122. Tiedemann K, Malmstrom A, Westergren-Thorsson G. Cytokine regulation of proteoglycan production in fibroblasts: separate and synergistic effects. *Matrix Biol* 1997: **15**: 469–478.
123. Tobita M, Uysal AC, Ogawa R, Hyakusoku H, Mizuno H. Periodontal tissue regeneration with adipose-derived stem cells. *Tissue Eng A* 2008: **14**: 945–953.
124. Tokiyasu Y, Takata T, Saygin E, Somerman M. Enamel factors regulate expression of genes associated with cementoblasts. *J Periodontol* 2000: **71**: 829–839.
125. Tsuboi R, Rifkin DB. Recombinant basic fibroblast growth factor stimulates wound healing in healing-impaired *db/db* mice. *J Exp Med* 1990: **172**: 245–251.
126. Tsukada K. 'If you encounter decubitus'. Important points on treating decubitus. *Medical Asahi* 1994: **10**: 28–36 (in Japanese).
127. Ueda M, Yamada Y, Kagami H, Hibi H. Injectable bone applied for ridge augmentation and dental implant placement: human progress study. *Implant Dent* 2008: **17**: 82–90.
128. Usui H, Shibayama M, Ohbayashi N, Konishi M, Takada S, Itoh N. Fgf18 is required for embryonic lung alveolar development. *Biochem Biophys Res Commun* 2004: **322**: 887–892.
129. Volpi N, Schiller J, Stern R, Soltes L. Role, metabolism, chemical modifications and applications of hyaluronan. *Curr Med Chem* 2009: **16**: 1718–1745.
130. Wada Y, Yamamoto H, Nanbu S, Mizuno M, Tamura M. The suppressive effect of enamel matrix derivative on osteocalcin gene expression of osteoblasts is neutralized by an antibody against TGF-β. *J Periodontol* 2008: **79**: 341–347.
131. Wang Y, Spatz MK, Kannan K, Hayk H, Avivi A, Gorivodsky M, Pines M, Yayon A, Lonai P, Givol D. A mouse model for achondroplasia produced by targeting fibroblast growth factor receptor 3. *Proc Natl Acad Sci USA* 1999: **96**: 4455–4460.
132. Wang H, Yoshiko Y, Yamamoto R, Minamizaki T, Kozai K, Tanne K, Aubin JE, Maeda N. Overexpression of fibroblast growth factor 23 suppresses osteoblast differentiation and matrix mineralization *in vitro*. *J Bone Miner Res* 2008: **23**: 939–948.
133. Weibrich G, Kleis WK, Hafner G. Growth factor levels in the platelet-rich plasma produced by 2 different methods: curasan-type PRP kit versus PCCS PRP system. *Int J Oral Maxillofac Implants* 2002: **17**: 184–190.
134. Weigel PH, Frost SJ, McGary CT, LeBoeuf RD. The role of hyaluronic acid in inflammation and wound healing. *Int J Tissue React* 1988: **10**: 355–365.
135. Wieman TJ, Smiell JM, Su Y. Efficacy and safety of a topical gel formulation of recombinant human platelet-derived growth factor-BB (becaplermin) in patients with chronic neuropathic diabetic ulcers. A phase III randomized placebo-controlled double-blind study. *Diabetes Care* 1998: **21**: 822–827.
136. Xu J, Liu Z, Ornitz DM. Temporal and spatial gradients of Fgf8 and Fgf17 regulate proliferation and differentiation of midline cerebellar structures. *Development* 2000: **127**: 1833–1843.
137. Yamada Y, Ueda M, Hibi H, Baba S. A novel approach to periodontal tissue regeneration with mesenchymal stem cells and platelet-rich plasma using tissue engineering technology: a clinical case report. *Int J Periodontics Restorative Dent* 2006: **26**: 363–369.
138. Yassibag-Berkman Z, Tuncer O, Subasioglu T, Kantarci A. Combined use of platelet-rich plasma and bone grafting with or without guided tissue regeneration in the treatment of anterior interproximal defects. *J Periodontol* 2007: **78**: 801–809.
139. Yukna RA, Mellonig JT. Histologic evaluation of periodontal healing in humans following regenerative therapy with enamel matrix derivative. A 10-case series. *J Periodontol* 2000: **71**: 752–759.
140. Zheng W, Wang S, Ma D, Tang L, Duan Y, Jin Y. Loss of proliferation and differentiation capacity of aged human periodontal ligament stem cells and rejuvenation by exposure to the young extrinsic environment. *Tissue Eng A* 2009: **15**: 2363–2371.
141. Zhou M, Sutliff RL, Paul RJ, Lorenz JN, Hoying JB, Haudenschild CC, Yin M, Coffin JD, Kong L, Kranias EG, Luo W, Boivin GP, Duffy JJ, Pawlowski SA, Doetschman T. Fibroblast growth factor 2 control of vascular tone. *Nature Med* 1998: **4**: 201–207.

SEMINAR セミナー

口腔疾患と全身とのかかわり

北村正博
Masahiro KITAMURA
大阪大学大学院口腔分子免疫制御学講座
歯周病分子病態学歯周病診断制御学准教授

村上伸也
Shinya MURAKAMI
大阪大学大学院口腔分子免疫制御学講座
歯周病分子病態学歯周病診断制御学教授

1 はじめに

全身の健康状態は，口腔にも影響を与える．すなわち，全身疾患が歯周病やう蝕(虫歯)などの口腔内に発症する歯科疾患に影響を与えたり，全身疾患の部分病変が口腔内に現れる場合がある．しかしながら近年，全身から口腔への方向とは逆に，歯科疾患が全身の健康を脅かしていることが明らかとなってきている．ここでは，歯や歯周組織に発症する歯科疾患ならではの発症メカニズムや臨床症状を紹介しつつ，口腔疾患と全身との相互関係について解説する．

2 歯科疾患の特徴

1. 歯及び歯周組織の特徴

歯の周囲は，歯の歯冠部が歯肉(歯ぐき)から口腔内に突出した，身体の他の部位では見られない解剖学的形態を示している．また組織学的に見ても，歯髄(歯の神経)を象牙質とエナメル質が取り囲む構造を持つ歯の歯根部分を，歯槽骨や歯肉などの歯周組織が被覆し歯を支持する独特の構造を有している(図1)．

2. 歯科における2大疾患：歯周病とう蝕

口腔内には様々な疾患が発症するが，歯周病とう蝕は，その罹患率の高さから歯科における2大疾患と呼ばれている．

歯周病は，歯と歯肉の境界部に存在する *Porphyromonas gingivalis* などの歯周病原菌が原因となり歯周組織が破壊される炎症性疾患で，我が国では成人の約80%が罹患している．[1] 歯周病に罹患していない健康な状態では，歯と歯肉の接合部には1～2 mmの歯肉溝が存在する．この歯肉溝付近に歯周病原細菌が集まったプラークが付着した状態が持続すると，プラーク由来の起炎物質によりまず歯肉の発赤・腫脹が生じ，歯肉溝が深くなりポケットが形成される．このような状態を，歯周病の初期段階である歯肉炎と呼ぶ．歯肉炎が長期間放置されると，歯

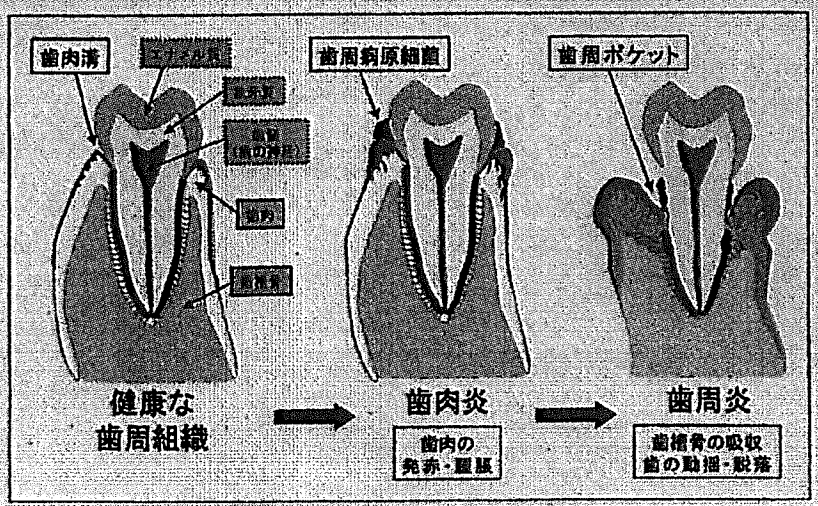

図1 歯と歯周組織の構造と歯周病の進行

と歯肉の接合部が傷害され，歯の周囲にさらに深い歯周ポケットが形成されて，歯槽骨などの深部歯周組織に炎症が波及した歯周炎へと進行する．そして歯周炎がさらに進行し重症化すると，歯を支持している歯槽骨の吸収が大きくなり歯が動揺し，最終的には歯が脱落することとなる(図1)．

一方，う蝕は歯の表面に存在する*Streptococcus mutans*などのう蝕原細菌が食物中の糖類を分解して産生する酸により，歯質が脱灰されて生じる歯の実質欠損である．一般に，う蝕がエナメル質に限局している間は無痛であることが多いが，象牙質に達すると歯髄を刺激して歯痛が生じるようになる．そして，う蝕が歯髄に達すると歯髄に細菌感染が起こり，歯髄炎を併発して激しい自発痛が生じる場合も少なくない．その後，歯髄が壊死して歯髄腔内の細菌感染が持続すると，それが慢性の感染源となり歯根の先端部(根尖)に根尖性歯周炎を併発することもある．

3．歯科疾患の感染症としての特徴と特殊性

歯周病，う蝕，そしてう蝕から併発する根尖性歯周炎では，その原因菌が生体外と考えられる歯の表面，歯周ポケット内あるいは歯の内部(歯髄腔)に存在することから，生体の免疫系の働きによる排除機構を十分受けない．そのため，多くの細菌感染症と異なり，これらの歯科疾患が重症化し歯が脱落することを除いて，感染源を人為的に除去しない限り自然治癒することはない．また，これらの歯科疾患の原因菌の多くは口腔内の常在菌で，原因菌が自ら産生した菌体外多糖に被覆され，共生・集合した細菌バイオフィルム[2]を形成する共通の特徴を有している．この細菌バイオフィルムは生体の細菌排除機構に対するバリアとして機能するだけではなく，内部の細菌の細胞壁の肥厚や代謝活性の低下をもたらし，免疫系や抗菌薬に対する抵抗性の獲得による歯科疾患の難治化の誘因になっている．

3 全身疾患の口腔への影響：全身疾患に伴う口腔症状と歯科疾患への影響

歯周病は，細菌の集合体であるプラークが原因となり発症するため，歯周病原菌に対する生体防御機能を減弱させるような全身疾患を有する患者では，歯周病の発症や進行のリスクが高くなると考えられる．現在のところ，歯周病の発症や進行に影響を与える全身疾患としては，糖尿病，白血病，後天性免疫不全症候群(AIDS)，骨粗鬆症などが代表例として挙げられる．[3~7] そして，生体防御機構に影響を与える肥満，喫煙，ストレス，妊娠などの全身的要因も歯周病発症のリスク因子になると考えられている．[5,6] また，全身疾患に伴って口腔内に症状が出現

表1 全身疾患に伴って現われる口腔症状

口腔症状		全身疾患
歯肉や口腔粘膜に出現する軟組織病変	Koplik斑	麻疹(はしか)
	歯肉などの口腔粘膜に自然出血，潰瘍形成，壊死	白血病，好中球減少症，再生不良性貧血などの血液疾患
	Hunter舌炎	悪性貧血
	口腔粘膜の再発性アフタ	ベーチェット病
	口唇，口腔粘膜及び舌の小水疱	手足口病
	口唇や口腔粘膜のメラニン色素沈着	Addison病，Peutz-Jeghers症候群
	口腔粘膜の神経線維腫	von Recklinghausen病
	びらん形成，ゴム腫，口蓋部の穿孔	梅毒
	歯肉出血，歯の動揺・脱落	ビタミンC欠乏症(壊血病)
	口腔乾燥症	シェーグレン症候群，糖尿病，甲状腺及び副腎皮質機能異常
歯や顎骨に出現する硬組織病変	歯の萌出遅延，移動，傾斜	Paget病，大理石病
	顎骨の成長不良，不正咬合，歯の萌出遅延・形態異常	ビタミンD欠乏症(くる病)
	ハッチンソンの歯	先天性梅毒
	歯の萌出異常，エナメル質や象牙質の形成不全	外胚葉異形成症，頭骨頭蓋異形成症，ダウン症などの遺伝性疾患

することがある(表1).[9,10] これらの口腔症状は，全身疾患の部分病変が口腔内に現われたものであるが，これらを初発症状として歯科医を訪れ，全身的な原疾患が発見されることもある．外胚葉異形成症，鎖骨頭蓋異形成症，ダウン症などの遺伝性疾患，乳幼児期の代謝障害や栄養障害などでは，歯の萌出異常や形成不全などが見られることがある.[9~11] そのためこれらの疾患の罹患者では，う蝕の罹患性が著しく高まる．また，唾液の分泌が減少する口腔乾燥症やシェーグレン症候群などでも，う蝕原細菌が産生する酸に対する唾液の緩衝作用が十分働かないため，う蝕が多発しやすい．

4 薬の副作用として現れる口腔症状

1. 薬物性歯肉肥大[4,5]

カルシウム(Ca)拮抗薬は，降圧薬として広く用いられている．しかしながら，Ca拮抗薬の副作用として線維性歯肉肥大があることは意外に知られていない．Ca拮抗薬は比較的高齢者に服用者が多く，歯肉肥大により口腔清掃不良を招きやすいため，重度の歯周病を発症していることが多い．Ca拮抗薬の副作用として歯肉肥大が認められた場合には，歯科受診を勧めるとともに，作用機序の異なる降圧剤への変更を検討することが望ましい．

抗てんかん薬であるフェニトイン服用者にも，歯肉肥大が認められることがある．Ca拮抗薬服用者の歯肉肥大と同様に，プラークによる歯科疾患の発症に対する予防が重要である．そして，他の抗てんかん剤への変更が望ましいが，それが困難なケースでは定期的に歯肉切除を行う場合もある．

また免疫抑制薬のシクロスポリンも，歯肉肥大を誘発することが報告されている．臨床的には，フェニトインにより誘発された歯肉肥大と類似しているが，歯肉が不整なギザギザした形態を示すことが多い．そしていずれの薬であれ薬物性歯肉肥大が認められた場合において，口腔衛生指導や歯石除去などの歯周病治療を行うことは，炎症の軽減を図る上で重要である．

2. 口腔乾燥症[12]

唾液の分泌低下には様々な原因が考えられるが，薬の副作用として口腔乾燥が生じる場合がある．降圧薬(α_1遮断薬)，副交感神経遮断薬，抗うつ薬，抗精神病薬などの服用で口腔乾燥感を自覚するケースが多いが，それ以外にもかなり多くの薬が口腔乾燥を引き起こすと考えられている．口腔乾燥症が発症すると，う蝕や歯周病などの歯科疾患の発症リスクが上昇することに加え，口腔内の灼熱感，味覚異常，義歯の不適合などが生じる．そのため食物摂取が困難となったり，食塊形成が障害されることで，嚥下障害が生じることもある．

3. 薬物性の歯の形成不全及び着色

歯の形成期にテトラサイクリン系の抗菌薬を投与された場合，歯の象牙質の着色(暗い黄色，灰色，茶色，時に青味がかった帯状紋様)やエナメル質の形成不全が生じることがある．テトラサイクリン系抗菌薬は，日本では1960年代に多く使用されたため，その当時に出生した人にこの抗菌薬が原因と見られる歯の着色や形成不全が認められることがある．7歳未満の小児(胎児期を含む)へのテトラサイクリン系抗菌薬の投与は，できるだけ避けるべきである.[11]

5 口腔疾患の全身への影響

1. 全身的な慢性炎症巣や細菌感染源としての歯科疾患

歯科疾患による炎症やその原因となる細菌感染は，局所に限局した小規模なものと考えがちであるが，実際にはかなり大きな炎症巣や細菌感染源が口腔には存在する．このことは，歯周病を例に挙げて考えると理解しやすい．例えば，親知らずを除いた28本の歯の周囲に5 mmの歯周ポケットが形成されたケースを想定してみると，ポケットに面する上皮の面積は大人の掌に相当する約72 cm^2にも及ぶ．そしてその炎症上皮と接するポケット内には，プラーク1 mg当たり10^8~10^9個もの高密度の細菌が生息しているのであるから，歯の周囲に限局した疾患である歯周病のイメージより，はるかに大きな慢性炎症巣と細菌感染源が恒常的に存在していることとなる(図2)．

2. 歯科疾患の全身への影響

近年，全身疾患が歯科疾患に影響を及ぼすリスク

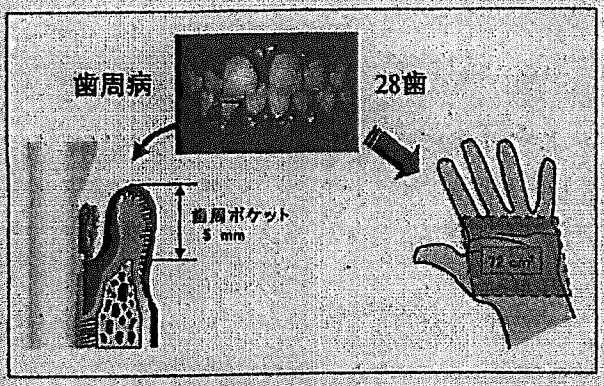

図2 慢性炎症巣や細菌感染源としての歯周病

ファクターであることが明確になる一方で，歯科疾患が全身の健康を脅かしていることが明らかとなってきた(図3).[13]

1) 細菌性心内膜炎

歯科で，歯周ポケット測定，歯石除去，抜歯などの出血を伴う可能性のある検査や治療を行うと，口腔内の細菌が血液中に侵入して一時的な菌血症が生じることがある.[14] 通常ではすぐに菌は消失して問題となることはないが，先天性心疾患や人工弁置換術を受けた人では細菌性心内膜炎を起こすリスクが高くなり，免疫低下が認められるような人では敗血症を併発することもある．そのため，細菌性心内膜炎のハイリスク群に対しては，菌血症が生じる可能性のある上記の歯科的処置を行う際の細菌性心内膜炎に対する標準的予防法として，成人でアモキシリン2.0 gの単回経口投与(小児用量は50 mg/kgで成人用量を超えない用量)が，「感染性心内膜炎の予防と治療に関するガイドライン」[15]で推奨されている．また，う蝕原細菌は細菌性心内膜炎の主要な原因菌として知られていることから，口腔衛生状態を良好に保ち，う蝕予防に努めることは細菌性心内膜炎の予防にもつながると考えられる．そして，歯周病などにより口腔内に炎症が存在する場合には，口腔細菌が口腔内から血液中に侵入しやすくなることから，歯周病の発症を抑制することも細菌性心内膜炎の予防には重要である．

2) 誤嚥性肺炎

誤嚥性肺炎は高齢者では死に直結することから，介護や医療の分野で特に予防に心掛けなければならない疾患である．近年，口腔細菌が誤嚥性肺炎の病巣から検出され，肺炎の誘因として歯科疾患が注目されている．一般に，要介護高齢者や口腔機能障害を持つ人は極めて口腔衛生状態が悪く，また歯周病やう蝕の罹患率も高く，義歯を入れている場合は義歯の衛生管理が十分なされていないことが多い．そして，それらの人では睡眠中に無症候性の誤嚥をくり返していることが多く，口腔細菌が呼吸器に侵入し肺炎を引き起こすと考えられている．介護施設の入居者に口腔ケアを行うと，肺炎の発症率や発熱日数などが減少したとの報告があり，口腔衛生状態の改善が肺炎の予防に重要であることが示唆されている.[16]

3) 心臓血管疾患

疫学研究から，歯周病患者において心臓血管疾患の発症リスクが1.2~2.5倍程度増加することが明

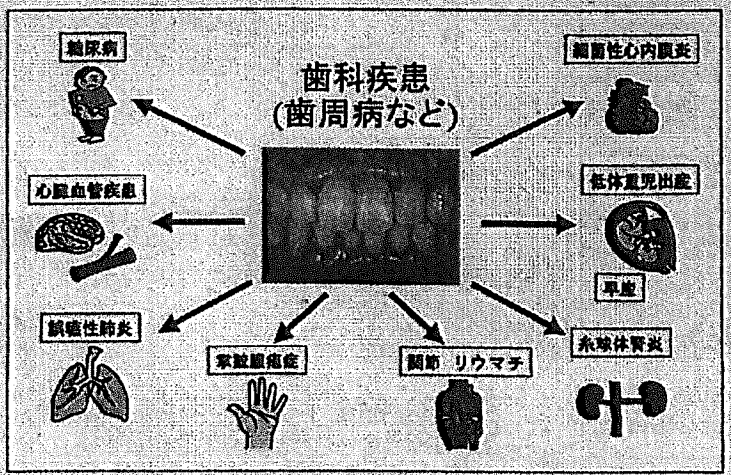

図3 歯周病などの歯科疾患がリスク因子となる全身疾患

らかとなり，歯周病が心臓血管疾患のリスクファクターとして注目されている.[4,17,18] 現在のところ，歯周病変で産生される炎症性サイトカインや口腔細菌が，血行性に心臓や血管へ移行し，動脈の粥状硬化(アテローム性動脈硬化症)を引き起こすのではないかと考えられている.

4) 糖尿病

糖尿病が歯周病の発症や進行のリスクファクターとなることは，以前より一般的に認められている.[9] しかしながら近年，糖尿病の重症度の指標であるヘモグロビンA1cが歯周治療を行うことにより低下することが報告され，歯周病が糖尿病のリスクファクターとなる可能性が示唆されている.[19] これまでの研究結果から，歯周病に罹患した歯周組織で産生されるTNF-αが肝臓における糖代謝に影響を与え，インスリン抵抗性を亢進させることが，歯周病が糖尿病に影響を与えるメカニズムの1つと考えられている.[20]

5) 早産，低体重児出産

歯周病と早産(妊娠37週未満)や低体重児(2,500 g未満)出産との関連については，関連を支持する報告と否定する報告の両者が存在する．近年それらのメタ解析が行われ，歯周病に罹患した妊婦は，早産や低体重児を出産するリスクが2～3倍高く，歯周治療により早産や低体重児出産が減少することが示され，両者の関連を肯定する結果が示されている.[21] 現在のところ，歯周組織の炎症に伴って産生されたプロスタグランジンE_2(PGE_2)が血行性に胎盤や子宮に移行して，子宮の収縮を誘発し早産や低体重児出産を引き起こすのではないかと推測されている.

6) その他の全身疾患

その他，歯科疾患の原因菌やその菌体成分が体内に侵入すると，それが抗原となり抗体が産生される．そして，抗原抗体反応が起こり形成された抗原-抗体複合体が原因となり，糸球体腎炎や関節炎が発症することがあるといわれている．さらに皮膚科領域の掌蹠膿疱症においても，歯科疾患がその原病巣となり得る可能性が示唆されている.[5]

6 おわりに

近年，全身疾患が歯科疾患に影響を及ぼすメカニズムが明確になる一方，口腔内に発症する歯科疾患が全身の健康を脅かしていることが明らかとなり，口腔疾患と全身との相互関係に関する知見が集積されつつある．特に歯周病の分野では，歯周病が全身の健康状態と密接に関連し合っていることを示唆する証拠がいち早く蓄積され，歯周病と全身との相互関係を基盤としたペリオドンタル・メディスン(歯周医学)という新しい学問分野が創世されている.[11]

今後，更にペリオドンタル・メディスンの考え方を口腔疾患全体に普及させ，口腔疾患と全身との相互関係に関する双方向的知見の集積と理解が期待される.

引用文献

1) 花田信弘ほか，"平成17年歯科疾患実態調査，" 歯科疾患実態調査報告解説検討委員会編，医歯薬出版，東京，2007，pp.34.
2) 森島清二，寒比須繁之，"歯周病と全身の健康を考える，" ライオン歯科衛生研究所編，バイオフィルム感染症としての歯周病，東京，医歯薬出版，2004，pp.38-45.
3) 伊藤公一ほか，"歯周病の分類，歯周病の診断と治療指針，" 日本歯周病学会編，医歯薬出版，東京，2007，pp.2-5.
4) 村上伸也，北村正博，"歯周病治療のストラテジー，" 吉江弘正，宮田 隆編，医歯薬出版，東京，2002，pp.163-174.
5) 北村正博，大阪府歯科医師会雑誌，610，12-24(2001).
6) 村上伸也ほか，"糖尿病患者に対する歯周治療ガイドライン，" 日本歯周病学会編，日本歯科医学会，東京，2009，pp.12-15.
7) 野口俊英，稲垣幸司，医学のあゆみ，232，182-188(2010).
8) 雪石 聡，永田英樹，医学のあゆみ，232，198-202(2010).
9) 宮崎 正，"口腔外科学，" 医歯薬出版，東京，2007，pp.41-43，72-83，177-195，476-482，.
10) 栄川敏彌，"臨床口腔診断学，" 国際医書出版，東京，1994，pp.490-500.
11) 大嶋 隆，"小児の歯科治療 シンプルなベストを求めて，" 大阪大学出版会，大阪，2009，pp.48-49，163-175，185-200.
12) 波郷 茂，"唾液 歯と口腔の健康，" 医歯薬出版，東京，2008.
13) 北村正博，村上伸也，医学のあゆみ，232，161-166(2010).
14) 村上伸也ほか，"糖尿病患者に対する歯周治療ガイドライン，" 日本歯周病学会編，日本歯科医学会，東京，2009，pp.52-57.
15) 感染性心内膜炎の予防と治療に関するガイドライン(2008年改訂版)，循環器病の診断と治療に関するガイドライン，2007年度合同研究班報告，日本循環器病学会ホームページ，2008.
16) 米山武義，医学のあゆみ，232，194-197(2010).
17) 村上伸也，北村正博，感染・免疫・炎症，33，84-87(2003).
18) 山崎和久，本田朋之，医学のあゆみ，232，176-180(2010).
19) 村上伸也ほか，糖尿病患者に対する歯周治療ガイドライン，日本歯周病学会編，日本歯科医学会，東京，2009，pp.30-35.
20) 西村英紀，曽我賢彦，医学のあゆみ，232，167-171(2010).
21) 和泉雄一，長谷川清，医学のあゆみ，232，172-175(2010).

M. Kitamura[1], M. Akamatsu[2], M. Machigashira[3], Y. Hara[4], R. Sakagami[5], T. Hirofuji[6], T. Hamachi[7], K. Maeda[7], M. Yokota[8], J. Kido[9], T. Nagata[9], H. Kurihara[10], S. Takashiba[11], T. Sibutani[12], M. Fukuda[13], T. Noguchi[13], K. Yamazaki[14], H. Yoshie[14], K. Iwroi[15], T. Arai[16], T. Nakagawa[17], K. Ito[18], S. Oda[19], Y. Izumi[19], Y. Ogata[20], S. Yamada[21], H. Shimauchi[22], K. Kunimatsu[23], M. Kawanami[24], T. Fujii[25], Y. Furuichi[26], T. Furuuchi[27], T. Sasano[27], E. Imai[28], M. Omae[29], S. Yamada[1], M. Watanuki[2], and S. Murakami[30]*

[1]Department of Periodontology, Division of Oral Biology and Disease Control, Osaka University Dental Hospital; [2]Clinical Development Department, Kaken Pharmaceutical Co., Ltd.; [3]Department of Periodontology, Kagoshima University Dental Hospital; [4]Department of Periodontology, Nagasaki University Hospital, Attached School of Dentistry; [5]Section of Periodontology, Department of Odontology, Fukuoka Dental College Medical and Dental Hospital; [6]Section of General Dentistry, Department of General Dentistry, Fukuoka Dental College Medical and Dental Hospital; [7]Division of Oral Rehabilitation, Department of Periodontology, Kyushu University Hospital; [8]Division of Periodontology, Operative Dentistry and Endodontics, Department of Cariology and Periodontology, Kyushu Dental College Hospital; [9]Department of Periodontology and Endodontology, Tokushima University Hospital; [10]Division of Frontier Medical Science, Department of Periodontal Medicine, Hiroshima University Hospital; [11]Department of Pathophysiology - Periodontal Science, Biopathological Science, Okayama University Hospital; [12]Department of Periodontology, Division of Oral Infections and Health Science, Asahi University Dental Hospital; [13]Department of Periodontology, Aichigakuin University Dental Hospital; [14]Division of Periodontology, Department of Oral Biological Science, Niigata University Medical and Dental Hospital; [15]Department of Oral and Maxillofacial Surgery, Machida Municipal Hospital; [16]Department of Periodontics and Endodontics, Tsurumi University Dental Hospital; [17]Department of Dentistry and Oral Surgery, Keio University Hospital; [18]Department of Periodontology, Nihon University School of Dentistry Dental Hospital; [19]Section of Periodontology, Department of Hard Tissue Engineering, Tokyo Medical and Dental University, University Hospital, Faculty of Dentistry; [20]Department of Periodontology, Nihon University Dental Hospital at Matsudo; [21]Department of Periodontology, Tokyo Dental College Chiba Hospital; [22]Division of Periodontology and Endodontology, Department of Oral Biology, Tohoku University Hospital; [23]Division of Periodontology, Department of Conservative Dentistry and Oral Rehabilitation, Iwate Medical University Hospital, Dental Center; [24]Department of Periodontology and Endodontology, Division of Oral Health Science, Hokkaido University Hospital; [25]Department of General Dentistry, Health Sciences University of Hokkaido Hospital; [26]Division of Periodontology and Endodontology, Department of Oral Rehabilitation, Dental and Medical Clinic, Health Sciences University of Hokkaido; [27]Division of Oral Diagnosis, Department of Oral Medicine and Surgery, Tohoku University Graduate School of Dentistry; [28]Department of Nephrology, Nagoya University Graduate School of Medicine; [29]Department of Oral and Maxillofacial Surgery, Izumisano Municipal Hospital, Rinku General Medical Center; and [30]Department of Periodontology, Osaka University Graduate School of Dentistry, 1-8 Yamadaoka, Suita, Osaka 565-0871, Japan; *corresponding author, ipshinya@dent.osaka-u.ac.jp

J Dent Res 90(1):35-40, 2011

FGF-2 Stimulates Periodontal Regeneration: Results of a Multi-center Randomized Clinical Trial

ABSTRACT

The efficacy of the local application of recombinant human fibroblast growth factor-2 (FGF-2) in periodontal regeneration has been investigated. In this study, a randomized, double-blind, placebo-controlled clinical trial was conducted in 253 adult patients with periodontitis. Modified Widman periodontal surgery was performed, during which 200 μL of the investigational formulation containing 0% (vehicle alone), 0.2%, 0.3%, or 0.4% FGF-2 was administered to 2- or 3-walled vertical bone defects. Each dose of FGF-2 showed significant superiority over vehicle alone ($p < 0.01$) for the percentage of bone fill at 36 wks after administration, and the percentage peaked in the 0.3% FGF-2 group. No significant differences among groups were observed in clinical attachment regained, scoring approximately 2 mm. No clinical safety problems, including an abnormal increase in alveolar bone or ankylosis, were identified. These results strongly suggest that topical application of FGF-2 can be efficacious in the regeneration of human periodontal tissue that has been destroyed by periodontitis.

KEY WORDS: periodontitis, tissue regeneration, fibroblast growth factor-2.

DOI: 10.1177/0022034510384616

Received April 19, 2010; Last revision June 29, 2010; Accepted July 3, 2010

A supplemental appendix to this article is published electronically only at http://jdr.sagepub.com/supplemental.

INTRODUCTION

Periodontitis progressively destroys periodontal tissues and ultimately can lead to the loss of the affected teeth (Socransky and Haffajee, 2002; Ezzo and Cutler, 2003), and the ideal goal of periodontal treatment is complete regeneration of the tissues lost to periodontitis. Unfortunately, while conventional treatments, which mechanically remove the bacterial biofilm, show some success in suppressing the progression of periodontitis, they rarely actively induce periodontal regeneration. Thus, several surgical techniques, including bone grafting, guided tissue regeneration (GTR) treatment, and topical application of platelet-rich plasma or enamel matrix derivatives, have been developed in an attempt to accomplish this goal. Recently, the efficacy of recombinant human growth factors in periodontal regeneration is winning attention from researchers. Their biological properties have been extensively evaluated, and their consistent quality would be expected to reduce variations in regenerative responses.

Fibroblast growth factor (FGF)-2 exhibits potent angiogenic activity and mitogenic ability on mesenchymal cells within the periodontal ligament and has been reported to be effective in regenerating periodontal tissue in animal models (Takayama *et al.*, 2001; Murakami *et al.*, 2003; Kao *et al.*, 2009). Importantly, exploratory Phase 2A study showed that FGF-2 significantly improved the percentage of bone fill compared with vehicle alone, with about 2 mm CAL regained (Kitamura *et al.*, 2008).

The purpose of this study, the largest study in the field of periodontal regenerative therapy, was to clarify the efficacy and safety of FGF-2 and to determine the optimal dose for clinical use.

MATERIALS & METHODS

Study Design

A multi-center, randomized, double-blind, placebo-controlled, dose-finding study was conducted at 24 dental hospitals in Japan from September 2005 to March 2008.

This trial was conducted in accordance with the Good Clinical Practice Guidelines, and the protocol was reviewed and approved by the institutional review boards of each hospital.

Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan) designed the study, and the academic authors participated in the development of the study design and protocol. Kaken also performed data gathering and analysis.

Eligibility of Patients

This study included male or female adult participants (age ≥ 20 yrs) with periodontitis, diagnosed as having 2- or 3-walled vertical periodontal tissue defects, 3 mm or deeper, apical to the remaining alveolar bone crest. In addition, patients' tooth mobility had to be Degree 2 or less, and the width of the attached gingivae had to be sufficient for GTR.

Patients were excluded if they had a malignant tumor or history thereof, severe diabetes (with a 6.5% or higher serum level of hemoglobin A1c), or hypersensitivity to protein drugs. Pregnant or nursing women were excluded. Patients with a consciousness disorder or severe disorders of the kidneys, liver, blood, or circulatory system were also excluded. All participants gave their written informed consent.

Randomization

Randomization was independently performed by the Registration Center (Adjust Co., Ltd., Sapporo, Japan). At each hospital, participants were randomly assigned to a block size of four, to receive 1 of 4 treatments: vehicle alone, 0.2% FGF-2, 0.3% FGF-2, or 0.4% FGF-2. Group assignment was not revealed until breaking of the blind.

Disallowed Medication and Procedures

The use of a calcium antagonist or adrenal cortical steroid (equivalent to 20 mg/day of Predonin) was disallowed within 4 wks after study drug administration. A surgical operation in the vicinity of the tooth selected for this study was disallowed within 36 wks after study drug administration.

Investigational Drug

This trial used recombinant human FGF-2 (Code No. KCB-1; Kaken Pharmaceutical Co., Ltd., Tokyo, Japan) produced by genetic recombination that transformed *Escherichia coli* with the human gene FGF-2. A gel-like investigational formulation was adopted for this study to improve drug administration to the region of alveolar bone defects. Before administration, the operators mixed freeze-dried FGF-2 with 3% hydroxypropyl cellulose, a colorless, viscous solution. The prepared investigational formulation (Code No. KCB-1D), containing 0% (vehicle alone), 0.2%, 0.3%, or 0.4% FGF-2, was administered within 24 hrs after preparation.

Study Intervention

All flap operations were performed in accordance with the modified Widman procedure, during which 200 μL of the investigational formulation were administered to the bone defect region. No specific root conditioning was performed. At 1, 2, and 4 wks after administration, the same clinical inspections were performed as before administration, and serum anti-FGF-2 antibodies were measured at 2 and 4 wks after administration. At 12, 24, and 36 wks following administration, standardized radiographs were taken, and periodontal tissues were inspected. In addition, ten patients from each group were randomly selected, and their serum FGF-2 levels were measured at 1, 2, 4, and 24 hrs after administration.

After breaking of the blind, at 72 wks after administration, a follow-up survey on bone fill, clinical attachment level (CAL), and adverse effects was conducted of all participant groups.

Outcome Measurements

The primary outcome was the percentage of bone fill shown by radiographs at 36 wks after administration. The geometrically standardized radiography used photograph indicators (Cone Indicator-II; Hanshin Technical Laboratory, Nishinomiya, Japan). Five doctors specializing in dental radiology at the Department of Oral Diagnosis at Tohoku University Graduate School of Dentistry (Sendai, Japan) independently measured the percentage of bone fill using methods described previously (Kitamura *et al.*, 2008). The median of the 5 measurements taken from the same image was then selected for efficacy analysis.

The secondary outcome was the CAL regained at 36 wks after administration. CAL, defined as the distance between the control point (the cement-enamel junction or margin of the restorative material) and the bottom of the gingival sulcus, was measured by investigators at each hospital. Prior to the initiation of baseline measurements, intra- and inter-examiner calibrations were performed on patients at each facility to ensure reproducibility and consistency by each investigator. All examiners used PCP-UNC-15 periodontal probes (Hu-Friedy, Chicago, IL, USA). Additionally, probing depth, bleeding on probing, gingival index, tooth mobility, gingival recession, plaque index, and width of keratinized gingivae were monitored during the study.

Sample Size Calculation

On the basis of the results from the exploratory study (Kitamura *et al.*, 2008), it was calculated that 49 participants were required for each group, assuming percentage bone fill of 24% in 'vehicle alone administered' participants and 58% in participants at either 0.2%, 0.3%, or 0.4% FGF-2 administration, a statistical power of 90%, and a two-sided type I error rate of 2.5% (for comparison of each FGF-2 group with the 'vehicle alone' group). Assuming that about ten participants in each group would be excluded due to discovery of bone defects non-conforming to inclusion criteria during flap operation, or due to withdrawal of consent, enrollment of 60 participants in each group was planned.

Statistical Analysis

SAS version 8.2 software (SAS Institute Inc., Cary, NC, USA) was used. For statistical comparison of the 3 dose groups in terms of efficacy endpoints with the 'vehicle alone' group, the Dunnett option was used, based on the Mixed procedure in the SAS system, in which adjusted p-values were computed for multiple comparisons, and analysis of the percentage of bone fill during follow-up was performed by repeated-measures analysis of variance with the Mixed procedure. Analysis was based on

the intention-to-treat (ITT) principle, with all participants included in their assigned groups.

RESULTS

Enrollment and Baseline Characteristics of the Participants

Patient flow through the study is shown in Fig. 1. Of 307 patients screened from August 2005 to April 2006, 267 patients were randomly assigned to one of the four groups. The characteristics at randomization are shown in Table 1. Fisher's exact probability test found no important imbalance regarding them.

Because 14 participants withdrew from the study before flap operation, 253 participants received the investigational drug. The safety and efficacy analyses were carried out on 253 and 249 participants, respectively.

Primary Outcome

All FGF-2 groups were significantly better than the 'vehicle alone' group ($p < 0.01$) for the primary outcome, the percentage of bone fill at 36 wks, in the ITT population (Table 2). Radiographs of a FGF-2-administered participant are shown in Fig. 2. Although all the groups showed improvement with time, significant differences were observed even at 12 wks in both the 0.3% and 0.4% FGF-2 groups (Table 2). Analysis of dose-response patterns by a maximum contrast method (Wakana *et al.*, 2007) showed that the percentage of bone fill at 36 wks reached a plateau in the 0.3% FGF-2 group.

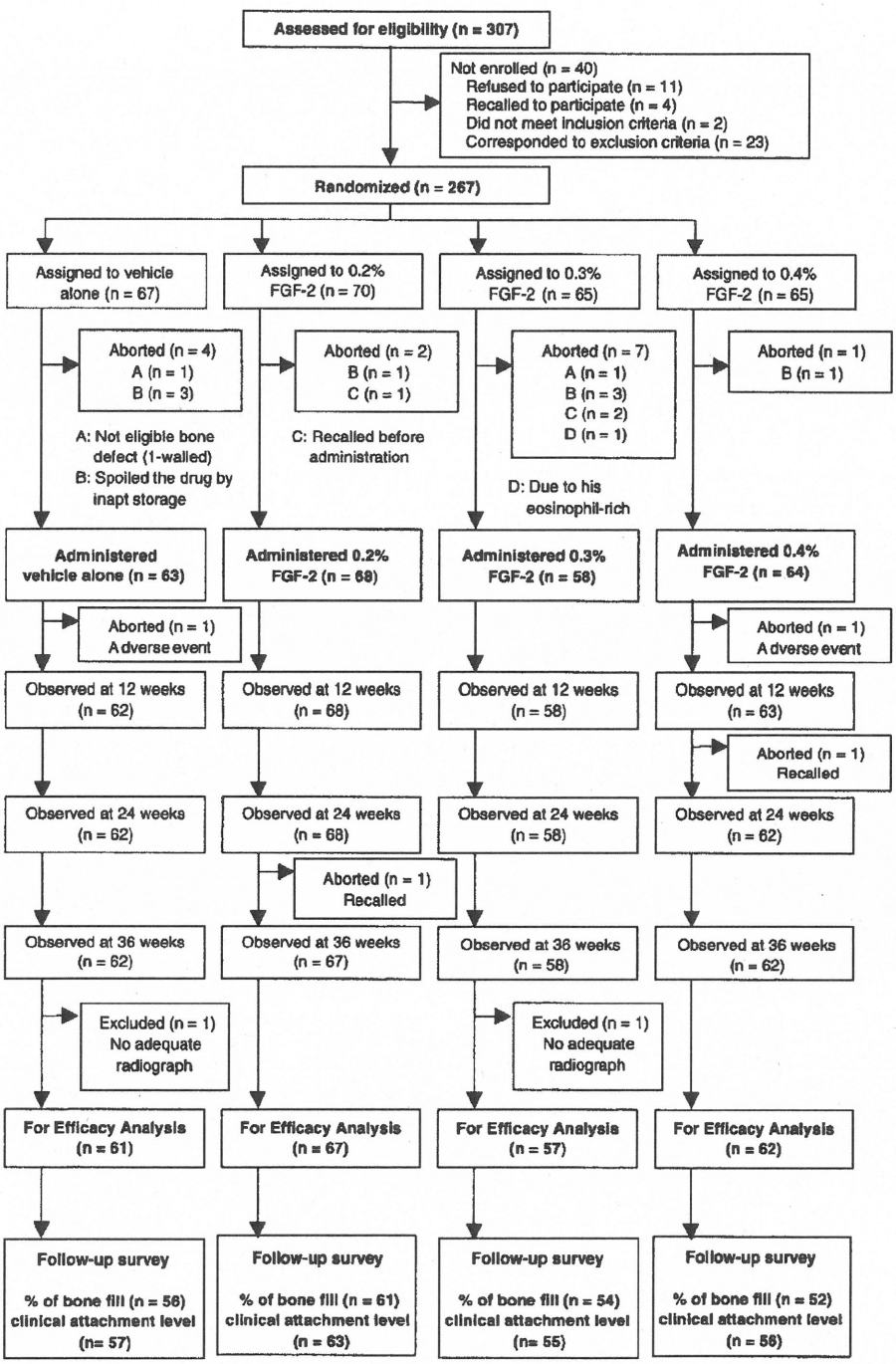

Figure 1. Patient flow diagram.

Secondary Outcome

The CAL regained after FGF-2 administration is shown in Table 2. No significant difference was observed among these 4 groups in the CAL regained, with all scoring around 2 mm.

Periodontal Inspections

Regarding probing depth, bleeding on probing, gingival index, tooth mobility, gingival recession, plaque index, and width of keratinized gingivae, no significant differences were observed among the 4 groups.

Safety

Adverse effects are listed in the Appendix Table. In addition, 10 participants from each of the groups were randomly selected, and blood samples were drawn. For 24 hrs after administration, the serum FGF-2 level was very low and

Table 1. Patient Characteristics

Item	Classification	Vehicle Alone	0.2% FGF-2	0.3% FGF-2	0.4% FGF-2
Numbers of patients		61	68	57	63
Sex (% of patients)	Male	42.6	47.1	36.8	50.8
	Female	57.4	52.9	63.2	49.2
Age (yrs)	Mean (SD)	52.2 (11.5)	53.2 (11.8)	52.8 (11.5)	52.5 (10.7)
Smoking habit (% of patients)	Yes	13.1	26.5	21.1	28.6
	No	86.9	73.5	78.9	71.4
Bone defect on x-rays (mm)	Mean (SD)	5.0 (1.8)	4.8 (1.6)	4.8 (1.7)	4.7 (1.6)
Probing depth (mm)	Mean (SD)	5.8 (1.6)	5.7 (1.5)	5.6 (1.4)	5.7 (1.4)
Width of keratinized gingiva (mm)	Mean (SD)	5.1 (2.3)	4.5 (2.2)	4.6 (2.1)	4.7 (1.9)
Bleeding on probing (% of patients)–		26.2	33.8	24.6	34.9
	+	73.8	66.2	75.4	65.1
Gingival index (% of patients)	0	26.2	39.7	33.3	27.0
	1	34.4	23.5	28.1	41.3
	2	37.7	36.8	38.6	31.7
	3	1.6	0.0	0.0	0.0
Mobility (% of patients)	0	59.0	73.5	61.4	54.0
	1	37.7	20.6	26.3	39.7
	2	3.3	5.9	12.3	6.3
Plaque index (% of patients)	0	68.9	70.6	64.9	69.8
	1	29.5	27.9	33.3	28.6
	2	1.6	1.5	1.8	1.6

Each oral inspection was performed only at the test site around a participant tooth.

Table 2. Efficacy Endpoints

	Vehicle Alone			0.2% FGF-2				0.3% FGF-2				0.4% FGF-2			
	No. of patients	Mean	SD	No. of patients	Mean	SD	p value	No. of patients	Mean	SD	p value	No. of patients	Mean	SD	p value
% bone fill															
12 wks	59	4.65	13.93	67	6.44	17.08	0.925	57	15.89	23.96	0.009	63	13.34	24.18	0.049
24 wks	59	12.06	19.65	66	18.25	23.43	0.391	57	31.73	24.30	< 0.001	61	34.74	33.04	< 0.001
36 wks	61	15.11	21.90	67	33.24	33.15	0.003	57	50.58	31.46	< 0.001	61	46.56	36.09	< 0.001
72 wks*	56	15.86	22.14	61	39.11	37.32	< 0.001	54	52.15	38.12	< 0.001	52	48.85	34.14	< 0.001
CAL regained															
12 wks	61	1.59	1.52	67	1.81	1.51	0.758	57	1.85	1.26	0.670	63	1.79	1.72	0.795
24 wks	61	1.74	1.67	67	1.99	1.60	0.751	57	2.22	1.69	0.294	62	2.19	1.88	0.320
36 wks	61	1.79	1.51	67	2.12	1.74	0.555	57	2.32	1.68	0.224	62	2.23	1.89	0.349
72 wks*	57	2.12	1.72	63	2.48	1.79	0.572	55	2.35	1.78	0.850	56	2.46	1.91	0.636

*Data were obtained after breaking of the blind.

almost the same as before administration. Increased serum anti-FGF-2 antibodies were not observed in any participants after administration.

Follow-up Survey

Two hundred forty-six participants underwent the follow-up survey, since three individuals discontinued participation in the study (Fig. 1). Of these participants, none had an abnormal increase in alveolar bone exceeding the cement-enamel junction or an equivalent control point or ankylosis. The follow-up survey found that both bone fill and CAL regained at 72 wks were retained at the 36-wk level in all groups (Table 2).

DISCUSSION

The present study was designed as a Phase 2B study, with the purpose of clarifying efficacy and safety and to determine the optimal dose of FGF-2 for clinical use.

Regarding the percentage of bone fill at 36 wks, all FGF-2 groups were clearly superior to the 'vehicle alone' group. When the missing data were imputed by the last-observation-carried-forward method, the same results were produced (data not shown). A relative difference of 35% was observed between the 0.3% FGF-2 group and the 'vehicle alone' group in this primary outcome, which is in agreement with the assumed difference in the sample-size calculation. These results strongly

suggest that FGF-2 induced new alveolar bone formation at 36 wks.

The percentage of bone fill at 36 wks reached a plateau in the 0.3% FGF-2 group. In the 0.3% FGF-2 group, the percentage of bone fill began to increase at 12 wks after administration and continued to increase at the same rate up to 36 wks. However, a non-significant change (only 1.6%) in the percentage of bone fill was observed between 36 and 72 wks.

Several lines of evidence suggest that the following 2 mechanisms for the investigational drug enhanced periodontal tissue regeneration. First, FGF-2 directly stimulated proliferation of mesenchymal progenitor cells in the periodontal ligament while maintaining multi-lineage potential. The periodontal ligament has heterogeneous cell populations, and researchers have predicted the existence of some progenitor cells that can differentiate into cementoblasts or osteoblasts (Lekic *et al.*, 2001; Murakami *et al.*, 2003; Shimono *et al.*, 2003). Interestingly, a recent study reported that some cells within the ligament express STRO-1 and CD146 mesenchymal stem cell markers. Such cells, according to the study, differentiate into cementoblast-like cells, adipocytes, and collagen-forming cells. It is expected that FGF-2 induces clonal expansion of such cell populations. Second, FGF-2 stimulated angiogenesis and the production of various types of extracellular matrix, such as hyaluronan and osteopontin (Shimabukuro *et al.*, 2005; Terashima *et al.*, 2008), which plays a crucial role in creating the local environment desirable for periodontal tissue regeneration.

Approximately 2 mm of CAL had already been regained in all the groups at 12 wks after administration, when bone fill was only 15.89% improved, less than half of the improvement at 36 wks, even in the 0.3% FGF-2 group. However, no significant differences between groups were observed regarding CAL regained at 36 wks after administration. This non-significance was considered to be caused by the difference in healing patterns between the FGF-2 groups and the 'vehicle alone' group. Conventional periodontal surgery, which corresponds to the 'vehicle alone' group, usually causes long junctional epithelial attachments, while gingival epithelial cells migrate along the gingival connective tissue down to the root surface (Caton and Zander, 1976; Bowers *et al.*, 1982; Wikesjö and Nilvéus, 1991). This pattern of healing in the 'vehicle alone' group (epithelial attachment) does not require neogenesis of alveolar bone, cementum on the root surface, or fibrous attachment, but maintains resistance to probing force for a period of time. Because manual probing cannot precisely distinguish fibrous attachment from epithelial attachment, the difference in the pattern of healing cannot be reflected in CAL regained between the groups.

The safety analysis indicated that the frequency of adverse effects was not associated with group allocation (Fisher's exact test). Clinical examination showed that no FGF-2 entered the circulation, and that FGF-2 was not associated with antibody production. These results suggest little possibility that FGF-2 will cause systemic adverse effects after topical application. Although oral inspection revealed changing color of gingiva, gingival swelling, bleeding, and protracted gingival wound healing, the severity was slight, and all of these disappeared by 36 days after administration. In addition, no serious adverse effects were reported. Therefore, none of the present results suggests any clinical safety issues related to FGF-2 administration to patients.

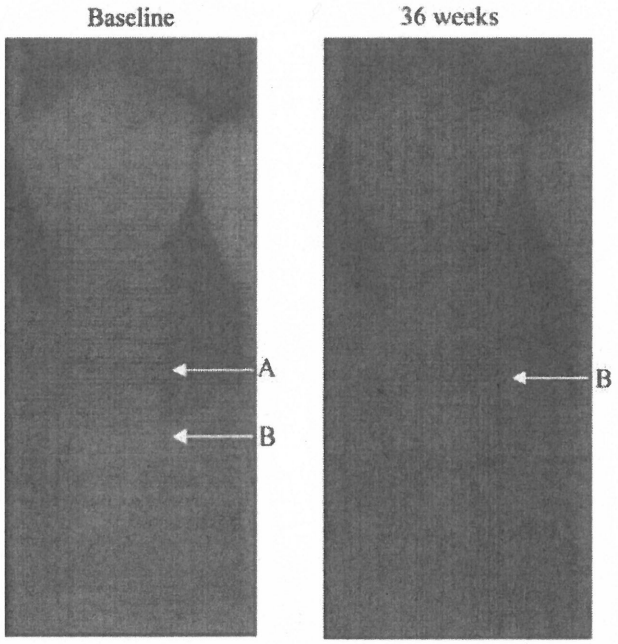

Figure 2. Radiographic outcome of a FGF-2-administered individual. A 0.3 % FGF-2-administered 39-year-old woman. The arrows indicate the remaining alveolar bone crest or the bottom of the bone defect. The depth of the intraosseous defect before administration was measured at 3.0 mm on the x-ray. The radiographs clearly show that the bone defect was filled with the newly generated alveolar bone at 36 wks after administration. The percentage of bone fill at 36 wks was 69.14%, with 3 mm CAL regained.

Since the selection criteria were almost the same as those for general periodontal surgery, the efficacy results of this study are considered applicable to the general population of periodontitis patients. However, the occurrence of rare adverse effects cannot be completely addressed, because the scale of periodontitis patients in Japan is much larger than that in this study.

It has been reported that GTR treatment showed about 34% bone fill at 6 mos after surgery (Kilic *et al.*, 1997), and that enamel matrix derivatives (Zetterström *et al.*, 1997) and platelet-derived growth factor-BB plus beta-tricalcium phosphate (GEM-21S) (Nevins *et al.*, 2005) showed 31% and 57% bone fill, respectively, at 3 yrs and 6 mos after surgery. In this clinical trial, 0.3% FGF-2 achieved 50.6% bone fill at 9 mos. This suggests comparability with the current treatments in alveolar bone regeneration.

Thus far, histological observation has yet to be performed, due to ethical reasons. To overcome this limitation, we conducted a series of animal studies. These animal studies demonstrated that topical application of FGF-2 into artificially prepared intra-osseous defects in alveolar bones induced significant periodontal tissue regeneration. Furthermore, histological analyses revealed new cementum with Sharpey's fibers, new functionally oriented periodontal ligament fibers, and new alveolar bone (Takayama *et al.*, 2001; Murakami *et al.*,

2003; Kao *et al.*, 2009). These clinical and non-clinical studies suggest that topical application of FGF-2 is effective for the regeneration of human periodontal tissue that has been destroyed by periodontitis.

ACKNOWLEDGMENTS

We thank all the investigators and particularly all the participants for their important contributions to the study. S. Murakami received research grants from Kaken. M. Watanuki and M. Akamatsu are employees and stockholders of Kaken.

REFERENCES

Bowers GM, Schallhorn RG, Mellonig JT (1982). Histologic evaluation of new attachment in human intrabony defects. A literature review. *J Periodontol* 53:509-514.

Caton J, Zander HA (1976). Osseous repair of an infrabony pocket without new attachment of connective tissue. *J Clin Periodontol* 3:54-58.

Ezzo PJ, Cutler CW (2003). Microorganisms as risk indicators for periodontal disease. *Periodontol 2000* 32:24-35.

Kao RT, Murakami S, Beirne OR (2009). The use of biologic mediators and tissue engineering in dentistry. *Periodontol 2000* 50:127-153.

Kilic AR, Efeoglu E, Yilmaz S (1997). Guided tissue regeneration in conjunction with 10 hydroxyapatite-collagen grafts for intrabony defects. A clinical and radiological evaluation. *J Clin Periodontol* 24:372-383.

Kitamura M, Nakashima K, Kowashi Y, Fujii T, Shimauchi H, Sasano T, *et al.* (2008). Periodontal tissue regeneration using fibroblast growth factor-2: randomised controlled phase II clinical trial. *PLoS One* 3:e2611.

Lekic P, Rojas J, Birek C, Tenenbaum H, McCulloch CA (2001). Phenotypic comparison of periodontal ligament cells *in vivo* and *in vitro*. *J Periodontal Res* 36:71-79.

Murakami S, Takayama S, Kitamura M, Shimabukuro Y, Yanagi K, Ikezawa K, *et al.* (2003a). Recombinant human basic fibroblast growth factor (bFGF) stimulates periodontal regeneration in class II furcation defects created in beagle dogs. *J Periodontal Res* 38:97-103.

Murakami Y, Kojima T, Nagasawa T, Kobayashi H, Ishikawa I (2003b). Novel isolation of alkaline phosphatase-positive subpopulation from periodontal ligament fibroblasts. *J Periodontol* 74:780-786.

Nevins M, Giannobile WV, McGuire MK, Kao RT, Mellonig JT, Hinrichs JE, *et al.* (2005). Platelet-derived growth factor stimulates bone fill and rate of attachment level gain: results of a large multicenter randomised controlled trial. *J Periodontol* 76:2205-2215.

Shimabukuro Y, Ichikawa T, Takayama S, Yamada S, Takedachi M, Terakura M, *et al.* (2005). Fibroblast growth factor-2 regulates the synthesis of hyaluronan by human periodontal ligament cells. *J Cell Physiol* 203:557-563.

Shimono M, Ishikawa T, Ishikawa H, Matsuzaki H, Hashimoto S, Muramatsu T, *et al.* (2003). Regulatory mechanisms of periodontal regeneration. *Microsc Res Tech* 60:491-502.

Socransky SS, Haffajee AD (2002). Dental biofilms: difficult therapeutic targets. *Periodontol 2000* 28:12-55.

Takayama S, Murakami S, Shimabukuro Y, Kitamura M, Okada H (2001). Periodontal regeneration by FGF-2 (bFGF) in primate models. *J Dent Res* 80:2075-2079.

Terashima Y, Shimabukuro Y, Terashima H, Ozasa M, Terakura M, Ikezawa K, *et al.* (2008). Fibroblast growth factor-2 regulates expression of osteopontin in periodontal ligament cells. *J Cell Physiol* 216:640-650.

Wakana A, Yoshimura I, Hamada C (2007). A method for therapeutic dose selection in a phase II clinical trial using contrast statistics. *Stat Med* 26:498-511.

Wikesjö UM, Nilvéus R (1991). Periodontal repair in dogs. Healing patterns in large circumferential periodontal defects. *J Clin Periodontol* 18: 49-59.

Zetterström O, Andersson C, Eriksson L, Fredriksson A, Friskopp J, Heden G, *et al.* (1997). Clinical safety of enamel matrix derivative (EMDOGAIN) in the treatment of periodontal defects. *J Clin Periodontol* 24(9 Pt 2): 697-704.

歯周組織再生療法を理解しよう
－理論からメインテナンスまで－

大阪大学大学院歯学研究科
口腔分子免疫制御学講座　歯周病分子病態学
北村正博

1. はじめに

近年、病気や外傷などで失われた組織や臓器を蘇らせようとする“再生医療”に対する期待が高まってきています。このような状況の中、歯科領域では、1980年代初めに他の医療分野に先立ち歯周組織再生誘導法（GTR法）[1]がいち早く登場し実用化されました。その後、術式の簡便なエナメルマトリクスタンパクを用いた歯周組織再生療法[2]が開発され、平成20年4月に実施されたGTR法の一部健康保険導入と合わせて、我国の歯科臨床の場で一定の成果をあげています。

ここでは、GTR法をはじめとする歯周組織再生療法の理論と臨床応用の実際について概説します。

2. 歯周組織再生療法の理論的背景

歯肉剥離掻爬術などの従前の歯周外科処置では、その治癒過程において、歯肉上皮の再生速度が歯根膜や歯槽骨などの他の歯周組織に比べ速く歯周組織の再生が期待される歯根面に歯肉上皮の再生速度が早期に到達します。そのため、これらの歯周外科処置では、歯根面には長い上皮性付着が形成され本来の歯周組織に見られるセメント質や歯根膜の再生を伴う線維性付着はわずかしか形成されず、ポケットは減少はするものの失われた歯周組織の再生はほとんど認められません（図1）。

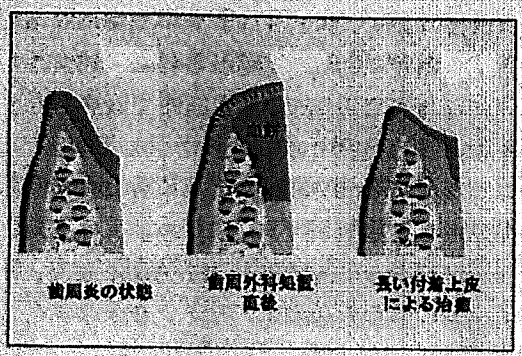

図1　従来の歯周外科処置後におこる治癒

GTR法は、歯周外科処置後の治癒過程に見られるこの歯肉上皮の歯根面に沿った根尖方向への増殖を人工膜で遮断し、歯根膜、セメント質および歯槽骨といった歯周組織が再生する空間を確保（スペースメーキング）する概念のもと考案されました（図2）。

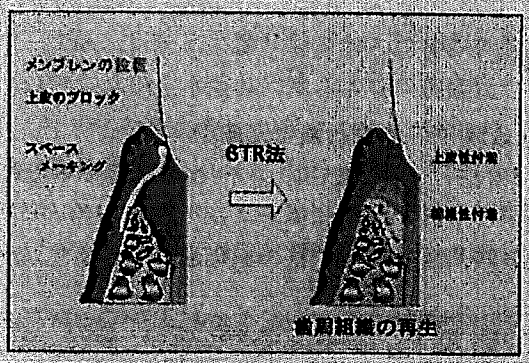

図2　歯周組織再生療法（GTR法）

一方、エナメルマトリクスタンパク（EMD：enamel matrix derivative）を用いた歯周組織再生療法は、歯の発生過程のプロセスを再現して、歯周組織再生を図ることを目指したものです。すなわち、歯根形成時に内外エナメル上皮が接合し形成されたヘルトヴィッヒの上皮鞘から分泌されるEMDは、歯小嚢の未分化間葉細胞からセメント芽細胞への分化を誘導し、歯根象牙質表面にセメント質を形成します。そして、このセメント質の形成が引き金となり、歯根膜、歯槽骨が形成され、歯周組織が完成します。そこで、この歯の発生過程におけるEMDの働きに注目し、歯周病により破壊された歯周組織において、歯周外科手術時にEMDを局所投与することにより、歯周組織再生を図る歯周組織再生誘導材料（エムドゲイン®）が開発されました。

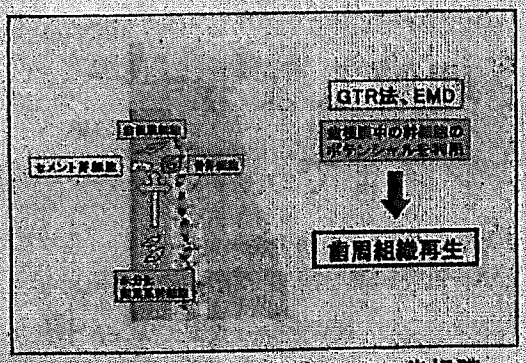

図3　幹細胞の供給源としての歯根膜

GTR法とEMDは、使用する材料が人工膜とゲル状製剤という点で差異がありますが、共に歯根膜中に存在する幹細胞のポテンシャルを利用するという共通点があり、歯根膜中の未分化間葉系幹細胞のセメント芽細胞、骨芽細胞および歯根膜細胞への分化を促進することにより歯周組織再生を効果的に生じさせます（図3）。

3. GTR法の治療計画と臨床例

3壁性および2壁性の深さ4mm以上の垂直性骨欠損と上顎大臼歯類側および下顎大臼歯のII度の根分岐部病変がGTR法の適応症となります。

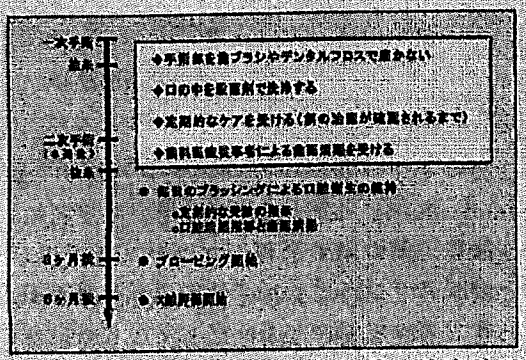

図4　非吸収性膜を用いたGTR法の治療計画

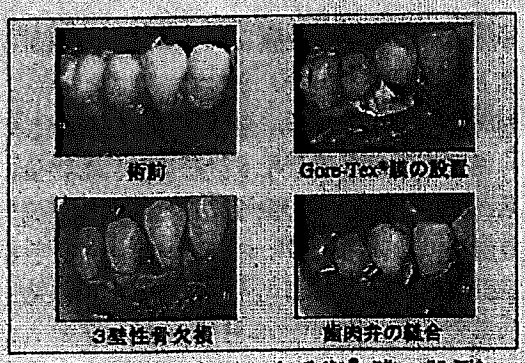

写真1　GTR法（一次手術：膜の設置）

23歳、男性。下顎右側第一小臼歯遠心部の3壁性骨欠損にGore-Tex®膜を用いたGTR法を行った。

図4に非吸収性膜であるGore-Tex®膜を使用したGTR法の治療計画を示します。GTR膜を設置する一次手術では、通常の歯肉剥離掻爬術の術式とほぼ同様に、局所麻酔、歯肉溝切開、歯肉弁剥離、肉芽組織除去、ルートプレーニングを行い、GTR膜を設置し（写真1）、膜を完全に被覆するように歯肉弁を復位し縫合します。一次手術後6~8週間でGTR膜を除去する二次手術を行いますが、一次手術から二次手術の間は、GTR膜が歯肉弁の下に埋入された状態となっていることから、定期的な来院によるプラークコントロールを図るとともに、手術部のブラッシングやフロスの使用を制限するなどの指導を徹底する必要があります。そして、GTR膜除去後、少なくとも3ヵ月程度はプロービングを避けるようにします。

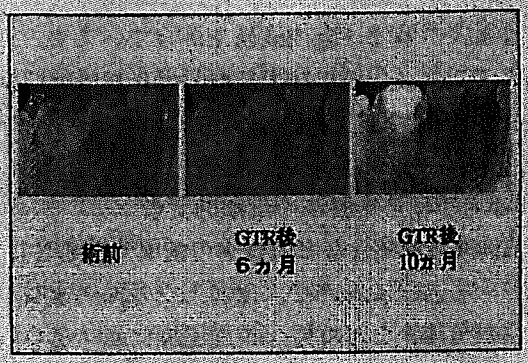

写真2　GTR法（X線検査の経過）

23歳、男性。下顎右側第一小臼歯遠心部に著明な歯槽骨の再生が認められる。

また、写真2にGore-Tex®膜を使用したGTR法の術前後のX線写真を示しますが、X線的な歯槽骨の再生は術後6ヵ月後程度から著明に観察されるようになります。図5に日本国内で販売されている主な非吸収性膜と吸収性膜を示します。GTR法でコラーゲンやポリ乳酸などを成分とする吸収性膜を使用した場合、GTR膜は徐々に生体内で吸収されるため膜を除去する二次手術が不要で、膜の露出などによる術後感染のリスクが少なく、

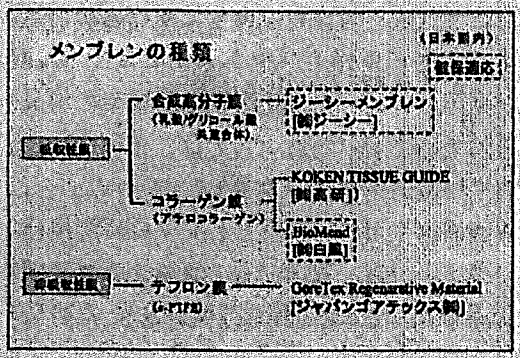

図5　GTR法で用いられる非吸収性膜と吸収性膜

歯肉退縮が少ない利点があります。しかしながら、膜の強度が非吸収性膜に比べて劣るため、スペースメーキングが困難となります（図6）。このように、非吸収性膜と吸収性膜には性状や術式に違いがありますが、アタッチメントの獲得や歯槽骨増加など歯周組織再生量の比較では、両者に差が認められないとする報告が多くあります。

非吸収性膜と吸収性膜

	長所	短所
非吸収性	・Space makingが確実	・2次手術が必要 ・歯肉退縮量が多い
吸収性	・2次手術不要 ・歯肉退縮量が少ない	・複雑な形態の骨欠損の場合、Space makingが不確実 ・メンブレンが早期に露出すると、早く吸収されてしまう

図6　非吸収性膜と吸収性膜の特徴

4. EMDの治療計画と臨床例

現在市販されているエムドゲイン®ゲルは、健康な6ヵ月齢の幼若ブタの歯胚からEMDを抽出・精製し、熱処理を加えたゲル状の歯周組織再生誘導材料です。EMDは、エナメル質やセメント質の形成に関与するタンパクの総称で、その成分にはその大部分を占めるアメロジェニンをはじめ数種類のタンパク質の存在が知られています。現在のところ、エムドゲイン®ゲルの適応症は、歯周ポケットの深さが6mm以上で、X線的に深さ4mm以上、幅2mm以上の垂直性骨欠損を有する中程度または重度歯周炎となっています。そして、EMDを用いた歯周組織再生療法とGTR法は切開、歯肉弁剥離、縫合などは手技的にほぼ同様ですが、EMDを用いた歯周組織再生療法では、歯周組織欠損部に人工膜を設置する代わりに、同部の根面をリン酸やEDTAで処理しエムドゲイン®ゲルを塗布します。これまでに、エムドゲイン®ゲルは、GTR法とほぼ同程度の歯周組織再生能を有していることが示されています。

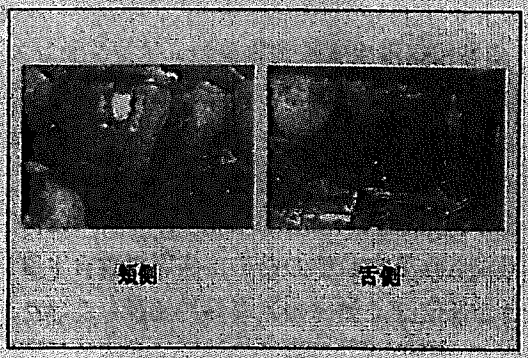

写真3　EMD適応症例
（エムドゲイン®投与）

24歳、女性。下顎右側第一大臼歯の近心部から舌側部に及ぶ根分岐部病変を含む2壁性骨欠を損にエムドゲイン®を適応した。

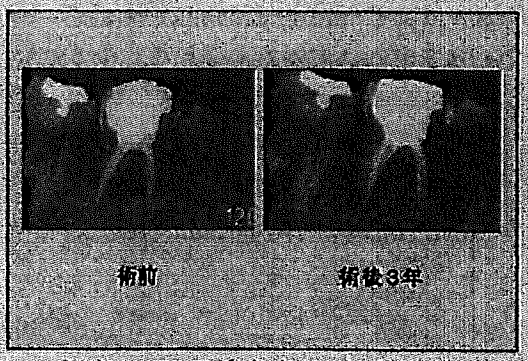

写真4　EMD適応症例（X線写真）

24歳、女性。下顎右側第一大臼歯の近心部と根分岐部病変に著明な歯槽骨の再生が認められる。

そして、EMDを用いた歯周組織再生療法は、GTR法と比べ手技的に簡便で、複雑な骨吸収を伴う多数歯にわたる歯周組織欠損に対応可能であることから、臨床的に応用範囲が広いと考えられます。また、エムドゲイン®ゲルは、ゲル状を示し、GTR法に比べ術後の創傷治癒が早期に生じることから、術後の疼痛が少ないという特徴がありま

す。エムドゲイン®ゲルの臨床例を写真3に示します。この症例は、根分岐部病変を伴う複雑な骨欠損を伴った症例でしたが、エムドゲイン®ゲルの投与により著明な歯周組織再生が認められました(写真4)。

5. 歯周組織再生療法実施後のメインテナンス

歯肉剥離掻爬術などの従前の歯周外科処置で長い上皮性付着が形成されるのに対して、歯周組織再生療法では、本来の歯周組織に見られるセメント質や歯根膜の再生を伴う線維性付着（新付着）が形成されます。そのため、歯周組織再生療法を受けた部位は、従来の歯周外科処置を受けた部位に比べ、プラークや咬合性外傷に対する抵抗性が高いと考えられます。しかしながら、これまでに歯周組織再生療法の予後について検討した研究において、術後のプラークコントロールや患者のコンプライアンスが再生療法の予後に影響を与えることが明らかになっています。従って、通常の歯周外科処置後と同様に、定期的にリコールし、スケーリング、ルートプレーニング、局所抗菌療法などからなる“SPT：Supportive periodontal therapy”を実施することが、歯周組織再生療法後の歯周組織の病状安定には重要であると考えられます。

6. まとめ－歯周組織再生療法の将来展望

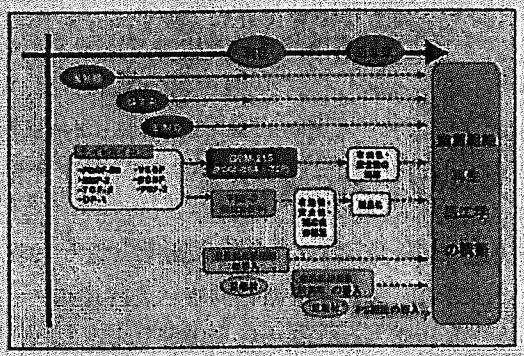

図7　歯周組織再生療法の確立のための取り組みの変遷

GTR法やEMDを用いた歯周組織再生療法が開発され、それまで対応に苦慮していた垂直性骨欠損を有する歯周病変の予後に対する考え方が一変しました。しかしながら、現状の歯周組織再生療法では、水平性骨欠損をはじめ重度の歯周組織欠損への適応はまだまだ困難です。

そこで近年、歯周組織再生に歯周組織再生能を有するシグナル分子（サイトカイン）、幹細胞および足場材といった再生医工学的要素の導入が試みられています（図7）。例えば、米国では、血小板由来増殖因子とβ-リン酸3カルシウムの合剤が、歯周組織再生用“材料”としてすでに実用化されています。一方、我国でも、塩基性線維芽細胞増殖因子を用いた歯周組織再生療法[3]の開発を目指した臨床治験が展開されています。このように、さらに簡便で、さらに重篤な歯周組織欠損への適応可能な歯周組織再生療法の開発競争が行われる中、平成22年11月に長年GTR法に用いられてきたGore-Tex®膜の製造中止が発表されました。このことは、サイトカイン治療や細胞治療など、次世代の歯周組織再生療法の時代の到来を予感させるものではないでしょうか。

参考文献

1）歯周病の検査・診断・治療計画の指針2008、NPO日本歯周病学会編，医歯薬出版，p27-28，2009.

2）北村正博，村上伸也：歯周疾患治療薬、歯科薬理学（石田　甫、五十嵐治義編）、医歯薬出版、第5版，p299-300、東京，2005.

3）村上伸也，島袋善夫，北村正博，山田　聡，野崎剛徳，橋川智子，柳田　学：サイトカインを用いた歯周組織再生療法の開発，FGF-2によるPeriodontal Tissue Engineering，生命歯科医学のカッティングエッジ（米田俊之編）、大阪大学出版会，大阪，57-66，2008.

TISSUE ENGINEERING: Part C
Volume 17, Number 2, 2011

DOI: 10.1089/ten.tec.2010.0139

Transplantation of Human Adipose Tissue-Derived Multilineage Progenitor Cells Reduces Serum Cholesterol in Hyperlipidemic Watanabe Rabbits

Hanayuki Okura, Ph.D.,[1,2] Ayami Saga, M.S.,[1] Yuichi Fumimoto, M.D., Ph.D.,[1] Mayumi Soeda, V.M.D.,[1] Mariko Moriyama, Ph.D.,[1,3] Hiroyuki Moriyama, Ph.D.,[3] Koji Nagai, M.D.,[1,4] Chun-Man Lee, M.D., Ph.D.,[1] Shizuya Yamashita, M.D., Ph.D.,[5] Akihiro Ichinose, M.D., Ph.D.,[4] Takao Hayakawa, Ph.D.,[3] and Akifumi Matsuyama, M.D., Ph.D.[1]

Familial hypercholesterolemia (FH) is an autosomal codominant disease characterized by high concentrations of proatherogenic lipoproteins and premature atherosclerosis secondary to low-density lipoprotein (LDL) receptor deficiency. We examined a novel cell therapy strategy for the treatment of FH in the Watanabe heritable hyperlipidemic (WHHL) rabbit, an animal model for homozygous FH. We delivered human adipose tissue-derived multilineage progenitor cells (hADMPCs) via portal vein and followed by immunosuppressive regimen to avoid xenogenic rejection. Transplantation of hADMPCs resulted in significant reductions in total cholesterol, and the reductions were observed within 4 weeks and maintained for 12 weeks. ^{125}I-LDL turnover study showed that the rate of LDL clearance was significantly higher in the WHHL rabbits with transplanted hADMPCs than those without transplanted. After transplantation hADMPCs were localized in the portal triad, subsequently integrated into the hepatic parenchyma. The integrated cells expressed human albumin, human alpha-1-antitrypsin, human Factor IX, human LDL receptors, and human bile salt export pump, indicating that the transplanted hADMPCs resided, survived, and showed hepatocytic differentiation *in vivo* and lowered serum cholesterol in the WHHL rabbits. These results suggested that hADMPC transplantation could correct the metabolic defects and be a novel therapy for inherited liver diseases.

Introduction

Familial hypercholesterolemia (FH) is characterized by premature and accelerated development of atherosclerotic lesions caused by elevated levels of cholesterol-rich lipoproteins in plasma. The disease is caused by mutations in the low-density lipoprotein (LDL) receptor gene that result in a significant decrease in receptor-mediated uptake of lipoproteins from the circulation.[1–3] Patients homozygous for defects in LDL receptors have serum cholesterol levels 5–10 times those of normal and suffer as early as the first two decades of life from complications such as coronary artery disease.[4,5] In homozygous FH patients, conventional drug therapy cannot treat the condition, and therapeutic recourses are limited to chronic plasmapheresis or orthotopic liver transplantation.[1] Although liver transplants lower LDL levels, the procedure is life threatening; in addition, donor livers are in short supply. Cellular transplantation has been proposed to provide functional LDL receptors for the treatment of hypercholesterolemia. Transplantation of allogenic and xenogenic hepatocytes has been shown to be effective in lowering serum cholesterol in the Watanabe heritable hyperlipidemic (WHHL) rabbit,[6–9] which is an animal model for homozygous FH. Further, a number of gene therapy approaches have shown some promises in animal models and human,[10–13] and the therapies will cure a number of patients with FH in near future. As an alternative to whole-organ transplantation and/or gene therapy, we have investigated the ability of human adipose tissue-derived multilineage progenitor cells (hADMPCs) to differentiate into hepatocytes *in vitro* and to replace critical liver functions[14] as well as previous reports,[15,16] because the *in vitro* differentiation of hADMPCs into various kinds of cell types in now well reported and hADMPCs can be easily and safely obtained in large

[1]Department of Somatic Stem Cell Therapy and Health Policy, Foundation for Biomedical Research and Innovation, Kobe, Japan.
[2]Research Fellow of the Japan Society for the Promotion of Science, Tokyo, Japan.
[3]Pharmaceutical Research and Technology Institute, Kinki University, Osaka, Japan.
[4]Department of Plastic Surgery, Kobe University Hospital, Kobe, Japan.
[5]Division of Cardiology, Department of Internal Medicine, Osaka University Graduate School of Medicine, Osaka Japan.

quantities without serious ethics issues.[17,18] In this study, we are investigating whether hADMPCs could differentiate into hepatocytes *in vivo* and replace critical liver functions as considerable therapeutic potential for cellular replacement.

Materials and Methods

Cells

hADMPCs were prepared as described previously[19] with some modifications.[14,17,18] Adipose tissues from human subjects were resected during plastic surgery in five subjects (four males and one female, age, 20–60 years) as excess discards. Ten to 50 g of subcutaneous adipose tissue was collected from each subject. All subjects provided informed consent. The protocol was approved by the Review Board for Human Research of Kobe University Graduate School of Medicine, Osaka University Graduate School of Medicine, and Foundation for Biomedical Research and Innovation. After five to six passages, the hADMPCs were used for transplantation. Human cryopreserved hepatocytes were purchased from Invitrogen (Lot number: HuP81) and cultured as indicated by the manufacturer's protocol. Human adipose tissue-derived fibroblastic cells were obtained according to previous report.[20]

Flow cytometric analysis

hADMPCs isolated from adipose tissue were characterized by flow cytometry. Cells were detached from culture dishes by 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) and suspended in Dulbecco's phosphate-buffered saline (DPBS; Nacalai Tesque) containing 0.1% fetal bovine serum. Aliquots (5×10^5 cells) were incubated for 30 min at 4°C with fluorescein isothiocyanate-conjugated mouse monoclonal antibodies to human CD31 (BD PharMingen), CD105 (Ancell Corporation), CD133 (R&D Systems), phycoerythrin-conjugated mouse monoclonal antibodies to human CD29, CD34, CD45, CD73 (BD PharMingen), CD44, or CD166 (Ancell). Isotype-identical antibodies served as controls. Further, the cells were incubated with mouse monoclonal antibodies against human stage-specific embryonic antigen-4 (from Chemicon International, Inc.), ABCG-2, or CD117 (BD PharMingen) with nonspecific mouse antibody used as a negative control. After washing with DPBS, cells were incubated with phycoerythrin-labeled goat anti-mouse Ig antibody (BD PharMingen) for 30 min at 4°C. After three washes, cells were resuspended in DPBS and analyzed by flow cytometry using a FACSCalibur flow cytometer and CellQuest Pro software (BD Biosciences).

Adipogenic, osteogenic, and chondrogenic differentiation procedure

For adipogenic differentiation, cells were cultured in the differentiation medium (Zen-Bio, Inc.). After 3 days, half of the medium was changed with adipocyte medium (Zen-Bio) every 2 days. Five days after differentiation, adipocytes were characterized by microscopic observation of intracellular lipid droplets by Oil Red O staining. Osteogenic differentiation was induced by culturing the cells in Dulbecco's modified Eagle's medium containing 10 nM dexamethasone, 50 mg/dL ascorbic acid 2-phosphate, 10 mM β-glycerophosphate (Sigma), and 10% fetal bovine serum. Differentiation was examined by Alizarin red staining. For Alizarin red staining, the cells were washed three times and fixed with dehydrated ethanol. After fixation, the cells were stained with 1% Alizarin red S in 0.1% NH_4OH (pH 6.5) for 5 min and then washed with H_2O. For chondrogenic differentiation, hADMPCs were first trypsinized and 2×10^5 cells were centrifuged at 400 *g* for 10 min. The resulting pellets were cultured in the chondrogenic medium (alpha-minimum essential medium (alpha-MEM) supplemented with 10 ng/mL transforming growth factor-β, 10 nM dexamethasone, 100 μM ascorbate, and 10 μL/mL 100×ITS Solution) for 14 days. For Alcian Blue staining, nuclear counterstaining with Weigert's hematoxylin was followed by 0.5% Alcian Blue 8GX for proteoglycan-rich cartilage matrix.

hADMPC transplantation and immunosuppression regimen

WHHL rabbits (8 weeks old; purchased from Kitayama-labes, Inc.) were anesthetized with pentobarbital (50 mg/kg). An incision distal and parallel to the lower end of the ribcage was made. The peritoneum was incised, and hADMPCs ($n=5$) or human adipose tissue-derived fibroblastic cells ($n=3$) (3×10^7 cells) suspended in 3 mL of Hanks' balanced salt solutions (HBSS) (20°C) or 3 mL of control saline ($n=6$) were infused in 5 min into the portal vein via a 18-gauge Angiocath™ (BD). The immunosuppression regimen (Fig. 1A) consisted of the following: (1) intramuscular injection of cyclosporin A (6 mg/kg/day) daily from the day before surgery to sacrifice; (2) intramuscular injection of rapamycin (0.05 mg/kg/day) daily from the day before surgery to sacrifice; (3) methylprednisolone at 3 mg/kg/day (days 1–7), followed by tapering to 2 mg/kg/day (days 8–14), 1 mg/kg/day (days 15–21) and 0.5 mg/kg/day (day 22 to the time at sacrifice); (4) intravenous injection of cyclophosphamide (20 mg/kg/day) at days 0, 2, 5, and 7; (5) ganciclovir (2.5 mg/kg/day intramuscular injection (i.m.)) was also administrated to avoid viral infection in the immunocompromised host.

DNA extraction and quantification of human-derived cells

Total DNA of WHHL rabbit liver, which was obtained at the time just after hADMPC transplantation, and 2, 4, 8, and 12 weeks after transplantation, were isolated using a NucleoSpin Tissue kit (Macherey-Nagel) according to the manufacturer's instructions. hADMPCs and rabbit hepatocytes were mixed at the ratios of 100:0 (100%), 10:90 (10%), 1:99 (1%), 0.1:99.9 (0.1%), 0.01:99.99 (0.01%), and 0.001:99.999 (0.001%), and DNA was isolated. Seven hundred nanograms of each samples of extracted DNA was quantified by real-time polymerase chain reaction (PCR) using the ABI Prism 7900 Sequence Detection System (Applied Biosystems), primers for the 82 bp *Alu* amplicon (forward, 5′-GTCAGGAGATCGA GACCATCCC; reverse, 5′-CCACTACGCCCGGCTAATTT), and SYBR Green (TOYOBO) dye using a previously published protocol.[21,22] Reactions were performed in quadruplicate and the *Alu* levels were calculated by the standard curve.

Assay for lipid profiling

Serum samples were obtained from nonfasting rabbits before and after transplantation. Serum total cholesterol was measured in each sample using assay kits from Wako Pure Chemical Industries. Serum lipoproteins were analyzed by an on-line dual enzymatic method for simultaneous quantification of cholesterol and triglycerides by high-performance

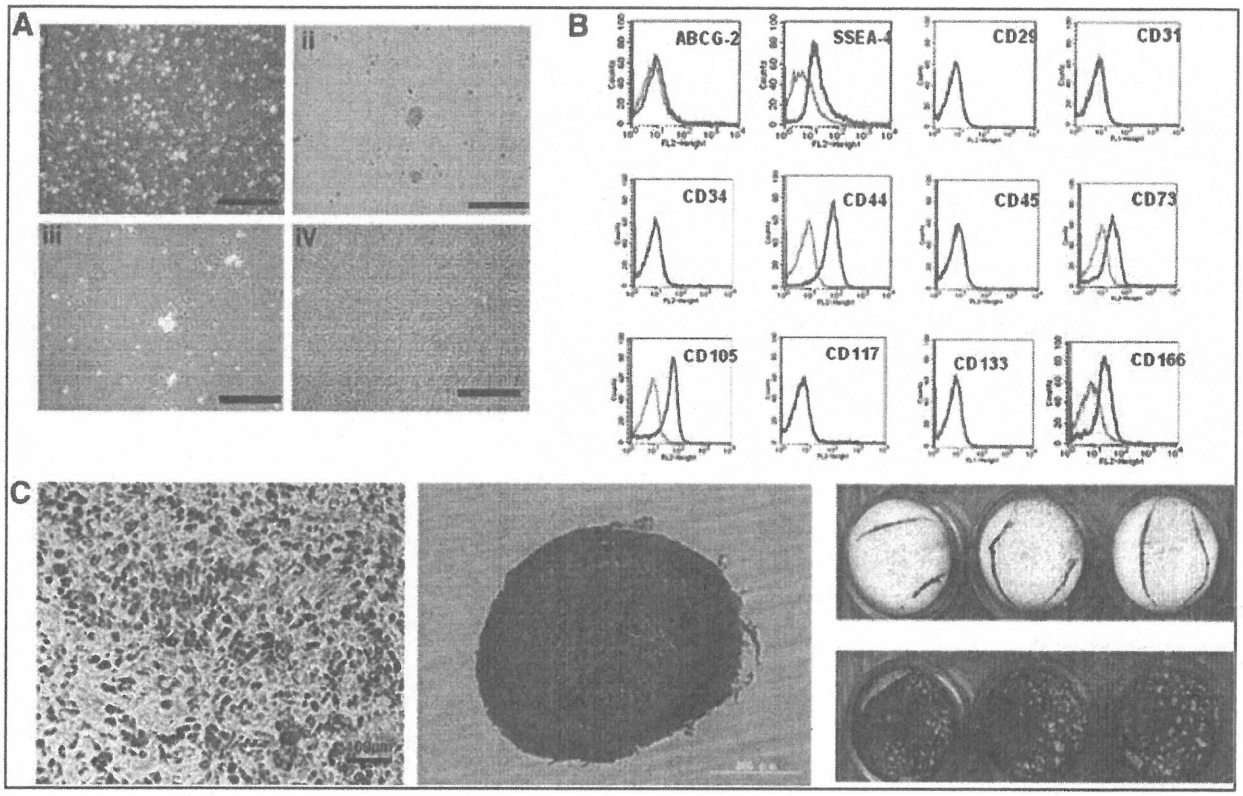

FIG. 1. **(A)** Morphological characters of human adipose tissue-derived multilineage progenitor cells (hADMPCs). The cells obtained from adipose tissue were seeded and incubated for 24 h **(i)**. After incubation, the adherent cells were treated with ethylenediaminetetraacetic acid solution, and the resulting suspended cells were replated at a density of 10,000 cells/cm^2 on human fibronectin-coated dishes (BD BioCoat) **(ii, iii)**. Within two to three passages after the initial plating of the primary culture, hADMPCs appeared as a monolayer of large flat cells (25–30 μm in diameter). As the cells approached confluence, they assumed a more spindle-shaped, fibroblastic morphology **(iv)**. i) Bar = 499 μm, ii) bar = 201 μm, iii) bar = 502 μm and iv) bar = 202 μm. **(B)** Cell surface markers expressed on hADMPCs. The cells were negative for markers of the hematopoietic lineage (CD45) and of hematopoietic stem cells, ABCG-2, CD34, and CD133. They were also negative for CD31, an endothelial cell-associated marker, and the surface antigen c-Kit (CD117). However, they stained positively for a number of surface markers characteristic of mesenchymal and/or neural stem cells, but not embryonic stem (ES) cells, including CD29, CD44 (hyaluronan receptor), CD73, CD105 (endoglin), and CD166. hADMPCs also were positive for stage-specific embryonic antigen (SSEA)-4. **(C)** Adipocytic, chondrocytic, and osteocytic differentiation potentials of hADMPCs. Adipocytic differentiation potential of hADMPCs was confirmed by Oil Red O staining (the left panel) (bar = 100 μm). Chondrocytic differentiation potential of hADMPCs was estimated by extracellular matrices with Alcian Blue staining (the middle panel). Osteogenic differentiation potential of hADMPCs was confirmed by Alizarin red S staining for mineralized nodules (the right panel).

liquid chromatography at Skylight Biotech, according to the procedure as described.[23]

Immunohistochemical staining of WHHL rabbit liver sections

The WHHL livers were harvested and fixed immediately with 10% formalin. They were placed into optimal cutting temperature compound (Sakura Finetechnical Co.), frozen immediately, and then sectioned at 7 μm thickness. The sections were then incubated with blocking solution (Blocking one; Nacalai Tesque) for 1 h. The samples were incubated with rabbit anti-human-specific albumin antibody (MBL), rabbit anti-human-specific alpha 1 anti-trypsin antibody, and rabbit anti-LDL receptor antibody, followed by Alexa Fluor 488-labeled goat anti-rabbit IgG (Molecular Probes). To show the colocalization of human CD90 and albumin, the samples were incubated with the rabbit anti-human CD90 monoclonal antibody (Epitomics, Inc.) and then with Alexa Fluor 488-labeled goat anti-rabbit IgG (Molecular Probes), and washed extensively. Then, the specimens were incubated with rabbit anti-human-specific albumin antibody (MBL), followed by Alexa Fluor 546-labeled goat anti-rabbit IgG (Molecular Probes). The treated sample was examined with a BioZero laser scanning microscope (Keyence).

PCR analysis of WHHL rabbit liver for human liver-specific genes

Total RNAs of WHHL rabbit liver, hADMPCs, and human hepatocytes were isolated using an RNAeasy kit (Qiagen). After treatment with DNase, the cDNA was synthesized using Superscript III RNase H-minus Reverse Transcriptase (Invitrogen). Real-time PCR was performed using the ABI Prism 7900 Sequence Detection System (Applied Biosystems). About 20×Assays-on-Demand™ Gene Expression Assay Mix for human alpha-1-antitrypsin (Hs01097800_m1), human albumin (Hs00609411_m1), human factor 9, human GATA-binding

protein 4 (GATA4) (Hs00171403_m1), human hepatocyte nuclear factor 3 beta (Hs00232764_m1), human LDL receptor (Hs00181192_m1), and human glyceraldehyde-3-phosphate dehydrogenase (Hs99999905_m1) were obtained from Applied Biosystems. It was confirmed that human detectors and rabbit detectors do not cross-react with the other species. TaqMan® Universal PCR Master Mix, No AmpErase® UNG (2×), was also purchased from Applied Biosystems. Reactions were performed in quadruplicate and the mRNA levels were normalized relative to human glyceraldehyde-3-phosphate dehy-

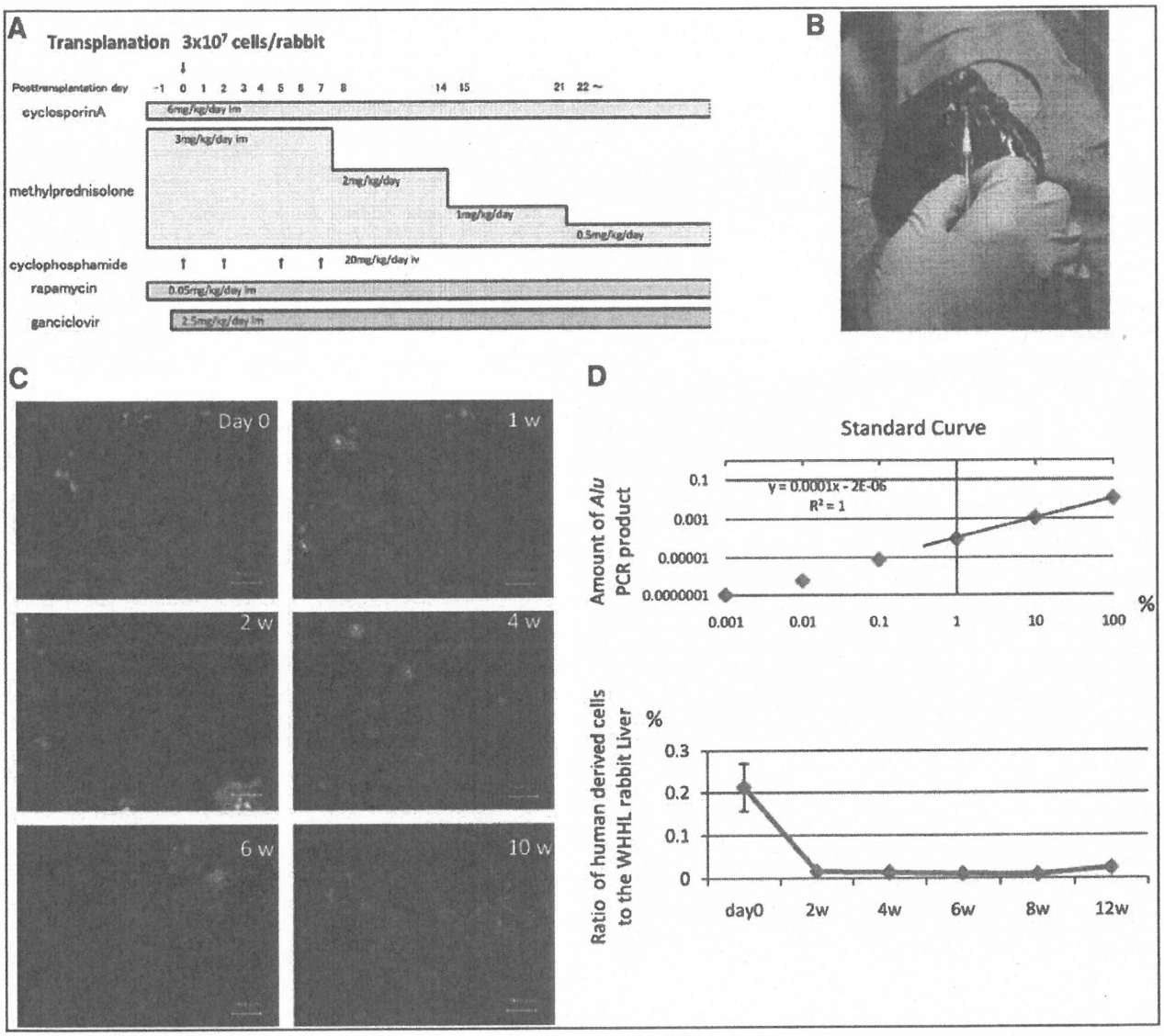

FIG. 2. **(A)** Immunosuppression regimen. Cyclosporin A (6 mg/kg/day) and rapamycin (0.05 mg/kg/day) were administered intramuscularly daily from the day before surgery to sacrifice. Methylprednisolone was administered at 3 mg/kg/day (days 1–7), 2 mg/kg/day (days 8–14), 1 mg/kg/day (days 15–21), and 0.5 mg/kg/day (day 22 to sacrifice). Cyclophosphamide (20 mg/kg/day) was injected intravenously at days 0, 2, 5, and 7. Ganciclovir (2.5 mg/kg/day) was also injected intramuscularly to avoid viral infection in the immunocompromised host. **(B)** Surgical procedure. Watanabe heritable hyperlipidemic (WHHL) rabbits were anesthetized with pentobarbital. An incision was made distal and parallel to the lower end of the ribcage. The peritoneum was incised and hADMPCs, and human adipose tissue-derived fibroblastic cells (hADFCs) (3×10^7 cells/rabbit) or controls were infused into the portal vein using an 18-gauge Angiocath. **(C)** Localization of transplanted hADMPCs in the WHHL liver. At the day of and 1, 2, 4, 6, and 10 weeks after transplantation of DiI-labeled hADMPCs via the portal vein, the WHHL rabbit liver was examined histologically. DiI-fluorescent labeled-hADMPCs resided and distributed in the portal area at the day of transplantation. One to 2 weeks after transplantation, the DiI-stained hADMPCs-derived cells were localized near the portal areas. Four weeks after transplantation some of the DiI-stained cells resembled innate hepatocytes morphologically. Six and 10 weeks after transplantation, DiI-positive transplanted cells were dispersed in a centrilobular direction, resembling the mature innate hepatocytes. Bars = 100 μm. **(D)** Quantification of repopulation of the transplanted cells in the liver. The ratios of human-derived cell repopulation were examined by analyzing an *Alu* repetitive DNA sequence at the day of and 2, 4, 8, and 12 weeks after transplantation. In upper panel the standard curve was indicated, and in lower panel the ratio of repopulation of human cells was shown in time course after transplantation of hADMPCs.