ると考えられる抗酸化酵素関連遺伝子(superoxide dismutase 2、superoxide dismutase 1/3)は歯肉由来の線維芽細胞の方が1.5～2.8倍の高発現を示していた。歯肉細胞は、皮膚よりも組織採取が主義的に簡単であり、患者的にも受け入れられやすい場合が大きい。また、酵素処理によって自動培養装置への投入もしやすいため、皮膚の瘢痕・皺などの修復に対しての細胞治療においては、自動培養装置とも相性が良く、外科的手技も勘弁で、抗加齢的な治療効果が期待される歯肉細胞の有効性が示唆されるものであった。

図6：皮膚由来・歯肉由来の線維芽細胞の遺伝子発現プロファイル比較(Gene Ontoloty解析)

GO hierarchy	Gene Title	Gene Symbol	p-value	Regulation (up: gingival, down: dermal)
Aging	T-box 2	TBX2	0.002	up
Oxidoreductase activity	steroid-5-alpha-reductase, alpha polypeptide 2	SRD5A2	0.038	down
	retinol dehydrogenase 5 (11-cis/9-cis)	RDH5	0.016	down
	prostaglandin-endoperoxide synthase 1	PTGS1	0.003	down
	EH-domain containing 3	EHD3	0.002	down
	cytochrome P450, family 26, subfamily B, polypeptide 1	CYP26B1	0.010	down
	24-dehydrocholesterol reductase	DHCR24	0.015	up
	lectin, galactoside-binding, soluble, 3 binding protein	LGALS3BP	0.000	up
	glutathione peroxidase 3 (plasma)	GPX3	0.021	up
	aldehyde dehydrogenase 2 family (mitochondrial)	ALDH2	0.004	up
	NAD(P)H dehydrogenase, quinone 1	NQO1	0.019	up
	fatty acid desaturase 2	FADS2	0.038	up
	dehydrogenase/reductase (SDR family) member 3	DHRS3	0.000	up
	cytochrome P450, family 27, subfamily A, polypeptide 1	CYP27A1	0.000	up
	hydroxysteroid (17-beta) dehydrogenase 2	HSD17B2	0.000	up
	superoxide dismutase 3, extracellular	SOD3	0.004	up
	flavin containing monooxygenase 1	FMO1	0.020	up
	aldo-keto reductase family 1, member B10 (aldose reductase)	AKR1B10	0.002	up
	cytochrome P450, family 7, subfamily B, polypeptide 1	CYP7B1	0.029	up
	prostaglandin I2 (prostacyclin) synthase	PTGIS	0.044	up
	fatty acid desaturase 1	FADS1	0.013	up
	hydroxysteroid (17-beta) dehydrogenase 14	HSD17B14	0.011	up
Antioxidant activity	prostaglandin-endoperoxide synthase 1	PTGS1	0.003	down
	glutathione peroxidase 3 (plasma)	GPX3	0.021	up
	superoxide dismutase 3, extracellular	SOD3	0.004	up
Extracellular matrix	TIMP metallopeptidase inhibitor 3 (Sorsby fundus dystrophy, pseudoinflammatory)	TIMP3	0.011	down
	tenascin C (hexabrachion)	TNC	0.006	down
	matrix metallopeptidase 1 (interstitial collagenase)	MMP1	0.011	down
	matrix metallopeptidase 12 (macrophage elastase)	MMP12	0.003	down
	latent transforming growth factor beta binding protein 2	LTBP2	0.029	down
	thrombospondin 4	THBS4	0.045	down
	fibroblast growth factor 1 (acidic)	FGF1	0.015	down
	secreted phosphoprotein 1	SPP1	0.041	down
	gamma-aminobutyric acid (GABA) B receptor, 2	GABBR2	0.043	down
	collagen, type VIII, alpha 1	COL8A1	0.025	down
	collagen, type X, alpha 1(Schmid metaphyseal chondrodysplasia)	COL10A1	0.005	down
	matrix metallopeptidase 14 (membrane-inserted)	MMP14	0.005	up
	lectin, galactoside-binding, soluble, 3 binding protein	LGALS3BP	0.000	up
	transforming growth factor, beta-induced, 68kDa	TGFBI	0.017	up
	EGF-containing fibulin-like extracellular matrix protein 1	EFEMP1	0.025	up
	fibulin 2 (congenital contractural arachnodactyly)	FBN2	0.022	up
	collagen, type V, alpha 1	COL5A1	0.024	up
	fibronectin leucine rich transmembrane protein 2	FLRT2	0.003	up
	gamma-aminobutyric acid (GABA) A receptor, epsilon	GABRE	0.010	up
	superoxide dismutase 3, extracellular	SOD3	0.004	up
	glypican 3	GPC3	0.001	up
	collagen, type IV, alpha 1	COL4A1	0.000	up
	spondin 2, extracellular matrix protein	SPON2	0.002	up
	CD248 molecule, endosialin	CD248	0.001	up
	fibronectin leucine rich transmembrane protein 3	FLRT3	0.016	up
	ADAM metallopeptidase with thrombospondin type 1 motif, 1	ADAMTS1	0.009	up

D．考察

本研究を通じて、我々が開発した自動培養装置の幅広い適応能があることが、線維芽細胞に続く、長期のMSC培養によって確認された。自動培養装置で培養・骨分化の誘導を行ったMSCは、in vivoにおいても充分にその機能性を維持していたため、本自動培養装置は幹細胞の培養にも適応できると強く考えられた。今後は、脂肪や神経などへの分化への応用が期待される。

また遺伝子発現解析を通じ、臨床的に利用しやすい歯肉組織の細胞において、治癒効果への関連性のある遺伝子発現が確認されたということは、自動培養装置との相性・治療効果への影響などを考え合わせた提案を行うことが、今後より多くの施設で自動培養装置を導入することにつながるのではないかと考えられた。

E．結論

本研究を通じて、再生医療の実用化を推進するための安全性向上のための自動培養装置の性能を、より幅広く検討することができた。結果、本研究で開発された自動培養装置は分化しきった細胞だけでなく、未分化な細胞も充分に分化能を維持したまま大量に培養することが可能であることが確認でき、今後の装置としての発展性を大きく期待するものであった。

F．研究発表

1．論文発表　　なし

2．学会発表

R. Kato, W. Yamamoto, Y. Tomita, M. Nakatochi, Y. Tomita, M. Okochi, H. Honda, H. Kagami, K. Ebisawa, M. Ueda: Development of morphological process-control analysis for the automation of processes in regenerative medicine, Biochemical Engineering XV (Engineering Biology from Biomolecules to Complex Systems), QuebecCity, Canada, July, 2007

G．知的財産権の出願・登録状況（予定を含む。）

1．特許取得　　なし

2．実用新案登録　　なし

Ⅲ. 研究成果の刊行に関する一覧表

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（上田　実）

H. Agata, I Asahina, Y. Yamazaki, M. Uchida, Y. Shinohara, M. J. Honda, H. Kagami, M. Ueda. Effective Bone Engineering with Periosteum-derived Cells. J. Dent Res 86(1), 79-83, 2007

D. Mizuno, H. Hideaki, H. Mizuno, J. Mase, K. Usami, M. Ueda. Bone regeneration of dental implant dehiscence defects using cultured periosteum membrane. Clinical Oral Implants Research, 2007 (Accepted)

Masaki J. Honda, Shuhei Tsuchiya, Yoshinori Sumita, Hiroshi Sagara, Minoru Ueda. The sequential seeding of eqithelial and mesenchymal cells for tissue-engineered tooth regeneration. Biomaterials, 28, 680-689, 2007

Hideyuki Nakashima, Kazunori Hamamura, Toshiaki Houjou, Ryo Taguchi, Noriyuki Yamamoto, Kenji Mitsudo, Iwai Tohnai, Minoru Ueda, Keiko Furukawa and Koichi Furukawa. Overexpression of caveolin-1 in a human melanoma cell line results of ganglioside GD3 from lipid rafts and alteration of leading edges, leading to attenuation of malingnant properties. Cancer Sci, vol. 98, no. 4, 512-520, 2007.

Kazunori Shimizu, Akira Ito, Manabu Arinobe, Yosuke Murase, Yoshihisa Iwata, Yuji Anrita, Hideaki Kagami, Minoru Ueda, and Hiroyuki Honda. Effective Cell-Seeding Technique Using Magnetite Nanoparticles and Magnetic force onto Decellularized Blood Vessels for Vascular. Tissue Engineering.

Kazunori Shimuzu, Akira Ito, Manabu Arinobe, Yosuke Murase, Yoshihisa Iwata, Yuji Narita, Hideaki Kagami, Minoru Ueda, and Hiroyuki Honda. Effective Cell-Seeding Technique Using Magnetite Nanoparticles and Magnetite Nanoparticles and Magnetic Force onto Decellularizad Blood Vessels for Vascular Tissue Engineering. JOURNAL OF BIOSCIENCE AND BIOENGINEERING, Vol. 103, No. 5, 472-478, 2007.

Kazuhiko Kinoshita, Hideharu Hibi, Yoichi Yamada, Minoru Ueda. Promoted New Bone

Formation in Maxillary Distraction Osteogenesis Using a Tissue-engineered Osteogenic Material. Journal of Cranifacial Surgery (Accepted).

Treatment of Human Infrabony Periodontal Defects by Grafting Human Cultured Porous Hydroxyapatite Granules: Three Case Reports. Journal of the International Academy of Periodontology (Accepted).

D. Iejima, Y. Sumita, H. Kagami, A. Ando, M. Ueda. Odontoblast marker gene expression in enhanced by a CC-chemokine family protein MIP-3α in human mesenchymal stem cells, Archives of oral biology vol. 52, 924-931, 2007.

三ッ口秀幸, 上田実, 平田仁 神経再生誘導管の有効性試験 骨軟部吸収性材料フォーラム 2007 (抄録集), 22

上田実 再生医療の成り立ちと実用化に向けた新たな取り組み 美容医療への応用ティッシュエンジニアリング 2007, 275-285.

上田実 実用化に向かう歯槽骨の再生医療 J. I. C. D., 2006, Vol. 37, No. 1, 28-32.

上田実 特集にあたって－運動器の再生医療の最新情報 THE BONE, Vol. 21, No. 4, 17-18 (413-414), 2007.

上田実 "Mesenchymal stem cells: building blocks for molecular medicine in the 21st century" 翻訳 THE BONE, Vol. 21, No. 4, 19-26 (415-422), 2007.

山田陽一、上田実 骨の再生 (歯槽骨) THE BONE, Vol. 21, No. 4, 51-56 (447-452), 2007.
上田実 老年医学領域における再生医療 医学のあゆみ Vol. 222, No. 5, 311-317, 2007.

上田実 「再生医療と美容」(上田実編) 南山堂, 2007.

上田実 「再生医療とインプラント」(上田実編) クインテッセンス出版, 2007.

<分担研究者>

（各務　秀明）

Agata H, Kagami H, Watanabe N, Ueda M. Effect of ischemic culture conditions on the survival and differentiation of porcine dental pulp-derived cells. *Differentiation* in press

Mizuno D, Kagami H*, Mizuno H, Mase J, Usami K & Ueda M. Bone regeneration of dental implant dehiscence defects using cultured periosteum membrane. *Clin. Oral Imp. Res.* in press

Tonomura A, Sumita Y, Ando Y, Iejima D, Kagami H*, Honda MJ, Ueda M. Differential inducibility of human and porcine dental pulp derived cells into odontoblasts. *Connect. Tissue Res.* 48, 229-238, 2007

Iejima D, Sumita Y, Kagami H*, Ando Y, Ueda M. Odontoblast marker gene expression is enhanced by a CC-chemokine family protein MIP-3□ in human mesenchymal stem cells. *Arch. Oral Biol.* 52, 924-931, 2007

Shimizu K, Ito A, Arinobe M, Murase Y, Iwata Y, Narita Y, Kagami H, Ueda M, Honda H. Effective Cell-Seeding Technique Using Magnetite Nanoparticles and Magnetic Force onto Decellularized Blood Vessels for Vascular Tissue Engineering. *J. Bioscien Bioeng.* 103, 472-478, 2007

Agata H, Asahina I*, Yamazaki Y, Uchida M, Shinohara Y, Honda M, Kagami H, Ueda M. Effective bone engineering using periosteum-derived cells. *J. Dent Res.* 86, 79-83, 2007

（紀ノ岡正博）

Mee-Hae Kim, Masahiro Kino-oka, Masaya Kawase, Kiyohito Yagi, and Masahito Taya: "Synergistic Effect of D-glucose and EGF Display on Dynamic Behaviors of Human Epithelial Cells", J. Biosci. Bioeng., Vol. 104, No. 5, pp. 428-431 (2007)

Mee-Hae Kim, Masahiro Kino-oka, Masaya Kawase, Kiyohito Yagi, and Masahito Taya: "Glucose Transporter Mediation Responsible for Morphological Change of Human Epithelial Cells on Glucose-Displayed Surface", J. Biosci. Bioeng., in press.

(本多裕之)

Akira Ito, Masatake Fujioka, Tatsuro Yoshida, Kazumasa Wakamatsu, Shousuke Ito, Toshiharu Yamashita, Kowichi Jimbow, Hiroyuki Honda : 4-S-Cysteaminylphenol-loaded magnetite cationic liposomes for combination therapy of hyperthermia with chemotherapy against malignant melanoma, Cancer Science, 98(3),424-430(2007)

Akira Ito, Hiroyuki Honda,Takeshi Kobayashi : Cancer immunotherapy based on intracellular hyperthermia using magnetite nanoparticles: a novelconcept of"heat-controlled necrosis" with heat shock protein expression, Cancer Immunol Immunother, 55(3),320-38(2007)

Kazunori Shimizu, Akira Ito, Jong-kook Lee, Tatsuro Yoshida, Keiko Miwa, Hisaaki Ishiguro, Yasushi Numaguchi, Toyoaki Murohara, Itsuo Kodama, Hiroyuki Honda : Construction of multilayered cardiomyocyte sheets using magnetite nanoparticles and magnetic force, Biotechnology and Bioengineering, 96(4),803-809(2007)

kousuke Ino, Akira Ito, Hirohito Kumazawa, Hideaki Kagami, Minoru Ueda, Hiroyuki Honda : Incorporating of capillary-lake structures into dermal cell sheets constructed by magnetic force-bases tissue engineering, Journal of Chemical Engineering Japan, 40(1), 51-58(2007)

C. Kaga, M. Okochi, M. Nakanishi, H. Hayashi, R. Kato, H. Honda: Screening of a novel octamer peptide, CNSCWSKD, that induces caspase-dependent cell death, Biochemical and Biophysical Research Communications (2007) 362(4),:1063-1068.

加藤竜司、山本若菜、各務秀明、上田実、本多裕之:「再生医療における細胞品質管理を目指した細胞画像データへの多変量解析の有効性」, ソフトウェアバイオロジー, 生物工学会出版音 (2007) in press

(木全弘治)

Sugaya N, Habuchi H, Nagai N, Ashikari-Hada S, Kimata K. 6-*O*-sulfation of heparan sulfate differentially regulates various FGFs-dependent signaling in culture. J Biol Chem. 2008: **283**: in press

Morita H, Yoshimura A, Kimata K. The role of heparan suflate in the glomerular

basement membrane. Kidney International 2008: 73: 247-248

Kobayashi T, Habuchi H, Tamura K, Ide H, Kimata K. Essential role of heparan sulfate 2-O-sulfotransferase in chick limb bud patterning and development. J Biol Chem. 2007: 282: 19589-19597

Habuchi H, Nagai N, Sugaya N, Atsumi F, Stevens RL, Kimata K. Mice deficient in heparan sulfate 6-O-sulfotransferase-1 exhibit defective heparan sulfate biosynthesis, abnormal placentation and late embryonic lethality. J Biol Chem. 2007: 282: 15578-15588

Kurup S, Wijnhoven TJM, Jenniskens GJ, Kimata K, Habuchi H, Li J-P, Lindahl U, van Kuppervelt TH, Spillmann D. Characterization of anti-heparan sulfate phage-display antibodies A04B08 and HS4E4. J Biol Chem. 2007: 282: 21032- 21042

Yamaguchi T, Ohtake S, Kimata K, Habuchi O. Molecular cloning of squid N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase and synthesis of a unique chondroitin sulfate containing E-D hybrid tetrasacchari de structure by the recombinant enzyme. Glycobiology 2007: 17: 1365-1376

Nagai N, Habuchi H, Kitazume S, Toyoda H, Hashimoto Y, Kimata K. Regulation of heparan sulfate 6-O-sulfation by beta -secretase activity. J Biol Chem. 2007: 282: 14942-14951

神村圭介、中藤博志、木全弘治. ヘパラン硫酸の微細構造に秘められた機能- 1 : 線虫 ショウジョウバエを用いた解析 実験医学 羊土社 2007: 27 No. 7: 1049-1053

小林孝、羽渕弘子、木全弘治. ヘパラン硫酸の微細構造に秘められた機能-2 : 脊椎動物におけるヘパラン硫酸O-硫酸転移酵素による形態形成制御 実験医学 羊土社 2007 : 27 No. 7 : 1054-1059

羽渕弘子, 羽渕脩躬, 木全弘治. ヘパラン硫酸プロテオグリカンと形態形成 メディカルレビュー社 2007 : 5 No. 1: 75-79

幡野その子、渡辺秀人、木全弘治. プロテオグリカンの生物学 ティッシュエンジニアリング 2007 日本医学館 2007: 82-87

単行本

Kimata K, Habuchi O, Habuchi H, Watanabe H. Knockout mice and proteoglycans in *COMPRIHENSIVE GLYCOSCIENCE.* Elsevier, (2007) Vol. 3, Chapter 4.10, 159-191

J. Esko, U. Lindahl, K. Kimata: Proteoglycans and sulfated glycosaminoglycans. Essentials of Glycobiology, 2nd edition. Edited by A. Varki, C. Bertozzi, R. Cummings, Etzler M, Esko J, Freeze H, Hart G, Stanle P, Cold Spring Harbor Laboratory (2007)

Kusafuka K, Watanabe H, Kimata K, Hiraki Y, Shukunami C, Kameya T.
Minute pleomorphic adenoma of the submandibular gland in patients with oral malignancy: a report of two cases with histological and immunohistochemical examination. Histopathology 2007: 51: 1258-1261.

Minamisawa T, Suzuki K, Maeda H, Shimokata S, Sugiura N, Kimata K. Hirabayashi
Characterization of isomeric unsulfated glycosaminoglycan oligosaccharides by mass spectrometry/ mass spectrometry. J Mass Spectrom Soc Jpn. 2007 Jan: 55(1): 1-6.

Koyama H, Hibi T, Isogai Z, Yoneda M, Fujimori M, Amano J, Kawakubo M, Kannagi R, Kimata K, Taniguchi S, Itano N. Hyperproduction of hyaluronan in neu-induced mammary tumor accelerates angiogenesis through stromal cell recrutitment:possible involvement of versican/PG-M. Am J Pathol. 2007 Mar: 170: 1086-99.

Sakai K, Kimata K, Sato T, Gotoh M, Narimatsu H, Shinomiya K, Watanabe H. Chondroitin sulfate N-acetylgalactosaminyltransferase-1 play a critical role in chondoroitin sulfate synthesis in cartilage. J Biol Chem. 2007: 282: 4152-4161

RESEARCH REPORTS
Biomaterials & Bioengineering

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Effective Bone Engineering with Periosteum-derived Cells

ABSTRACT

Bone augmentation *via* tissue engineering has generated significant interest. We hypothesized that periosteum-derived cells could be used in place of bone marrow stromal cells (which are widely used) in bone engineering, but the differences in osteogenic potential between these 2 cell types are unclear. Here, we compared the osteogenic potential of these cells, and investigated the optimal osteoinductive conditions for periosteum-derived cells. Both cell types were induced, *via* bFGF and BMP-2, to differentiate into osteoblasts. Periosteal cells proliferated faster than marrow stromal cells, and osteogenic markers indicated that bone marrow stromal cells were more osteogenic than periosteal cells. However, pre-treatment with bFGF made periosteal cells more sensitive to BMP-2 and more osteogenic. Transplants of periosteal cells treated with BMP-2 after pre-treatment with bFGF formed more new bone than did marrow stromal cells. Analysis of these data suggests that combined treatment with bFGF and BMP-2 can make periosteum a highly useful source of bone regeneration.

KEY WORDS: marrow stromal cells, periosteal cells, osteogenic potential, bFGF, BMP-2.

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INTRODUCTION

Bone defects that do not heal spontaneously need bone reconstruction for the recovery of bone function. While autografting is the gold standard in bone reconstructive surgery, autografts involve donor site morbidity. Artificial bone substitutes are also utilized; however, results are inconsistent, because these materials lack osteogenic potential. Recently, tissue engineering has attracted considerable attention (Young *et al.*, 2005). Because it requires only a small amount of tissue from the patient, bone reconstruction by this technique is less invasive and is safer than conventional methods. Bone marrow stromal cells have multi-lineage differentiation potential and can therefore differentiate into cells with an osteogenic phenotype. Accordingly, they have been frequently used for bone reconstruction (Luria *et al.*, 1987; Haynesworth *et al.*, 1992; Matsubara *et al.*, 2005). Periosteum-derived cells have also been used recently (Breitbart *et al.*, 1998; Perka *et al.*, 2000). There is a growing requirement for dentists to regenerate alveolar bone as a regenerative therapy for periodontitis and in implant dentistry. Concerning the donor site, it is easier for general dentists to harvest periosteum than marrow stromal cells, because they can access the mandibular periosteum during routine oral surgery. However, the differences in osteogenic potential between marrow stromal cells and periosteal cells remain unclear. In the present study, we compared the osteogenic potential of periosteum-derived cells and marrow stromal cells, after treatment with basic fibroblast growth factor (bFGF) and bone morphogenetic protein-2 (BMP-2), both of which have significant effects on osteogenesis (Rosen and Thies, 1992; Marie, 2003). Recently, Fakhry *et al.* (2005) showed that the combined use of bFGF and BMP-2 greatly enhances the osteogenic potential of chick embryonic calvaria-derived cells. Therefore, using BMP-2 and bFGF under the conditions described by Fakhry, with modifications, we attempted to determine the optimal osteo-inductive conditions for human mandibular periosteum-derived cells.

MATERIALS & METHODS

Cell Cultures

The study conformed to the tenets of the Declaration of Helsinki, and the protocol was approved by the Ethical Committees of both Kitasato University and the Institute of Medical Science, The University of Tokyo. All subjects provided written informed consent.

Human periosteal tissue (1 cm^2) was obtained from the mandibular angle of six patients (ages [yrs] 18, 19, 20, 21, 21, 24; gender, one male and five females) during the course of oral surgery. The excised sections of tissue were plated into 10-cm dishes (TPP, Zollstrasse, Trasadingen, Switzerland) containing α-MEM (Kohjin Bio, Saitama, Japan), supplemented with 10% fetal bovine serum (JRH Bioscience, Lenexa, KS, USA) and 1% penicillin-streptomycin-glutamine (serum-conditioned α-MEM, Invitrogen, Carlsbad, CA, USA), and were

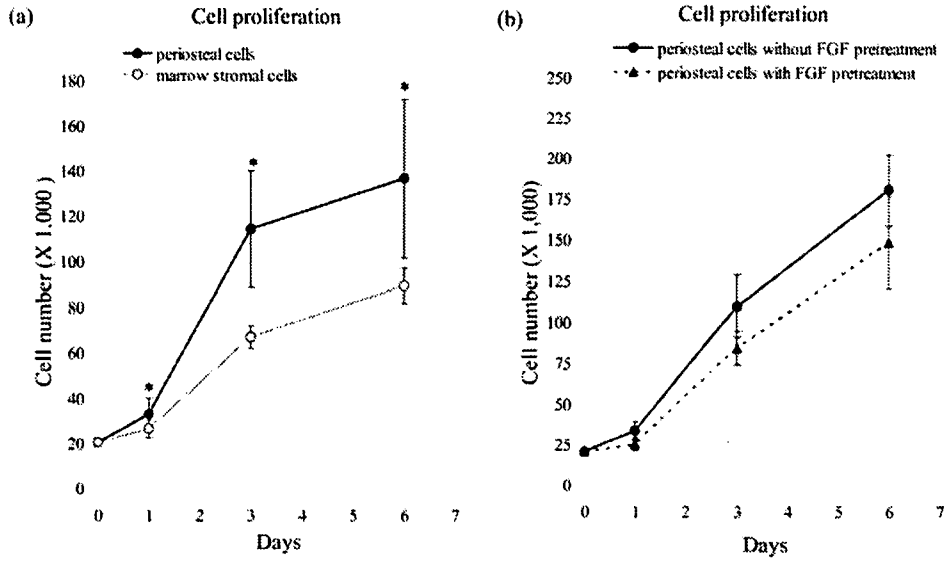

Figure 1. Periosteal cells (closed circles) and marrow stromal cells (open circles) were plated in 12-well plates at a density of 2.0 x 10^4 cells/well. Cell numbers were counted directly (**a**). FGF-pre-treated periosteal cells are indicated by closed triangles, and non-pre-treated periosteal cells are indicated by closed circles (**b**). Values are means ± standard deviation for 3 cultures. Asterisk indicates significant difference in the number of periosteal cells, compared with marrow stromal cells on the same day; $p < 0.05$.

cultured at 37°C in 5% CO_2. The medium was replaced every 2 days. When the cultures reached 90% confluence, cells were passaged and re-plated in 150-cm^2 flasks (Corning, Big Flats, NY, USA).

Bone marrow stromal cells were obtained by iliac aspiration from three male volunteers (ages [yrs] 29, 33, 48), and were seeded in flasks and maintained in serum-conditioned α-MEM. The following day, floating cells were removed, and the medium was replaced with fresh medium. Passages were performed when cells reached 90% confluence. To ensure phenotypic uniformity, we used each cell lineage at the same passage (from 2 to 6) in the subsequent experiments.

Pre-treatment with Basic FGF

Cells were pre-treated with bFGF under conditions described elsewhere (Fakhry *et al.*, 2005), with modifications. Both cell types were cultured in serum-conditioned α-MEM in dishes to 60% confluence. At that point, the serum-conditioned α-MEM was replaced with medium containing 1 ng/mL recombinant human bFGF (donated by Professor Y. Tabata, Kyoto University, Kyoto, Japan) and 100 μM ascorbic acid (Wako, Osaka, Japan), and the cells were cultured for 2 more days. Then, the cells were detached with trypsin-EDTA and re-plated (FGF pre-treatment).

Cell Proliferation Assay

On day 0, each cell type was plated at a density of 2.0 x 10^4 cells/mL/well in 12-well plates (Greinerbio-one, Kremsmuenster, Austria) containing serum-conditioned α-MEM. Cell numbers were counted directly in triplicate, and the medium was replaced with fresh serum-conditioned α-MEM on days 1 and 3.

Alkaline Phosphatase Activity Assay

On day 0, both cell types (with and without bFGF pre-treatment) were plated at a density of 1.0 x 10^5 cells/mL/well in 12-well plates containing serum-conditioned α-MEM. On day 1, either 1, 5, or 10 ng/mL bFGF or 30, 100, or 300 ng/mL recombinant human BMP-2 (donated by Astellas Pharma Inc., Tokyo, Japan) was added to each well, along with 100 μM ascorbic acid. On day 4, the medium was replaced with fresh medium containing identical growth factors. On day 7, cells were harvested and extracted with 20 mM HEPES (Dojindo, Kumamoto, Japan) buffer (pH 7.5) containing 1% Triton X-100 (Wako). Alkaline-phosphatase-specific (ALP) activity was assayed as described previously (Asahina *et al.*, 1993). ALP activity is expressed as μmol p-nitrophenol/min/μg protein.

Reverse-transcription/Polymerase Chain-reaction

For RNA preparation, on day 0, both cell types (with and without FGF pre-treatment) were plated in 10-cm dishes containing serum-conditioned α-MEM. Cells were treated with or without 100 ng/mL BMP-2, 100 μM ascorbic acid for the following 6 days. Media were completely replaced on days 1 and 4. On day 7, total RNA was extracted with the use of TRIZOL reagent (Invitrogen). RNA samples (1 μg) were reverse-transcribed with Superscript III® reverse-transcriptase and oligo-dT primers (Invitrogen), according to the manufacturer's protocol. For PCR amplification, we used primer sequences that have been described previously (Wordinger *et al.*, 2002; Kamata *et al.*, 2004). We analyzed gene expression of glyceraldehyde-3'-phosphate dehydrogenase (GAPDH), type I collagen (Col I), ALP, osteopontin (OP), osteocalcin (OC), BMP-2, BMP-4, and BMP receptors (BMPr) IA, IB, and II. After amplification, samples were analyzed by electrophoresis on a 1.5% agarose gel, and were visualized by ethidium bromide staining.

Transplantation of Cells into Immunodeficient Mice

For each transplantation, 1 x 10^6 harvested cells (with or without pre-treatment), in 1 mL of serum-conditioned α-MEM, were mixed with 50 mg of β-tricalcium phosphate (β-TCP) granules (Osferion®; Olympus, Tokyo, Japan) in a 14-mL polypropylene tube (Becton Dickinson, Franklin Lakes, NJ, USA). For the ensuing 6 days, cells were cultured in media with 100 ng/mL BMP-2 and 100 μM ascorbic acid, or without these additives as a control. The medium was replaced with fresh identical medium on day 4. After culture, each cell mixture was transplanted into a 6-week-old female BALB/cAJcl-nu/nu mouse (Nihoncrea, Tokyo, Japan). Five subcutaneous pockets were created in the back of each mouse, under anesthesia with diethyl ether, and the cell mixture was transplanted. As a negative control, β-TCP alone was prepared and implanted into the mice. The transplants were harvested after 4 wks. NIH guidelines for the care and use of laboratory animals were observed in all procedures.

Histomorphometric Analysis of the Transplants

The harvested samples were fixed in 4% formaldehyde, decalcified, and embedded in paraffin wax. Then, 5-μm-thick sections were prepared from the middle of each transplant and

stained with hematoxylin and eosin. The volume of newly formed bone was analyzed with Scion Image software (NIH, Bethesda, MD, USA), as described previously (Alsberg *et al.*, 2001); the bone area is expressed as the percentage of total area (27.2 mm^2).

Statistical Analysis

Results are expressed as mean values ± standard deviation. Statistical analysis of differences between groups was performed with Student's *t* test.

RESULTS

Cell Proliferation

Proliferation of periosteal cells was significantly greater than that of bone marrow stromal cells (Fig. 1a). Although the number of periosteal cells was similar to that of marrow stromal cells on day 1, periosteal cells were twice as numerous as marrow stromal cells on day 3. Periosteal cells proliferated much faster than did marrow stromal cells, and had reached 100% confluence by day 6. Up to and including passage 6, all periosteal cells (regardless of donor) proliferated faster than the fastest-proliferating marrow stromal cells; in later passages, both cell types proliferated slightly slower (data not shown). FGF-pre-treated periosteal cells proliferated slightly slower than non-pre-treated periosteal cells (Fig. 1b), but they still proliferated faster than marrow stromal cells.

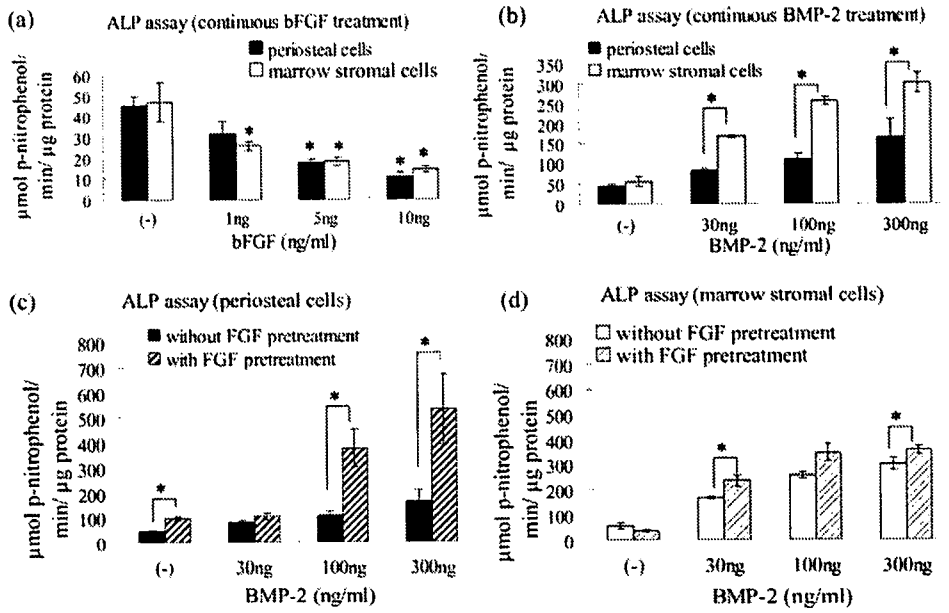

Figure 2. Periosteal cells (black) and marrow stromal cells (white) were plated in 12-well plates at a density of 1.0 x 10^5 cells/well. Then, the cells were incubated with bFGF (**a**) or BMP-2 (**b**) for 6 days. Alkaline phosphatase activity was analyzed on day 7 of culture. In (a), asterisk indicates significant difference ($p < 0.05$), compared with the control (-). FGF-pre-treated periosteal cells are indicated by black hatched bars; non-pre-treated periosteal cells are indicated by black bars; FGF-pre-treated marrow stromal cells are indicated by white hatched bars; non-pre-treated marrow stromal cells are indicated by white bars (**c,d**). Values are means ± standard deviation for 3 cultures. In b-d, asterisk indicates significant difference ($p < 0.05$) between paired conditions.

Alkaline Phosphatase Activity

Recombinant human bFGF had an inhibitory effect on ALP activity in both cell types, in a dose-dependent manner. Continuous exposure to bFGF (6 days) did not induce osteogenic differentiation in either cell type (Fig. 2a). Treatment with BMP-2 significantly enhanced the ALP activity of marrow stromal cells, but did not enhance the ALP activity of periosteal cells (Fig. 2b). FGF pre-treatment enhanced the responsiveness of periosteal cells to BMP-2; at doses of 100 and 300 ng/mL BMP-2, the ALP activity of FGF-pre-treated cells was about 3 times higher than that of the non-pre-treated cells (Fig. 2c). This effect of FGF pre-treatment on the BMP-2 induced increase in ALP activity was weaker for bone marrow cells than for periosteal cells (Fig. 2d).

Reverse-transcription/Polymerase Chain-reaction Assay

Gene expression of periosteal cells and marrow stromal cells was examined by RT-PCR (Fig. 3). Control periosteal cells expressed only Col I and GAPDH. Treating periosteal cells with BMP-2 enhanced their expression of ALP, OP, BMP2, BMP4, BMPrIA, BMPrIB, and BMPrII. FGF pre-treatment of periosteal cells enhanced their expression of ALP, OP, BMP2, BMP4, BMPrIA, and BMPrIB. Combined FGF pre-treatment and BMP-2 treatment of periosteal cells enhanced their expression of ALP, BMP2, BMP4, BMPrIA, BMPrIB, and BMPrII.

Control marrow stromal cells expressed Col I, GAPDH,

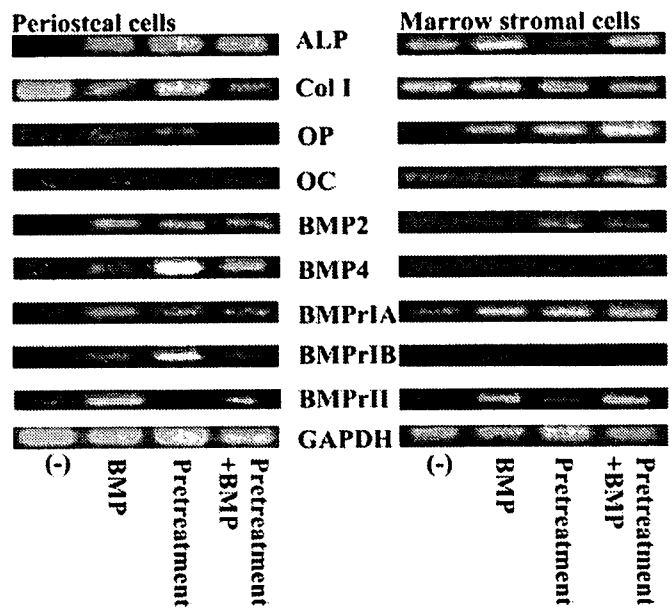

Figure 3. RT-PCR analysis of periosteal cells and marrow stromal cells. For 6 days, each cell type was cultured in serum-conditioned α-MEM alone (-) or in serum-conditioned α-MEM containing BMP-2 (BMP). To evaluate the effect of FGF pre-treatment, we pre-treated some cells with bFGF for 2 days (Pre-treatment), and treated some cultures with BMP after bFGF pre-treatment (Pre-treatment + BMP).

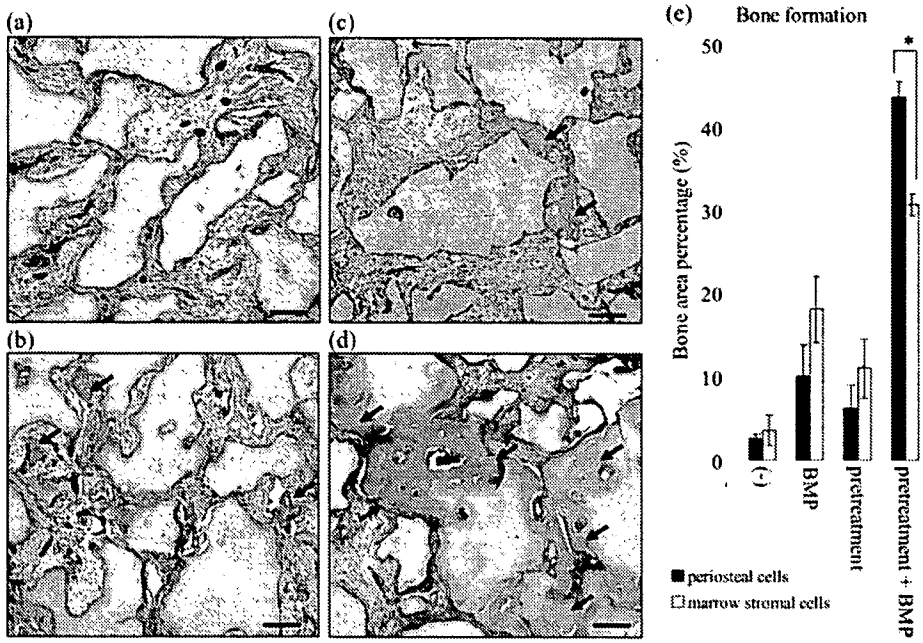

Figure 4. Hematoxylin and eosin staining demonstrating *in vivo* bone formation. Transplants of untreated control periosteal cells (-) formed little bone (**a**). Transplants of periosteal cells that received BMP-2 treatment alone (BMP) (**b**) or received FGF pre-treatment alone (pre-treatment) (**c**) also formed new bone. Periosteal cells that were treated with BMP-2 after FGF pre-treatment (pre-treatment + BMP) formed significant amounts of new bone (**d**). Bar indicates 300 μm. Arrow indicates new bone. The difference in new bone volume between periosteal cells (black) and marrow stromal cells (white) was determined by histomorphometric analysis (**e**). Asterisk indicates significant difference ($p < 0.05$) between paired conditions. Values are means ± standard deviation for 3 sections of each sample.

ALP, OC, BMPrIA, and BMPrII. Treating marrow stromal cells with BMP-2 enhanced their expression of ALP, OP, BMPrIA, BMPrIB, and BMPrII. FGF pre-treatment of marrow stromal cells enhanced their expression of OP, OC, BMP2, BMPrIA, and BMPrII, but it inhibited their expression of ALP. Combined FGF pre-treatment and BMP-2 treatment of marrow stromal cells enhanced their expression of ALP, OP, OC, BMP2, BMPrIA, and BMPrII.

Transplantation of Periosteal Cells

Control periosteal cells had formed little new bone at 4 wks after transplantation (Fig. 4a). Implantation of β-TCP alone did not cause formation of any new bone (data not shown). Periosteal cells treated with BMP-2 alone or FGF pre-treatment alone also formed new bone (Figs. 4b, 4c); however, the amount of newly formed bone was less than that of periosteal cells treated with BMP-2 after FGF pre-treatment. Periosteal cells treated with BMP-2 after FGF pre-treatment had formed significant ectopic new bone at 4 wks after transplantation (Fig. 4d).

Histomorphometric analysis showed that marrow stromal cells formed slightly more new bone than did periosteal cells under control conditions (untreated), after treatment with BMP-2 alone, and after FGF pre-treatment alone; however, these differences were not statistically significant. Periosteal cells treated with BMP-2 after FGF pre-treatment generated significantly more newly formed bone than did marrow stromal cells treated with BMP-2 after FGF pre-treatment. Periosteal cells treated with BMP-2 after FGF pre-treatment formed 3 times as much new bone as did periosteal cells treated with BMP-2 without FGF pre-treatment (Fig. 4f).

DISCUSSION

In this study, we compared the osteogenic potential and clinical usefulness of periosteal cells with those of marrow stromal cells.

The proliferation rate of periosteal cells was much greater than that of marrow stromal cells (Fig. 1a). Reports indicate that primary cultures of human bone cells obtained from different donors can have very different proliferation rates (Im *et al.*, 2004). We observed differences in proliferation rate among the present donors, but all of the present periosteal cells proliferated faster than did the present marrow stromal cells. Also in the present study, both cell types retained high expansion potential at later passages (Sakaguchi *et al.*, 2005). Thus, the use of periosteal cells may shorten the cell culture period, thereby reducing both cost and the risk of contamination.

In the present study, we used periosteal cells obtained from young adults. Aging reportedly affects the mitogenic activity of osteoprogenitor cells (Tanaka *et al.*, 1999). There is a need for studies comparing the bone-forming ability of periosteal cells between old and young donors.

Many *in vitro* studies have indicated that bFGF has a mitogenic effect on osteoprogenitor cells (Tanaka *et al.*, 1999; Shimoaka *et al.*, 2002). In contrast, the present transient bFGF treatment inhibited the proliferation of periosteal cells, although the effect was not statistically significant (Fig. 1b). The discrepancy between these studies may be due to the method of FGF treatment. In previous studies, the bFGF treatment was of longer duration than in the present study. Cells committed to the osteoblast lineage have been found to have lower proliferative potential than uncommitted cells (Malaval *et al.*, 1999). Although the present continuous treatment with bFGF inhibited ALP activity (Fig. 2a), consistent with results from a previous study (Kalajzic *et al.*, 2003), cessation of bFGF treatment may induce osteoblastic differentiation of periosteal cells. This hypothesis is consistent with the present data regarding osteoblastic gene expression of cells pre-treated with FGF. FGF pre-treatment increased the expression of molecules considered early-stage markers of osteogenic differentiation, such as ALP (Fig. 3) (Ryoo *et al.*, 2006).

BMP-2 and bFGF have been found to induce osteogenic differentiation (Canalis *et al.*, 1988; Yamaguchi *et al.*, 1991), but, in the present study, the response to BMP-2 was greater for marrow stromal cells than for periosteal cells. Control periosteal cells showed less ALP activity and expressed fewer osteogenic markers than control marrow stromal cells. This

suggests that marrow stromal cells are further differentiated along the osteoblastic cell lineage than are periosteal cells. Previous studies indicated that transient treatment with FGF, followed by treatment with BMP-2, enhanced osteogenic differentiation *in vitro* (Hanada *et al.*, 1997; Fakhry *et al.*, 2005). Similarly, in the present study, FGF pre-treatment enhanced responsiveness to BMP-2, and this enhancement was much stronger in periosteal cells than in marrow stromal cells. This suggests that the enhancement of osteogenic potential after FGF pre-treatment was due to an increase in the number of cells in the pre-osteoblast or osteoblast lineage.

Consistent with previous *in vitro* studies, the present combination of FGF pre-treatment and BMP-2 treatment enhanced the bone-forming potential of periosteal cells, which formed a greater volume of new bone than did marrow stromal cells *in vivo*. Thus, periosteal cells cultured under conditions that promote osteogenesis may be as useful for bone tissue engineering as are marrow stromal cells, and offer the advantage of greater proliferation.

ACKNOWLEDGMENTS

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REFERENCES

Alsberg E, Anderson KW, Albeiruti A, Franceshi RT, Mooney DJ (2001). Cell-interactive alginate hydrogels for bone tissue engineering. *J Dent Res* 80:2025-2029.

Asahina I, Sampath TK, Nishimura I, Hauschka PV (1993). Human osteogenic protein-1 induces both chondroblastic and osteoblastic differentiation of osteoprogenitor cells derived from newborn rat calvaria. *J Cell Biol* 123:921-933.

Breitbart AS, Grande DA, Kessler R, Ryaby JT, Fitzsimmons RJ, Grant RT (1998). Tissue engineered bone repair of calvarial defects using cultured periosteal cells. *Plast Reconstr Surg* 101:567-574.

Canalis E, Centrella M, McCarthy T (1988). Effects of basic fibroblast growth factor on bone formation *in vitro*. *J Clin Invest* 81:1572-1577.

Fakhry A, Ratisoontorn C, Vedhachalam C, Salhab I, Koyama E, Leboy P, *et al.* (2005). Effects of FGF-2/-9 in calvarial bone cell cultures: differentiation stage-dependent mitogenic effect, inverse regulation of BMP-2 and noggin, and enhancement of osteogenic potential. *Bone* 36:254-266.

Hanada K, Dennis JE, Caplan AI (1997). Stimulatory effects of basic fibroblast growth factor and bone morphogenetic protein-2 on osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells. *J Bone Miner Res* 12:1606-1614.

Haynesworth SE, Goshima J, Goldberg VM, Caplan AI (1992). Characterization of cells with osteogenic potential from human marrow. *Bone* 13:81-88.

Im GI, Qureshi SA, Kenny J, Rubash HE, Shanbhag AS (2004). Osteoblast proliferation and maturation by bisphosphonates. *Biomaterials* 25:4105-4115.

Kalajzic I, Kalajzic Z, Hurley MM, Lichtler AC, Rowe DW (2003). Stage specific inhibition of osteoblast lineage differentiation by FGF2 and noggin. *J Cell Biochem* 88:1168-1176.

Kamata N, Fujimoto R, Tomonari M, Taki M, Nagayama M, Yasumoto S (2004). Immortalization of human dental papilla, dental pulp, periodontal ligament cells and gingival fibroblasts by telomerase reverse transcriptase. *J Oral Pathol Med* 33:417-423.

Luria EA, Owen ME, Friedenstein AJ, Morris JF, Kuznetsow SA (1987). Bone formation in organ cultures of bone marrow. *Cell Tissue Res* 248:449-454.

Malaval L, Liu F, Roche P, Aubin JE (1999). Kinetics of osteoprogenitor proliferation and osteoblast differentiation in vitro. *J Cell Biochem* 74:616-627.

Marie PJ (2003). Fibroblast growth factor signaling controlling osteoblast differentiation. *Gene* 316:23-32.

Matsubara T, Suardita K, Ishii M, Sugiyama M, Igarashi A, Oda R, *et al.* (2005). Alveolar bone marrow as a cell source for regenerative medicine: differences between alveolar and iliac bone marrow stromal cells. *J Bone Miner Res* 20:399-409.

Perka C, Schultz O, Spitzer RS, Lindenhayn K, Burmester GR, Sittinger M (2000). Segmental bone repair by tissue-engineered periosteal cell transplants with bioresorbable fleece and fibrin scaffolds in rabbits. *Biomaterials* 21:1145-1153.

Rosen V, Thies RS (1992). The BMP proteins in bone formation and repair. *Trends Genet* 8:97-102.

Ryoo HM, Lee MH, Kim YJ (2006). Critical molecular switches involved in BMP-2-induced osteogenic differentiation of mesenchymal cells. *Gene* 366:51-57.

Sakaguchi Y, Sekiya I, Yagishita K, Muneta T (2005). Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. *Arthritis Rheum* 52:2521-2529.

Shimoaka T, Ogasawara T, Yonamine A, Chikazu D, Kawano H, Nakamura K, *et al.* (2002). Regulation of osteoblast, chondrocyte, and osteoclast functions by fibroblast growth factor (FGF)-18 in comparison with FGF-2 and FGF-10. *J Biol Chem* 277:7493-7500.

Tanaka H, Ogasa H, Barnes J, Liang CT (1999). Actions of bFGF on mitogenic activity and lineage expression in rat osteoprogenitor cells: effect of age. *Mol Cell Endocrinol* 150:1-10.

Wordinger RJ, Agarwal R, Talati M, Fuller J, Lambert W, Clark AF (2002). Expression of bone morphogenetic proteins (BMP), BMP receptors, and BMP associated proteins in human trabecular meshwork and optic nerve head cells and tissues. *Mol Vis* 8:241-250.

Yamaguchi A, Katagiri T, Ikeda T, Wozney JM, Rosen V, Wang EA, *et al.* (1991). Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation in vitro. *J Cell Biol* 113:681-687.

Young CS, Abukawa H, Asrican R, Ravens M, Troulis MJ, Kaban LB, *et al.* (2005). Tissue-engineered hybrid tooth and bone. *Tissue Eng* 11:1599-1610.

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Bone regeneration of dental implant dehiscence defects using a cultured periosteum membrane

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Key words: culture, dehiscence defect, GBR, membrane, periosteum, titanium implant

Abstract

Objectives: This study aimed to demonstrate the feasibility of a cultured periosteum (CP) membrane for use in guided bone regeneration at sites of implant dehiscence.

Material and methods: Four healthy beagle dogs were used in this study. Implant dehiscence defects (4 × 4 × 3 mm) were surgically created at mandibular premolar sites where premolars had been extracted 3 months back. Dental implants (3.75 mm in diameter and 7 mm in length) with machined surfaces were placed into the defect sites (14 implants in total). Each dehiscence defective implant was randomly assigned to one of the following two groups: (1) PRP gel without cells (control) or (2) a periosteum membrane cultured on PRP gel (experimental). Dogs were killed 12 weeks after operation and nondecalcified histological sections were made for histomorphometric analyses including percent linear bone fill (LF) and bone-to-implant contact (BIC).

Results: Bone regeneration in the treatment group with a CP membrane was significantly greater than that in the control group and was confirmed by LF analysis. LF values in the experimental and the control groups were 72.36 ± 3.14% and 37.03 ± 4.63%, respectively ($P<0.05$). The BIC values in both groups were not significantly different from each other. The BIC values in the experimental and the control groups were 40.76 ± 10.30% and 30.58 ± 9.69%, respectively ($P=0.25$) and were similar to native bone.

Conclusion: This study demonstrated the feasibility of a CP membrane to regenerate bone at implant dehiscence defect.

In dental implant surgery, buccal dehiscence defects are common when alveolar bone volume is inadequate (Ofer et al. 2005). Buccal bone is relatively thin and bone resorption after tooth extraction in buccal areas is faster and more prevalent compared with lingual bone (Nevins et al. 2006).

Guided bone regeneration (GBR) is an established surgical technique for correcting buccal dehiscence defects (Tae-Ju et al. 2003). Various barrier membranes have been used in GBR procedures and can be classified into two types: absorbable and nonabsorbable.

Expanded polytetrafluoroethylene (e-PTFE) is a typical nonabsorbable membrane, which has been one of the most-used materials for GBR. Although an e-PTFE membrane is a proven material for GBR, additional surgery for membrane removal is often required and frequent complications, such as membrane exposure and infection, have been reported (Blumenthal 1993).

Collagen membrane, derived from porcine and bovine skin, is a commonly used absorbable membrane (Parodi et al. 1998). Although collagen membrane is considered to be biocompatible, it is not possible to

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completely avoid contamination with pathogens such as animal viruses or prions (Cassinelli et al. 2006). Polyglycolic acid, polylactic acid or copolymers of the two are examples of biosynthetic absorbent membranes. An advantage of these biosynthetic absorbable membranes is that they have minimal risk of infection; however, they metabolize into acidic compounds, which can be detrimental to bone regeneration (Linhart et al. 2001).

Each of these materials is currently used in clinical practice; however, the benefits are closely offset by potential hazardous effects. Furthermore, unlike osteogenic cell-populated membranes, acellular membranes do not have the ability to form new bone. Together, these factors have contributed to growing interest in studying regeneration techniques using autologous cells (Schmelzeisen et al. 2003).

The periosteum is comprised of two tissue layers: the outer fibroblast layer that provides attachment to soft tissue, and the inner cambial region that contains a pool of undifferentiated mesenchymal cells, which support bone formation (Squier et al. 1990). Recently, studies have reported the existence of osteogenic progenitors, similar to mesenchymal stem cells (MSCs), in the periosteum (Tenenbaum & Heersche 1985; Zohar et al. 1997). Under the appropriate culture conditions, periosteal cells secrete extracellular matrix and form a membranous structure (Mizuno et al. 2006). The periosteum can be easily harvested from the patient's own oral cavity, where the resulting donor site wound is invisible. Owing to the above reasons, the periosteum offers a rich cell source for bone tissue engineering.

Our group has previously demonstrated bone regeneration using a cultured periosteum (CP) membrane in a critical-sized rat calvaria bone defect (Mase et al. 2006). CP has also been shown to regenerate bone in a surgically created furcation bone defect using a canine model (Mizuno et al. 2006). Considering the biocompatibility of CP and its capacity for alveolar bone regeneration, it should be useful to investigate the potential of CP for bone regeneration around an implant site. The purpose of this study was to investigate the potential of CP to regenerate bone to mitigate implant dehiscence defects.

Material and methods

Animals

Four healthy female Beagle dogs designated as No. 1, 2, 3, and 4 (Oriental Kobo, Nagoya, Japan) with no periodontal disease, weighing approximately 10 kg, were used. All animal experiments were approved by the 'Animal Experiment Advisory Committee of Nagoya University School of Medicine' and performed in accordance with the 'Guidelines for Animal Experimentation of Nagoya University.'

Harvest of periosteum and periosteal cell culture

Under general and local anesthesia, the periosteum (approximately 10 × 10 mm) was aseptically harvested from the buccal side of the mandibular body in four dogs. The harvested periosteum was cut into 3 × 3 mm pieces. The tissues were placed directly on a six-well plate and cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Culture medium was composed of Medium 199 (Invitrogen-Gibco, Carlsbad, CA, USA) with 10% (v/v) fetal bovine serum (FBS) (SAFC Biosciences Inc., Lenexa, KS, USA), antibiotics (Invitrogen-Gibco) containing 150 U/ml penicillin G sodium, 150 mg/ml streptomycin, 375 μg/ml amphotericin B and 25 mg/l L-ascorbic acid (Sigma-Aldrich, Tokyo, Japan) (all final concentrations). The medium was changed every 2 days. Typically, 6 weeks of culture was sufficient to obtain appropriate CP thickness for grafting.

PRP gel preparation

PRP gels were made according to the manufacturer's instructions using the ReGen PRP-Kit 2® (kindly supplied by Regen Lab, Mollens, Switzerland). Briefly, 10 ml of venous peripheral blood was collected. Blood was first centrifuged at 1500 *g* for 8 min. All plasma was aspirated into another test tube, and then a second centrifugation at 1600 *g* for 10 min was performed. Then, the volume of plasma in excess was discarded with the 10 ml syringe to obtain the PRP volume required to cover the implant surface. PRP was polymerized with 10% $CaCl_2$. The consistency of polymerized PRP was relatively soft and used as a gel but not in a membranous form.

Surgical procedures

Before creating the defect and placing the implant, the second, third and fourth mandibular premolars (P_2–P_4) were extracted bilaterally under general anesthesia (i.m. injection of 4 mg/kg ketamine hydrochloride and 0.04 mg/kg atropine for induction with intravenous (i.v.) administration of 1 mg/kg sodium pentothal for maintenance). After 12 weeks of healing, buccal dehiscence defects were created under general and local anesthesia. During surgeries, the animals received lactated Ringer's solution i.v. After midcrestal and vertical split-thickness incisions of the alveolar mucosa, mucomembranous flap elevation was performed supraperiosteally, and the periosteum at the operation region was removed completely.

A uniform crestal bone defect was prepared using a fissure bur under saline irrigation. The dimensions of the dehiscence defects were 4 mm in height from the top of the crestal bone, 3 mm in depth from the surface of the buccal bone, and 4 mm in width mesiodistally (Fig. 1a). After creating the defect, a titanium threaded implant (Nobelbiocare AB™, Göteborg, Sweden machine surface) 3.75 mm in diameter and 7 mm in length were placed in each bone defect (Fig. 1b). The intervals between two adjacent dehiscence defects were set more than 7 mm to prevent possible effects from the treatment of neighboring implant. No. 1, 2, and 3 animals had received two implants in each mandibular quadrant bilaterally. On the other hand, No. 4 animal had received only two implants unilaterally because of incomplete healing after tooth extraction in the opposite mandibular quadrant. Two types of treatments (PRP gel placement only or PRP gel with an autologous CP graft) were randomly assigned to each bone defect. Groups with an autologous CP graft had a cultured periosteal membrane placed over the PRP gel directly, covering the defect and implant completely (Fig. 1c). Primary wound closure was performed with 4-0 Vicryl (Ethicon, Somerville, NJ, USA) using vertical mattress sutures. To prevent postsurgical infection, antibiotics were administered for 4 days and 0.2% chlorhexidine mouth irrigation was performed for 7 days. Throughout the study, the dogs were fed with a softened, high-calorie diet to prevent mechanical trauma and weight reduction.

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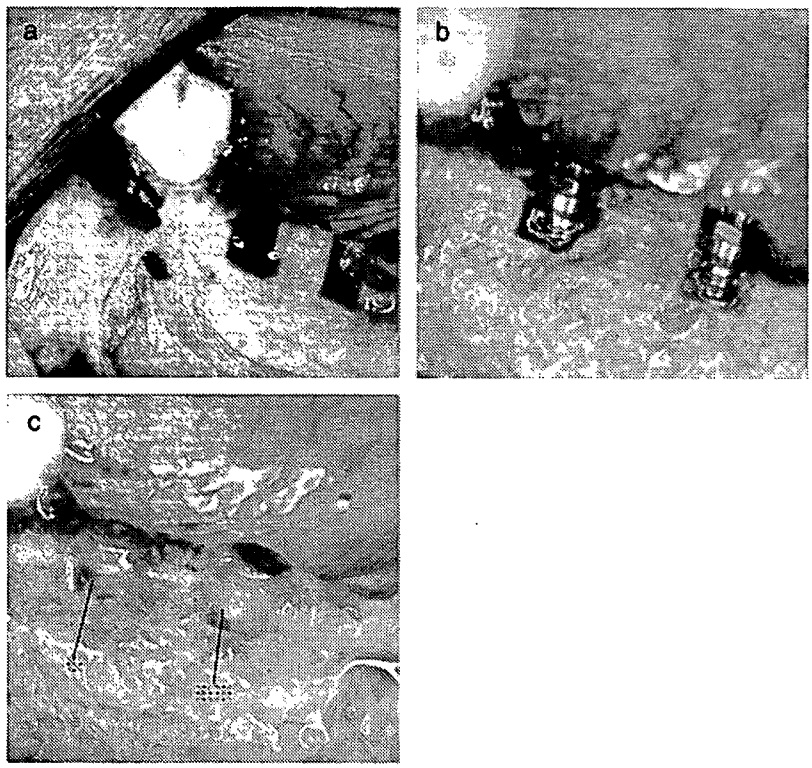

Fig. 1. Photographs demonstrating the surgical procedures used in this study. (a) Defects (4 × 4 × 3 mm) were surgically created at the mandibular premolar sites. (b) Implant placement at the created defects before cover screws were set. (c) Implant dehiscence defect was covered with a cultured periosteum (CP) membrane on PRP gel (CP group, ※※ right). Defect covered with PRP gel (control group, ※ left).

Histomorphometric analysis

Animals were killed with an overdose of pentobarbital (65 mg/kg, i.v.) 12 weeks after operation. The soft tissues around implants were completely removed, and then samples including dental implants were harvested using an electrical saw from the top of the mandibular crest to the bottom of the mandible and fixed with 70% ethanol. These specimens were dehydrated using ascending grades of alcohol, infiltrated and embedded in methyl methacrylate for nondecalcified sectioning. Upon polymerization, blocks were sawed, ground, and bucco-lingual sections of approximately 40 μm thickness were obtained using the Exact Cutting-Grinding System (Exact Apparatebau, Norderstedt, Germany). Sections were then stained with toluidine blue and microscopic images were analyzed (Osteoplanll, Carl Zeiss, Thornwood, NY, USA). The following histomorphometric parameters were evaluated:

(1) Percent linear bone fill (LF: new bone height divided by original defect length) was calculated according to the following formula:

$$LF(\%) = (A - A')/A \times 100$$

where A is the original defect length indicating the linear distance between the defect base and the top surface of the fixture (4 mm), and A' is the postoperative defect length indicating the linear distance between the postoperative defect base and the top surface of the fixture.

(2) Percent new bone–implant contact (BIC) was calculated according to the following formula:

$$BIC(\%) = C/B \times 100$$

where B is the total length of the thread before graft operation and C is the sum of new BIC.

Statistical analysis

For comparison between two treatment groups, a two-tailed Student's t-test was used. P-values <0.05 were considered to be statistically significant.

Results

At 12 weeks, membrane and implant exposure was observed in six of 14 implant sites (bilateral mandibular quadrant of No. 1 animal including two PRP and two CP sites and unilateral mandibular quadrant of No. 2 animal including one PRP and one CP sites). Because of the possible contamination risk, these six implant sites were excluded from histological and histomorphometrical analyses.

Macroscopic findings

At 12 weeks after operation, PRP and CP had completely disappeared in both the control and the CP group. Compared with the control group, the bone regeneration in the CP group was evident and the thread of the dental implants was almost covered with the regenerated bone, while most of the thread in the control group was exposed (Fig. 2a and b).

Histological observations

The CP group demonstrated relatively thick and dense lamellar bone formation from the bottom to the top of the created defect (Fig. 3a and c). Thick layers of woven bone were attached to the implant surface and osteoblast-like cells were observed around the surface. Although abundant neovasucularization was observed in the bone matrix, inflammatory cells were rarely observed. The border between the regenerated and original bone was not clear. In the control group, 12-week specimens exhibited thin cortical bone formation at the dehiscence defect (Fig. 3b and d). Scarce woven bone was observed between the implant surface and the cortical bone, and few osteocytes and osteoclasts were observed within the bone matrix and at the surface, respectively.

Histomorphometric analyses

LF was significantly higher in the CP group than in the control group (Fig. 4a). The mean LF values were 72.36 ± 3.14% and 37.03 ± 4.63% in the CP and control groups, respectively ($P<0.05$). In contrast, there was no significant difference in BIC (Fig. 4b). The mean BIC values were 40.76 ± 10.30% for the CP group and 30.58 ± 9.69% for the control group.

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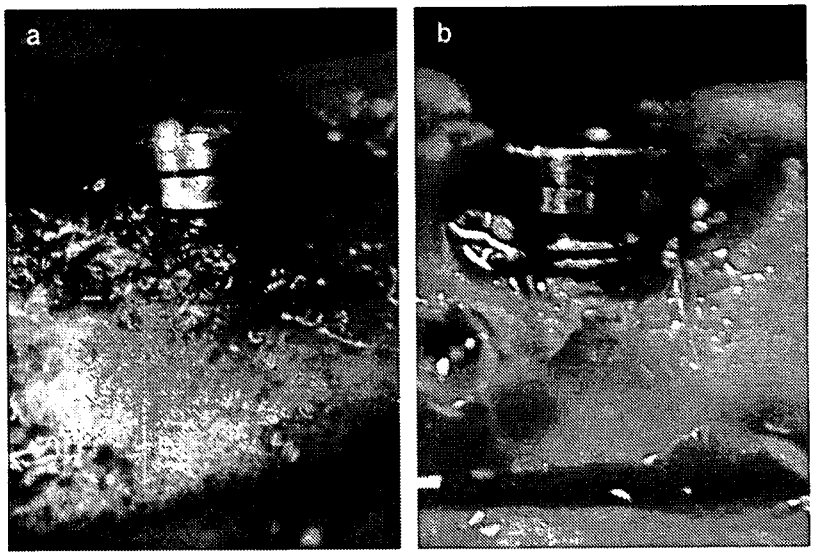

Fig. 2. Macroscopic observations at 12 weeks postoperation. The buccal dehiscence defect was almost completely covered with regenerated bone in the cultured periosteum group (a). Implant thread was partially covered with regenerated bone in the control group (b).

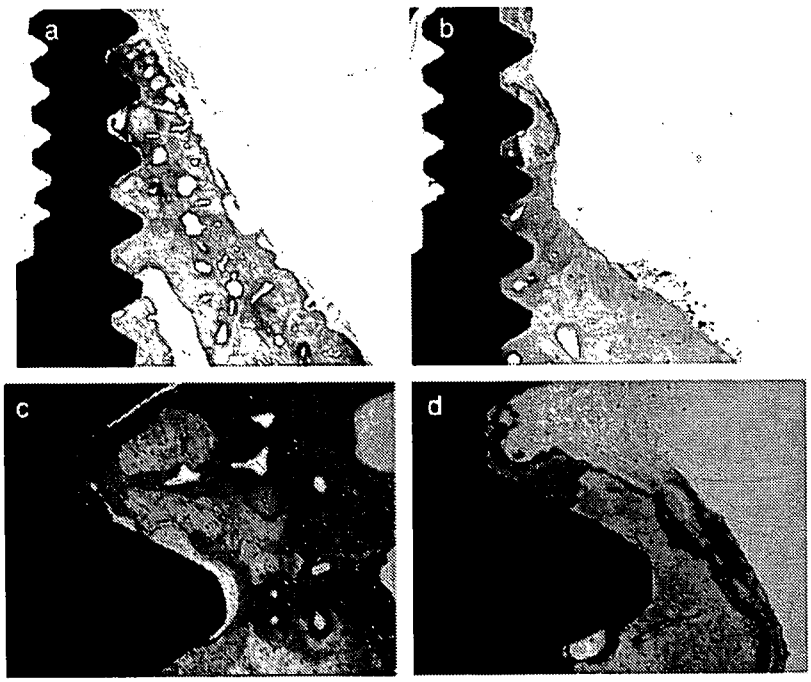

Fig. 3. Bright-field photomicrographs showing toluidine blue staining of the nondecalcified sections at 12 weeks postoperation. Thick, dense lamellar bone formation near both the bottom and the top of created defects was observed in the cultured periosteum group (a, c). Thin cortical bone formation along the implant thread was observed in the control group (b, d). Original magnification × 20 (a, b), × 100(c, d).

Discussion

In this study, we present evidence that CP can influence regeneration of alveolar bone at implant dehiscence defects. These findings confirm results from previous studies using a critical-sizeed bone defect of rat calvaria (Mase et al. 2006) and furcation defects in canine premolars (Mizuno et al. 2006).

The amount of bone at the implant dehiscence defect was significantly greater in the CP group than the control group, suggesting that new bone formed via the CP graft. Histological analysis revealed that the regenerated bone was integrated to the native mature bone. The role of the transplanted cells was not directly evaluated in this study; however, MSCs, which can differentiate into osteogenic cells, were found in periosteal tissue (Nakahara et al. 1990). Earlier, we reported that CP cells express elevated osteoblast markers and alkaline phosphatase activity (Mizuno et al. 2006). Considering the nature and potential of CP cells, it is feasible that the grafted CP cells perpetuated the bone regeneration observed in this study. However, the role of the transplanted CP may not be limited to bone formation. MSCs derived from the bone marrow also express various growth factors such as VEGF, which induce neovascularization to the transplanted tissue (Shintani et al. 2001; Raida et al. 2006). There is also evidence that MSCs interact with immunocompetent cells, which may be related to cell recruitment, adhesion, and differentiation (Xiao-Xia et al. 2005). The influence of transplanted CP may also involve growth factors introduced into the local environment by the CP; however, these factors have yet to be characterized.

In this study, the soft tissues around the dental implants were stripped before histological analyses in order to evaluate the level and three-dimensional extent of bone regeneration. On the other hand, this resulted in loss of information about the soft tissue including the periosteum and grafted CP. Because the information about those soft tissues is also valuable, it should be important to investigate the fate of those tissues in future experiments.

The consensus tissue engineering paradigm includes cells, scaffolds, and bioactive molecules. For periodontal therapy, there are several reports based on this tissue engineering paradigm that incorporate various polymers such as collagen and gelatin as a scaffold material (Ripamonti et al. 2001; Jin et al. 2003; Taba et al. 2005). However, natural biodegradable materials cannot eliminate the possible risk of infection and degraded products may interfere with the regeneration process. Instead of culturing cells on natural biodegradable scaffolds, we have been able to stimulate periosteal cells to form their own matrix and generate a cell-populated membrane *in vitro* (Mizuno et al. 2006). This method creates a CP membrane durable enough to be held by forceps, making it feasible to

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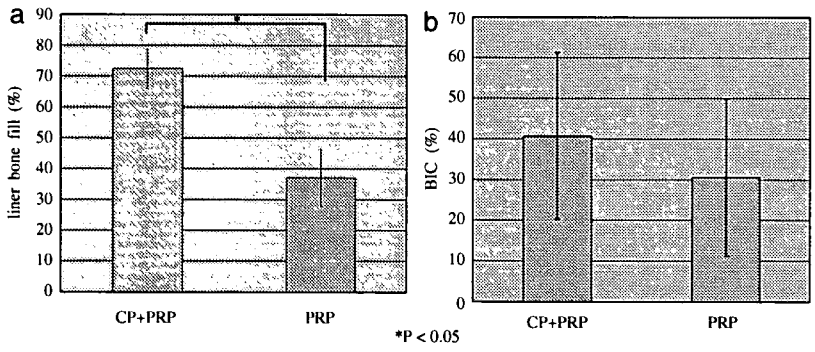

Fig. 4. Histomorphometric analyses. (a) Linear bone fill (LF). LF was significantly higher in the cultured periosteum (CP) group as compared with the control group ($P<0.05$). (b) Bone-to-implant contact (BIC). No statistically significant differences were found among the CP and control groups.

transplant CP membranes without the need for a biodegradable support. Furthermore, the thickness of the CP is approximately 200 μm, which may be beneficial to cells, allowing oxygen and nutrients to diffuse into the transplanted tissue. A major disadvantage of using CP for clinical treatment might be the time period required for tissue culture. Four to 6 weeks is a typical time frame for obtaining CP with enough mechanical strength to be transplanted. Furthermore, the cultured period would likely differ for each patient and may be unpredictable at the beginning of culture. This uncertainty makes it difficult to formulate a treatment schedule in advance. To overcome this problem, we have investigated the potential of CP cryopreservation (Mase et al. 2006). In this study, the optimal preincubation protocol for CP was investigated and it was found that CP could be successfully cryopreserved under specific conditions without loss of osteogenic potential. Cryopreseravation of CP should increase the usefulness of these approaches in future clinical applications.

On average, CP consists of 20–30 cellular layers with autologous matrices and shows enough mechanical strength to be held with forceps. However, the thickness can differ in each culture and some of the periosteum cultures showed fragility even under identical culture conditions. These differences may be due to variability in the originally harvested cells. At present, little is understood about the formation mechanism of the membranous structure by periosteal cells, making it difficult to manage the individual variation in CP membranes. To increase the usefulness of this approach, it would be important to investigate the underlying mechanisms of membrane formation and the factors that influence CP structure. Those studies would contribute to the routine, stable generation of CP and, in turn, facilitate industrialization of this technique in the future.

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References

Blumenthal, N.M. (1993) A clinical comparison of collagen membranes with e-PTFE membranes in the treatment of human mandibular buccal class II furcation defects. *Journal of Periodontology* **64**: 925–933.

Cassinelli, C., Cascardo, G., Morra, M., Draghi, L., Motta, A. & Catapano, G. (2006) Physical–chemical and biological characterization of silk fibroin-coated porous membranes for medical applications. *International Journal of Artificial Organs* **29**: 881–892.

Jin, Q.M., Anusaksathien, O., Webb, S.A., Rutherford, R.B. & Giannobile, W.V. (2003) Gene therapy of bone morphogenetic protein for periodontal tissue engineering. *Journal of Periodontology* **74**: 202–213.

Linhart, W., Peters, F., Lehmann, W., Schwarz, K., Schilling, A.F., Amling, M., Rueger, J.M. & Epple, M. (2001) Biologically and chemically optimized composites of carbonated apatite and polyglycolide as bone substitution materials. *Journal of Biomedical Materials Research* **54**: 162–171.

Mase, J., Mizuno, H., Okada, K., Sakai, K., Mizuno, D., Usami, K., Kagami, H. & Ueda, M. (2006) Cryopreservation of cultured periosteum: effect of different cryoprotectants and pre-incubation protocols on cell viability and osteogenic potential. *Cryobiology* **52**: 182–192.

Mizuno, H., Hata, K., Kojima, K., Bonassar, L.J., Vacanti, C.A. & Ueda, M. (2006) A novel approach to regenerating periodontal tissue by grafting autologous cultured periosteum. *Tissue Engineering* **12**: 1227–1335.

Nakahara, H., Bruder, S.P., Haynesworth, S.E., Holecen, J.J., Baber, M.A., Goldberg, V.M. & Caplan, A.I. (1990) Bone and cartilage formation in diffusion chambers by subcultured cells derived from the periosteum. *Bone* **11**: 181–188.

Nevins, M., Camelo, M., De Paoli, S., Friedland, B., Schenk, R.K., Parma-Benfenati, S., Simion, M., Tinti, C. & Wagenberg, B. (2006) A study of the fate of the buccal wall of extraction sockets of teeth with prominent roots. *International Journal of Periodontics and Restorative Dentistry* **26**: 19–29.

Ofer, M., Sandu, P., Zvi, A. & Nemcovsky, C.E. (2005) Healing of dehiscence-type defects in implants placed together with different barrier membranes: a comparative clinical study. *Clinical Oral Implants Research* **16**: 210–219.

Parodi, R., Carusi, G., Santarelli, G. & Nanni, F. (1998) Implant placement in large edentulous ridges expanded by GBR using a bioresorbable collagen membrane. *International Journal of Periodontics and Restorative Dentistry* **18**: 266–275.

Raida, M., Heymann, A.C., Gunther, C. & Niederwieser, D. (2006) Role of bone morphogenetic protein 2 in the crosstalk between endothelial progenitor cells and mesenchymal stem cells. *International Journal of Molecular Medicine* **18**: 735–739.

Ripamonti, U., Crooks, J., Petit, J.C. & Rueger, D.C. (2001) Periodontal tissue regeneration by combined applications of recombinant human osteogenic protein-1 and bone morphogenetic protein-2. A pilot study in Chacma baboons (*Papio ursinus*). *European Journal of Oral Sciences* **109**: 241–248.

Schmelzeisen, R., Schimming, R. & Sittinger, M. (2003) Making bone: implant insertion into tissue-engineered bone for maxillary sinus floor

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augmentation – a preliminary report. *Journal of Cranio-Maxillofacial Surgery* **31**: 34–39.

Shintani, S., Murohara, T., Ikeda, H., Ueno, T., Sasaki, K., Duan, J. & Imaizumi, T. (2001) Augmentation of postnatal neovascularization with autologous bone marrow transplantation. *Circulation* **103**: 897–903.

Squier, C.A., Ghoneim, S. & Kremenak, C.R. (1990) Ultrastructure of the periosteum from membrane bone. *Journal of Anatomy* **171**: 233–239.

Taba, M. Jr., Jin, Q., Sugai, J.V. & Giannobile, W.V. (2005) Current concepts in periodontal bioengineering. *Orthodontics and Craniofacial Research* **8**: 292–302.

Oh, T.-J., Meraw, S.J., Lee, E.-J., Giannobile, W.V. & Wang, H.-L. (2003) Comparative analysis of collagen membranes for the treatment of implant dehiscence defects. *Clinical Oral Implants Research* **14**: 80–90.

Tenenbaum, H.C. & Heersche, J.N. (1985) Dexamethasone stimulates osteogenesis in chick periosteum *in vitro*. *Endocrinology* **117**: 2211–2217.

Xiao-Xia, J., Yi, Z., Bing, L., Shuang-Xi, Z., Ying, W., Xiao-Dan, Y. & Ning, M. (2005) Human mesechymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. *Blood* **105**: 4120–4126.

Zohar, R., Jaro, S., Christopher, A. & McCulloch, G. (1997) Characterization of stromal progenitor cells enriched by flow cytometry. *Blood* **90**: 3471–3481.

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Glucose transporter mediation responsible for morphological change of human epithelial cells on glucose-displayed surface

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Running head: MORPHOLOGICAL CHANGES ON GLUCOSE-DISPLAYED SURFACE

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Abstract

Cellular morphology is one of the important factors to coordinate cell signaling. In the present study, the morphological variation via glucose transporter (GLUT)-mediated anchoring was investigated in the cultures of human mammary epithelial cells in the presence or absence of insulin on the surfaces with the changed ratios of D- and L-glucose displayed. With increasing ratio of D-glucose displayed on the surfaces, the cells showed the stretched shape in the culture with 10 $\mu g/cm^3$ insulin, reaching the highest extent of cell stretching at 100% D-glucose display, while the round shaped cells were dominant at 0% D-glucose display. In the absence of insulin, on the other hand, the extent of cell stretching depicted a concave profile in terms of the ratio of D-glucose displayed, the extent being the highest at 50% D-glucose display. Blocking of integrin $\alpha_5\beta 1$ or GLUTs1 and 4 on the cells with corresponding antibodies revealed that the primary mechanism for cell attachment was based on integrin-mediated binding, and that GLUTs1 and 4 contributed largely to morphological changes of cells. In confocal microscopic examination, moreover, it was found that GLUT4 localization occurred in response to the D-glucose display as well as insulin addition. In the absence of insulin, GLUT4 spots were extensively observed in cell body whether the D-glucose was displayed or not. However, in the presence of insulin, the broad distribution of GLUT4 appeared on the basal and apical sides of cells at 100% D-glucose display, in contrast with its localization only on the apical cell side at 0% D-glucose display. These results suggest that the quantitative balance of GLUTs on the cytoplasmic membrane and D-glucose displayed on the surface determines the cell morphology, as explained by a "receptor saturation" model.