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 remakable 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 cementblasts occurs in an earlier stage than does differentiation into osteoblasts: Perhaps adhesion of MSC on the surface of denuded dentine enhanced cementblast 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

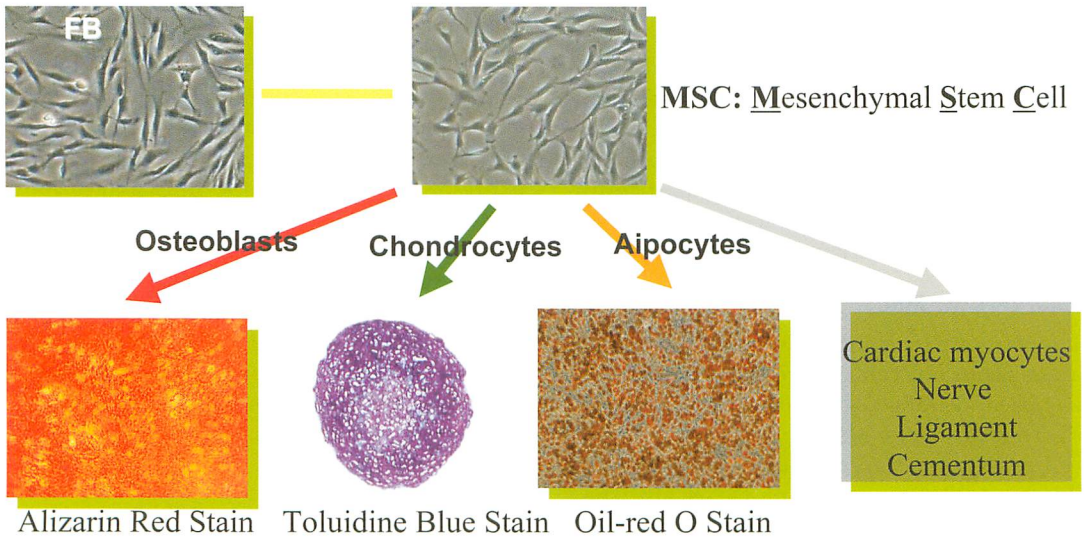

Fig. 1 Multi-lineage differentiation potentials of MSC expanded using the super-expansion method. FGF-2 was added to MSC cultures, or MSC were seeded and maintained on ECM-coated dishes. These MSC retained osteogenic, adipogenic and chondrogenic potentials throughout many mitotic divisions.

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

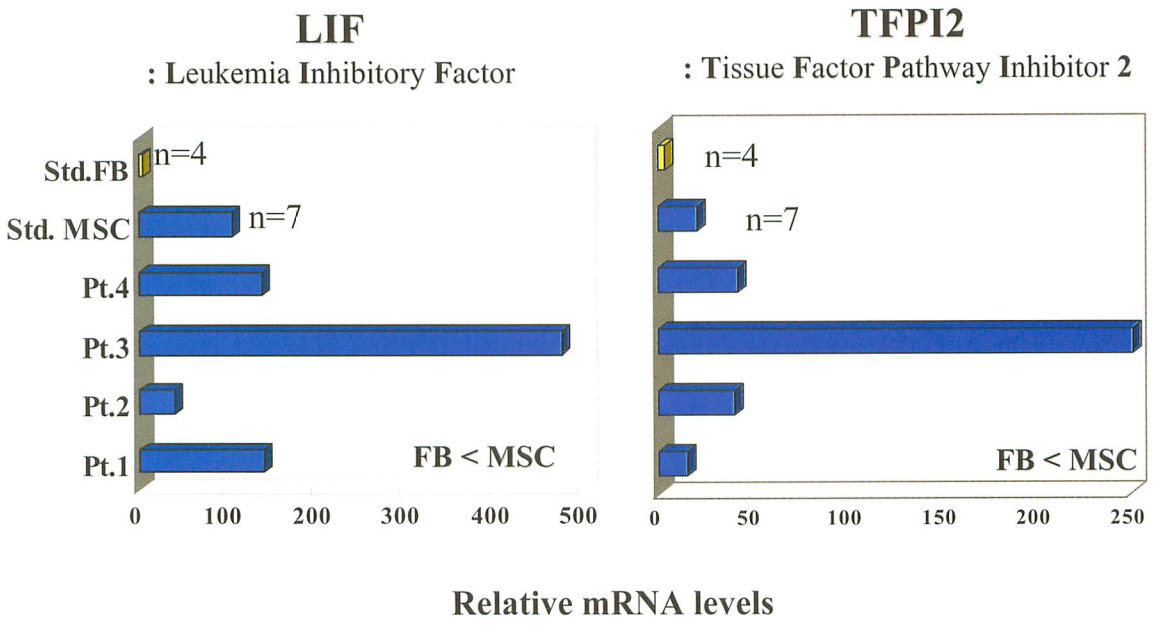

Fig. 2 The use of MSC marker genes in clinical studies. The expression levels of the genes in transplantable MSC from four patients were similar to those in standard MSC.

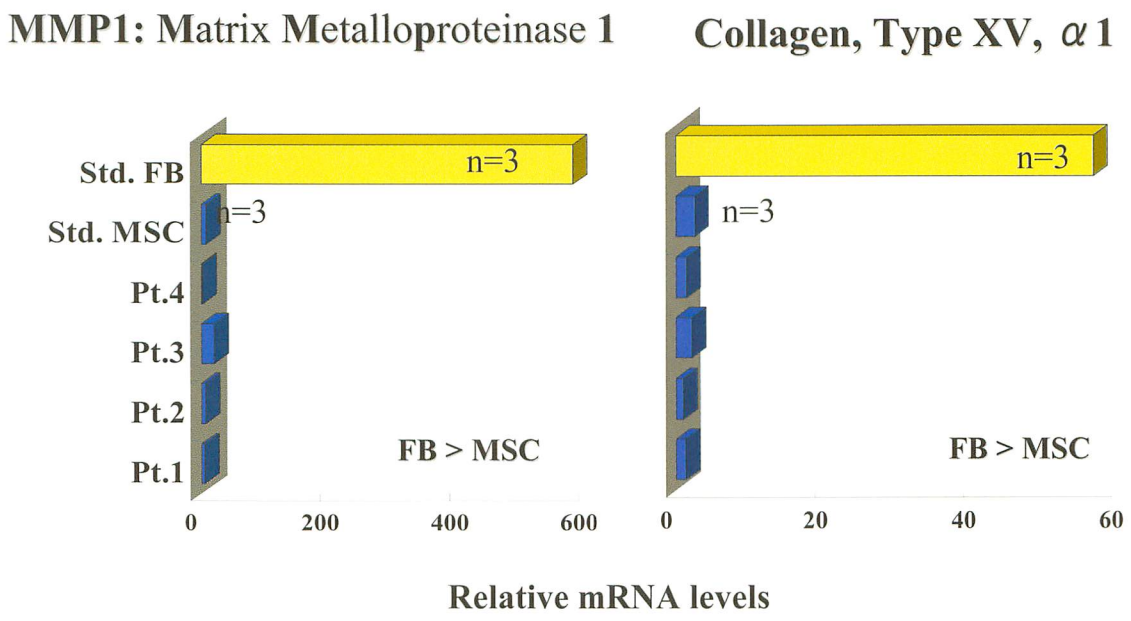

Fig. 3 MSC marker genes were used in clinical studies to confirm the absence of fibroblasts in transplantable cells.

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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