ity of cultured corneal epithelial cell sheets in rabbit. Cornea 24, 735-41, 2005.

Seko K, Kagami H, Senga K, Ozeki K, Mizutani H, Ueda M. Effects of Ovariectomy and estrogen replaceme nt on rat oral mucosa. Maturitas 50, 44-51, 2005.

M. Tatebe, R. Nakamura, H. Kagami, K. Okada, M. Ueda. Gene-environment interaction involved in oral carcino genesis: molecular epidemiological study for Differentiation of transplanted mesenchymal stem cells in a large osteochondral defect in rabbit. Cytotherapy vol. 7 (6) , 520-530, 2006.

Y Murase, Y. Narita, H. Kagami, K. Miyamoto, Y. Ueda, M. Ueda, T murohara. Evaluation of Compliance and Sti ffness of Decellularized Tissues as Scaffolds for Tissue-Engineered Small Caliber Vascular Grafts Usin g Intravascular Ultrasound. ASAIO, 450-455, 2006.

D. Mizuno, H. Kagami, H. Mizuno, J. Mase, K. Usami, M. Ueda. Bone regeneration of dental implant dehiscence d efects using cultured periosteum membrane. Clinical Oral Implants Research, 2007, in press.

Honda MJ, Shinohara Y, Sumita Y, Tonomura A, Kagami H, Ueda M. Performance of collagen sponge as a 3-D scaffold for tooth-tissue engineering. Biomaterials. 27: 3238-3248, 2006.

Honda MJ, Shinohara Y, Sumita Y, Tonomura A, Kagami H, Ueda M. Shear stress facilitates tissue-enginee red odontogenesis. Bone, 39 (1) 125-33, 2006.

Ohno K, Hattori T, Kagami H, and Ueda M. Effects of Preceding Sialadenitis on Development of A utoimmunity against Salivary Gland. Oral Diseases, 13: 158-162, 2006.

Matsunuma H, Kagami H, Narita Y, Hata K-I, Ono Y, Ohshima S, and Ueda M. The potential of bone marr ow-derived mononuclear cells for enhancing angiogenesis in ureteral acellular matrix. Tissue Engineeri ng. 12, 509-518, 2006.

Agata H, Kagami H, Watanabe N, Ueda M. Effect of ischemic culture conditions on the survival and diffe rentiation 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 dehiscenc edefects 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 huma n 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-S eeding Technique Using Magnetite Nanoparticles and Magnetic Force onto Decellularized Blood Vessels fo r 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 eng ineering using periosteum-derived cells. *J. Dent Res.* 86, 79-83, 2007

山田陽一、各務秀明、上田 実 幹細胞を応用した歯周組織再生 「再生歯科のテクニックとサイエンスー歯周・審美・インプラントー」 第1編 歯周組織・再生 吉江弘正/宮本泰和編 クインテッセンス出版株式会社、2005

各務秀明　唾液による健康づくり一明日からの臨床に取り組む一　Part 3 唾液のはたらき　１２.唾液腺の再生医療は可能か？　日本歯科評論　増刊２００５　株式会社ヒョーロンパブリシャーズ　201-208, 2005

各務秀明, 杉戸孝行, 上田実　増殖因子および培養細胞を用いた唾液腺の再生 口科誌 54(2): 211-215, 2005

本田雅規, 小原孝之, 住田吉慶, 各務秀明, 魚返拓利, 上田実　歯の再生ーイヌ下顎骨への歯胚細胞移植　「再生医療」　4(1) : 85-89, 2005

各務秀明, 渡辺真紀, 外村明子, 安藤由典, 本田雅規, 上田実　歯および歯胚由来細胞を用いた再生医療とその可能性　「再生医療」　4(1):79-83, 2005

(紀ノ岡正博)

Norihiko Hata, Yuka Agatahama, Masahiro Kino-oka, and Masahito Taya: "Relations between Individual Cellular Motions and Proliferative Potentials in Successive Cultures of Human Keratinocytes", Cytotechnology, Vol. 47, No. 1-3, pp. 127 - 131 (2005)

M.-H. Kim, M. Kino-oka, M. Kawase, K. Yagi, M. Taya: "Response of Human Epithelial Cells on Culture Surfaces with Varied Roughnesses Prepared by Immobilizing Dendrimers with/without D-Glucose Display", J. Biosci. Bioeng., Vol. 103, No. 2, pp. 192 -199, 2007.

紀ノ岡正博、田谷正仁: "移植を前提とした組織培養工程における細胞評価"、 医工学治療、Vol. 17, No. 4, pp. 203-206 (2005)

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)

Tomokatu Hongo, Mariko Kajikawa, Seiichi Ishida, Shogo Ozawa, Yoichi Ishikawa, Takeshi Kobayashi and Hiroyuki Honda : Three-dimensional high density culture of HepG2 cells in a 5-ml radial-flow bioreactor for construction of artificial liver, Journal of Bioscience and Bioengineering, 99(3), 237-244 (2005)

Ryuji Kato, Hideo Nakano, Hiroyuki Konishi, Katsuya Kato, Yuichi Koga, Tsuneo Yamane, Takeshi Kobayashi and Hiroyuki Honda : Novel strategy for protein exploration High-throughput screening assisted with fuzzy neural network, Journal of Molecular Biology, 351, 683-692 (2005)

Hiro Takahashi and Hiroyuki Honda : A new reliable cancer diagnosis method using boosted fuzzy classifier with SWEEP operator method, Journal of Chemical Engineering of Japan, 38, 763-773 (2005)

Akira Ito, Eri Hibino, Hiroyuki Honda Chiaki Kobayashi, Hiroko Terasaki, Hideaki Kagami, Minoru Ueda and Takeshi Kobayashi: Construction and delivery of tissue-engineering human retinal pigment epithelial cell sheets using magnetite nanoparticles and magnetic force, Tissue Engineering, 11(3/4), 489-496 (2005)

Akira Ito, Kousuke Ino, Masao Hayashida, Takeshi Kobayashi, Hiroshi Matsumura, Hideaki Kagami, Minoru Ueda and Hiroyuki Honda : A novel methodology for fabrication of tissue-engineered tubular constructs using magnetite nanoparticles and magnetic force, Tissue Engineering, 11(9/10), 1553-1561 (2005)

Akira Ito, Eri Hibino, Kazunori Shimizu, Takeshi Kobayashi, Yoichi Yamada, Hideharu Hibi and Minoru Ueda and Hiroyuki Honda, Magnetic force-based mesenchymal stem cell expansion using antibody-conjugated magnetoliposomes, Journal of Biomedical, Materials Research: Part B-Applied Biomaterials, 75(2), 320−327 (2005)

Yasuyuki Tomita, Hiroyuki Asano, Hideo Izawa, Mitsuhiro Yokota, Takeshi Kobayashi and Hiroyuki Honda: "Classification Method for Predicting the Development of Myocardial Infarction by Using the Interaction between Genetic and Environmental Factors", IPSJ Digital Courier, Vol. 2, pp. 691-709, 2006.

Hiro Takahashi, Hiroyuki Honda : Prediction of peptide binding to MHC classII molecules using boosted fuzzy classifier with sweep operator method, Journal of Bioscience and Bioengineering, 101(2), 137-141, 2006.

Mina Okochi, Mari Nakanishi, Ryuji Kato, Takeshi Kobayashi, Hiroyuki Honda : High-throughput screening of cell death inducible short peptides from TNF-related apoptosis-inducing ligand sequence, FEBS Letters, 580, 885-889, 2006.

Kazunori Shimizu, Akira Ito and Hiroyuki Honda : Enhanced cell-seeding into 3-D porous scaffolds use of magnetic nanoparticles, Journal of Biomedical Materials Research Part B, 77(2), 265-272, 2006.

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

N. Kamiya, H. Watanabe, H. Habuchi, H. Takagi, T. Shinomura, K. Shimizu, K. Kimata. Versican/PG-M Regu lates Chondrogenesis as an Extracellular Matrix Molecule Crucial for Mesenchymal Condensation. J Biol Chem 2006;281:2390-400.

Y. Inoue, M. Yoneda, J. Zhao, O. Miyaishi, A. Ohno-Jinno, T. Kataoka, Z. Isogai, K. Kimata, M. Iwaki, M. Zako. Molecular cloning and characterization of chick spacrcan. J Biol Chem 2006.

K. Sakai, K. Kimata, T. Sato, M. Gotoh, H. Narimatsu, K. Shinomiya, H. Watanabe. Chondroitin sulfate N -acetylgalactosaminyltransferase-1 plays a critical role in chondroitin sulfate synthesis in cartilage. J Biol Chem , 282:4102-4161, 2006.

K. Matumoto, N. Kamiya, K. Suwan, F. Atumi, K. Shimizu, T. Shinomura, Y. Yamada, K. Kimata, H. Watan abe. Versican/PG-M aggregates i cartlage: identification and characterization. J Biol Chem, 281:18257-1 8263, 2006.

N. Kamiya, H. Watanabe, H. Habuchi, H. Takagi, T. Shinomura, K. Shimizu, K. Kimata. Versican/PG-M Regu lates Chondrogenesis as an Extracellular Matrix Molecule Crucial for Mesenchymal Condensation. J Biol Chem , 281:2390-400, 2006.

A. Sakai, N. Akifusa, N. Itano, K. Kimata, T. Kawamura, T. Koseki, T. Takehara, T. Nishihara. Potentia l role of high molecular weight hyaluronan in the anti-Candida activity of human oral epithelial cells. M edical Mycology, 45:73-79, 2007.

N. Sugiura, S. Shimokata, H. Watanabe, K. Kimata. MS analysis of chondroitin polymerization:effects of Mn2+ ions on the stability of UDP-sugars and chondroitin synthesis. Analytical Biochemistry, in press, 2007.

T. Minamisawa, K. Suzuki, H. Maeda, S. Shimokata, N. Sugiura, K. Kimata. Characterization of isomericu nsulfated glycosaminoglycan oligosaccharides by mass spectrometry/ mass spectrometry. J. Mass Spectr m. Soc. Jpn, 55:1-6, 2007.

L. Zhuo, A. Kanamori, R. Kannagi, N. Itano, J. Wu, M. Hamaguchi, N. Ishiguro, K. Kimata. SHAP Potentia tes the CD44-mediated leukocyte adhesion to the hyaluronan substratum. J Biol Chem, 281:20303-20314, 20 06.

Y. Inoue, M. Yoneda, J. Zhao, O. Miyaishi, A. Ohno-Jinno, T. Kataoka, Z. Isogai, K. Kimata, M. Iwak i, M. Zako. Molecular cloning and characterization of chick spacrcan. J Biol Chem, 281:10381-10388, 200 6.

K. Kimata, O. Habuchi, H. Habuchi, E. Watanabe. Konckout mice and preteoglycans (Chapter3. 11). Comprehensiv e Glycoscience-From Chemistry to Systems Biology. Elsevier, in press, 2006.

H. Habuchi, O. Habuchi, K. Uchimura, K. Kimata, T. Muramatsu. Determination of substrate specificity of s ulfortansferases and glycosyltransferases (proteoglycans). Methods in Enzymology. Glycomics. AcademicPr ess, 225-242. vol 416, 2006.

Sugaya N, Habuchi H, Nagai N, Ashikari-Hada S, Kimata K. 6-*O*-sulfation of heparan sulfate differential ly 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 tetrasaccharide 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

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

澤井崇博, 板野直樹, 木全弘治. マウス初代軟骨細胞におけるbone morphogenetic protein-2のヒアルロン酸合成酵素発現制御. 愛知医科大学医学会雑誌 2005;33:1-6.

蛯沢克己, 木全弘治, 渡辺秀人. プロテオグリカン. ティッシュエンジニアリング2005. 日本医学館, 2005:25-30. (日本組織工学会編集)

卓麗聖, 木全弘治. 第1編 糖鎖を科学する 第1章 糖鎖のしくみ 第3節プロテオグリカン 1 ヒアルロン酸の構造と機能. 糖鎖科学の新展開. NTS, 2005:40-47.

渡辺秀人, 木全弘治. 3. 糖鎖遺伝子研究 1. 糖転移酵素 1.17 コンドロイチン硫酸合成関連遺伝子群. 未来を拓く糖鎖科学. 金芳堂, 2005:172-174.

板野直樹, 木全弘治. 3. 糖鎖遺伝子研究 1 糖転移酵素 1.19 ヒアルロン酸合成酵素. 未来を拓く糖鎖科学. 金芳堂, 2005:176-177.

木全弘治. 4. 糖鎖機能解析 7 再生医療・移植 7.1 組織再生におけるプロテオグリカン. 未来を拓く糖鎖科学. 金芳堂, 2005:303-309.

羽渕弘子, 木全弘治. 4. 糖鎖機能解析 9 発生・分化・形態形成 9.6 形態形成におけるヘパラン硫酸プロテオグリカンの機能. 未来を拓く糖鎖科学. 金芳堂, 2005:339-432.

渡邊裕規, 渡辺秀人, 木全弘治. 連載 軟骨代謝の研究ー基礎と臨床ー最近の進歩<10> 基礎ー軟骨におけるプロテオグリカンの役割 CLINICAL CALCIUM 第16巻6号. 医療ジャーナル社 , 146-149. 2006.

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. THE LUNG perspectives, 15:75-81, 2007.

坂井顕一郎, 木全弘治, 渡辺秀人. 細胞接着と細胞増殖を制御するプロテオグリカン. 再生医療の基礎シリーズ2 再生医療のための細胞生物学. コロナ社, 49-75, 2007.

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)

特許出願

- 特願2005-000740 腱または靭帯組織再生器具, 上田 実
- 特願2005－045417 ヒト歯髄細胞の分化誘導法及び象牙質再生用組成物, 株式会社日立メディコ/名古屋大学, 上田実, 各務秀明
- PCT/JP2005/013679 特願2004－215556 間葉系幹細胞から象牙芽細胞への分化誘導方法, 上田実, 各務秀明, 安藤由典, 家島大輔
- 特願2005－306471 組織形成用複合材料およびその製造方法, 上田実, 各務秀明, 岡田邦彦, 水野裕和, 宇佐見一公

・特願2005－298161　移植材料及び骨質改善剤，上田実，各務秀明，山田陽一，岡田邦彦，上嶋伸知，八島明弘，高後友之
・特願2005－102404　細胞の増殖又は分化方法
・特願2005―203999　細胞の播種方法
・特願2005－222886　ヒト歯胚細胞の判別及び選択方法
・特願2005-045417　象牙質再生用組成物の製造方法，株式会社日立メディコ/名古屋大学，安藤由典，外村明子，各務秀明，上田実
・特願2005-102404　培養上清を用いた細胞培養技術，株式会社日立メディコ/名古屋大学，家島大輔，小原孝之，安藤由典，外村明子，各務秀明，上田実
・特願2005-48527　象牙質の再生方法及びこれに用いる移植物，上田実/日本特殊陶業/日立メディコ，小原孝之，安藤由典，外村明子，各務秀明，上田実
・特願2005-143443　骨組織形成用細胞の調整方法、及び骨組織形成用細胞の利用，名古屋大学/オステオジェネシス株式会社，上田実，山田陽一，島伸行
・特願2005-190374　皮膚組織改善財，名古屋大学，蛯沢克己，上田実，各務秀明，岡田邦彦，加藤竜司，ﾏｽﾞﾘｻﾞﾑ･ｱﾌﾞﾄﾞｩﾙ
・特願2006-000075　移植のための組織組成物，東京大学，上田実
・特願2006-008521　自動培養装置
・出願準備中　自動培養装置

・特願2005-190374 2005/6/29　皮膚組織改善材及びその利用
上記のＰＣＴ：PCT/JP2006/308317　2006/6/28

・特願2006-226332 2006/8/23　培養スケジュール管理装置及び培養スケジュール管理プログラム

・特願2006-008521 2006/1/17　自動培養装置

・特願2006-279476 2006/10/13　自動培養装置

・特願2006-45813　発明の名称　コンドロイチン硫酸合成促進

・特願2007-212116　加藤竜司、山本若菜、本多裕之、他：「細胞品質予測モデル」（発明登録済→特許出願中）

Morimichi Ohya
Yoichi Yamada
Ryotaro Ozawa
Kenji Ito
Makoto Takahashi
Minoru Ueda

Sinus floor elevation applied tissue-engineered bone

Comparative study between mesenchymal stem cells/platelet-rich plasma (PRP) and autogenous bone with PRP complexes in rabbits

Authors' affiliations:
Morimichi Ohya, Ryotaro Ozawa, Kenji Ito, Makoto Takahashi, Minoru Ueda, Department of Oral and Maxillofacial Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan
Yoichi Yamada, Center for Genetic and Regenerative Medicine, Nagoya University School of Medicine, Nagoya, Japan

Correspondence to:
Prof. Minoru Ueda
Department of Oral and Maxillofacial Surgery
Nagoya University Graduate School of Medicine
65 Tsuruma-cho
Showa-ku
Nagoya 466-8550
Japan
Tel.: +81 52 744 2348
Fax: +81 52 744 2352
e-mail: mueda@med.nagoya-u.ac.jp

Date:
Accepted 20 September 2004

To cite this article:
Ohya M, Yamada Y, Ozawa R, Ito K, Takahashi M, Ueda M. Sinus floor elevation applied tissue-engineered bone. Comparative study between mesenchymal stem cells/platelet-rich plasma (PRP) and autogenous bone with PRP complexes in rabbits.
Clin. Oral Impl. Res. 16, 2005; 622–629
doi: 10.1111/j.1600-0501.2005.01136.x

Key words: bone regeneration, mesenchymal stem cells (MSCs), platelet-rich plasma (PRP), sinus floor elevation (SFE), tissue engineering

Abstract: In the present study, we compared bone regeneration ability in sinus floor elevation between a tissue engineering method using mesenchymal stem cells (MSCs) and platelet-rich plasma (PRP), and a promising new method using particulate cancellous bone and marrow (PCBM) and PRP. Bilateral sinus floor elevation procedures were performed in 18 adult Japanese white rabbits. MSCs/PRP or PCBM/PRP complexes were grafted to each maxillary sinus in the same rabbits. The MSCs were isolated from rabbit iliac crest marrow, and PRP was obtained from peripheral blood. PCBM were collected from the rabbit iliac crest and mixed with PRP. The animals were sacrificed at 2, 4, and 8 weeks after transplantation, and the bone formation ability of each implant was evaluated histologically and histometrically. According to the histological observations, both sites (MSCs/PRP and PCBM/PRP) showed well newly formed bone and neovascularization at 2 and 4 weeks. However, at 8 weeks, the lamellar bone was observed to be occupied by fatty marrow in large areas in both sites. There was no significant difference in bone volume or augmented height between MSCs/PRP and PCBM/PRP groups each week, but there were significant differences in bone volume and augmented height between 2 and 8 weeks in PCBM/PRP or MSCs/PRP groups and in bone volume between 4 and 8 weeks in the PCBM/PRP group ($P<0.05$). These results suggest that the MSCs/PRP complex may well be used for bone regeneration in sinus floor elevation, compared with the PCBM/PRP complex.

The dental restoration of the posterior maxilla using osseointegrated implants is one of the most problematic in implant dentistry. Patients with edentulous posterior maxillae often present with atrophy of the alveolar ridge and pneumatization of the maxillary sinuses following teeth loss. This situation limits the volume of bone available for implant placement. These clinical problems might be improved partly by utilizing sinus floor elevation procedures with autogenous bone grafts, which are considered as an ideal graft material and the gold standard to which other graft materials are compared. The bone grafts harvested from the iliac crest also have shown excellent survival with implants loaded and functional (Kent & Block 1989). However, the bone grafts have to injure the normality organization besides the operation field, and it involves morbidity (Boyne et al. 1980; Tatum 1986; Younger & Chapman 1989; Lundgren et al. 1996). Other autogenous approaches such as intraoral bone grafts generally result in less morbidity, but the bone volume offers less bone availability than the iliac crest as a donor site. Nowadays, bone substitutes such as hydroxyapatite, β-tricalcium phosphate ceramics, or coral scaffolds (Kadiyara et al. 1997b; Petite et al. 2000) are used to provide alternatives to

autogenous bone for the improvement of the bone volume. But in human specimens, there were problems where graft particles were still present after 4 years in the sinus floor elevation (Leonardis & Pecosa 1999; Piatelli et al. 1999). So it has been said that an ideal bone substitute should have the following characteristics: it should be biocompatible and be replaced by newly formed bone, and it should have osteoinductive properties (Jensen et al. 1996).

Langer & Vacanti (1993) have called the process involving the morphogenesis of new tissues formed from isolated cells and biocompatible polymers and growth factors 'tissue engineering'. This form of bone regeneration by autogenous cell transplantation is one of the most promising treatment concepts being developed, since it eliminates the problems of donor site morbidity for autologous grafts, the immunogenicity of allogenic grafts, and loosening of alloplastic implants (Vacanti 1988; Vacanti et al. 1988). In this study, we used mesenchymal stem cells (MSCs) as the isolated cells, and platelet-rich plasma (PRP) as the growth factors and scaffold. MSCs have been thought to be multipotent cells that can replicate as undifferentiated cells and that have the potential to differentiate into lineages of mesenchymal tissue including bone, cartilage, fat, tendon, muscle, and marrow stroma (Caplan 1991; Owen & Friedenstein 1998; Pittenger et al. 1999), and have received widespread attention because of their potential utility in tissue engineering applications. PRP is believed to result in early consolidation and graft mineralization in approximately half the time that it would take using an autogenous graft alone (Canalis 1981; Cho et al. 1995; Marx et al. 1998; Anitua 1999) and promote a 15–30% increase in the trabecular bone density (Marx et al. 1998).

So we have attempted to regenerate bone in a sinus floor elevation as for bone grafts with minimal invasiveness and good plasticity, and to provide a clinical alternative to autogenous bone grafts using MSCs/PRP complexes. Further, there are no reports that sinus floor elevation applied MSCs/PRP complexes and there have been comparative studies with autogenous bone grafts/PRP complexes.

In this study, we compared the bone regeneration ability in sinus floor elevation with the MSCs/PRP groups and particulate cancellous bone and marrow (PCBM)/PRP groups. This research will be useful as a preliminary study for dental implant installation and as an improved method for maxillary sinus augmentation with minimal invasiveness.

Material and methods

Rabbit animal model and sinus augmentation procedure

This study used 18 adult female Japanese white rabbits that weighed 3.1–3.5 kg. A skin incision was made in the cheek a few millimeters above the inferior border of the incisive bone and maxilla. The subcutaneous tissue and the muscles were dissected to expose the maxillary periosteum, which was incised and elevated dorsally. A round diamond bur was used to draw the outline of the trap door (5 mm × 5 mm with the distal vertical osteotomy line 2 mm mesial from the first molar) in the lateral antral wall of the maxilla. The trap door was removed, and the antral membrane was elevated carefully to avoid perforation. Exposure of the maxillary sinus was performed bilaterally, and the MSCs/PRP complex was placed into the left maxillary sinus, and the PCBM/PRP complex was placed into the right one. The periosteum and skin flap were replaced, sutured, and allowed to heal (Fig. 1a).

MSCs isolation and cultivation, PRP, PRP gel preparation, and MSCs/PRP admixture and PCBM/PRP admixture preparation

The PRP and the gel preparation were carried out according to the same method developed by Yamada et al. (2004c). At first, approximately 30 ml whole blood was drawn from the Japanese white rabbits into centrifuge tubes. The blood was first centrifuged in a standard laboratory centrifuge machine, Himac CT (Hitachi Koki, Hitachi, Japan), for 5 min at 203 g. Subsequently, the yellow plasma (containing the buffy coat, which contained the platelets and leukocytes) was taken up into a neutral monovette with a long cannula. A second centrifugation at 1050 g. for 5 min was performed to combine the platelets into a single pellet and the plasma supernatant, which was platelet-poor plasma (PPP) and contained relatively few cells, was removed. The resulting pellet of platelets, the buffy coat/plasma fraction (PRP), was re-suspended in the residual 3 ml of plasma and used in the platelet gel. We evaluated the whole blood, PRP, PPP, and blood platelets. The platelet counts in the PRP and PPP were measured in Beckman Coulter GEN*S™, and Beckman Coulter STKS ERE™ (Beckman Coulter, Fullerton, CA, USA). Platelet counts yielded a mean value of 227,000, with a range of 214,000–250,000. The PRP mean platelet count was 864,000 with a range of 834,000–892,000. These values confirmed the platelet sequestration ability of the process, which shows that the concentration was 383% above the baseline platelet counts. The PRP was stored at room temperature in a conventional shaker until use. Bovine thrombin in powder form (10,000 U) was dissolved in 10 ml 10% calcium chloride in a separate sterile cup. Next, 600 µl PRP was aspirated into a 1 ml syringe, while in a second 1 ml syringe 100 µl of the thrombin/calcium chloride mixture was aspirated. Here the cells resuspended directly into PRP. The two syringes were connected with a T connector and the plungers of the syringes were pushed and pulled alternatively, allowing the air bubble to transverse the two syringes. Within 5–30 s, the contents assumed a gel-like consistency as the thrombin affected the polymerization of the fibrin to produce an insoluble gel.

The MSCs were isolated from the rabbits left iliac crest marrow aspirates (10 ml) according to the previously reported method (Kadiyara et al. 1997a, 1997b). Briefly, the basal medium, low-glucose Dulbecco's modified Eagles medium, and growth supplements (50 ml of mesenchymal cell growth supplement, 10% fetal bovine serum, 10 ml of 200 mM L-glutamine, and 0.5 ml of penicillin–streptomycin mixture containing 25 U of penicillin and 25 µg streptomycin) were purchased from Cambrex® Inc. (Walkersville, MD, USA). Three supplements for inducing osteogenesis, Dexamethasone (Dex), sodium α-glycerophosphate (α-GP), and L-ascorbic acid 2-phosphate (AsAP) were purchased from Sigma Chemical Co. (St Louis, MO, USA). The cells were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. In culture, the MSCs trypsinized and were used for

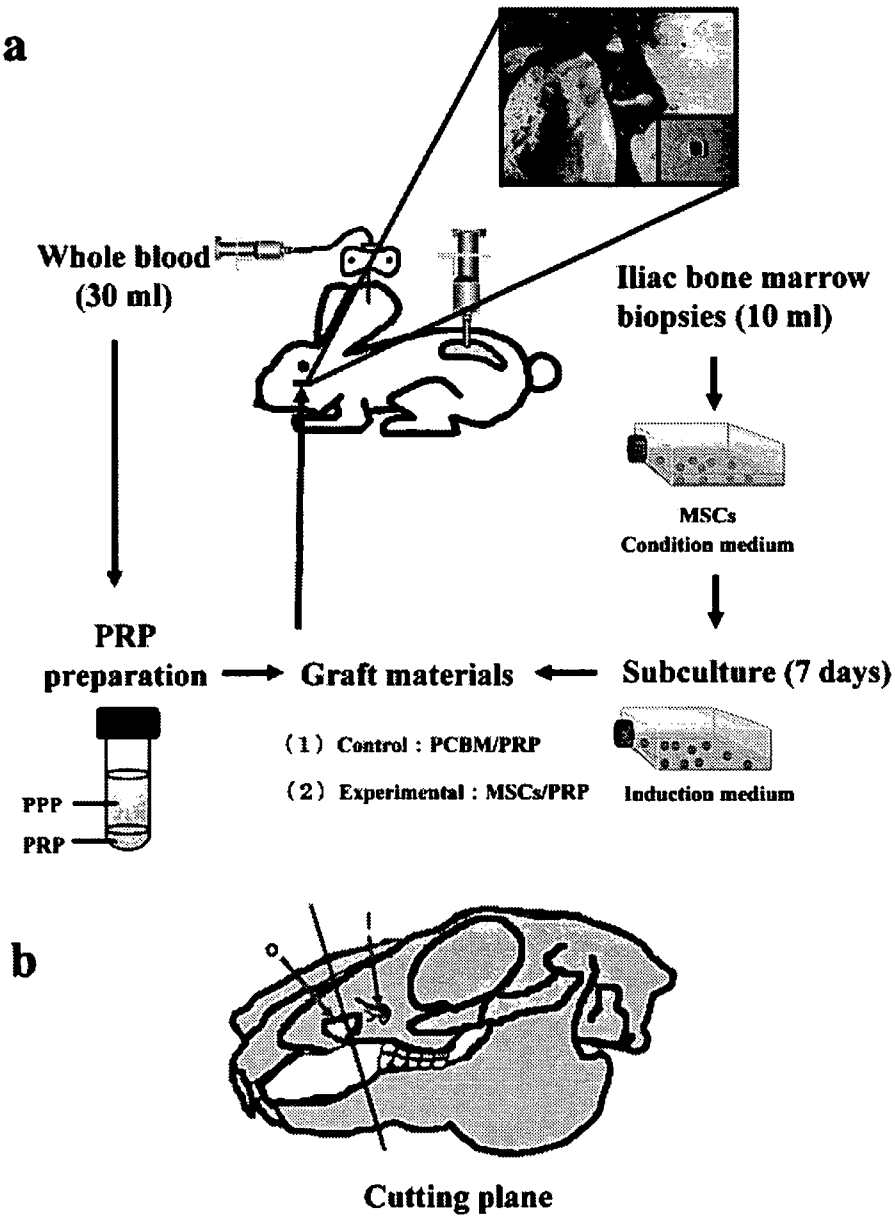

Fig. 1. (a) Scheme of experimental protocol. (b) Portion grafted to rabbit maxillary sinus. The cutting plane is standardized in the transverse plane at the rostrocaudal midpoint of the osteotomy window (O). I, infraorbital nerve and artery.

implanting. Then, 600 µl PRP and MSCs (1×10^7 cells/ml) were aspirated into a 1 ml syringe, while in another 1 ml syringe 100 µl of the thrombin/calcium chloride mixture was aspirated. Also, the graft volumes were standardized using a mold (5 mm × 5 mm × 5 mm), and the gel was implanted into the maxillary sinus.

PCBM was harvested from the right iliac crest of the animal from which the PCBM/PRP complex was made by mixing with PRP. The graft volumes were standardized using a mold (5 mm × 5 mm × 5 mm) (Fig. 1a). The groups were implanted into the maxillary sinus area.

Histological observation

The rabbits were sacrificed at 2, 4, or 8 weeks after the transplantation. The maxillae were dissected and cut into smaller blocks that included the nasal and sinus cavities. Implants were fixed in buffered 10% formaldehyde and decalcified, embedded in paraffin, sectioned at 4 µm thickness, and stained with hematoxylin and eosin (Fig. 1b). They were analyzed each six sections per operative site.

Histomorphometric analysis

Each image of the calcified specimens at the rostrocaudal midpoint of the antral wall was copied on a color reversal film, digitized as a 256 × 256 array of 8-bit density values, and transferred to a microcomputer for analysis (NIH Image, version 1.61, National Institutes of Health). The augmented area was defined as the area that was enclosed within the bone labels in the residual maxilla and nasal wall, the sinus membrane, and the antral wall. The volume of total bone (newly formed bone and grafted bone) in the augmented area and augmented height (the maximal height of the augmented space) were quantified using this computer-based image-analysis system.

Statistical analysis

Group means and standard deviations were calculated for each measured parameter. The data were compared using the paired, two-tailed Student's *t*-test for the total bone between the PCBM/PRP and MSCs/PRP groups. A *P*-value of <0.05 indicated statistical significance.

Results

Histological observation of the MSCs/PRP and PCBM/PRP groups

The elevated sinus membrane had no perforation and maintained the form of the ciliated epithelium and serous glands in all animals at each week.

At 2 weeks after transplantation, the PCBM/PRP site showed a trabeculae of newly formed bone that were composed of woven bone around the grafted bone. Most of the lacunae around the osteocytes were large and all trabeculae were embedded in fibrovasculae tissue. In the MSCs/PRP site, newly formed trabeculae were observed with abundant osteocyte, cuboidal osteoblasts, fibrovascular tissue, and many blood vessels (Fig. 2a–d).

At 4 weeks after transplantation in the PCBM/PRP and MSCs/PRP sites, the trabeculae were more mature than at 2 weeks; lamellar bone structure was apparent, woven bones decreased, and the bone lacuna became narrow in comparison with 2 weeks (Fig. 3a–d).

At 8 weeks in both sites, cortical bone formation was observed under the elevated membrane and at the lateral sinus wall. Trabeculae with clear lamellar structures were embedded in the fatty marrow in both sites (Fig. 4a–d).

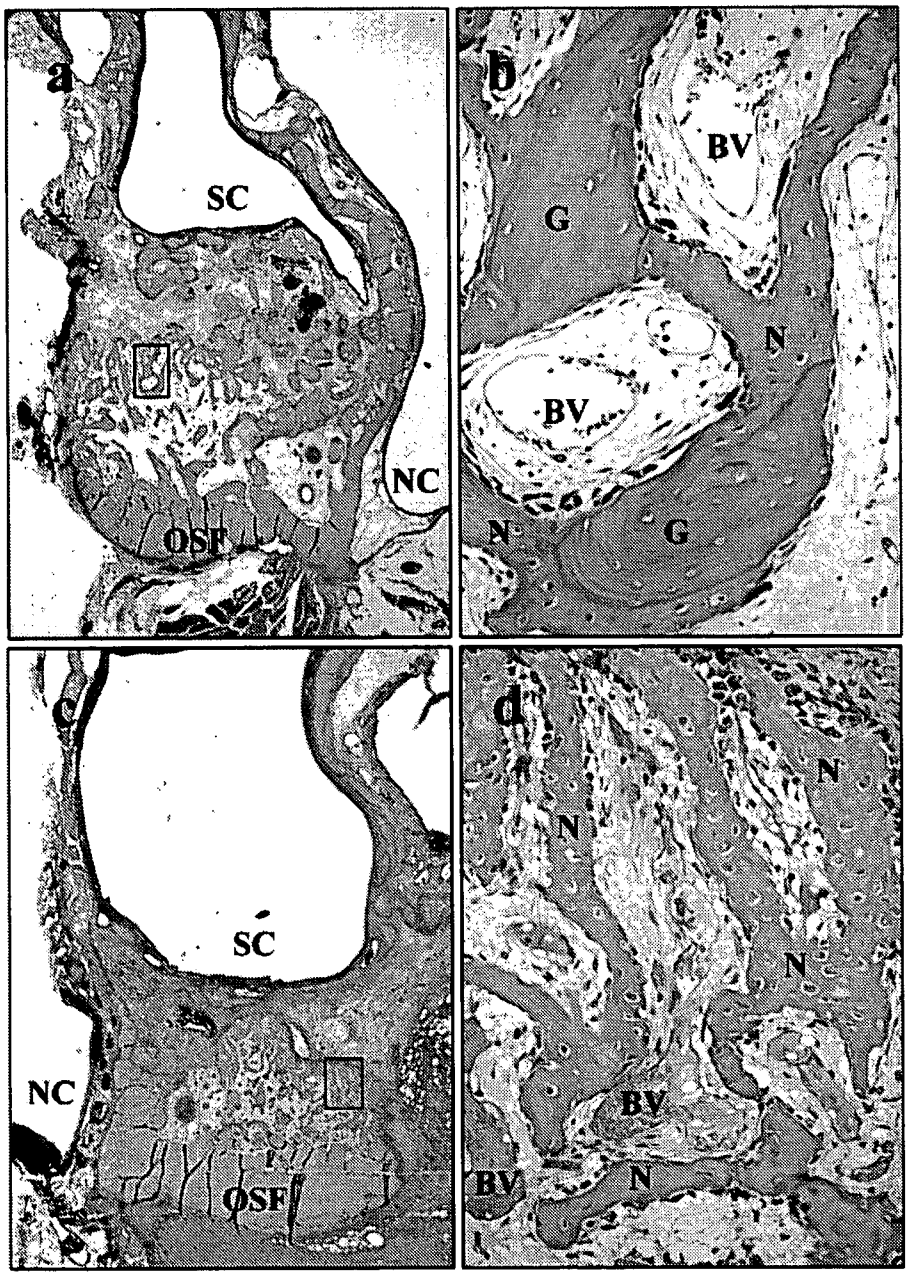

Fig. 2. Histologic evaluation of PCBM/PRP and MSCs/PRP sites at 2 weeks. Section from the (a) PCBM/PRP site (magnification × 3.125); (b) PCBM/PRP site (magnification × 50); (c) MSCs/PRP site (magnification × 3.125). (d) MSCs/PRP site (magnification × 50). SC, sinus cavity; NC, nasal cavity; OSF, original sinus floor; BV, blood vessels; N, newly formed bone; G, grafted bone; PCBM, particulate cancellous bone and marrow; PRP, platelet-rich plasma; MSCs, mesenchymal stem cells. The sections were stained by hematoxylin and eosin.

Histomorphometric analysis

Bone volume

The volumes of total bone PCBM/PRP and MSCs/PRP groups were 35 ± 5.2% and 29.1 ± 4.4% at 2 weeks, 28.6 ± 3.4% and 24.1 ± 3.6% at 4 weeks, and 20.6 ± 4% and 20.9 ± 4.1% after 8 weeks, respectively. There was no difference in bone volume between MSCs/PRP and PCBM/PRP groups each week. But there were significant differences in bone volume between 2 and 8 weeks in PCBM/PRP or MSCs/PRP groups and between 4 and 8 weeks in the PCBM/PRP group ($P<0.05$) (Fig. 5a).

Augmented height

The augmented heights of PCBM/PRP and MSCs/PRP groups were 2.1 ± 0.5 and 1.7 ± 0.2 mm at 2 weeks, 1.7 ± 0.6 and 1.5 ± 0.3 mm at 4 weeks, and 1.3 ± 0.4 and 1.3 ± 0.3 mm after 8 weeks, respectively. There was no difference in augmented height between MSCs/PRP and PCBM/PRP groups each week. But there were significant differences in augmented height between 2 and 8 weeks in PCBM/PRP or MSCs/PRP groups ($P<0.05$) (Fig. 5b).

Discussion

Generally, atrophy of the alveolar ridge is because of tooth loss, and autogenous bone grafts are used for the bone regeneration, with dental implants utilized for altitude absorbing of the upper jaw bone (Boyne et al. 1980; Tatum 1986; Lundgren et al. 1996). In posterior maxilla restoration using osseointegrated implants, sinus floor elevation is often also undertaken by an autogenous bone graft.

Autogenous bone graft is considered the ideal graft to satisfy the following criteria: (1) low risk of infection, (2) low antigenicity, (3) the ability to produce bone by osteoinduction and osteoconduction, and (4) easy correction (Block & Kent 1997). However, the preferred autogenous material causes specific problems such as its limited supply, attendant donor-site morbidity, and the occasional unsuitability for the proposed reconstruction because of poor tissue quality, or the extremely difficulty in shaping the graft (Laurie et al. 1984; Summers & Eisenstein 1984; Younger & Chapman 1989). Therefore, in this study we use tissue engineering technology with minimal invasiveness for bone regeneration using MSCs as the autogenous cell and PRP as growth factors and scaffold. It is one of the most promising treatment concepts being developed, since it may be possible to eliminate the problems of donor site morbidity for autologous grafts (Vacanti 1988; Vacanti et al. 1988). The PRP also contains growth factors such as platelet-derived growth factor, transforming growth factors-α, and insulin growth factor PRP is also reported to promote wound healing, bone formation, and has attracted attention for use with autogenous bone (PCBM) in clinical cases (Marx et al. 1998; Wiltfang et al. 2003).

MSCs have been thought to be multipotent cells that can replicate as undifferentiated cells, including bone, cartilage, fat, tendon, muscle, and marrow stroma, and have received widespread attention because

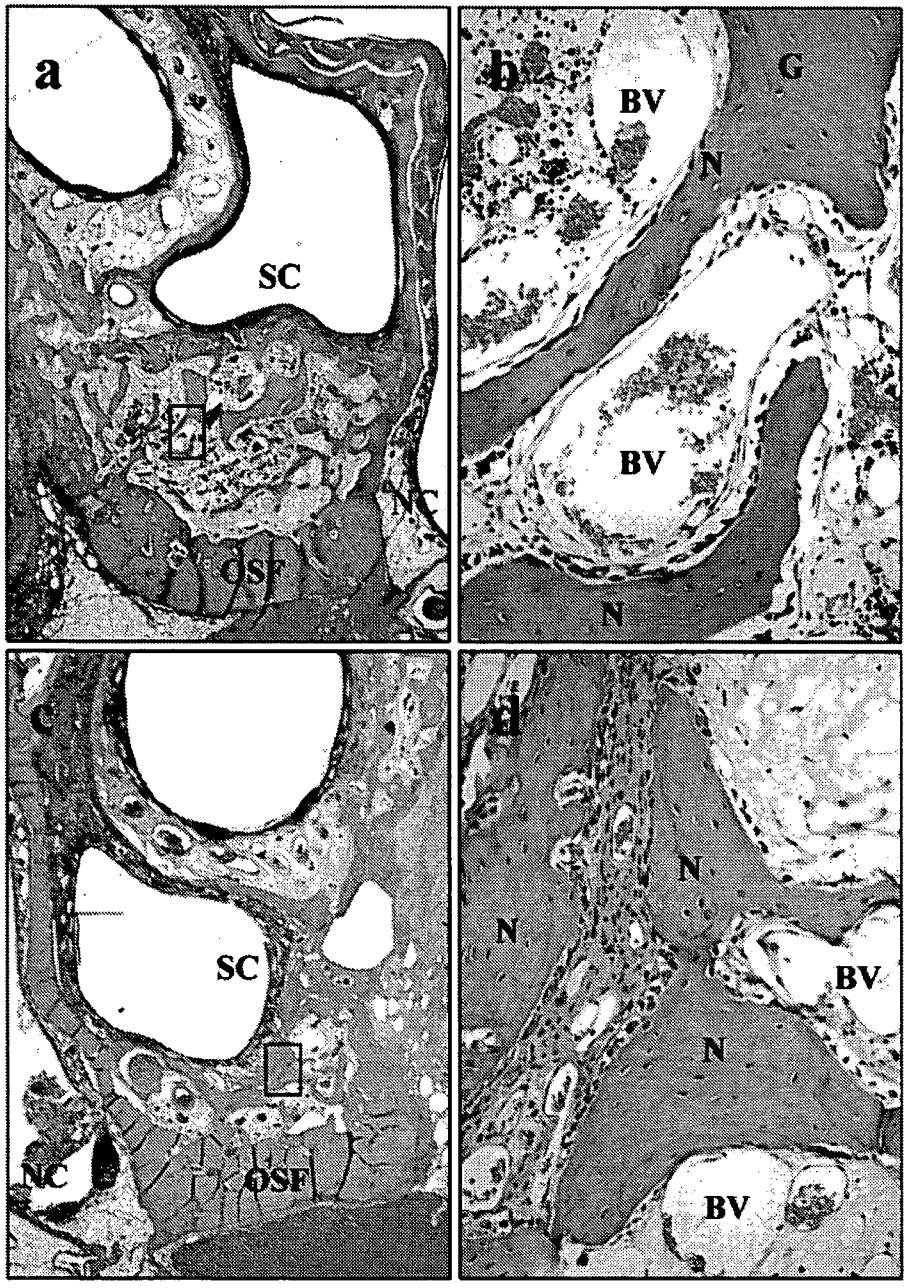

Fig. 3. Histologic evaluation of PCBM/PRP and MSCs/PRP sites at 4 weeks. Section from the (a) PCBM/PRP site (magnification × 3.125); (b) PCBM/PRP site (magnification × 50); (c) MSCs/PRP site (magnification × 3.125); (d) MSCs/PRP site (magnification × 50). SC, sinus cavity; NC, nasal cavity; OSF, original sinus floor; BV, blood vessels; N, newly formed bone; G, grafted bone; PCBM, particulate cancellous bone and marrow; PRP, platelet-rich plasma; MSCs, mesenchymal stem cells. The sections were stained by hematoxylin and eosin. Original magnification of (a) and (c), × 3.125 for all prints.

of their potential utility in tissue engineering applications (Yoshikawa et al. 1996, 1998; Pittenger et al. 1999; Noshi et al. 2001; Yamada et al. 2004a, 2004b). Artificial bone utilizing MSCs and PRP-like gel, which had a low immune rejection response from autogenous blood, was reported in a study using the mandible of the dog (Yamada et al. 2004c). However, the study did not investigate spaces such as the maxillary sinus area for dental implant installation. Thus, we examined the ability of sinus floor elevation using the tissue engineering method with MSCs/PRP complexes, and the promising PCBM/PRP complexes.

According to the histological analysis, woven bone was observed in both sites (MSCs/PRP and PCBM/PRP sites) at the early stage of 2 weeks after the transplant, and the maturity of the newly formed bone was observed at 4 weeks time dependently. However, 8 weeks after grafting, most newly formed or transplant bones were absorbed in both sites and its fatty marrow was found in the sinus floor area, and blood vessel structure was hardly observed. Therefore, it would be difficult to support implants in the posterior maxilla at 8 weeks, because it was a bone volume of around 20% with all that fatty tissue. The results of Watanabe et al. (1999) or Wada et al. (2001), which our experimental model is based upon, also showed fatty marrow in the entire the maxillary sinus at 8 weeks after the transplant when a block autogenous bone or PCBM without PRP is transplanted in the maxillary sinus floor of the rabbit. The fact that fatty marrow was formed might be because of the fatty marrow characteristic of the given animal. It is probable that the adipocyte cells were in a space where the general hematopoiesis function declined and therefore thrived when hematopoiesis cells decreased, and was not promoted by a physiological condition such as the addition of time. Conversely, when hematopoiesis cells increase, the necessary space for adipocyte cells is reduced (Tavasori & Crosby 1970; Tavassoli 1976a, 1976b). In this experiment, we observed new bone formation with newly abundant blood vessels in the early stages (2 and 4 weeks) in both groups (Figs 2 and 3), after which, the blood vessel space might be in the adipocyte cells. This stage may be a beneficial period to load cells compared with an earlier period prior to the remodeling of the bone. This procedure may be applicable to dental implants, for example, one-stage procedures that combine both dental implant installation and the bone graft, and in fact this method was successful in clinical situations (Ueda et al. 2005; Yamada et al. 2004a, 2004b), because new bone formation and active remodeling were observed when the bone was mechanically stimulated (Lanyon & Rubin 1984; Rubin & Lanyon 1987), such as by micromovement between 50 and 150 μm that starts bone formation (Szmukler-Moncler et al. 1998, 2000).

According to histomorphometric analysis, bone volume and augmented height showed peaks as early as 2 weeks and decreased over time after transplant in this study (Fig. 5a, b), whereas Wada et al. (2001) reported that newly formed bone

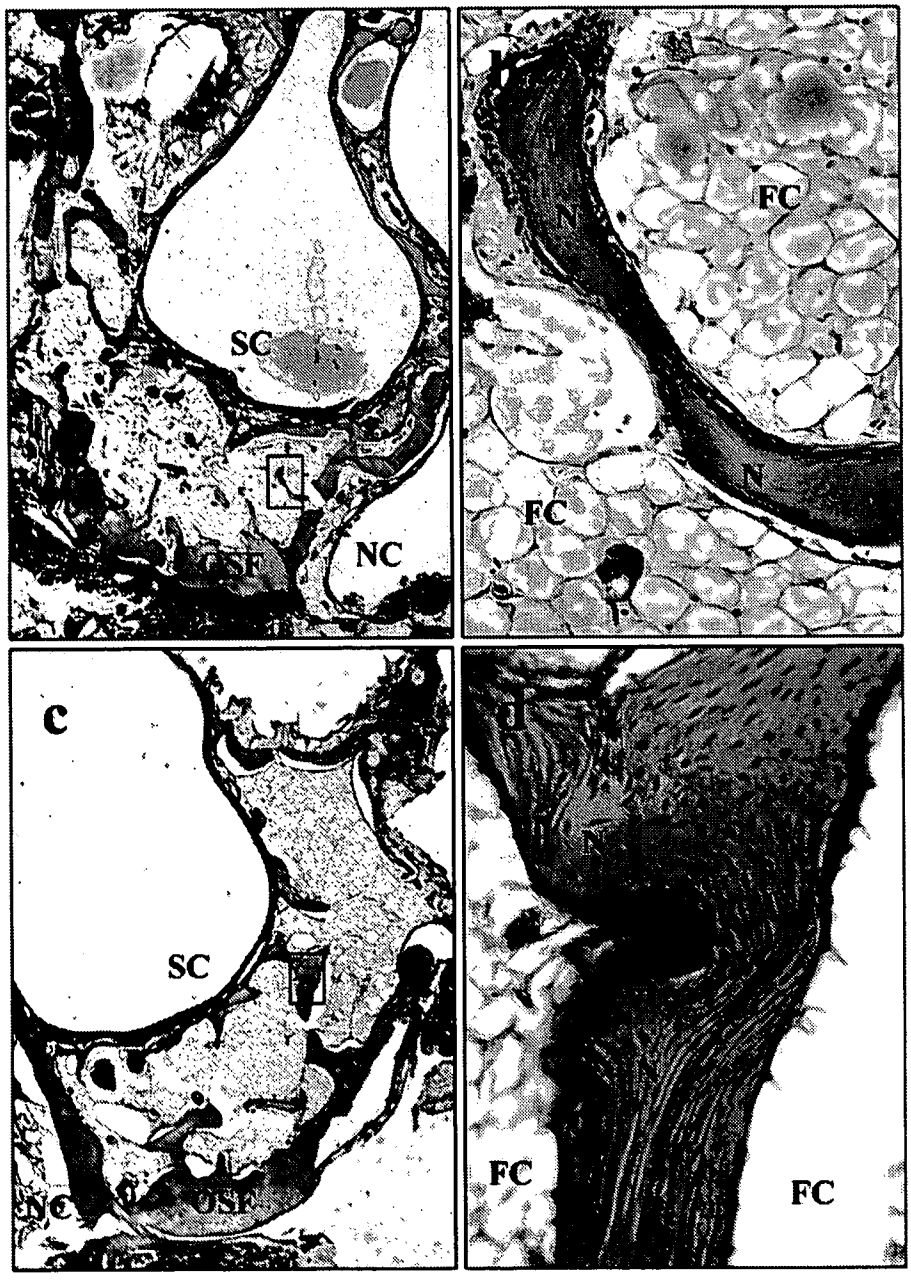

Fig. 4. Histologic evaluation of PCBM/PRP and MSCs/PRP sites at 8 weeks. Section from the (a) PCBM/PRP group (magnification × 3.125); (b) PCBM/PRP site (magnification × 50); (c) MSCs/PRP site (magnification × 3.125). (d) MSCs/PRP site (magnification × 50). SC, sinus cavity; NC, nasal cavity; OSF, original sinus floor; BV, blood vessels; N, newly formed bone; G, grafted bone; FM, fatty marrow; PCBM, particulate cancellous bone and marrow; PRP, platelet-rich plasma; MSCs, mesenchymal stem cells. The sections were stained by hematoxylin and eosin.

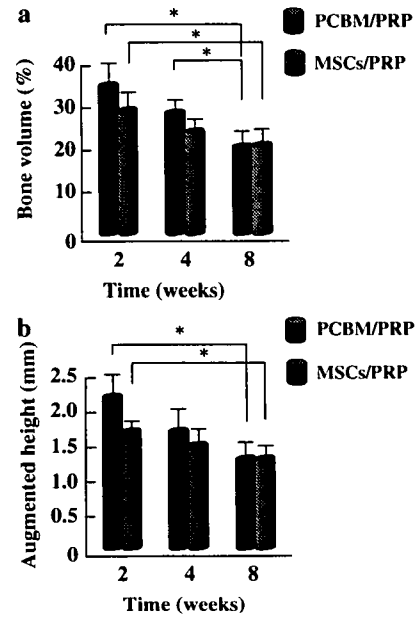

Fig. 5. Histomorphometrical evaluation. Bone volume (a) was 35 ± 5.2%, 28.6 ± 3.4%, 20.6 ± 4% in the PCBM/PRP group (blue) and 29.1 ± 4.4%, 24.1 ± 3.6%, 20.9 ± 4.1% in the MSCs/PRP group (red) at 2, 4, and 8 weeks after transplantation, respectively. Asterisks indicate significant differences ($^*P<0.05$). Augmented height (b) was 2.1 ± 0.5, 1.7 ± 0.6, 1.3 ± 0.4 mm in the PCBM/PRP group (blue) and 1.7 ± 0.2, 1.5 ± 0.3, 1.3 ± 0.3 mm in the MSCs/PRP group (red) at 2, 4, and 8 weeks after transplantation, respectively. Asterisks indicate significant differences ($^*P<0.05$). PCBM, particulate cancellous bone and marrow; PRP, platelet-rich plasma; MSCs, mesenchymal stem cells.

volume reaches a peak at 4 weeks and the volume showed the same value as our value at 8 weeks in transplanted PCBM without PRP to the same sinus floor elevation model. Thus, the early peak in the present study might be because of a bone-promoting effect by PRP, which is known to enhance the formation of new bone and accelerate wound healing (Marx et al. 1998). The use of PRP might provide optimal conditions for more rapid and effective bone regeneration of the grafted bone or MSCs. On the other hand, the bone absorption rates in MSCs/PRP or PCBM/PRP groups at 4 weeks from 2 weeks showed about 5% or 6.4%, respectively. This might be related to the process of bone formation such as the MSCs/PRP group induced bone tissue formation, which then MSCs self-organized according to the surrounding environment, although osteogenesis was induced from the surroundings of the grafted bone in the PCBM/PRP group.

In summary, the MSCs/PRP complex had well-formed newly formed bone and neovascularization, compared with the PCBM/PRP complex. The findings of this experimental study indicate that the use of a mixture of MSCs /PRP complex yielded good results in osteogenesis and bone volume comparable with that achieved by autogenous bone, and PCBM in sinus floor elevation. Therefore, this application of this promising new sinus floor elevation method for dental implants with tissue engineering technology deserves further study.

Acknowledgments: The authors wish to thank Drs Hideharu Hibi, Ken-ichiro Hata, Keisuke Wada, Kazuyo Watanabe, and members of the Department of Oral and Maxillofacial

Surgery, Nagoya University Graduate School of Medicine for their generous assistance, encouragement, and contributions to the completion of this study. The authors are also indebted to Drs Hideaki Kagami, Kunihiko Okada, Yuki Ito, Kana Ichihashi, and members of the Department of Tissue Engineering, Nagoya University School of Medicine for their help and discussion. This work was partly supported by the Japan Society for the Promotion of Science (JSPS), KAKENHI (16390583) and The Ministry of Education, Culture, Sports, Science and Technology (MEXT), KAKENHI (15791163).

Résumé

La possibilité de régénérer l'os dans l'épaississement du sinus a été comparée entre une méthode de travail du tissu en utilisant des cellules souches primaires mésenchymateuses (MSC) et du plasma riche en plaquettes (PRP) et une nouvelle méthode utilisant de l'os cortical et spongieux (PCBM) et PRP. L'épaississement bilatéral des sinus a été effectué chez 18 lapins blancs japonais adultes. Les complexes MSC / PRP ou PCBM/PRP ont été placés dans chaque sinus de ces lapins. Les MSC étaient isolés à partir de la moelle de la crête iliaque du lapin et le PRP était obtenu du sang périphérique. Le PCBM étaient collectés de la crête iliaque du lapin et mélangés au PRP. Les animaux ont été sacrifiés deux, quatre et huit semaines après la transplantation et l'habilité à former de l'os de chaque implant a été évaluée tant histologiquement que histométriquement. Suivant les observations histologiques, les deux sites montraient de l'os néoformé ainsi qu'une néovascularisation après deux et quatre semaines. Cependant, après huit semaines, l'os lamellaire était occupé par une moelle grasse dans des grandes aires des deux sites. Il n'y avait aucune différence significative dans le volume osseux ou la hauteur d'épaississement entre les deux groupes, mais il y avait des différences significatives dans le volume osseux et la hauteur d'épaississement entre deux et huit semaines dans les deux groupes et dans le volume osseux entre les semaines 4 et 8 dans le groupe PCBM/PRP ($p<0,05$). Le complexe MSC/PRP peut aussi bien être utilisé pour la régénération osseuse dans l'épaississement sinusal que le complexe PCBM/PRP.

Zusammenfassung

In dieser Arbeit verglichen wir die Knochenregenerationsfähigkeit von zwei verschiedenen Methoden zur Sinusbodenelevation. Einmal behandelte man das Gewebe mit mesenchymalen Stammzellen (MSCs), vermischt mit plättchenangereichertem Plasma (PRP), und einmal, eine vielversprechende neue Methode, mit zerkleinertem spongiösem Knochen und Knochenmark (PCBM), vermischt mit PRP. Bei 18 erwachsenen japanischen weissen Kaninchen führte man beidseits eine Sinusbodenelevation durch. Auf der einen Seite baute man den Sinus maxillaris mit MSCs/PRP, auf der anderen Seite desselben Kaninchens mit PCBM/PRP auf. Die MSCs hatte man aus dem Knochenmark des Beckenknochens, das PRP aus venösem Blut desselben Kaninchens gewonnen. PCBM hatte man vom Beckenkamm des Kaninchens entnommen und es mit PRP gemischt. Die Tiere opferte man 2, 4 und 8 Wochen nach der Transplantation und untersuchte dann histologisch und histometrisch die Knochenregenerationsfähigkeit jedes Implantates. Den histologischen Beobachtungen zufolge zeigten beide Seiten (MSCs/PRP und PCBM/PRP) nach 2 und 4 Wochen neu gebildeten Knochen und Neueinsprossungen von Gefässen. Nach 8 Wochen aber beobachtete man auf beiden Seiten, dass der lamelläre Knochen in grossen Teilen mit fetthaltigem Knochenmark belegt war. Zu keinem Zeitpunkt fand man zwischen den beiden Gruppen (MSCs/PRP und PCBM/PRP) signifikante Unterschiede bezüglich Knochenvolumen oder Höhe des Augmentates. Wohl aber bestanden signifikante Unterschiede bezüglich Knochenvolumen oder Höhe des Augmentates zwischen Woche 2 und 8 bei beiden Gruppen (MSCs/PRP und PCBM/PRP) und bezüglich Knochenvolumen zwischen Woche 4 und 8 bei der PCBM/PRP-Gruppe ($P<0.05$). Diese Resultate lassen vermuten, dass ein Gemisch von MSCs/PRP genausogut wie ein Gemisch von PCBM/PRP zur Förderung der Knochenregeneration bei der Sinusbodenelevation verwendet werden kann.

Resumen

En el presente estudio, homos comparado la habilidad de regeneración en la elevación del seno maxilar entre un método de ingeniería tisular usando células madre mesenquimales (MSCs) y plasma rico en plaquetas (PRP), y un prometedor nuevo método usando hueso esponjoso particulado y médula (PCBM) y PRP. Se llevaron a cabo procedimientos de elevación del seno bilateralmente en 18 conejos blancos japoneses jóvenes. Se injertaron complejos MSCs/PRP o PCBM/PRP en cada seno maxilar en el mismo conejo. Se aislaron las MSCs de la médula de la cresta iliaca del conejo, y el PRP se obtuvo de la sangre periférica. El PCBM se recolectó de la cresta iliaca y se mezcló con el PRP. Los animales se sacrificaron a las 2, 4 y 8 semanas tras el transplante, y se evaluó la habilidad de formación de hueso de cada implante histológica e histometricamente. De acuerdo con las observaciones histológicas, ambos lados (MSCs/PRP y PCBM/PRP) mostraron hueso neoformado y neovascularización a las semanas 2 y 4. De todos modos, a las 8 semanas, el hueso lamelar se observó ocupado por medula grasa en amplias áreas de ambos lados. No hubo una diferencia significativa en el volumen óseo o en la altura aumentada entre los grupos MSCs/PRP y PCBM/PRP alrededor de cada semana, pero si hubo diferencias significativas en el volumen óseo y altura aumentada entre las semanas 2 y 8 en los grupos PCBM/PRP o MSCs/PRP y en el volumen óseo entre las semanas 4 y 8 en el grupo PCBM/PRP ($P<0.05$). Estos resultados sugieren que el complejo MSCs/PRP puede muy bien ser usado para regeneración ósea en elevación del seno, comparado con el complejo PCBM/PRP.

要旨

本研究では上顎洞挙上術において間葉幹細胞（ＭＳＣ）と多血小板血漿（ＰＲＰ）を用いた組織工学の方法と、顆粒状海綿骨と骨髄（ＰＣＢＭ）及びＰＲＰを用いた新規の方法の骨再生能を比較した。日本家兎成獣１８羽において両側上顎洞挙上術を行った。各家兎の各側上顎洞に**MSC/PRP**あるいは**PCBM/PRP**の複合体を移植した。**MSC**は家兎の腸骨稜骨髄から分離し、**PRP**は末梢血から得た。**PCBM**は家兎の腸骨稜から採取し、**PRP**と混ぜた。動物は移植後２、４、８週間後に屠殺し、各インプラントの骨形成能を組織学及び組織形態計測によって評価した。組織像の観察によると両側（**MSC/PRP**及び**PCBM/PRP**）とも２週間後と４週間後に、新生骨の良好な形成と新生血管を示した。しかし８週間後には両側とも層状骨は広範にわたって脂肪性骨髄によって占められていた。どの週にも**MSC/PRP**と**PCBM/PRP**の群間に骨量または造成した骨高径の有意差は認められなかったが、**PCBM/PRP**または**MSC/PRP**群の間に２週間後と８週間後の間には骨量と造成した骨高径に有意差が認められた。また**PCBM/PRP**群において４週間後と８週間後の間に骨量に有意差が認められた（$p<0.05$）。これらの結果は、**MSC/PRP**の複合体は**PCBM/PRP**複合体に比べて上顎洞挙上術における骨再生にとって有効であることを示唆している。

References

Anitua, E. (1999) Plasma rich in growth factors: preliminary results of use in the treatment of future sites for implants. *International Journal of Oral & Maxillofacial Implants* **14**: 529–535.

Block, M.S. & Kent, J.N. (1997) Sinus augmentation for dental implants: the use of autogenous bone. *Journal of Oral and Maxillofacial Surgery* **55**: 1281–1286.

Boyne, P.J., James, R.A. & Linda, L. (1980) Grafting of the maxillary sinus floor with autogenous marrow and bone. *Journal of Oral and Maxillofacial Surgery* **38**: 613–616.

Canalis, E. (1981) Effect on platelet-derived growth factor DNA and protein synthesis in cultured rats calvaria. *Metabolism* **30**: 970–975.

Caplan, A.I. (1991) Mesenchymal stem cells. *Journal of Orthopaedic Research* **9**: 641–650.

Cho, M.I. , Lin, W.L. & Genco, R.J. (1995) Platelet-derived growth factor modulated guided tissue regenerative therapy. *Journal of Periodontology* **66**: 522–528.

Jensen, S.S., Aaboe, M., Pinholt, E.M., Hjørting-Hansen, E., Melsen, F. & Ruyter, I.E. (1996) Tissue reaction and material characteristics of four bone substitutes. *International Journal of Oral & Maxillofacial Implants* **11**: 55–66.

Kadiyara, S., Jaiswal, N. & Bruder, S. (1997a) Culture-expanded bone marrow-derived mesenchymal stem cells can regenerate a critical-size segmental bone defect. *Tissue Engineering* **3**: 173–185.

Kadiyara, S., Young, R.G., Thiede, M.A. & Bruder, S.P. (1997b) Culture expanded canine mesenchymal stem cells possess osteochondrogenic potential in vivo and in vitro. *Cell Transplantation* **6**: 125–134.

Kent, J.N. & Block, M.S. (1989) Simultaneous maxillary sinus floor bone grafting and placement hydroxylapatite-coated implants. *Journal of Oral and Maxillofacial Surgery* **47**: 238–242.

Langer, R. & Vacanti, J.P. (1993) Tissue engineering. *Science* **260**: 920–926.

Lanyon, L.E. & Rubin, C.T. (1984) Static vs dynamic loads as an influence on bone remodeling. *Journal of Biomechanics* **17**: 897–905.

Laurie, S.W.S., Kaban, L.B., Mulliken, J.B. & Murray, J.E. (1984) Donor site morbidity after harvesting rib and iliac bone. *Plastic and Reconstructive Surgery* **73**: 933–938.

Leonardis, D. & Pecora, G.E. (1999) Augmentation of the maxillary sinus with calcium sulfate: one year clinical report from a prospective longitudinal study. *International Journal of Oral & Maxillofacial Implants* **14**: 869–878.

Lundgren, S., Moy, P., Johansson, C. & Nilsson, H. (1996) Augmentation of the maxillary sinus floor with particulated mandible. *International Journal of Oral & Maxillofacial Implants* **11**: 760–766.

Marx, R.E., Carlson, E.R., Eichstaedt, R.M., Schimmele, S.R., Stauss, J.E. & Georgeff, K.R. (1998) Platelet-rich plasma, growth factor enhancement for bone grafts. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology Endodontology* **85**: 638–646.

Noshi, T., Yoshikawa, T., Dohi, Y., Ikeuchi, M., Horiuchi, K., Ichijima, K., Sugimura, M., Yonemasu, K. & Ohgushi, H. (2001) Recombinant human bone morphogenic protein-2 potentiates the in vivo osteogenic ability of marrow/hydroxyapatite composites. *Artificial Organs* **25**: 201–208.

Owen, M. & Friedenstein, A.J. (1998) Stromal stem cells: marrow derived osteogenic precursors. *Ciba Foundation Symposium* **136**: 42–60.

Petite, H., Viateau, V., Bensaid, W., Meunier, A., de Pollak, C. & Bourguignon, C. (2000) Tissue-engineered bone regeneration. *Nature Biotechnology* **18**: 959–963.

Piatelli, M., Favero, G.A., Scarano, A., Orsini, G. & Piatelli, A. (1999) Bone reactions to anorganic bovine bone (Bio-Oss) used in sinus augmentation procedures. *International Journal of Oral & Maxillofacial Implants* **14**: 835–840.

Pittenger, M.F., Mackay, A.M., Beck, C.B., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S. & Marshak, D.R. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284**: 143–147.

Rubin, C.T. & Lanyon, L.E. (1987) Osteoregulatory nature of mechanical stimuli: function as a determinant for adaptive remodeling in bone. *Journal of Orthopaedic Research* **5**: 300–310.

Summers, B.N. & Eisenstein, S.M. (1984) Donor site pain from the ileum: a complication of lumbar spine fusion. *Journal of Bone and Joint Surgery–British volume* **71**: 677–680.

Szmukler-Moncler, S., Piattelli, A., Faver, GA. & Dubruille, J.H. (2000) Considerations preliminary to the application of early and immediate loading protocols in dental implantology. *Clinical Oral Implants Research* **11**: 12–25.

Szmukler-Moncler, S., Salama, H., Reingewirtz, Y. & Dubruille, J.H. (1998) Timing of loading and effect of micromotion on bone–dental implant interface: review of experimental literature. *Journal of Biomedical Materials Research* **43**: 192–203.

Tatum, H. (1986) Maxillary and sinus implant reconstructions. *Dental Clinics of North America* **30**: 207–229.

Tavassoli, M. (1976a) Ultrastructural development of bone marrow adipose cell. *Acta Anatomica* **94**: 65–77.

Tavassoli, M. (1976b) Marrow adipose cells. Histochemical identification of labile and stable components. *Archives of Pathology & Laboratory Medicine* **100**: 16–18.

Tavassoli, M. & Crosby, W.H. (1970) Bone marrow histogenesis: a comparison of fatty and red marrow. *Science* **169**: 291–293.

Ueda, M., Yamada, Y., Ozawa, R. & Okazaki, Y.A. (2005) Clinical report of injectable tissue-engineered bone applied for alveolar augmentation with simultaneous implant placement. *The International Journal of Periodontics and Restorative Dentistry* **2**: 129–137.

Vacanti, J.P. (1988) Beyond transplantation. *Archives of Surgery* **123**: 545–549.

Vacanti, J.P., Morse, M.A., Saltzman, W.M., Domb, A.J., Perez-Atayde, A. & Langer, R. (1988) Selective cell transplantation using bioabsorbable artifical polymers as matrices. *Journal of Pediatric Surgery* **23**: 3–9.

Wada, K., Niimi, A., Watanabe, K., Sawai, T. & Ueda, M. (2001) Maxillary sinus floor augmentation in rabbits. *The International Journal of Periodontics and Restorative Dentistry* **21**: 253–263.

Watanabe, K., Niimi, A. & Ueda, M. (1999) Autogenous bone grafts in the rabbit maxillary sinus. *Oral Surgery Oral Medicine Oral Pathology* **88**: 26–32.

Wiltfang, J., Schlegel, K.A., Schultze-Mosgau, S., Nkenke, E., Zimmeremann, R. & Kessler, P. (2003) Sinus floor augmentation with α-tricalciumphosphate (α-TCP): does platelet-rich plasma promote its osseous integration and degradation? *Clinical Oral Implants Research* **14**: 213–218.

Yamada, Y., Ueda, M., Hibi, H. & Nagasaka, T. (2004a) Translational research for injectable tissue-engineered bone regeneration using mesenchymal stem cells and platelet-rich plasma – from basic research to clinical case study. *Cell Transplantation* **13**: 343–355.

Yamada, Y., Ueda, M., Naiki, T. & Nagasaka, T. (2004b) Tissue-engineered injectable bone regeneration for osseointegrated dental implants. *Clinical Oral Implants Research* **15**: 589–597.

Yamada, Y., Ueda, M., Naiki, T., Takahashi, M., Hata, K. & Nagasaka, T. (2004c) Autogenous injectable bone for regeneration with mesenchymal stem cells and platelet-rich plasma.-Tissue-engineered bone regeneration-. *Tissue Engineering* **10**: 955–964.

Yosikawa, T., Ohgushi, H., Akahane, M., Tamai, S. & Ichijima, K. (1998) Analysis of gene expression in osteogenic cultured marrow/hydroxyapatite construct implanted at ectopic sites: a comparison with the osteogenic ability of cancellous bone. *Journal of Biomedical Materials Research* **41**: 568–573.

Yosikawa, T., Ohgushi, H. & Tamai, S. (1996) Immediate bone forming capability of prefabricated osteogenic hydroxyapatite. *Journal of Biomedical Materials Research* **32**: 481–492.

Younger, E.M. & Chapman, M.W. (1989) Morbidity at bone graft donor sites. *Journal of Orthopaedic Trauma* **3**: 192–195.

Kenji Ito
Yoichi Yamada
Takahito Naiki
Minoru Ueda

Simultaneous implant placement and bone regeneration around dental implants using tissue-engineered bone with fibrin glue, mesenchymal stem cells and platelet-rich plasma

Authors' affiliations:
Kenji Ito, Takahito Naiki, Minoru Ueda, Department of Oral and Maxillofacial Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan
Yoichi Yamada, Center for Genetic and Regenerative Medicine, Nagoya University School of Medicine Nagoya, Japan

Correspondence to:
Assist. Prof. Yoichi Yamada
Center for Genetic and Regenerative Medicine
Nagoya University School of Medicine,
65 Tsuruma-cho
Showa-ku
Nagoya 466-8550
Japan
Tel.: +81 52 744 2348
Fax: +81 52 744 2352
e-mail: yyamada@med.nagoya-u.ac.jp

Date:
Accepted 5 October 2005

To cite this article:
Ito K, Yamada Y, Naiki T, Ueda M. Simultaneous implant placement and bone regeneration around dental implants using tissue-engineered bone with fibrin glue, mesenchymal stem cells and platelet-rich plasma. *Clin. Oral Impl. Res.* 17, 2006; 579–586
doi: 10.1111/j.1600-0501.2005.01246.x

Key words: injectable tissue-engineered bone, mesenchymal stem cells (MSCs), platelet-rich plasma (PRP), simultaneous implant placement, tissue engineering

Abstract: This study was undertaken to evaluate the use of tissue-engineered bone as grafting material for alveolar augmentation with simultaneous implant placement. Twelve adult hybrid dogs were used in this study. One month after the extraction of teeth in the mandible region, bone defects on both sides of the mandible were induced using a trephine bar with a diameter of 10 mm. Dog mesenchymal stem cells (dMSCs) were obtained via iliac bone biopsy and cultured for 4 weeks before implantation. After installing the dental implants, the defects were simultaneously implanted with the following graft materials: (i) fibrin, (ii) dMSCs and fibrin (dMSCs/fibrin), (iii) dMSCs, platelet-rich plasma (PRP) and fibrin (dMSCs/PRP/fibrin) and (iv) control (defect only). The implants were assessed by histological and histomorphometric analysis, 2, 4 and 8 weeks after implantation. The implants exhibited varying degrees of bone–implant contact (BIC). The BIC was 17%, 19% and 29% (control), 20%, 22% and 25% (fibrin), 22%, 32% and 42% (dMSCs/fibrin) and 25%, 49% and 53% (dMSCs/PRP/fibrin) after 2, 4 and 8 weeks, respectively. This study suggests that tissue-engineered bone may be of sufficient quality for predictable enhancement of bone regeneration around dental implants when used simultaneous by with implant placement.

The use of dental implants in oral rehabilitation is becoming a standard method of care in dentistry. In the case of insufficient bone volume, a procedure for augmentation is needed. The ability to augment the alveolar ridge has gradually expanded the scope of implant dentistry. During the past 10 years, alveolar augmentation techniques have become established treatment modalities. Dahlin et al. (1989) reported an experimental study on rabbits involving the formation of new bone around titanium implants using the membrane technique. In addition, various bone-grafting materials have been used for augmentation, including autogenous grafts, freeze-dried bone grafts, hydroxyapatite and xenografts (Hirsch & Ericsson 1991; Smiler et al. 1992). Although the results of these investigations indicate that augmentation is clinically successful for various graft materials, it is questionable whether these materials, except for autogenous bone, have adequate osteogenic potential and biomechanical properties (Moy et al. 1993; Wheeler et al. 1996). On the other hand, autogenous bone, which currently remains the material of choice, is available for bone reconstructive procedures (Wood & Moore 1989). However, its use is limited due to donor site morbidity and limited amounts of graft material available for harvesting.

To avoid these problems, we attempted to regenerate bone in a significant osseous

defect with minimal invasiveness, and to provide a clinical alternative to the graft materials described above. The new technology that we developed is called 'injectable tissue-engineered bone' (Yamada et al. 2003, 2004a), and involves the morphogenesis of new tissue using constructs formed from isolated cells with biocompatible scaffolds and growth factor, and was established based on tissue engineering concepts (Langer & Vacanti 1993). We previously reported that tissue-engineered bone induces excellent bone regeneration and promotes bone formation in a grafted area treated with platelet-rich plasma (PRP), which contains various growth factors (Yamada et al. 2004a).

Recently, for dental implant treatments, a two-step procedure, which allows for placement of the implants after primary healing and remodeling of the graft, has been recommended for patients with less than 5 mm of alveolar bone height in the posterior maxilla or alveolar ridge. On the other hand, the one-step procedure, which places dental implants simultaneously with the grafting for the patients who have at least 5 mm of alveolar bone to stabilize the implants (Jensen et al. 1990; Raghoebar et al. 1993; Marx 1994), offers the advantages of less surgical treatment and coordinated consolidation of the graft around the implants during healing, thus reducing both the number of surgical procedures and the healing time.

Therefore, we explored the application of tissue-engineered bone with plasticity for the one-step procedure and the ability of mesenchymal stem cells (MSCs), PRP and fibrin to increase the rate of bone formation and to enhance bone regeneration.

Material and methods

Canine animal model

All animal experiments were performed in strict accordance with protocols approved by the Institutional Animal Care Committee. After a period of housing, 12 adult hybrid dogs with a mean age of 2 years were operated on under general anesthesia. The first molar, premolars and the second and third premolars in the mandible region were extracted and were then allowed to heal for 1 month. Three bone defects each in the right and left mandible were prepared using a 10 mm diameter trephine bar. After ∅3.75 × 7 mm Brånemark implants (Nobel Biocare Norden AB, Gothenburg, Sweden) were installed into the defects, they were implanted using the following graft materials: fibrin glue (fibrin); dog mesenchymal stem cells and fibrin glue (dMSCs/fibrin); dMSCs, platelet-rich plasma and fibrin glue (dMSCs/PRP/fibrin); or control (defect only). Nonabsorbent membranes (Gore-Tex®, W.L. Gore & Associates, Flagstaff, AZ, USA) were used for preventing connective tissue prolapse in this experiment. The selection of the treatments and localizations was random. Then, we examined the resultant osteogenesis by histological and histomorphometric analyses.

MSCs isolation and cultivation

dMSCs were isolated from 10 ml samples of dog iliac bone marrow aspirates. Bone marrow cell isolation and expansion was performed according to previously published methods (Kadiyala et al. 1997). Briefly, basal medium (condition medium), low-glucose Dulbecco's modified Eagles medium (DMEM) and growth supplements consisting of 50 ml of mesenchymal cell growth supplement, 10 ml of 200 mM L-glutamine and 0.5 ml of a penicillin–streptomycin mixture containing 25 U of penicillin and 25 μg of streptomycin were purchased from Cambrex® Inc. (Walkersville, MD, USA). The three supplements used for inducing osteogenesis, dexamethasone (Dex), sodium β-glycerophosphate (β-GP) and L-ascorbic acid 2-phosphate (AsAP), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The cells were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. dMSCs were replated at a density of 3.1×10^3 cells/cm^3 in 0.2 ml/cm^2 condition medium. Differentiated dMSCs were identified by detecting alkaline phosphatase (ALP) activity using *p*-nitrophenylphosphatase as a substrate. In culture, dMSCs were trypsinized and used for implantation (Fig. 1).

Preparation of fibrin glue

Pasteurized fibrin glue (Bolheal, the Chemosero-Therapeutic Research Institute, Kumamoto, Japan) was prepared by mixing solutions A and B, where solution A consisted of 80 mg/ml fibrinogen and 75 U/ml fibrin-stabilizing factor XIII dissolved in 1 ml of plasmin inhibitor aprotinin (1000 kIM/ml) and solution B contained 250 U of thrombin dissolved in 1 ml of 40 μM $CaCl_2$. The solutions were mixed in a 1 : 1 (volume/volume) ratio. The clotting reaction between A and B produced a semirigid three-dimensional network in typical reactions at room temperature.

The measurement of ALP activity of MSCs mixed with various ratios of fibrin and PRP *in vitro* dMSCs isolated as described above were used. By mixing various ratios of fibrin and PRP (0%, 20%, 40%, 60%, 80% and 100% of PRP, where 100% means PRP only and 0% means fibrin only) with the dMSCs, we investigated the effect of this mixture as a scaffold for bone formation *in vitro* by measuring ALP activity. ALP activity was measured in the supernatant of the homogenate of the dMSCs/PRP/fibrin glue admixture in culture medium by using *p*-nitrophenylphosphatase as a substrate at 1, 3, 6, 9, 12 and 18 days *in vitro*. Briefly, an aliquot (5 μl) of the homogenate supernatant was added to 1 ml of 50 mM *p*-nitrophenyl phosphate containing 1 mM $MgCl_2$ and the mixture was incubated for 30 min at 37°C. Two milliliters of 0.2 N NaOH was added to stop the enzymatic reaction, and the absorption at 410 nm was measured spectrophotometrically. ALP activity was expressed as micromoles of *p*-nitrophenol released per composite after 30 min of incubation at 37°C. Each value is the mean value obtained from five independent experiments.

PRP preparation and injection of dMSCs and/or PRP /fibrin glue admixtures

Approximately 50 ml of whole blood was withdrawn from each dog into a centrifuge tube containing 10 ml of culture medium with 250 U/ml of preservative-free heparin. The blood was first centrifuged in a standard laboratory Himac CT centrifuge (Hitachi koki, Hitachi, Japan) for 5 min at 1100 rpm. Subsequently, the yellow plasma containing the buffy coat with platelets and leukocytes was taken up into a neutral monovette with a long cannula. A second centrifugation at 2500 rpm for 10 min was performed to combine the platelets into a single pellet, and the plasma supernatant, which was platelet-poor plasma (PPP) and contained relatively few cells, was removed. The resulting pellet of

platelets, termed the buffy coat/plasma fraction (PRP), was re-suspended in 5 ml of residual plasma and used for the platelet gel. Platelet counts for the PRP and PPP were measured using a BECKMAN COULTER GEN*S™ and STKS•RE™ (Beckman Coulter, Fullerton, CA, USA). The PRP was stored at room temperature in a conventional shaker until use. Five thousand units of bovine thrombin in powdered form was dissolved in 5 ml of 10% calcium chloride in a separate sterile cup. Next, 0.2 ml of PRP, 1×10^7 dMSCs and 0.15 ml of fibrin glue (Solution A) and 0.5 ml of air were aspirated into a 2.5 ml syringe, and in a second 2.5 ml syringe 33 µl of the thrombin/calcium chloride and 0.15 ml of fibrin glue (Solution B) mixture was aspirated. The two syringes were connected with a 'T' connector and the plungers of the syringes were alternatively pushed and pulled, thereby allowing the trapped air bubble to transverse the two syringes. Within 5–30 s, the contents assumed a gel-like consistency as the thrombin affected the polymerization of the fibrin to produce an insoluble gel. In the *in vivo* study, the gel was injected into the bone defect field using a 16-gauge needle attached to a 5 ml syringe. Samples were analyzed 2 ($n = 6$), 4 ($n = 6$), and 8 ($n = 6$) weeks after injection.

Histological and histomorphometric analyses

The dogs were killed 2, 4 and 8 weeks after dental implant insertion. Their mandibles were dissected and cut into smaller blocks, and block sections were fixed in 10% formaldehyde. The sections were then embedded in methylmethacrylate (Technovit 7200VLC, Kulzer GmbH, Wehrheim, Germany) and polymerized. They were next sectioned and ground to 10 µm thickness using an Exact Cutting–Grinding System (Exact Apparatebau, Norderstedt, Germany), and stained with toluidine blue. Histological analysis was performed to obtain a general description of the tissue surrounding the implants. Histomorphometrical analysis was performed by using a light microscope (Hitachi Tablet Digitizer HDG-1212D, Hitachi Seiko Ltd., Tokyo, Japan) connected to a PC equipped with a video and image analysis system (System Supply Co. Ltd., Ina, Japan). The following histomorphometrical analyses were carried out:

Bone–implant contact (BIC) (%) = (total length of bone contact/total length of implant surfaces) × 100.

Statistical analysis

Group means and standard deviations were calculated for each parameter. The data were compared using the paired two-tailed Student's t-test between the controls and the fibrin, dMSC/fibrin, dMSCs/PRP/fibrin groups. A P-value of <0.05 was considered to indicate statistical significance.

Results

The dependence of ALP activity on the mixing ratio between fibrin glue and PRP plus cells (dMSCs) *in vitro*.

The mean platelet count of the PRP used in this experiment was 1,155,000, with a range of 680,000–1,630,000. These values confirmed the platelet sequestration ability of the process, which showed that the concentration was 464% above the baseline platelet counts. We investigated the effect of PRP in fibrin glue on the production of ALP activity to find the optimum mixing ratio for the enhancement of bone

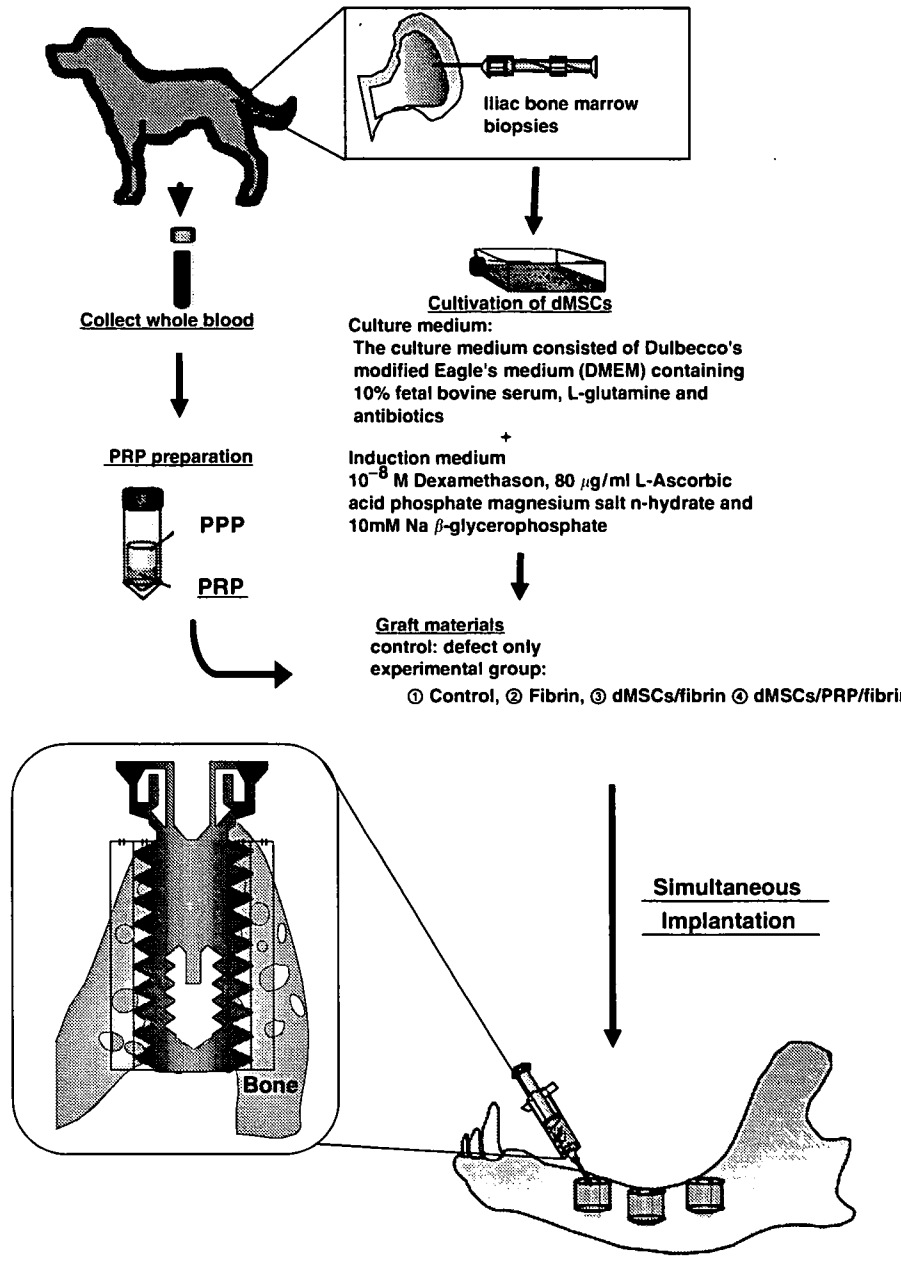

Fig. 1. Schematic of the experimental protocol.

formation. With the 0% and 20% mixing ratios, ALP activity was hardly observed. On the other hand, at ratios of 40% and above, the ALP activity sharply increased after 9 days (Table 1).

In vivo macro evaluation of the implants (with fibrin, dMSCs/fibrin, dMSCs/PRP/fibrin) compared with the control

Macroscopic findings showed that bone regeneration around the dental implants promoted by dMSCs/PRP/fibrin and dMSCs/fibrin resulted in a natural marginal bone level, but regeneration induced by fibrin or the control (defect only) was incomplete. The dental implant thread was exposed in the fibrin and control groups, but not in the dMSCs/PRP/fibrin and dMSC/fibrin groups (Fig. 2).

Histological findings and histomorphometric analysis of osseointegrated dental implants

All implants and guided bone regeneration (GBR) membranes healed uneventfully and remained stable throughout the experimental period, and experimental groups with membrane exposure were excluded. At 2 weeks, the control, fibrin and dMSCs/fibrin groups showed only very little new bone formation around the dental implants. The dMSCs/PRP/fibrin group showed partial bone regeneration on the implant surface (Fig. 3). At 4 weeks, the control and fibrin groups were not sufficiently regenerated in the buccal and lingual sites. At the dMSCs/fibrin site, the buccal wall site had a thin partial bony layer on the implant surface. At the dMSCs/PRP/fibrin graft site, excellent bone regeneration was seen in the buccal and lingual sites, and the bone regenerated by dMSCs/PRP/fibrin had a newly formed woven and lamellar structure (Fig. 4). At 8 weeks, the control and fibrin sites still possessed partial regenerated bone connective tissue around the dental implants. In the dMSCs/fibrin site, mature bone regeneration was gradually observed. In the dMSCs/PRP/fibrin site, bone regeneration was sufficient at the buccal site and also showed a mature lamellar structure (Fig. 5).

The implants exhibited varying degrees of bone-implant contact (BIC). The BIC was 17%, 19% and 29% (control), 20%, 22% and 25% (fibrin), 22%, 32% and 42% (dMSCs/fibrin) and 25%, 49% and 53% (dMSCs/PRP/fibrin) after 2, 4 and 8 weeks, respectively (Fig. 6). At 4 weeks, the BIC for dMSC/PRP/fibrin showed a markedly greater increase than the BIC in the other groups, and was about twice their levels. The BIC of dMSCs/PRP/fibrin was the highest among the graft materials and showed a significant increase ($P<0.05$) on the implant surface compared with the BIC of the control (Fig. 6).

Table 1. **The correlation between fibrin glue and PRP in ALP activity**

PRP/ fibrin (%)	Induction time:days					
	1	3	6	9	12	18
0	0.2	0.2	0.1	0.4	0.2	2
20	0.5	0.7	0.6	0.5	0.7	1.6
40	0.4	1.3	1.6	5.4	2.7	2.8
60	0.9	1.2	0.8	5	2.9	2.3
80	0.8	0.9	0.8	4.8	2.1	2.2
100	0.1	1.2	0.8	5.5	2.6	3.1

PRP, platelet-rich plasma; ALP, alkaline phosphatase.

Discussion

To prevent insufficient bone volume for dental implants, bone grafts have been used together with oral implants over the last 30 years or so. Various clinical investigations and case reports have indicated that (Lundgren et al. 1996; Valentini et al. 1998) autogenous bone has the best osteogenic potential and biomechanical properties of regenerated bone. However, the quantitative limitations of harvested autogenous bone often force the clinician to combine the autograft with other types of grafts to obtain an adequate amount of grafting material. Alternatives to autogenous bone grafts include inorganic bone minerals and biologically active synthetic materials. The osteoconductive properties of these synthetic materials have been demonstrated, and it has been shown that the bone formation occurs within the particles through gelation and corrosion phenomena arising from interfacial ion exchange between the particles of materials and surrounding tissue fluids in a series of animal studies (Schepers et al. 1993; Furusawa & Mizunuma 1997). However, these materials can not sufficiently induce bone regeneration due to inadequate osteoinductive ability.

Accordingly, recent tissue engineering approaches have attempted to create new bone based on the use of MSCs seeded onto porous ceramic scaffolds with osteoconductive properties (Boo et al. 2002). These attempts have yielded sub-optimal results due to the slow resorption rate of hydroxyapatite-based ceramics. Also, these delivery substances do not exhibit good plasticity and the cellular implantation procedure is complicated by problems associated with the delivery vehicles because the block materials do not have plasticity. Isogai et al. (2000) reported that a combina-

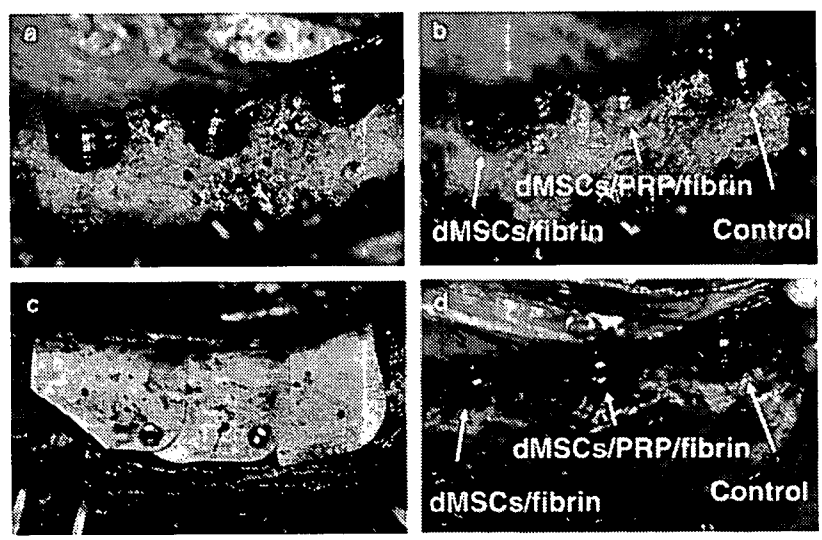

Fig. 2. Macroscopic observations of bone regeneration, (a) Experimental design of the dog mandible prepared using a 10 mm diameter trephine bar and simultaneously inserted dental implants. (b) Graft materials implanted in bone defects. (c) Barrier membranes placed after implantation. (d) New bone regeneration in the dog mesenchymal stem cells (dMSCs)/fibrin, dMSCs/platelet-rich plasma (PRP)/fibrin and control groups after 8 weeks. Bone regeneration promoted by dMSCs/PRP/fibrin and dMSCs/fibrin resulted in a natural marginal bone level, but bone regeneration induced by the control (defect only) was incomplete.

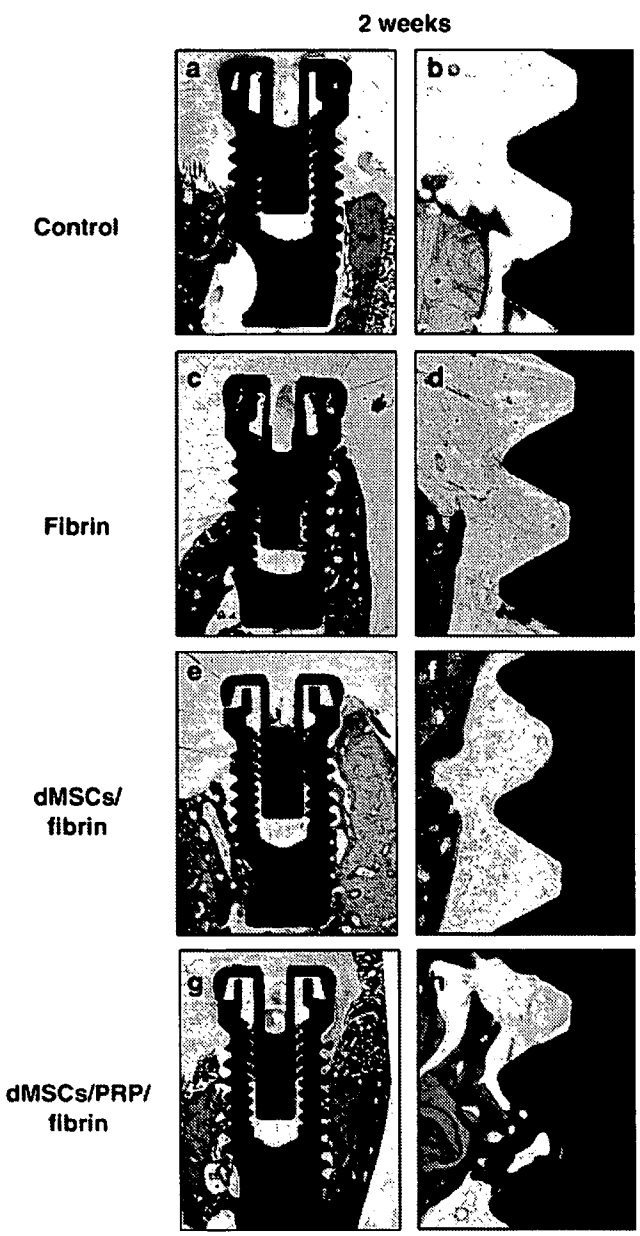

Fig. 3. Photographs of the histological section seen by light microscopy at 2 weeks. Non-decalcified ground sections surface stained with toluidine blue. Original magnification, × 12.5 for (a), (c), (e), (g) and × 250 for (b), (d), (f), (h). (a) In the control group, the buccal and lingual walls were not sufficiently regenerated for dental implants (lower magnification). (b) Control group (higher magnification). (c) Fibrin group (lower magnification). Most of the threads on the buccal aspect were exposed. (d) Fibrin group (higher magnification). (e) Dog mesenchymal stem cells (dMSCs)/fibrin group (lower magnification). The buccal wall reached about half the height of the natural marginal bone level. (f) dMSCs/fibrin group (higher magnification) (g) dMSCs/platelet-rich plasma (PRP)/fibrin group (lower magnification). Regenerated bone was partially observed. (h) dMSCs/PRP/fibrin group (higher magnification).

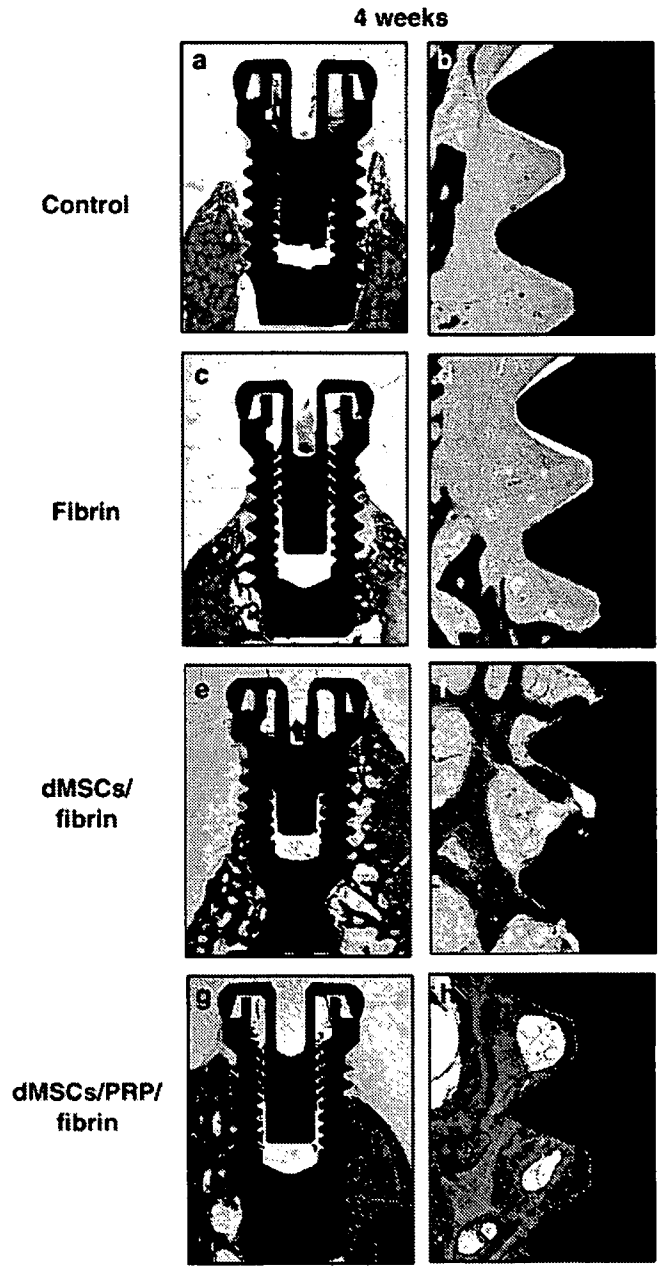

Fig. 4. Photographs of the histology sections seen by light microscopy at 4 weeks. Non-decalcified ground sections surface stained with toluidine blue. Original magnification, × 12.5 for (a), (c), (e), (g) and × 250 for (b), (d), (f), (h). (a) In the control group, the buccal and lingual walls were only slightly regenerated for dental implants (lower magnification). (b) Control group (higher magnification). (c) Fibrin group (lower magnification). Most of the threads were covered by soft tissue. (d) Fibrin group (higher magnification). (e) Dog mesenchymal stem cells (dMSCs)/fibrin group (lower magnification). Partial bone regeneration was observed along the buccal and lingual walls. (f) dMSCs/fibrin group (higher magnification). (g) dMSCs/platelet-rich plasma (PRP)/fibrin group (lower magnification). The regenerated buccal bone was composed of lamellar bone. (h) dMSCs/PRP/fibrin group (higher magnification).

tion of fibrin glue with delivery vehicle and cultured periosteal cells resulted in new bone formation at heterotopic sites in nude mice. In numerous reports about materials, fibrin was found to have hemostatic effects and to promote wound healing (Matras et al. 1978). In a bone regeneration study using the rabbit tibia, Bösch et al. (1977) and Schwarz et al. (1993) reported that fibrin stimulated neovascularization of bone with accelerated healing and earlier new bone formation. Additionally, the use of fibrin as an osteoconductive material has been recommended (Schlag et al. 1989). Therefore, we used fibrin as a scaffold, which is one of the three key factors in the tissue engineering concept (Langer & Vacanti 1993).

Fibrin glue, a composite of fibrinogen and thrombin, is a potentially suitable biological vehicle for cell transplantation