Journal of Oral Science, Vol. 46, No. 4, 207-213, 2004

Original

Differentiation of the human mesenchymal stem cells derived from bone marrow and enhancement of cell attachment by fibronectin

Naomi Ogura[§,‡], Masaru Kawada[†], Wei-Jen Chang[†], Qi Zhang[*], Sheng-Yang Lee[¶], Toshirou Kondoh[§,‡] and Yoshimitsu Abiko[†,‡]

Departments of [§]Oral Surgery and [†]Biochemistry and [‡]Research Institute of Oral Science, Nihon University School of Dentistry at Matsudo, Chiba, Japan
[*]Wuhan University, School of Stomatology,
[¶]Graduate Institute of Oral Rehabilitation Sciences and School of Dentistry, Taipei Medical University, Taipei, Taiwan

(Received 7 April and accepted 6 September 2004)

Abstract: The ability of human mesenchymal stem cells (hMSC) to differentiate into osteoblasts was examined through the use of osteogenic induction medium (MSCOIM) cultures. hMSC first attached to the dish surface and exhibited fibroblast-like spindle shapes, and after proliferation, formed cuboidal shapes. Calcium assays and the use of von Kossa and alizarin red S staining showed that hMSC were capable of mineralization when cultured in MSCOIM. Gene expressions of Cbfa-1 and BMP-4, which are markers for osteogenic differentiation, were also increased during the hMSC differentiation into osteoblasts. When compared to albumin (Alb)-coated dishes, microscopic observation documented enhanced cell attachment and spreading when hMSC were cultured on fibronectin (FN)-coated dishes. Adherent cell numbers also exhibited a greater increase on the FN-coated dishes during earlier culture stages than that seen for the Alb-coated dishes. These findings suggest that hMSC have the capability to differentiate into osteoblasts and that FN can stimulate the attachment and spreading of the hMSC. (J. Oral Sci. 46, 207-213, 2004)

Correspondence to Dr. Yoshimitsu Abiko, Department of Biochemistry, Nihon University School of Dentistry at Matsudo, 2-870-1 Sakaecho-nishi, Matsudo, Chiba 271-8587, Japan
Tel: +81-47-360-9328,
Fax: +81-47-360-9329.
E-mail: yabiko@mascat.nihon-u.ac.jp

Key words: mesenchymal stem cell; osteoblast; fibronectin.

Introduction

Bone formation comprises a complex set of events that begins with the recruitment and proliferation of osteoprogenitors followed by cell differentiation, osteoid formation, and mineralization. It has been recognized that bone is unique and has a vast potential for regeneration from stem cells (1,2). A number of reports have indicated there is a potential for mesenchymal stem cell (MSC) transplantation from bone marrow for a variety of skeletal disorders (3,4). The development of MSC differentiation from human bone marrow along specific lineages in combination with biomimetic scaffolds provides the possibility of tissue engineering for bone and cartilage (5). The ability of progenitor cells to attach to a scaffold surface during early stages is important in the development of new tissue structures (6,7).

The initial contact of the cells with the implant surface is an important event for adhesion of the bone cells that influence osseointegration. One strategy to improve osseointegration that has been tried involves the precoating of implants with an extracellular matrix protein for use as a scaffold, which enables specific cell-extracellular matrix interactions, along with the use of growth factors to facilitate the differentiation of the osteoblasts (8,9). Extracellular matrices, such as collagen type I and fibronectin (FN), are effective in promoting cellular

adhesion and spreading, and they possess a RGD (arginine-glycine-aspartic acid) sequence, which is recognized by integrins (10-12). Signaling occurs when integrins bind to the extracellular matrix and results in changes in the expression of select genes that control the initial preosteoblast/osteoblast adhesion, proliferation and maturation (10-12).

The aims of the present study were to examine the early stages of cell attachment and the spreading of human mesenchymal stem cells (hMSC) derived from bone marrow on surfaces that were modified by FN.

Materials and Methods

Cell culture

The Poietics™ Human Mesenchymal Stem Cell product (hMSC; Lot No. 9F1938) was obtained from Cambrex Bio Science Walkersville (East Rutherford, NJ, USA). The cells were cultured in MSC growth medium (MSCGM; the cell system components consisted of the MSC basal medium and the SingleQuots® growth supplements MCGS, which contained fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin; Cambrex Bio Science Walkersville) in a humidified incubator (Forma CO_2 incubator MIP-3326; Sanyo Electric Medical System, Japan) in the presence of 95% air and 5% CO_2 at 37˚C. In the experiment for osteogenic differentiation, hMSC from the 5th to 7th doubling passages were seeded at 3.1×10^3 cells/cm^2 in MSCGM. After 24 h, the media was changed to MSC osteogenic induction medium (MSCOIM; the cell system components consisted of MSC basal medium and the osteogenic SingleQuots® that contained FBS, L-glutamine, penicillin/streptomycin, dexamethasone, ascorbate, and β-glycerophosphate; Cambrex Bio Science Walkersville,). The media was changed twice a week. The schema for the culture is shown in Fig. 1.

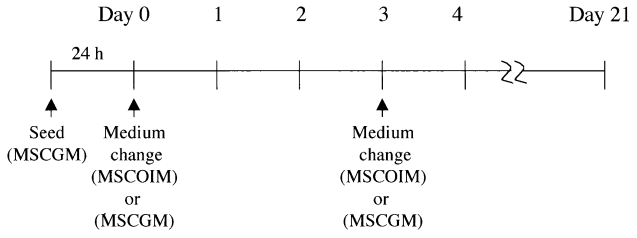

Fig. 1 Schema of hMSC culture. The cells were seeded at 3.1×10^3 cells/cm^2 in MSCGM. After 24 h (day 0), the media was changed to MSCOIM or MSCGM. The media was changed twice a week.

Microscopic observation

Morphology of hMSC was observed by phase-contrast microscopy and pictures were taken of the specimens (DP12, Olympus, Tokyo, Japan).

Histochemical analysis

Mineralization of the cell layer was examined through the use of alizarin red S or von Kossa staining. The cell layers were washed twice with phosphate-buffered saline (PBS) fixed with 10% formalin solution for 30 min, and then washed with pure water two times. For alizarin red S staining, the cells were placed in 1% alizarin red S (Kanto Chemical, Tokyo, Japan) solution for 10 min, washed with pure water three times and then air-dried. The von Kossa staining was performed according to the method of Jaiswal et al. (1). Briefly, the cell layers were placed in a 5% silver nitrate (Kanto Chemical) solution for 10 min, and kept in the dark. After washing with pure water, the cell layers were then exposed to bright light for 15 min.

Calcium assay

Calcium content was measured using a calcium assay kit (Sigma Diagnostics, St. Louis, MO, USA). Briefly, the cell layers were washed twice with PBS. Calcium in the matrix was dissolved with 0.5 M HCl, assay reagent was added, and then the sample was measured at a wavelength of 598 nm.

Alkaline phosphatase assay

The cell lysate was obtained through the use of a solubilizing solution (0.01% NaCl, 0.1% Triton X-100), incubated with *p*-nitrophenyl phosphate solution (Chemicon International, Tenecula, CA, USA) at 37˚C, and then 1 M NaOH was added to stop the reaction. The samples were measured at a wavelength of 415 nm. ALP activity was calculated per 10^5 cells, and was determined from a *p*-nitrophenol standard curve with 1 unit representing the release of 1 μmol of *p*-nitrophenol per min at 37˚C.

Real-time PCR

Total RNA was extracted using TRIZOL Reagent (Life Technologies, Gaithersburg, MD, USA) and the FastPrep FP120 Instrument (BIO 101, Vista, CA, USA). cDNA was synthesized using a GeneAmp RNA PCR kit (Perkin-Elmer, Norwalk, CT, USA). The real-time PCR was carried out using a DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland). The PCR mixture, which contained 20 pmol of forward and reverse primers and 2 μl of cDNA, was subjected to amplification with a DNA Engine Opticon 1 (MJ Research, San Francisco, CA, USA). The cycles were set at 95˚C for 10 min for preheating, followed by 40

cycles at 94˚C for 15 sec, 55˚C for 30 sec and 72˚C for 30 sec. The amplicons were detected directly by measuring the increase in fluorescence caused by the binding of the SYBR Green I dye to gene-specific, amplified, double-stranded DNA using a DNA Engine Opticon 1. Following the completion of the PCR reaction, the temperature was raised from the annealing temperature to 95˚C for a melting curve analysis.

The initial template concentration was derived from the cycle number at which the fluorescent signal crossed a threshold in the exponential phase of the PCR reaction (CT-value). The number of transcripts was determined based on the threshold cycles of Cbfa-1, BMP-4 and GAPDH. ΔCT (CT-Cbfa-1 or BMP-4 minus CT-GAPDH) designated the relative abundance of the Cbfa-1 or BMP-4 transcript. ΔΔCT (ΔCT-osteoinduction minus ΔCT-day 0) represented the relative n-value compared to the control. The 2^{-n} represented the difference in mRNA expressions in osteoinduction cells relative to day 0. The respective primer sequences were 5'-TGA GAG CCG CTT CTC CAA CC-3' (the forward primer for Cbfa-1); 5'-GCG GAA GCA TTC TGG AAG GA-3' (the reverse primer for Cbfa-1); 5'-AAG CGT AGC CCT AAG CAT-3' (the forward primer for BMP-4); 5'-CAT CCA GGT ACA GCA TGG-3' (the reverse primer for BMP-4); 5'-ATC ACC ATC TTC CAG GAG-3' (the forward primer for GAPDH); and 5'-ATC GAC TGT GGT CAT GAG-3' (the reverse primer for GAPDH).

Fibronectin coating

After pouring 500 μl of 6.25 μg/ml fibronectin (human plasma; KOKEN, Japan) or 50 μg/ml albumin (Alb, human; Funakoshi, Japan) in PBS into the wells of a 24-well tissue culture plate (Corning Cell Wells, Corning, NY, USA), the plates were then incubated at 37°C overnight for coating, after which the wells were washed twice with PBS.

Cell adhesion

For adhesion experiments, hMSC were plated at 4.5×10^4 cells/well in a 24-well plate. The cells were cultured in MSC basal medium that was supplied with L-glutamine and penicillin/streptomycin, and serum free in a CO_2 incubator, and then the cells were detached by 0.05% trypsin and 0.02% EDTA. Cell numbers were counted with a coulter counter (Model ZM, Electronics, UK).

Statistics

Multiple samples were collected for each measurement, and the results were expressed as means ± SD. A Student's *t*-test was used for the analysis of the difference.

Results

Differentiation of hMSC

We first examined the ability of hMSC to differentiate into osteoblasts using MSCOIM. Most of the cells that attached to the plastic surface exhibited a fibroblast-like spindle shape. They proliferated to form a uniform confluent cell monolayer and became cuboidal in shape 4 days after changing from MSCGM to the MSCOIM (Fig. 2). The hMSC cultured in MSCOIM stained weakly on day 10, but stained strongly on day 13 or 17 when exposed to alizarin red S, which indicates calcium deposition (Fig. 3). On day 21, formed calcium phosphate deposition was observed after von Kossa staining. In contrast these cells were not found in MSCGM cultures (Fig. 4). Calcium contents of the hMSC cell layers on day 21 were also found to have increased in MSCOIM cultures as compared to controls (MSCGM) (Fig. 5). We then measured ALP activity of hMSC cultured in MSCOIM or MSCGM. As shown in Fig. 6, there was increased ALP activity in the hMSC cultures on day 4, with the increase peaking on day 7. Furthermore, gene expression of Cbfa-1 and BMP-4 were examined by real-time PCR. Both Cbfa-1 and BMP-4 mRNA levels were significantly enhanced on culture day 12 as compared to day 0 (Table 1).

Cell adhesion

The hMSC attachment and spreading were also examined on FN-coated dishes and compared with Alb-coated dishes,

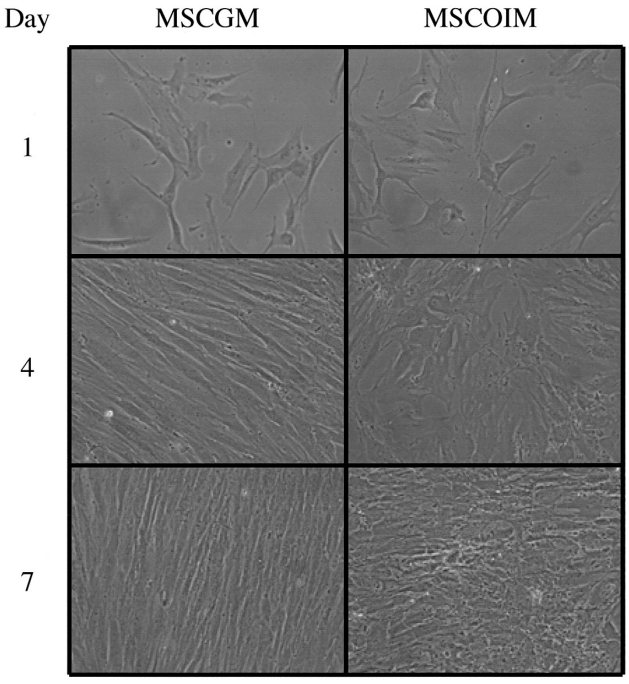

Fig. 2 Morphology of hMSC.

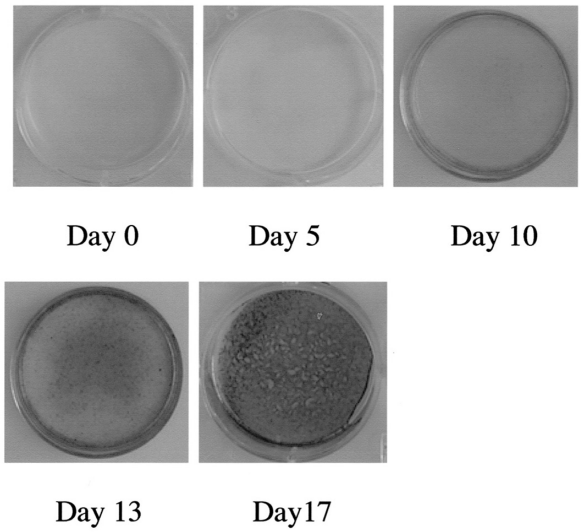

Fig. 3 Alizarin red S staining.

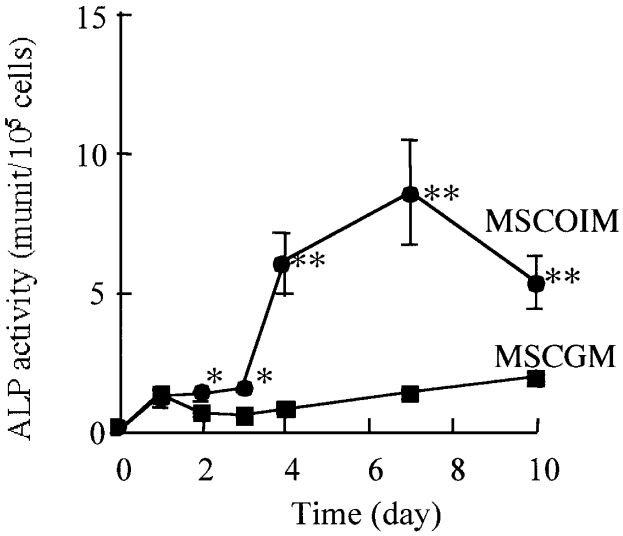

Fig. 6 ALP activity of hMSC. Values represent the means ± SD of the results from four wells. *, $P < 0.01$, **, $P < 0.005$ vs. MSCGM.

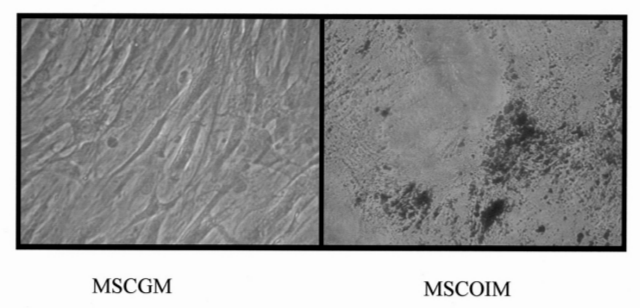

Fig. 4 von Kossa staining on day 21. hMSC were cultured with MSCGM or MSCOIM.

Table1 Gene expression of Cbfa-1 and BMP-4 in osteogenic induction of hMSC

	Cbfa-1 (Fold)	BMP-4 (Fold)
Day 0	1.0	1.0
Day 4	2.78	0.87
Day 8	1.44	0.53
Day 12	16.14	3.60

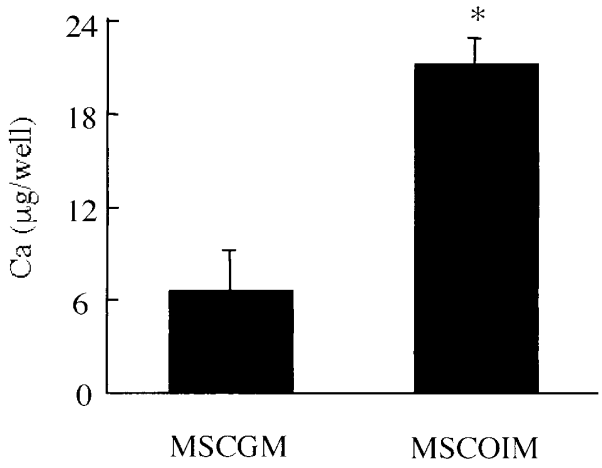

Fig. 5 Level of calcium deposition in the cell layers of hMSC. Values represent the means ± SD of the results from four wells. *, $P < 0.005$ vs. MSCGM.

which were used as controls. Enhanced cell attachment and spreading were observed in the FN-coated dishes as compared to the Alb-coated dishes (Fig. 7). To quantitatively confirm the number of adherent cells on the FN-coated and Alb-coated dishes, each were measured and compared. As shown in Fig. 8, hMSC rapidly adhered to the FN-coated dishes in the earlier stages, i.e., up to the 20 minute time point, and higher cell numbers were found in the samples obtained up to the 50 min point as compared to the Alb-coated dishes.

Discussion

Tissue engineering, which includes bed structures for osteogenesis in dental implantations, makes use of the advantage of combining cultured living cells and an implant body that can be used as a scaffold (10-12). Previous attempts to tissue engineer bone or cartilage reconstruction around implants have utilized osteoblasts or chondrocytes encapsulated in various hydrogels, which can survive *in*

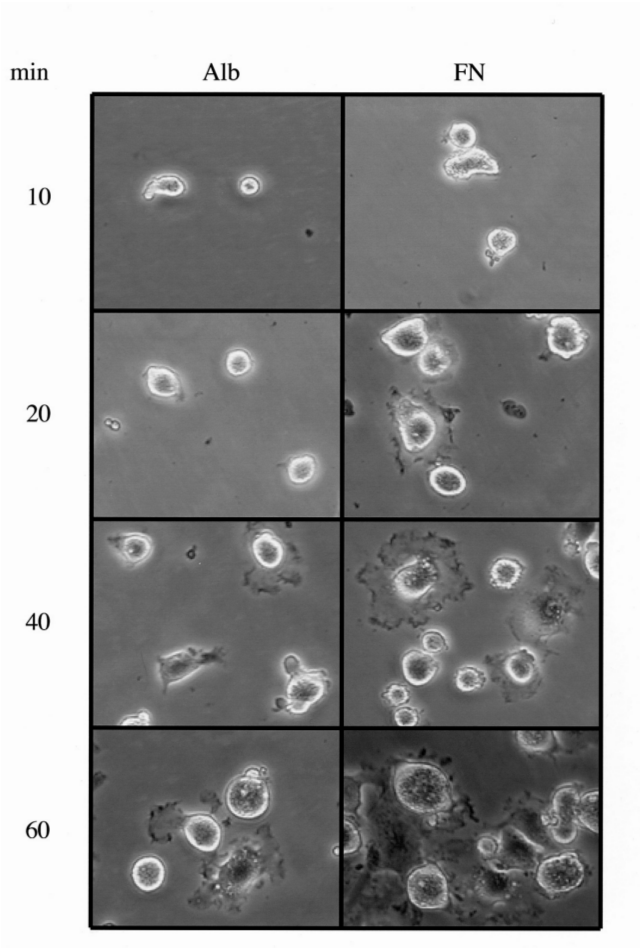

Fig. 7 Morphology of hMSC attachment in Alb- or FN-coated plates.

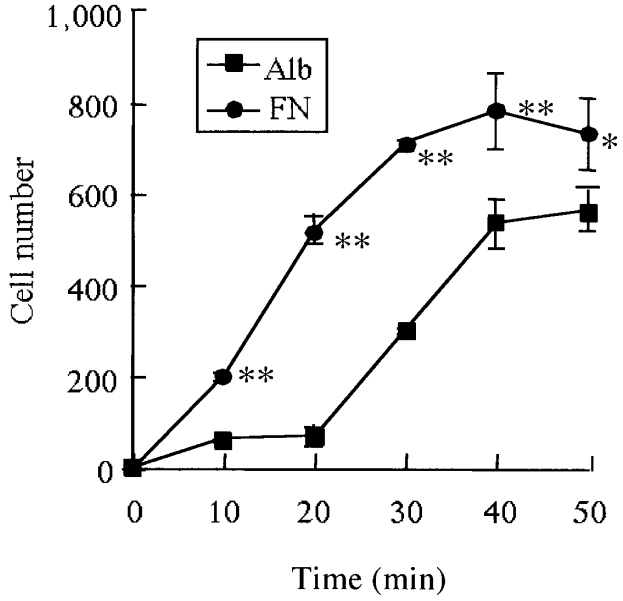

Fig. 8 Cell number attachment for hMSC in Alb- or FN-coated plates. Values represent the means ± SD of the results from four wells. *, $P < 0.01$, **, $P < 0.005$ vs. Alb.

vitro fabrication and be able to synthesize cell-associated scaffold extracellular matrices (13,14). For dental implantation, it is recommended that patients have transplantation of normal osteoblasts or chondrocytes that come from their own bodies. However, this may not always be possible. On the other hand, cultured MSC from bone marrow can be regarded as a bone progenitor/precursor cell population that can be derived from adults (6,7). It has the ability to proliferate extensively, and maintain its ability to differentiate into osteoblastic or chondrocytic cells *in vitro*. The objective of this study was to examine hMSC differentiation into the osteoblastic lineage and study the cell attachment that occurs during early stages when using FN as a scaffold.

In our preliminary experiments, the ability of hMSC to differentiate into osteoblasts was examined. Results obtained from the calcium assay and alizarin red S and von Kossa staining documented that mineralization occurred in the cell layers during osteoblast differentiation of the hMSC in the MSCOIM cultures. ALP activity and gene expression for Cbfa-1and BMP-4, which are markers for osteogenic differentiation, increased for hMSC during cultures with MSCOIM. These findings demonstrate that hMSC maintain the ability to differentiate into osteoblastic cells and thus it seems likely that MSCOIM is necessary in order to be able to induce differentiation and mineralization of hMSC. Therefore our data indicate that the osteogenic potential of hMSC-derived osteogenic precursors may be useful for autologous grafts.

For dental implantation, a stable primary stabilization and a subsequent osseointegration of the endoprotheses are of critical importance with regard to the overall clinical results. In order to accelerate and enhance the bone growth and to strengthen the osseous fixation, implant surfaces have been modified, and in particular, there has been an increased use in surface coatings of functional proteins. Numerous substances, such as growth factors (2,15,16) and extracellular matrices (3-5,16-18), are currently used to coat the surfaces of the biomaterial used in the implants. FN is a major extracellular matrix protein and is found in abundance in blood plasma. This protein is known to be involved in cell adhesion processes and contains the specific amino acid sequence for the RGD motif that is recognized by specific cell membrane receptors such as integrins (19).

In our study, the measurement of cell adhesion during the early stages showed that hMSC had enhanced cell attachment and spreading in FN-coated dishes as compared

to the Alb-coated control dishes. It has also been reported that in non-differentiation, expanded attached hMSC were uniformly positive for SH2, SH3, CD29, CD44, CD71, CD90, CD106, CD120a, and CD124 (7). CD29, which is β1 integrin, has been suggested to be important for cell adhesion in hMSC (20), although there have been no reports on the presence of α integrins (CD49a-f) in hMSC to the best of our knowledge. On the other hand, CD44 is not only hyaluronic acid but also a FN receptor, and it has been implicated in hematopoietic stem cell adhesion, proliferation, and mobilization (21). Enhanced cell attachment and spreading in FN-coated plates may be associated with CD29 and CD44 of the hMSC surface proteins.

The most frequent complication of dental implantation is bone resorption that is initiated by an inflammatory response at the implant site. Recent studies have demonstrated that titanium is the most frequent component of dental implants, and that this material is responsible for inducing apoptosis and suppressing expression of the osteoblastic phenotype in hMSC (22,23). Therefore, it is suggested that precoating dental implants with extracellular matrices such as FN is an important strategy when trying to improve the success rate for tissue regeneration.

The results obtained in this paper are very promising and we propose that the use of culture-expanded osteoprogenitor cells in conjunction with a FN-modified implant body will lead to significant improvements in grafting techniques.

Acknowledgments

We would like to thank Dr. Hideaki Nagura for suggestions with regard to this study.

This study was supported in part by a Grant from the Ministry of Education, Culture, Sports, Science, and Technology to promote 2001-multidisciplinary research projects (in 2001-2005).

References

1. Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP (1997) Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells *in vitro*. J Cell Biochem 64, 295-312
2. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284, 143-147
3. Cooper LF, Harris CT, Bruder SP, Kowalski R, Kadiyala S (2001) Incipient analysis of mesenchymal stem-cell-derived osteogenesis. J Dent Res 80, 314-320
4. Young CS, Terada S, Vacanti JP, Honda M, Bartlett JD, Yelick PC (2002) Tissue engineering of complex tooth structures on biodegradable polymer scaffolds. J Dent Res 81, 695-700
5. Alhadlaq A, Mao JJ (2003) Tissue-engineered neogenesis of human-shaped mandibular condyle from rat mesenchymal stem cells. J Dent Res 82, 951-956
6. Howlett CR, Evans MDM, Walsh WR, Johnson G, Steele JG (1994) Mechanism of initial attachment of cells derived from human bone to commonly used prosthetic materials during cell culture. Biomaterials 15, 213-222
7. Grzesiak JJ, Pierschbacher MD, Amodeo MF, Malaney TI, Glass JR (1997) Enhancement of cell interactions with collagen/glycosaminoglycan matrices by RGD derivatization. Biomaterials 18, 1625-1632
8. Yliheikkilä PK, Masuda T, Ambrose WW, Suggs CA, Felton DA, Cooper LF (1996) Preliminary comparison of mineralizing multilayer cultures formed by primary fetal bovine mandibular osteoblasts grown on titanium, hydroxyapatite, and glass substrates. Int J Oral Maxillofac Implants 11, 456-465
9. Shah AK, Lazatin J, Sinha RK, Lennox T, Hickok NJ, Tuan RS (1999) Mechanism of BMP-2 stimulated adhesion of osteoblastic cells to titanium alloy. Biol Cell 91, 131-142
10. Masi L, Franchi A, Santucci M, Danielli D, Arganini L, Giannone V, Formigli L, Benvenuti S, Tanini A, Beghè F, Mian M, Brandi ML (1992) Adhesion, growth, and matrix production by osteoblasts on collagen substrata. Calcif Tissue Int 51, 202-212
11. Cooper LF, Handelman B, McCormack SM, Guckes AD (1993) Binding of murine osteoblastic cells to titanium disks and collagen I gels: implications for alternative interpretations of osseointegration. Int J Oral Maxillofac Implants 8, 264-272
12. Degasne I, Baslé MF, Demais V, Huré G, Lesourd M, Grolleau B, Mercier L, Chappard D (1999) Effects of roughness, fibronectin and vitronectin on attachment, spreading, and proliferation of human osteoblast-like cells (Saos-2) on titanium surfaces. Calcif Tissue Int 64, 499-507
13. Weng Y, Cao Y, Silva CA, Vacanti MP, Vacanti CA (2001) Tissue-engineered composites of bone and cartilage for mandible condylar reconstruction. J Oral Maxillofac Surg 59, 185-190
14. Cassinelli C, Morra M, Bruzzone G, Carpi A, Di Santi G, Giardino R, Fini M (2003) Surface

chemistry effects of topographic modification of titanium dental implant surface: 2. *In vitro* experiments. Int J Oral Maxillofac Implants 18, 46-52
15. Cole BJ, Bostrom MPG, Pritchard TL, Sumner DR, Tomin E, Lane JM, Weiland AJ (1997) Use of bone morphogenetic protein 2 on ectopic porous coated implants in the rat. Clin Orthop 345, 219-228
16. Chaipinyo K, Oakes BW, van Damme M-P I (2002) Effects of growth factors on cell proliferation and matrix synthesis of low-density, primary bovine chondrocytes cultured in collagen I gels. J Orthop Res 20, 1070-1078
17. MacDonald, DE, Markovic B, Allen M, Somasundaran P, Boskey AL (1998) Surface analysis of human plasma fibronectin adsorbed to commercially pure titanium materials. J Biomed Master Res 41, 120-130
18. Geißler U, Hempel U, Wolf C, Scharnweber D, Worch H, Wenzel K-W (2000) Collagen type I-coating of Ti6Al4V promotes adhesion of osteoblasts. J Biomed Master Res 51, 752-760
19. Pierschbacher MD, Ruoslahti E (1984) Variants of the cell recognition site of fibronectin that retain attachment-promoting activity. Proc Natl Acad Sci USA 81, 5985-5988
20. Lee JW, Kim YH, Park KD, Jee KS, Shin JW, Hahn SB (2004) Importance integrin β1-mediated cell adhesion on biodegradable polymers under serum depletion in mesenchymal stem cells and chondrocytes. Biomaterials 25, 1901-1909
21. De Ugarte DA, Alfonso Z, Zuk PA, Elbarbary A, Zhu M, Ashjian P, Benhaim P, Hedrick MH, Fraser JK (2003) Differential expression of stem cell mobilization-associated molecules on muti-lineage cells from adipose tissue and bone marrow. Immunol Lett 89, 267-270
22. Wang ML, Tuli R, Manner PA, Sharkey PF, Hall DJ, Tuan RS (2003) Direct and indirect induction of apoptosis in human mesenchymal stem cells in response to titanium particles. J Orthop Res 21, 697-707
23. Wang ML Nesti LJ, Tuli R, Lazatin J, Danielson KG, Sharkey PF, Tuan RS (2002) Titanium particles suppress expression of osteoblastic phenotype in human mesenchymal stem cells. J Orthop Res 20, 1175-1184