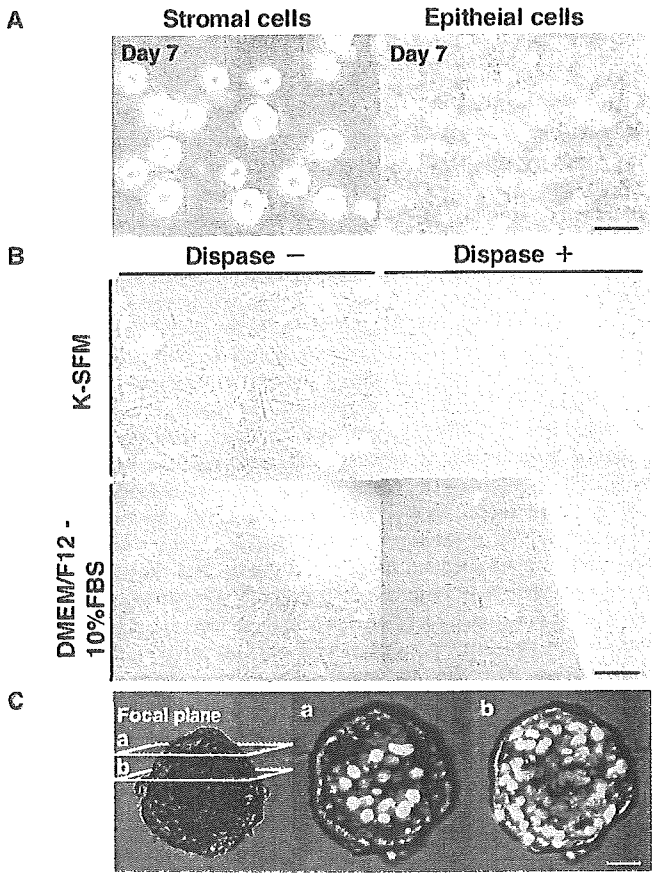

FIGURE 1. Sphere cells derived from the mouse corneal stroma. (**A**) Mouse corneal stroma and epithelium were separated by dispase treatment. Cells were cultured in DMEM/F12 supplemented with EGF and FGF2. After 7days' culture, spheres formed from stromal cells, but not from epithelial cells. (**B**) Mouse corneal discs were cultured in K-SFM or DMEM/F12 with 10% FBS. Epithelial cells migrated from intact corneal discs in K-SFM (*top left*) but not from dispase-treated (denuded) discs (*top right*). Expanding fibroblastic cells were still observed after dispase treatment. (**C**) Confocal images of the sphere in two different focal planes, a and b, as shown schematically (*left*). *Blue*: DAPI-stained nuclei. Scale bar: (**A**) 50 μm; (**B**) 100 μm; (**C**) 10 μm.

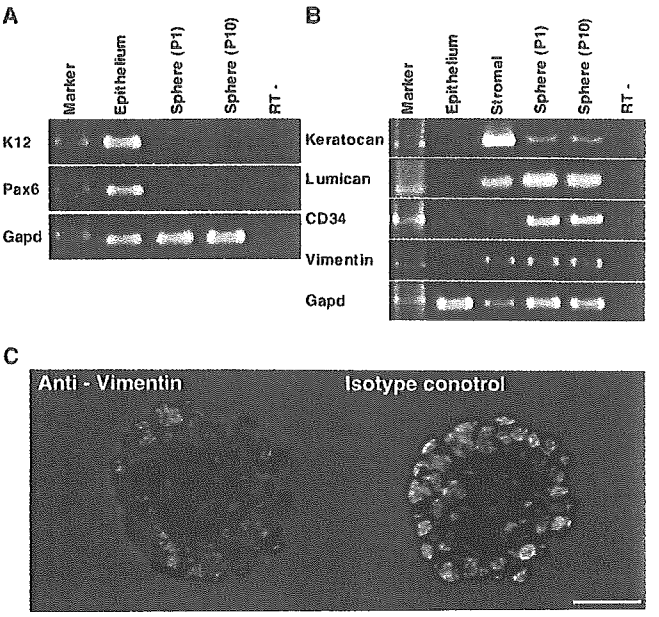

FIGURE 2. Sphere cells express keratocyte markers. (**A**, **B**) Total RNA was prepared from epithelial, stromal, and sphere cells. RT-PCR was performed with gene-specific primers. The epithelial markers K12 and Pax6 were not detected in the spheres (**A**). In contrast, the keratocyte markers keratocan, lumican, and CD34 were detected (**B**). Immunocytochemical analysis showed expression of the mesenchymal marker, vimentin, in spheres (**C**). *Blue*: nuclei of cells counterstained with DAPI. Scale bar, 50 μm.

Figures 2A and 2B, in addition to the mesenchymal intermediate filament vimentin, the expression of the genes described earlier were detected in the stromal spheres. On the contrary, K12 and Pax6, both of which are expressed in corneal epithelium,[40,42,55–58] were not detected in sphere cells (Fig. 2A).

Immunocytochemical analysis of spheres did not detect K12 and Pax6 expression (not shown), whereas vimentin staining was positive (Fig. 2C). These results show that sphere cells were of stromal, not epithelial, origin.

Characteristics of Sphere Cells

Sphere cells plated on collagen I-coated dishes in serum-free medium exhibited a dendritic morphology consistent with keratocytes (Fig. 3A).[3–10] RT-PCR showed that expression of keratocan and Aldh were retained under these conditions (Fig. 4). In contrast, the morphology of sphere cells subcultured in 10% serum were fibroblastic, and the expression of these genes was not detected (Fig. 4). Corneal sphere cells further differentiated to express α-SMA after exposure to TGF-β, which is consistent with the myofibroblast phenotype (Figs. 3C, 3D). Furthermore, when cells were subcultured on collagen gels in the presence of TGF-β, fibroblast-mediated gel contraction was observed (Fig. 5). Without TGF-β, contraction to 68.5% ± 1.75% of the original gel thickness was observed, whereas contraction was enhanced to 50.2% ± 3.96% or 29.4% ± 1.96% of the original thickness in the presence of 0.1 ng/mL or 1 ng of TGF-β, respectively ($P < 0.01$). TGF-β-dependent contraction was reduced to control levels when anti-TGF-β antibody was added to the medium.

Discussion

We successfully isolated and subcultured sphere-forming cells from the mouse corneal stroma, yielding a multifold increase in available cells for further experiments. Zhao et al.[40] have re-

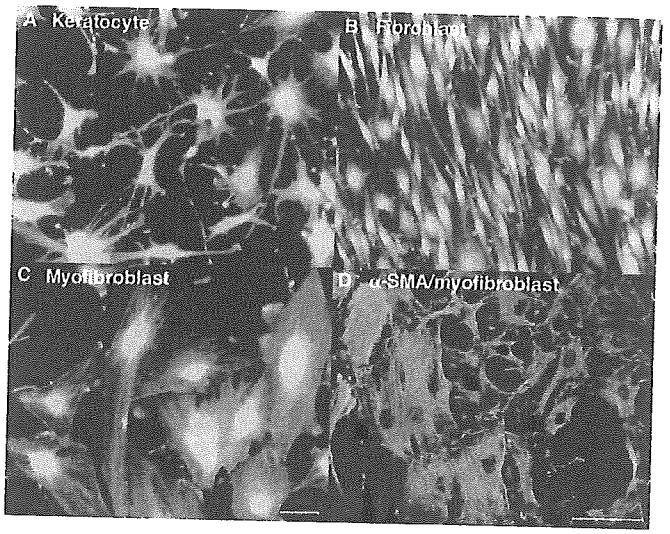

FIGURE 3. Phenotype of corneal stromal sphere cells stained by calcein-AM. Dissociated sphere cells were dendritic in SFM (**A**) and fibroblastic in adherent culture with medium containing 10% FBS (**B**). In the presence of TGF-β, morphology of adherent cells became myofibroblastic (**C**), and the cells expressed α-SMA, detected by immunocytochemistry (**D**, *green*). *Blue*: nuclei of cells counterstained with DAPI. Scale bar, 50 μm.

ported that cells present in limbus-derived spheres are derived from the limbal epithelium but not the stroma. However, the corneal sphere cells that we isolated did not express the epithelial markers K12 or Pax6 throughout the study, and furthermore, exhibited properties of corneal keratocytes when subcultured in serum-free medium. The morphology of the subcultured cells as shown in Figure 3 was similar to that of keratocytes in situ, and together with the expression of keratocan, lumican, Aldh, and CD34 in the subcultured cells, the

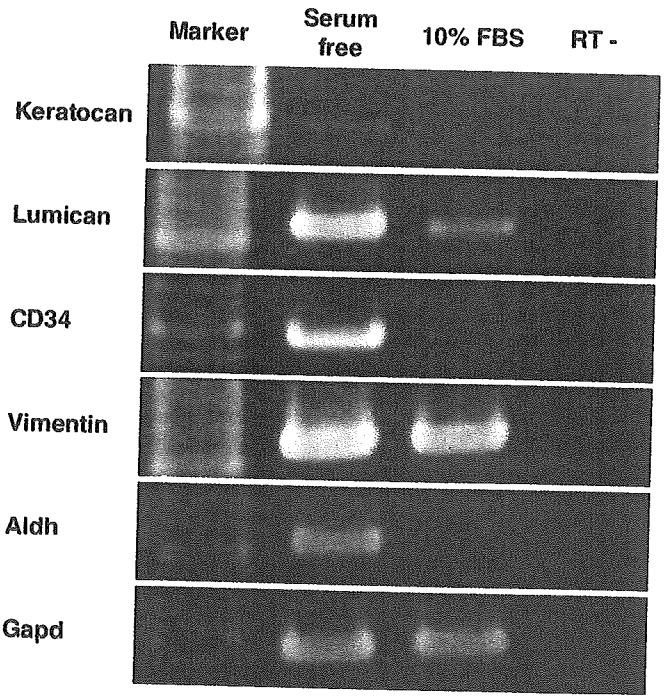

FIGURE 4. RT-PCR analysis of keratocyte markers expressed in sphere cells subcultured on plastic. Keratocan and Aldh were expressed only in cells in SFM, whereas lumican, CD34, and vimentin were also detected in cells cultured in the presence of 10% FBS.

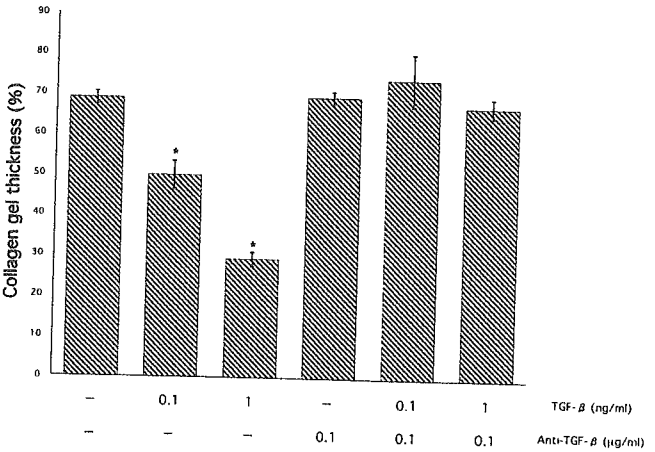

FIGURE 5. Collagen gel contraction assay of fibroblasts. Mouse corneal spheres were allowed to differentiate in 10% serum-containing medium. Dose-dependent TGF-β-induced collagen gel contraction was observed, which was inhibited by an anti-TGF-β antibody ($^*P < 0.01$).

collective evidence shows that these cells were of keratocyte origin. Although most genes were expressed during sphere cultures and maintained after adhesion to plastic dishes, keratocan, and Aldh were exclusively expressed in the keratocyte phenotype in serum-free medium (Fig. 4). Although the biological role of Aldh is not known, abundant expression of the water-soluble enzyme was shown to be expressed in the keratocyte phenotype, but not by the fibroblasts or myofibroblasts.[32,59]

Plated sphere cells can further be induced to differentiate into the fibroblast and myofibroblast phenotypes. Sphere cells seeded onto plastic in the presence of 10% serum exhibited the morphology and properties of stromal fibroblasts.[28] The transition to α-SMA-positive myofibroblasts by exposure to TGF-β, causing collagen gel contraction (Fig. 5), is also a functional property of stromal fibroblast primary cultures.[29,43,60] Therefore, subcultured sphere cells can be conditioned to express all three known phenotypes of keratocytes after expansion by sphere culture. Berryhill et al.[36] reported that the fibroblast phenotype can be partially restored to the keratocyte phenotype in terms of extracellular matrix production and morphology. However, biological functions, including Aldh activity, were not restored, suggesting that reversal to keratocyte phenotype after mass culture in serum-containing medium is not practical. Espana et al.[41] have reported the use of amniotic membrane (AM) as a substrate for keratocyte cultures in the presence of serum. They have shown that even with the use of serum, primary keratocytes maintained dendritic morphology on AM. The expression of keratocan and lumican was also present for up to five passages, which is a significant improvement over previous reports using artificial substrates. Still, the scarcity of keratocytes in tissue usually necessitates the use of human tissue or cells from larger animals, such as cows,[28,36] rabbits,[30] and rhesus monkeys.[61] Biochemical and molecular analysis of such cells are difficult due to the lack of available antibodies and genomic information.

During subcultures of spheres, cells that failed to form spheres were found attached to the dish as nondividing, fibroblast-like cells (data not shown). Although these cells may have had low viability, there may be a selection process that allows only cells with high growth potential to propagate as spheres. Once secondary spheres are successfully initiated, subsequent passages continue to produce spheres for at least 12 passages, the longest that we observed. To our surprise, cells from later passages continued to show the keratocyte phenotype when subcultured on plastic, suggesting the possible presence of

committed progenitor cells during the sphere culture stage. Many aspects of the keratocyte are still not understood, and the availability of cells from the mouse cornea should be a powerful tool in studying the biology of these cells.

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Transplantation of corneal endothelium with Descemet's membrane using a hyroxyethyl methacrylate polymer as a carrier

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SCIENTIFIC REPORT

Transplantation of corneal endothelium with Descemet's membrane using a hyroxyethyl methacrylate polymer as a carrier

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Aims: To evaluate the histology and function of Descemet's membrane transplanted with intact endothelium.
Methods: Japanese white rabbits and human eyebank eyes were used as donors and recipients of Descemet's membrane transplantation. Donor endothelium was hydrodissected by injecting indocyanine green from a limbal incision, and then processed as a corneal scleral button. A 6 mm diameter donor sheet was trephined, and folded in half using a 6 mm diameter polymer as a carrier. Recipient endothelium was also hydrodissected from the limbus using trypan blue to stain the Descemet's membrane. Continuous curvilinear descemetorhexis (CCD) was performed to remove a circular section of the Descemet's membrane using a 27 gauge cystotome. Donor tissue was inserted into the anterior chamber through a 5 mm limbal incision and apposed to the host stroma. Polymers were removed following transplantation. Similar surgical procedures were performed in both rabbits and eyebank eyes. Haematoxylin eosin stains were performed after 28 days in rabbits, and eyebank eyes were fixed immediately following surgery for endothelial cell counts.
Results: Rabbit control eyes demonstrated stromal oedema caused by loss of Descemet's membrane, whereas transplanted eyes had clear corneas. The mean (standard deviation) pachymetry of operated eyes was 376.6 (SD 32.5) μm compared with 389.6 (SD 25.1) μm in the unoperated eye. Mean endothelial density immediately following surgery in eyebank eyes was 2749 (SD 288) cells/mm^2.
Conclusions: Transplantation of Descemet's membrane by CCD produces a functional graft with an optically clear interface similar to control cornea.

Penetrating keratoplasty (PKP) has a long history as a surgical technique to treat irreversible opacification of the cornea. PKP is still the first choice of surgery for a wide range of diseases including bullous keratopathy, dystrophies, keratoconus, and trauma. However, damage spanning the entire thickness of the cornea from epithelium to endothelium is a relatively rare occurrence. For diseases other than bullous keratopathy, techniques such as deep lamellar keratoplasty (DLKP)[1 2] or epithelial sheet transplantation[3 4] are new alternatives to PKP in which only damaged tissue is surgically replaced. Similarly, the stroma and epithelium are not necessary in the treatment of bullous keratopathy, where only the endothelium is compromised by disease or injury. Several studies have shown the feasibility of transplanting posterior lamellar tissue instead of PKP.

One such technique is endothelial lamellar keratoplasty which uses a microkeratome to create a flap, followed by the replacement of posterior lamellar tissue taken from a donor cornea.[5 6] The use of a microkeratome leaves little opacification of the stromal interface, and a large flap covering the donor offers greater adhesive strength than running sutures used in PKP. Another method for transplanting endothelial tissue with a thin layer of stroma used as a carrier was reported by Melles *et al*.[7] The technique was further refined to allow transplantation through a small limbal incision—as opposed to a large wound reported in the original technique.[8] Initial clinical results are promising; however, manual lamellar incision of the stroma may cause mild haze in the stromal interface.

The adhesion between Descemet's membrane (DM) and posterior stroma is not anatomically strong, so the membrane can be peeled off without much effort. This is done when removing donor endothelium from a DLKP donor button before transplantation.[9] DM can also be detached by hydrodissection or viscodissection to obtain a smooth interface at the plane separating DM and stroma.[10] Our experience with DLKP by hydrodissection led to a new technique to obtain controlled sizes of DM by hydrodissecion followed by continuous curvilinear descemetorhexis (CCD). However, Descemet's membrane alone has the tendency to curl into a tube-like structure with the endothelial cells facing outwards. In order to control the plasticity of DM, we used polymers as a carrier during insertion and spreading of DM. In the present study, we show the histopathological and functional results of endothelial transplantation in rabbit and human eye bank eyes by this technique.

METHODS

Japanese white rabbits (female, 3 kg body weight, Shiraishi experimental animal breeding farm, Tokyo, Japan) were used as an animal model for endothelial transplantation. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research. Recipient animals (n = 4) were anaesthetised with 4 ml intramuscular ketamine and xylazine (1:7 mixture) and topical xylocain. To limit the extensive fibrin response observed in rabbits, pupils were expanded with topical tropicamide, and the anterior chamber was irrigated with heparin (5 U/ml). A 5 mm incision was made at the corneoscleral rim down to the plane of Schlemm's canal, and DM was hydrodissected by injecting 0.04% trypan blue into the supra Descemet space using a blunt 27 gauge needle (fig 1A–C). Following injection of air into the anterior

Abbreviations: CCD, continuous curvilinear descemetorhexis; DLKP, deep lamellar keratoplasty; DM, Descemet's membrane; HE, haematoxylin eosin; PKP, penetrating keratoplasty.

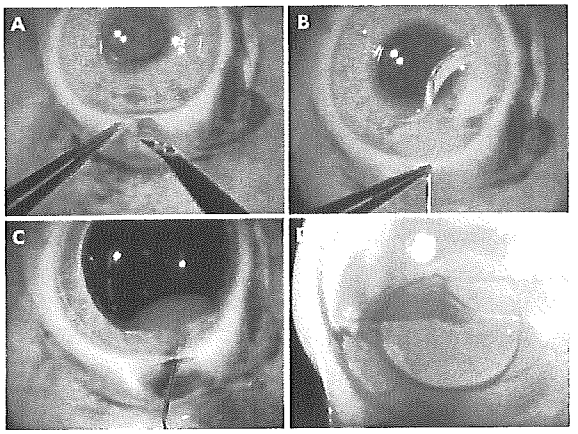

Figure 1 Surgical procedure for preparing the recipient shown in a rabbit eye. (A) An incision was made down to the plane of DM. (B), (C) Hydrodissection of DM is performed with balanced salt solution followed by trypan blue. (D) After removing trypan blue dye, a cystotome is inserted into the anterior chamber to perform CCD. The same procedure was performed in eyebank eyes.

chamber, CCD was performed to remove a circular section of the Descemet's membrane using a 27 gauge cystotome (fig 1D).

Two animals were sacrificed using an overdose of pentobarbital at the time of surgery to provide a total of four donor buttons. Following enucleation of the donor globe, a 3 mm incision was made at the corneoscleral rim, and DM was hydrodissected by injecting 6.25 mg/ml indocyanine green so that the donor and recipient DM could be differentiated by colour. After creating a corneal scleral button, a 6 mm diameter incision was made in the DM using a manual trephine, and the endothelium was coated with viscoelastic material to protect the cells from physical friction. The polymer/DM composite was folded in half using an 8 mm diameter hyroxyethyl methacrylate polymer (one day ACUVUE; Johnson and Johnson, Jacksonville, FL, USA) as a carrier (fig 2A). The polymer/DM with endothelial cells facing inward was grasped with capsulorrhexis forceps towards the leading edge of the polymer (fig 2B, C). Donor tissue with the polymer carrier was inserted into the anterior chamber filled with air through the 5 mm limbal incision, and apposed to the host stroma by expanding the polymer. Additional air is injected into the anterior chamber to further adhere the DM to the posterior stromal surface. Polymers were removed at the end of surgery. Control animals (n = 4) were operated by CCD alone without transplantation of donor endothelium, and another four rabbits served as sham negative control. All animals received topical antibiotics (levofloxacin) and steroids (betamethasone). After observing the rabbits for 28 days, animals were sacrificed and recipient corneas were fixed with 10% formalin over night. Paraffin sections were stained with haematoxylin and eosin.

Human eye bank eyes (five donors globes, five recipient globes) were obtained from the Northwest Lions Eyebank (Seattle, WA, USA). Donor DM were transplanted into five globes according to the procedure described above. Transplanted eyes were immediately processed into corneal scleral buttons for histological analysis of the transplanted endothelium. Following fixation with 4% paraformaldehyde, endothelial cell density was calculated by staining the nuclei of cells with 4', 6–diamidino–2-phenylindole dihydrochloride (DAPI, Dojindo Laboratories, Kumamoto, Japan) and analysed using the NIH Image software (National Institute of Health, Bethesda, MA, USA).

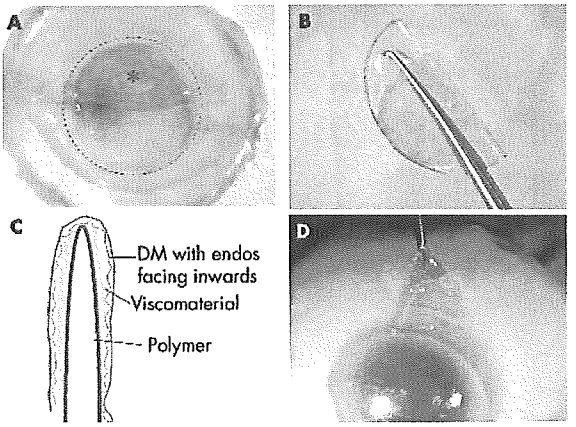

Figure 2 Preparation of donor tissue shown in an eyebank eye. (A) Donor DM is dissected with indocyanine green as described in figure 1, processed into a corneoscleral button, and trephined so that an incision is made in DM (dotted circle). Viscomaterial is coated on the endothelial surface and a hyroxyethyl methacrylate polymer carrier (*) is folded in half and placed on DM. (B) Capsulorrhexis forceps used to grasp the polymer/DM composite with endothelial cells facing inwards. (C) A cross sectional schematic view of polymer/DM shown in (B). (D) The polymer is removed after DM is placed in position with the use of air. The same procedure was performed in rabbits.

RESULTS

Endothelial transplantation in rabbits

The left eyes of four rabbits were transplanted with donor DM. Figure 3A shows a transplanted eye following surgery; the transplanted DM is stained green and the host DM is stained blue. Figure 3B shows the transplanted eye with a clear central cornea 28 days following surgery. Control eyes (fig 3C) had extensive stromal oedema with minimal view of the anterior chamber. The mean (standard deviation) pachymetry reading of transplanted eyes was 407.2 (SD 63.0) μm, which was not significantly different compared with sham operated controls with an average thickness of 391.2 (SD 20.8) μm. Positive control eyes operated by CCD alone were too oedematous to obtain readings with the pachymeter used in our study. The mean cell density in the rabbit eye 28 days after surgery was 2201.3 (SD 441.5) cells/

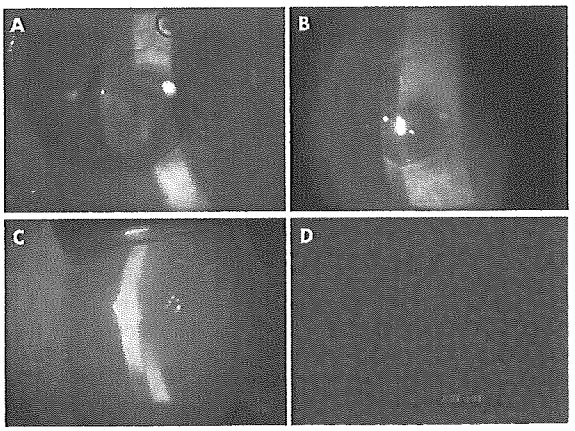

Figure 3 Postoperative eye in a rabbit with DM transplantation immediately following surgery (A) and after 28 days (B). Control eyes stripped of DM had extensive stromal oedema and opacification (C). Histology of endothelium within the graft shows a uniform layer of endothelium (D).

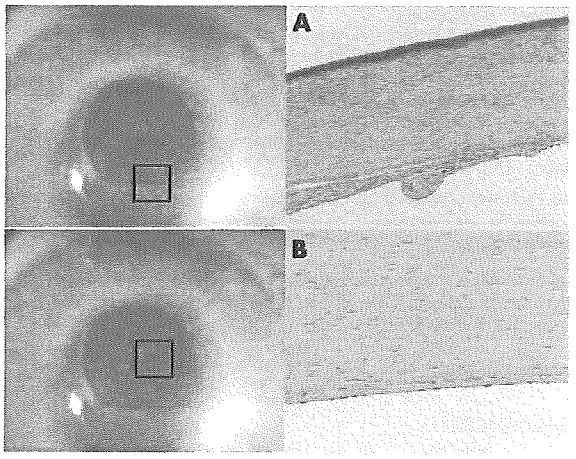

Figure 4 HE stains of the edge (A) and centre (B) of donor show a well apposed DM with slight curling of the edge. Original magnification (×100) for HE stains.

mm^2 (fig 3D) compared with 3180.5 (SD 98.2) cells/mm^2 in sham control. Cell loss was greater in rabbit eyes compared with eye bank eyes because of the very shallow anterior chambers in rabbits, which was an obstacle during surgical procedures.

Animals were sacrificed after 28 days, and the transplanted eyes were processed for histological examination. Haematoxylin eosin (HE) stains of the peripheral graft show a slightly curled edge of the transplanted DM (fig. 4A). The central graft is attached to the host stroma (fig 4B), which under high magnifications shows how the transplanted DM resembles DM in sham operated controls (fig 5). There was no observable infiltration of inflammatory cells in any of the operated eyes after 28 days.

Endothelial transplantation in human eyebank eyes

A total of five "donors" were transplanted into another five eyebank eyes using the same procedure explained in detail above. Corneal scleral rims were processed immediately following implantation to show the positioning of the graft (fig 6). Nuclear stains using DAPI revealed an endothelial cell density of 2749 (SD 288) cells/mm^2, suggesting that an adequate density of endothelial cells can be transplanted using this technique. No information was available as to the cell density of the eyes before the experiment; therefore the endothelial cell loss rate was not calculated.

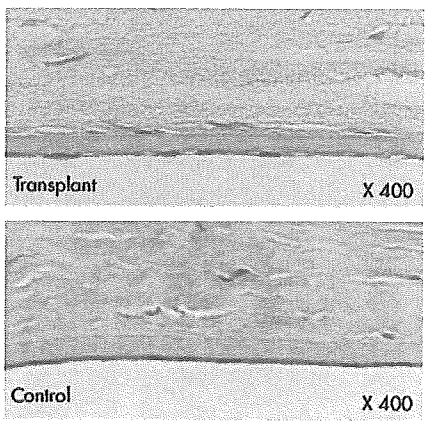

Figure 5 Higher magnification (×400) of the central graft reveals similar histological findings in both the transplanted eye and control.

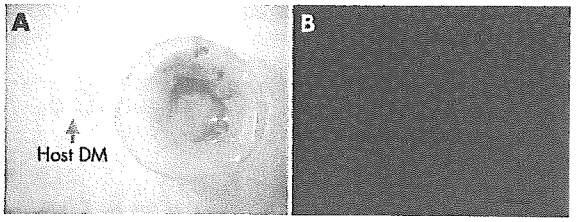

Figure 6 DM transplantation in human eyebank eye with green donor DM and blue recipient DM following CCD (A). Average endothelial density immediately following insertion of donor DM was 2749 (SD 288) cells/mm^2.

DISCUSSION

Surgical techniques in keratoplasty have changed over the past few years towards reducing tissue damage to a minimum, while transplanting only the layers of the cornea that are necessary for the particular patient. For stromal diseases such as keratoconus and the various dystrophies, DLKP can remove all of the stroma while leaving the host endothelium intact.[1 2] Epithelial transplants using amniotic membrane carriers are already in clinical use for the treatment of acute phase burns and Stevens Johnson syndrome with persistent epithelial defects.[11] The same technique can also be used to restore vision in the chronic stage of cicatricial disease such as ocular cicatricial pemphigoid, if the indications are chosen carefully.

Bullous keratopathy, however, is still mainly treated by PKP as the techniques involved in transplanting endothelium were believed to be technically difficult. Several experimental reports using animals have attempted to transplant endothelium by seeding the cells on hydrogel polymers,[12] or by using bovine DM as carriers.[13] More recently, clinical studies have described techniques to transplant the posterior stroma, including the endothelium, into bullous keratopathy patients. One technique is reported by Azar *et al*, who used a microkeratome to create a flap so that the posterior half of the donor cornea could be transplanted beneath the flap.[6] Our experience with the technique is favourable, with a clear optical interface between the host and donor. However, epithelial ingrowth can be a complication, and the need of a keratome can be costly. Another method described by Melles *et al* uses a spatula to dissect the host Descemet's membrane, after which a donor DM is inserted into the anterior chamber though a small corneal scleral incision.[7 8] The procedure seems promising in terms of refraction; however, stromal haze in the donor-host interface may occur when a spatula or knife is used to create a lamellar incision in the deep stroma. A custom made scraper was recently reported by the same group, which may solve these issues in the repicient stromal bed.[14]

The method described in this study uses hydrodissection to dissociate Descemet's membrane in both the donor and host, without the use of special instruments. This creates clear cut dissociation at the interface of DM and stroma, because fluid enters into the plane of weakest adhesion. The interface in this case is smoother than other methods using blades or spatula and, furthermore, does not interrupt the posterior stroma that contains keratocytes which may become activated to cause tissue reaction. The high magnification in figure 5 shows how the transplanted DM is closely apposed to the host stroma with little cellular infiltration or scarring in the posterior stroma. The grafts in rabbits show no interface

opacity under slit lamp examination, and the endothelial cell count is within an acceptable range. The average cell density in rabbits was lower than human eyebank eyes in our study, which was due to the fact that surgical manipulation was much more difficult in rabbits, which have very shallow anterior chambers and show fibrinotic response during surgery.

The rabbit data show that transplanted DM is functional, and that little tissue reaction is observed at the host-donor junction. However, as rabbit endothelial cells are known to proliferate in vivo, the same experiment was done using human eyebank eyes. The objective was to simulate a clinical situation, and also to assess the loss of endothelial cells during the procedure. As shown in figure 6, controlling the size and apposition of donor DM is possible, and the average endothelial cell density was above 2500 cells/mm^2. The preoperative endothelial density was not available; however, considering that the eyes were preserved in moist chambers without the use of storage medium, the results can be said to be more than adequate.

The largest obstacle to manipulating DM is the tendency for the 10 μm thick membrane to curl up with the endothelium facing outwards. Melles *et al* have developed an injector for the purpose of inserting the membrane into the anterior chamber.[10] We have shown that a hydroxyethyl methacrylate polymer can be used as a carrier by folding the DM in half, and also protect the graft from the loss of endothelial cells during insertion into the anterior chamber. The polymer can then be unfolded in the anterior chamber, and also be used to appose the DM to the stroma. The inserted DM is unfolded using a spatula inserted into the plane opposite the endothelium. Although the donor edge is not necessarily a perfect fit with the recipient bed (fig 4), the adhesion of donor DM is firm as evidenced from the fact that the membrane remains attached following paraffin fixation during HE stains. The report by Melles *et al* also shows that a perfect match is not necessary.

Transplantation of hydrodissected DM is an effective means to reproduce the normal anatomy of the posterior stroma. The hydrostatic pressure exerted by the endothelial pump is sufficient for the immediate attachment of the donor DM, and a polymer carrier offers protection and a means to insert DM through a small surgical wound. Encouraged by the results of this study, we are currently preparing a clinical trial based on the surgical technique described here.

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