能を有する単球やマクロファージを*ex vivo*で*GFP*遺伝子を結合させたゼラチンと共培養を行ったところ，細胞内で*GFP*遺伝子の発現を確認した（図❺）．*GFP*遺伝子を貪食させた細胞を下肢虚血モデルのマウスまたは腸管癌モデルマウスの静脈内へ投与したところ，病態部位へ特異的に集合することが観察された．心筋の虚血-再灌流傷害モデルまたは下肢虚血モデルにおいて，血管新生因子を発現する遺伝子を導入したマクロファージの静脈内投与により，血管新生を示唆する血流量の増加を観察できた．一部の虚血肢モデルでは，放射光による微小循環造影法によって微小血管の増加を確認した．本法の優れた点は，ウィルスベクターを用いないため感染の危険性が少ないことに加えて，移植したマクロファージや単球が走化性によって傷害部位特異的に集まるため遺伝子の導入効率が高まること，並びに患部へ凝集したこれらの細胞自身が，導入された遺伝子をもとにタンパク質を合成することである．しかもマクロファージや単球は線維芽細胞や平滑筋細胞とは異なり血管内へ投与しても凝集することがなく，血管内投与が可能である．

Ⅲ．遺伝子導入した血管内皮前駆細胞を用いた血管新生療法

われわれが考案したゼラチンを用いた細胞内遺伝子導入法は，貪食能を持つ細胞であれば基本的に適用可能であると考えられる．従って，貪食能を有する血管内皮前駆細胞へあらかじめ遺伝子を導入して生体内へ細胞移植すれば，血管内皮前駆細胞自身の血管新生能力に加えて導入した遺伝子による血管新生作用の相乗効果が期待される．下肢虚血モデルに対する治療法としては既に，機能強化の目的で血管内皮前駆細胞へVEGFの遺伝子を導入して投与する方法が報告されている[10) 11)]．さらにわれわれは，血管内皮前駆細胞の有するvasculogenesis，angiogenesisと補完的な作用を有する遺伝子を導入することで，より成熟した血管床を再構築することを目指しており，本法を難治性の循環障害；心筋梗塞，下肢虚血，そして原発性肺高血圧症の治療に適用できないかと考えた．原発性肺高血圧症とは何らかの原因により肺血管抵抗の上昇が起こり，その結果として右心室負荷が生じる病態である．血管内皮細胞の機能不全が本病態の素因である可能性が指摘されており[12) 13)]，現在の治療法としては血管拡張物質である一酸化窒素，プロスタサイクリン，またはアドレノメヂュリンの全身性投与における治療効果が確認されている．現在われわれは，これらの遺伝子を導入した血管内皮前駆細胞を用い，人為的肺高血圧症ラットに対する細胞移植治療の効果を検討中であり，良好な治療効果を得つつある．

■おわりに

以上のことから，われわれの開発した生体吸収性ゼラチンを用いた新しい遺伝子導入法は，従来の方法に比べて効率性，安全性，治療法の簡便性等において優れた方法であると言えよう．今後は臨床応用を目指すべく遺伝子導入効率および治療効果のさらなる改善はもとより，遺伝子の発現部位や発現時期を制御する技術の開発が必要であろう．機能的により成熟した組織や器官の再生を可能とするには複数の細胞や遺伝子を組み合わせる必要があり，生体吸収性ゼラチンを用いた遺伝子導入法がこのような次世代の遺伝子治療法の確立の足がかりとなることを期待する．

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特集　循環器内科学における先進治療

ゼラチン*
—遺伝子複合体を用いた血管新生療法—

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Key Words: gelatine, gene therapy

遺伝子治療は21世紀において進歩の見込まれる新しい治療法の一つで，その進展に大きな期待が寄せられている[1)]．遺伝子治療は遺伝性疾患であるADA(アデノシンデアミナーゼ)欠損症から開始され，現在，米国をはじめ全世界で，家族性高コレステロール血症，嚢胞性線維症，血友病，ゴーシェ病などの遺伝病，下肢の末梢動脈閉塞性疾患や心筋梗塞などの虚血性疾患，慢性関節リュウマチのような自己免疫疾患だけでなく，メラノーマ，悪性脳腫瘍，白血病，神経芽細胞腫，腎癌などの悪性疾患にまでその適応が広がってきている[2)3)]．そのためすでに，3,000人以上の患者に遺伝子治療が試みられており，なかでも虚血性疾患に対する血管新生療法はADA欠損症についで臨床における有用性が期待される分野の一つである[4)]．

現在行われている遺伝子治療は，欠損遺伝子や変異遺伝子のかわりに，あるいはサイトカイン，血管新生因子などの生体作用物質を導入する目的で，外来の野生型遺伝子を導入して行う原因治療である．したがって，遺伝子を生体内の細胞に組み込む遺伝子導入法が遺伝子治療においてもっとも重要な技術的根幹を担うことになる．

遺伝子導入法は，目的遺伝子を物理学的あるいは化学的方法により細胞の中に遺伝子を導入する方法(トランスフェクション)と，ウイルスベクターを用いて遺伝子を細胞の中に導入する方法(トランスダクション)に大別される．トランスフェクションには，微小注入法，電子銃法，電気穿孔法やリポソーム法などが含まれ，トランスダクションとしては，アデノウイルスやレトロウイルスなどのウイルスベクターを用いた方法がすでに報告されている．

しかしながら，現在でも遺伝子導入法には技術的な問題がまだ数多く残されている．とくに，ウイルスベクターの安全性の問題，標的細胞への遺伝子導入効率の向上の問題，遺伝子発現の制御の問題などさまざまな難問が数多くあり，遺伝子治療の臨床応用が制限されている．そのため，早急に安全性の高い高効率の遺伝子導入

* Gelatine—angiogenic therapy with gelatine : gene complex—.

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法を開発することが必要であり，現在でも世界中の施設で模索されている[5]．

一方で，上記疾患のなかでも虚血性疾患に対する血管新生療法の分野において，骨格筋が遺伝子導入効率が悪いものの，プラスミドの筋肉内注射により骨格筋がプラスミドを取り込み蛋白を発現させることが可能であるため，早くから下肢の末梢動脈閉塞性疾患において遺伝子治療の検討が行われた[6]．

下肢の末梢動脈閉塞性疾患は，趾のしびれや冷感にはじまりやがて歩行時疼痛(間歇性跛行)を生じるようになる[7]．さらに進行すると，虚血性潰瘍，安静時疼痛や組織壊死などの症状を呈し強い苦痛を伴う疾患である[8]．治療は，保存的療法(禁煙，保温，薬物療法，歩行療法など)，外科的治療〔経皮的血管拡張術(PTA：percutaneous transluminal angioplasty)，バイパス術など〕などが行われる[9]．これらの治療法の進歩により下肢切断にいたる症例は減少した[10]．しかし，いまだ多くの患者で強い症状があるにもかかわらず，末梢血管狭窄や血管炎があるために血管拡張術やバイパス術のような血行再建術の治療対象となりえず，いまだ全世界で年間100万人中300人前後の患者が下肢の切断術を受けている[11]．安静時疼痛や虚血性潰瘍がある患者で治療に反応しない症例では，その痛みのために日常活動が制限されるうえに，ただ下肢の切断を待つだけの状況になってしまう．このような状況下において難治性の末梢動脈閉塞性疾患に対する新たな治療として，血管新生因子を用いた遺伝子治療が追求されることはごく自然の流れであったように思われる．

血管新生療法を成功させるためには有用な血管新生因子を用いることが必要になる．これまでに，血管新生因子としてFGF(fibroblast growth factor)，VEGF(vascular endothelial growth factor)，HGF(hepatocyte growth factor)，EGF(epidermal growth factor)やPDGF-B(platelet-derived growth factor-B)などが報告されている[12,13]．

これらの血管新生因子が実際の生体における血管新生時にどの程度関与しているのかははっきりしていない．しかしながら，外来遺伝子補充療法という観点からその強い血管内皮細胞増殖作用が認められているFGF, VEGF, HGFなどを用いた治療法が早期からよく検討されている．これらの血管新生因子はそれぞれ血管内皮細胞増殖作用だけでなくさまざまな生物活性作用も有する．たとえばFGFで，線維芽細胞増殖，平滑筋細胞増殖や前立腺細胞増殖などの作用，HGFで肝細胞増殖作用などの血管新生とは異なった作用が報告されており，今後厳密には病態に応じた各遺伝子の使い分けが必要になると思われる．

血管新生因子を用いた血管新生療法は，1992年Baffour[14]らによりはじめて報告された．彼らは家兎の下肢虚血モデルを用い，下肢に1～3μgのFGF-2を連日筋肉内投与し血管新生が生じるか検討した．その結果，FGF-2の投与によりコントロール群に比し虚血肢で有意な血管新生が認められ，FGF-2の筋肉内投与が虚血肢における血管新生を増強することを報告した．その後Takeshitaら[15]は，VEGFを用いた血管新生療法を報告した．彼らは家兎の下肢虚血モデルを用い，500～1,000μgのVEGFを動脈内に投与し虚血肢に血管新生が生じることを示し，VEGFによる血管新生療法の有用性と血管新生因子の動脈内投与の有用性を明らかにした．その後，諸家により続々と血管新生因子を用いた血管新生療法の有効性が報告されたが，これらの報告はすべて遺伝子ではなく血管新生因子を用いた血管新生療法の報告であった．

ヒトへの応用を考えると大量のタンパク質の精製には莫大なコストがかかり，血管新生療法を広く普及させるときの障壁となる．すでに骨格筋に遺伝子が取り込まれることは報告されているので，Riessenら[16]は，ハイドロゲルポリマーを用いバルーンカテーテルにルシフェラーゼDNAを含有させることにより動脈内に直接遺伝子を導入することが可能か検討し，経動脈的に遺伝子を導入することが可能であることを示した．この成功により，VEGFのプラスミドを虚血肢の筋肉内投与あるいは血管内投与により遺伝子治療を行うことが可能となり，米国においてVEGFを用いたヒトへの血管新生療法が開始された[17]～[19]．

Isnerらは，下肢の安静時疼痛や難治性潰瘍を有する9人の末梢動脈閉塞性疾患患者の10肢(うち難治性潰瘍7肢)に*phVEGF*$_{165}$(plasmid human

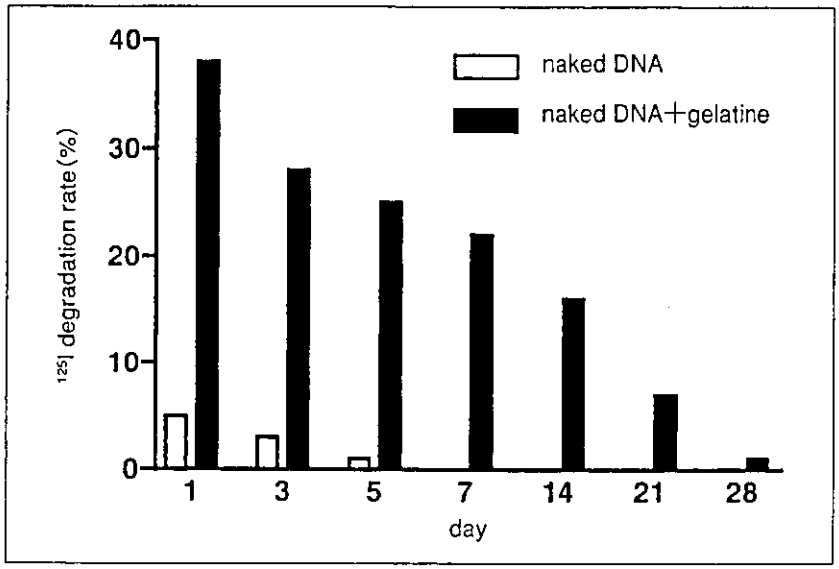

図 1　DNA degradation rate

$VEGF_{165}$)を投与し，臨床症状(疼痛や難治性潰瘍)や血流が改善するかどうか検討した．　治療後，疼痛は減少し7肢中4肢の難治性潰瘍が治癒し，下肢切断術が推奨されていた患者3人が下肢切断術を受けずに済み，実際の血管造影で血管新生が著明に生じていることが確認された．また，anckle-brachial indexやtoe-brachial indexも著明に改善していた．このように，VEGFによる重度の末梢動脈閉塞性疾患患者に対する治療は予想以上に好成績をあげ，安静時疼痛や難治性潰瘍を有する患者に対し新たな治療としてその選択肢が広がった．しかしながら，*pVEGF*投与中に血液中のVEGF濃度が上昇する可能性があり，その適応には十分な検討が必要である[20]．とくに，血管新生療法を受ける患者が癌や増殖性網膜症を有していないかなど十分確認する必要がある．

このような状況の中でわれわれは，どのようにしたら筋肉細胞内に投与した遺伝子の導入効率を上昇することができるか検討した．遺伝子の導入効率が低い理由の一つとして生体内にはさまざまな核酸分解酵素が存在しており，そのため生体内に投与された遺伝子がただちに分解されてしまう，あるいは生体内に投与した遺伝子がただちに拡散してしまうという機序が考えられている．実際にわれわれは，生体内に投与した遺伝子がどの程度まで筋組織内に残存することが可能か検討した．正常マウス下肢に^{125}IでラベルしたLacZ のnaked DNA 50μgを筋注し，その分解率を検討した．遺伝子投与1日後にすでに遺伝子は投与量の5％と大幅に減少していた．投与5日後には1％まで減少しており，7日後には遺伝子は筋肉内から完全に消失していた(図1)．この結果から考えると，遺伝子投与量は1週間に1回の筋肉内投与として有効治療量の約100倍の濃度を注射する必要があることになる．そこでわれわれは，少しでも生体内における遺伝子の分解の抑制をめざし遺伝子の徐放化について検討した．徐放化に際して，①生体内で緩除に遺伝子が分解されること，②遺伝子の活性を落とさないこと，③簡単に徐放化することができることなどが要求された．そこでわれわれは，ゼラチンを徐放化剤として利用することを考えた．ゼラチンはその構造，サイズや表面荷電を容易に変えることが可能な物質である[21]．われわれは，その表面荷電を陽性とし格子状とすることで，陰性荷電した遺伝子とイオン結合させ遺伝子の徐放化させることを考えた．われわれが実験で使用しているゼラチンは生体内のプロテアーゼで分解されるため，ヒトへの投与が可能である．そこでわれわれは，前述した方法と同様にしてゼラチンを用い遺伝子の分解率を比較検討した．その結果，遺伝子単独投与群と比較しゼラチンで徐放化した遺伝子群で

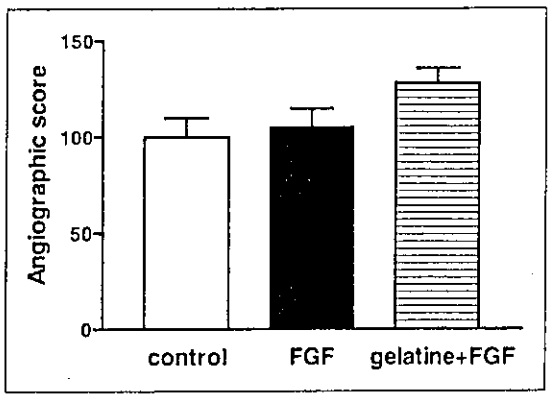

図2　Angiographic score after treatment

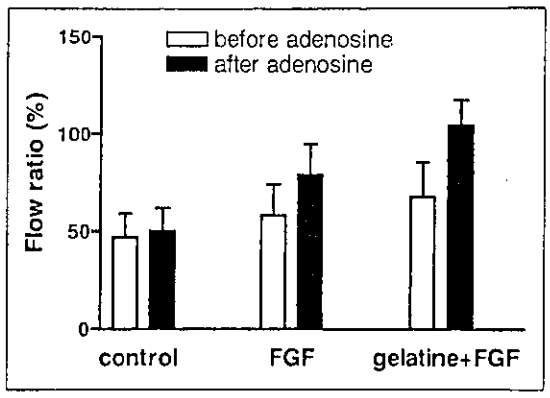

図3　Flow ratio before/after adenasine treatment

は，遺伝子投与1日後には38%に分解されるが，投与7日後でも22%遺伝子が残存し，投与14日後でも16%と1割以上の遺伝子が生体内に残存することが確認され，投与28日後でも1%残存していた(図1)．このことから，ゼラチンと遺伝子を結合させて徐放化することにより，遺伝子単独投与と異なり遺伝子投与量は2週間に1回の筋肉内投与として有効治療量の約10倍の濃度を注射するだけで治療効果を上げることができることになる．次に筋組織内での遺伝子の発現率に差があるか検討した．家兎下肢虚血モデルを作成し*pLacZ* 500μgおよびゼラチン＋*pLacZ* 500μgを虚血肢に筋肉内投与し組織中のLacZの発現を検討した．遺伝子単独投与群に比しゼラチン＋遺伝子群で，LacZの発現は1視野あたり平均約10倍の発現の増加が認められた．次に，実際に家兎下肢虚血モデルを用いてその治療効果について遺伝子単独投与群と比較検討した．下肢虚血モデルは，左大腿動脈を摘除したモデルを使用した．動脈を摘除後10日目に，血管新生因子である*pFGF-4* 500μgまたは，ゼラチン2mg＋*pFGF-4* 500μgを虚血部位に5カ所に分けて筋注し，コントロール群，遺伝子単独投与群，ゼラチン＋遺伝子投与群の3群で動脈摘除後38日目の血管新生効果を比較検討した．血管新生の評価は，血管造影後のangio-graphic scoreと健常肢に対する虚血肢の血流比で行った．血流の計測はマイクロスフェアーを用いて行った．虚血モデル作成後コントロール群において，angio-graphic scoreは100±10.0，遺伝子単独投与群で105±10.0，ゼラチン＋遺伝子投与群で128±8.00と，ゼラチン＋遺伝子投与群において，コントロール群や遺伝子単独投与群に比し血管造影上有意に血管新生を認めた(図2)．また，実際の血流もコントロール群で47.0±12.0%，遺伝子単独投与群で58.0±16.0%，ゼラチン＋遺伝子投与群で68.0±18.0%であり，ゼラチン＋遺伝子投与群において，コントロール群に比し有意な血流の増加を認めた(図3)．実際にわれわれの生体では，運動負荷時に局所でアデノシンや乳酸などの強い血管拡張物質が産生されて血管を拡張し，組織の酸素需要に対応できるようなシステムが作動している．そこで，血管新生療法によりできた血管がアデノシンに対して反応性をもつかどうか，アデノシンを投与後血流を計測し検討した．アデノシン投与後の血流はコントロール群および遺伝子単独投与群において，50.0±12.0%，79.0±16.0%とアデノシン投与前と比較して変化が認められなかった．しかし，ゼラチン＋遺伝子投与群での血流はアデノシン投与後105±13.0%とアデノシン投与前に比し有意に上昇しており，ゼラチンで遺伝子を徐放化することにより新生血管の生理学的反応性も高めることが明らかになった(図3)．

以上の点からゼラチンを用いた血管新生療法は遺伝子が徐放化されるだけでなく，その導入効率の改善や生理学的反応性をもつ血管の新生などさまざまな治療的長所を有し，今後ヒトへの臨床応用が期待される．

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REVIEW

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Tissue engineering for myocardial regeneration

Abstract Recent progress in stem cell biology has shown the possibility of implantable human myocardial cell sources. It has encouraged myocardial tissue engineering for rescuing damaged hearts. The present strategy is to repair not all of the myocardial tissue, but part of it. There are two approaches. The first is direct injection of dissociated cell suspensions via the pericardium, coronary arteries, or endocardium. Studies using animal models have found improved heart function after transplantation of various types of cells. Myoblasts or bone marrow cells have already been transplanted into patients suffering from severe ischemic heart disease. In direct transplantation of dissociated cells, it is difficult to control the shape, size, and location of the grafts. To solve these problems, further therapies to transplant tissue-engineered three-dimensional (3-D) heart grafts have been investigated. The most popular technique in tissue engineering is to use 3-D biodegradable scaffolds as alternatives to the extracellular matrix. On the basis of this concept, poly(glycolic acid)(PGA), gelatin, alginate, and collagen have been used as scaffolds to fabricate 3-D heart tissues. A new method consisting of layering cell sheets to construct 3-D tissues without any artificial scaffolds has also been applied to myocardial tissue engineering. Electrically communicative pulsatile heart tissues have been achieved both in vitro and in vivo by layering cardiomyocyte sheets. Although myocardial tissue engineering has rapidly progressed, there are several problems to be solved with regard to the source of myocardial cells, tissue reconstruction, neovascularization, and transplantation technology. Further interdisciplinary research will solve these problems, and transplantation of cells or engineered heart tissues will become one of the major treatments for severe heart failure in the near future.

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Introduction

Recently, stem cell biology and tissue engineering have rapidly progressed, encouraging research on cardiovascular tissue reconstruction from cultured cells to rescue patients suffering from heart failure. Stem cell biology has revealed that various types of stem cells exist in the body and that embryonic stem (ES) cells can differentiate into various cell lineages in vitro.[1] Many researchers are now trying to establish new methods of isolating stem cells and controlling their differentiation.[2,3] It is also crucial to grow enough cells for tissue reconstruction. On the other hand, tissue engineering was proposed by Langer and Vacanti in 1993. This is an interdisciplinary field of research that seeks methods of bioengineering three-dimensional (3-D) tissues.[4] The concept is that preparations of cells, extracellular matrix (ECM), and growth factors together lead to tissue reconstruction. Langer and Vacanti used 3-D biodegradable scaffolds including poly(glycolic acid) (PGA) and poly(lactic acid) (PLA) as alternatives to the ECM. Cartilage engineered by their technology is now clinically used. The use of 3-D scaffolds has been applied to the fabrication of almost all tissues and has promoted research in tissue engineering.

In cardiovascular tissue engineering, vascular grafts and heart valves have been fabricated by various technologies.[5,6] At first, investigators tried to construct hybrid vascular grafts, which are synthetic grafts seeded with endothelial cells for antithrombogenesis. Recently, based on Langer and Vacanti's concept, natural and synthetic biodegradable materials have been used as temporary scaffolds. Tissue-engineered vessel constructs with rupture strength of more than 2000 mmHg have been reported.[7,8] Bioengineered vascular grafts using PGA scaffolds and autologous vascular cells have been clinically implanted into several children with heart malformations, and their patency has been reported.[9] Several types of biodegradable scaffolds formed

into appropriate shapes have been used to construct heart valves as same as vascular grafts. Decellularized xenogenic valves have also been used as 3-D scaffolds. These tissue-based constructs may overcome the problems of prosthetic devices, including thromboembolic complications and reoperation due to the inability of the devices to grow.

In contrast to research on blood vessels and heart valves, research on cardiac muscle tissue engineering has been delayed because of the difficulty of establishing myocardial cell sources. However, recent progress in stem cell biology has shown the possibility of implantable human myocardial cell sources and has accelerated myocardial tissue engineering.[5,6] At present most researchers are attempting to repair not all of the myocardial tissue, but part of it. There are two approaches to rescue impaired hearts. The first is direct transplantation of dissociated cells, which regenerate and remodel in the surrounding tissue, resulting in restoration of heart function. The second is to fabricate 3-D heart grafts by tissue engineering technology and to transplant the grafts into damaged hearts. These approaches have been expected to result in new therapeutic strategies for severe heart failure, replacing conventional treatments. In this article, we focus on the rapid progress of myocardial tissue engineering and discuss present problems and future perspectives.

Cell sources and isolated cell transplantation

Research on direct transplantation of cells for impaired heart tissue has been conducted since the early 1990s. Soonpaa et al. first reported the possibility of isolated cardiomyocyte transplantation into myocardium.[10] They demonstrated that fetal mouse cardiomyocytes grafted into syngeneic host hearts survived, and they confirmed that nascent intercalated disks connected the engrafted cardiomyocytes and the host myocardium. Other studies demonstrated that engrafted fetal cardiomyocytes formed new tissues in myocardial infarction model animals, and some of them reported improvement in heart function.[11,12] Desirable effects of transplantation of heart cells into globally damaged hearts was also reported in a study using a dilated cardiomyopathic myocardium model.[13] Several studies found that transplantation of fetal rat cardiomyocytes was more effective than transplantation of neonatal or adult rat cardiomyocytes.[11,12] These reports suggest that less differentiated cells may have more regenerative power, leading to more efficient support for heart function.[14,15] From this point of view, ES cells may be hopeful cell sources. ES cells have the abilities to proliferate in an undifferentiated state and to differentiate into various types of cells in response to specific stimuli. It has already been reported that ES cells can differentiate into cardiomyocytes in vitro in both mice and humans.[16] Min et al. demonstrated that implantation of mouse ES cells improved cardiac function in infarcted rat hearts.[17] Recent progress in nuclear transfer technology raises the possibility of nonimmunoreactive ES cell production; therefore, ES cell-derived cardiomyocyte transplantation may be available in the near future. Although many researchers expect a great potential for ES cells, clinical application of human ES cells faces the same ethical problems as that of human fetal cardiomyocytes.

On the other hand, stem cells originating from bone marrow have been investigated as desirable cell sources in terms of ethical problems and immunoreactions. Makino et al. established a cardiomyogenic cell line from murine bone marrow stromal cells by using 5-azacytidine, which regulates the genes related to transdifferentiation.[18] Tomita et al. reported that rat bone marrow cells cultured with 5-azacytidine differentiated into cardiac-like muscle cells, and transplantation of these cells improved myocardial function in cryoinjured rat hearts.[19] Recently, Orlic et al. demonstrated that sorted bone marrow cells, which are lineage-negative and c-kit-positive cells, formed new myocardial tissue in infarcted hearts, restoring their function.[20] Surprisingly, these totipotent bone marrow cells differentiated into myocytes, endothelial cells, and smooth muscle cells without any specific agents. It has also been reported that human mesenchymal stem cells from bone marrow engrafted in the adult murine myocardium appear to differentiate into cardiomyocytes.[21] These reports indicate that multipotent bone marrow cells can differentiate and regenerate de novo tissues in response to the surrounding tissues. Thus, bone marrow may be the best source of cells for myocardial tissue engineering.

Myoblasts have also been investigated as potential cell sources instead of cardiomyocytes. They exist between skeletal myocytes and differentiate into muscle when tissues are damaged. They can be isolated from autologous muscle biopsies and can be expanded in vitro. One of their favorable features is that they are more resistant to ischemia than cardiomyocytes. Differentiated myotube formation and long-term survival of myoblast grafts in murine hearts were demonstrated by Koh et al.[22] Murry et al. reported that skeletal myoblasts regenerated new muscle tissue when grafted into injured hearts, and that this tissue contracted when stimulated electrically.[23] Taylor et al. found improvement of cardiac function after autologous skeletal myoblast transplantation into cryoinfarcted rabbit hearts.[24] Menasche et al. first reported the clinical application of myoblast transplantation for heart failure.[25] They implanted autologous myoblasts into the postinfarction scar during coronary artery bypass procedures and confirmed their contraction and viability in the grafted scar. A clinical trial of skeletal myoblast transplantation is now ongoing. In addition to skeletal myoblasts, transplantation of autologous smooth muscle cells prevented cardiac dilatation and improved ventricular function in hamsters with dilated cardiomyopathy.[26] These positive effects of transplanted cells have been considered to result from the inhibition of heart tissue remodeling, including wall thinning and heart dilatation, and also from neovascularization by growth factors secreted from the transplanted cells.

Dissociated cell transplantation has been performed by direct myocardial injection, coronary artery injection, or an intraventricular approach. The NOGA system (Biosense Webster) is used for the intraventricular approach. The

system incorporates a miniature location sensor and electrodes into a catheter that is inserted into the ventricle, recording intracardiac electrical activation and ventricular motion in real time. These data provide realistic 3-D dynamic heart images and serve as guidance for cell injection. Because methods of cell injection influence the efficacy of cell transplantation, improvements in these methods are indispensable in myocardial tissue engineering.

Construction of 3-D myocardial tissue

In direct transplantation of dissociated cells, it is difficult to control the shape, size, and location of the transplanted grafts. Therefore, research on methods of transplanting tissue-engineered functional heart grafts has begun.[4,5] As described above, the most popular approach of tissue engineering is to use 3-D biodegradable scaffolds as alternatives to ECM. The concept has also been applied to myocardial tissue engineering; two techniques are used to seed cells into scaffolds. One is to pour cells onto prefabricated, highly porous scaffolds (Fig. 1A). The other is to polymerize biodegradable molecules after mixing them with isolated cells (Fig. 1B). In the first technique, PGA, gelatin, or alginate has been used as a prefabricated scaffold. Freed's group reported that cultivation of neonatal rat cardiac myocytes on PGA scaffolds processed into 97% porous meshes resulted in contractile 3-D tissues.[27-29] Rotating bioreactors were used for seeding the cells; mixed cultivation had several advantages over static conditions, including higher cellularity of the construct, more aerobic cell metabolism, and more differentiated cells. Freed confirmed that the electrical conduction velocities in native heart tissues and the engineered constructs were comparable and showed the feasibility of using the constructs as in vitro models for electrophysiological studies. Li et al. developed tissue-engineered cardiac grafts using gelatin sponges.[30,31] The gelatin grafts, which had been seeded with fetal rat cardiomyocytes for 7 days, were sutured to cryoinjured rat hearts. The cells within the grafts survived and formed junctions with host hearts 5 weeks after operation. A gelatin patch was used to replace a right ventricular outflow tract.[32] After 12 weeks, the reconstructed tissue complemented the wall, even after the gelatin scaffold had been completely dissolved. Leor et al. also reported bioengineered heart grafts using porous alginate scaffolds with an average pore diameter of 100μm.[33] Fetal rat cardiac cells were seeded into the dry alginate scaffolds by dropping the cell suspension and were cultured for 4 days. The constructs were transplanted onto the scars of coronary-ligated rat hearts 7 days after operation. Histological studies showed almost

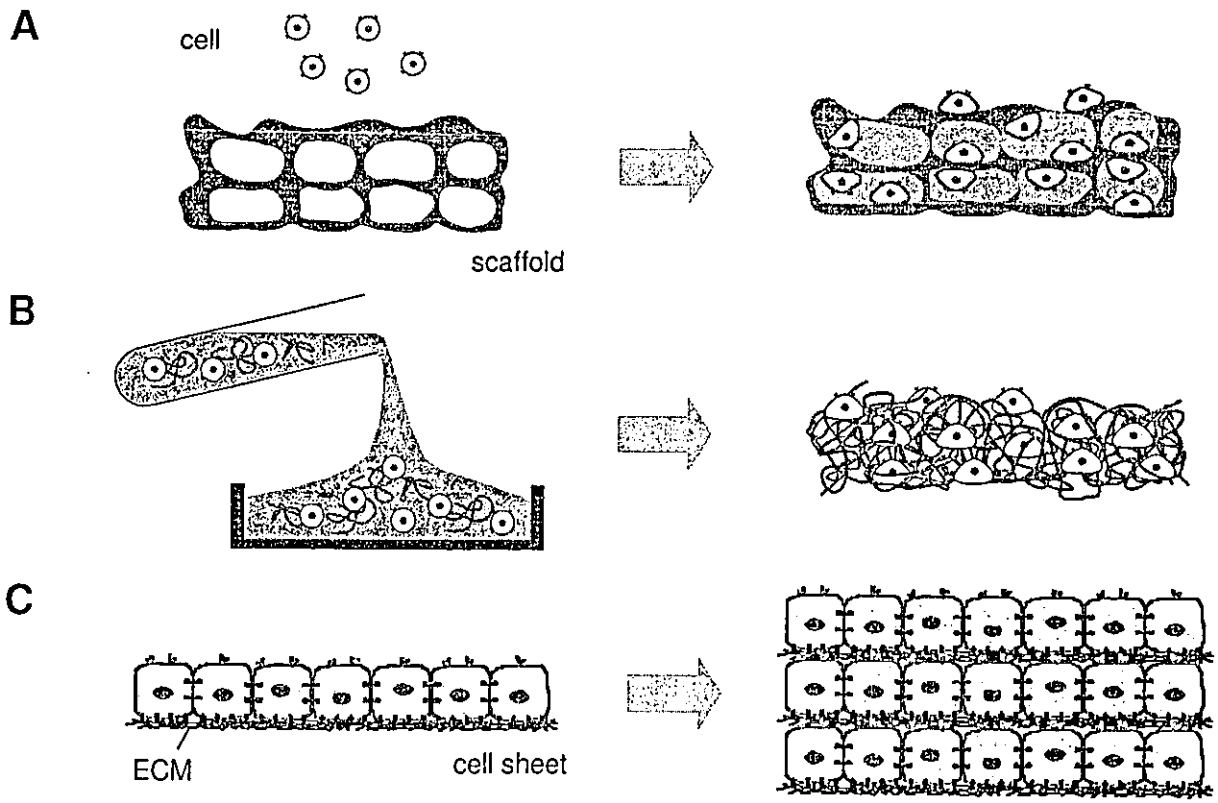

Fig. 1. Tissue engineering methodologies. A Isolated cells are poured onto prefabricated, highly porous scaffolds. The scaffolds are biodegraded, and extracellular matrix (*ECM*) occupies the space within the cells, leading to 3-D tissues. B A mixture of isolated cells and biodegradable molecules is poured into an appropriate mold, and then the molecules are polymerized. The construct is regenerated into tissues. C Intact cell sheets released from temperature-responsive culture surfaces are layered. Cell sheets adhere to each other via biological ECM, resulting in 3-D tissues containing no biodegradable scaffolds

complete disappearance of the scaffolds and the presence of transplanted myocytes 9 weeks after implantation. The grafts attenuated left ventricular dilatation and deterioration of heart function.

For the second technique of premixing cells and ECM alternatives, Eschenhagen's group used collagen gel as a scaffold.[34,35] Cardiac myocytes were mixed with ice-cold collagen solution and cast into silicone molds. By gelling at 37°C and culturing for 4 days, 3-D contractile heart tissues were fabricated. Eschenhagen established a system of measuring the force of the constructs isometrically and demonstrated typical features of native hearts, including a positive force-length relation (Frank-Starling mechanism), a high sensitivity to calcium, and a positive inotropic effect of isoprenaline. It has also been reported that chronic mechanical stretch guides cardiac myocytes into unidirectionally elongated cells and induces hypertrophy, resulting in improvement of contractile function.[36]

New approach in tissue engineering

In contrast to the technology using 3-D biodegradable scaffolds as alternatives for ECM, we have exploited a new method of tissue engineering that layers cell sheets for the construction of 3-D tissues (Fig. 1C). Cell sheets are obtained by using novel cell culture surfaces grafted with a temperature-responsive polymer, poly(*N*-isopropylacrylamide)(PIPAAm).[37,38] These culture surfaces are slightly hydrophobic and cell adhesive under culture conditions at 37°C and change reversibly to a hydrophilic and non-cell-adhesive state below 32°C due to rapid hydration and swelling of grafted PIPAAm. In contrast to cells that have undergone enzymatic digestion, including trypsinization, both cell-to-cell junctions and adhesive proteins within confluent cultured cells are completely preserved, leading to production of intact cell sheets by detachment from PIPAAm-grafted surfaces. Various types of cell sheets have been successfully obtained and transferred onto other surfaces.[39–42] 3-D tissues can be fabricated by layering cell sheets without any artificial scaffolds.

This technology has also been applied to myocardial tissue engineering.[43–45] Neonatal rat cardiomyocyte sheets detached from PIPAAm-grafted surfaces were overlaid to construct cardiac grafts. Layered cell sheets began to pulse simultaneously, and morphological communication via connexin 43 was verified between the sheets. Four-layer constructs were macroscopically observed to pulse spontaneously. In vivo, surface electrograms originating from the grafts transplanted into subcutaneous tissues of nude rats were detected (Fig. 2), and their spontaneous beating was macroscopically observed. Histological studies showed characteristic structures of heart tissue, including elongated cells, sarcomeres, desmosomes, and gap junctions (Figs. 3 and 4). Multiple neovascularization was also observed within contractile tissues. These results demonstrate that electrically communicative pulsatile 3-D cardiac constructs are achieved both in vitro and in vivo by layering cardiomyocyte sheets. In collaboration with Matsuda and Sawa's group at Osaka University, improvement of cardiac function in ischemic heart models by transplantation of layered cardiac grafts has been demonstrated.[46]

Problems and future perspectives

Although myocardial tissue engineering has rapidly progressed and has the possibility of repairing damaged hearts, there are several problems to be solved. As previously mentioned, the source of myocardial cells is one of the most critical problems. From the point of view of immunorejection and ethical problems, cardiomyocytes derived from bone marrow cells may be most desirable. But some breakthrough technologies in isolation, differentiation, and expansion of stem cells will be needed for clinical application. Recently, multipotent cells have been reported to differentiate into cardiomyocytes in specific environments in cardiac muscle tissue or in co-culture with

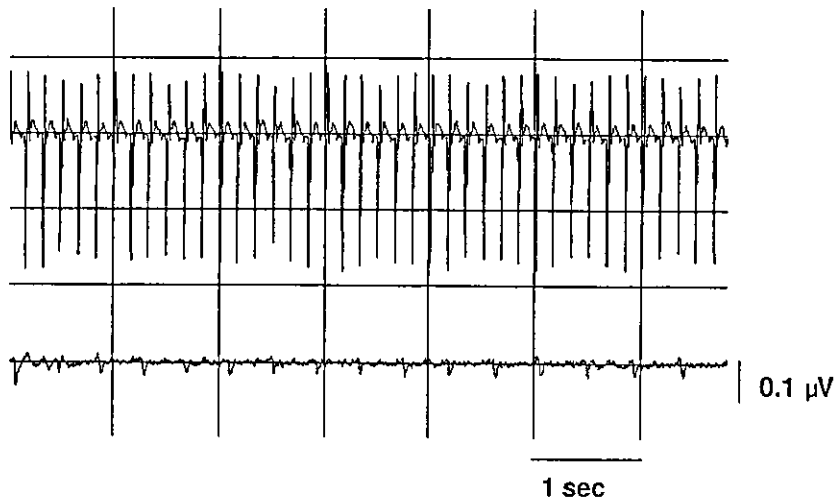

Fig. 2. Skin surface electrogram of transplanted cardiomyocyte sheets. *Upper tracing* shows the electrocardiogram of the host heart. *Lower tracing* shows the electrogram detected from the electrode set at the skin surface just above the graft transplantation site. Skin surface electrogram originating from transplanted cardiomyocyte sheets is detected independently of host electrocardiogram

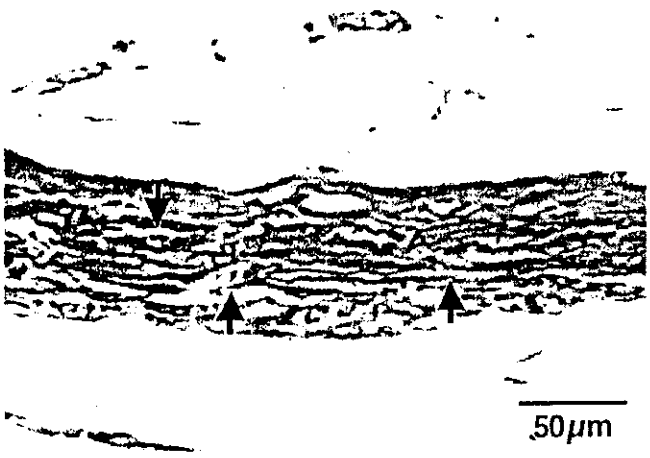

Fig. 3. Histology of transplanted cardiomyocyte sheets (three layers). Azan staining shows a striated cardiac tissue graft including elongated cardiomyocytes and multiple neovascularization (*arrows*)

Fig. 4. Transmission electron microscopic image of transplanted cardiac graft. Well-differentiated sarcomeres and characteristic cell-to-cell connections, including desmosomes (*arrows*), are shown

cardiomyocytes.[20,21,47] Although they may be candidates for myocardial cell sources, some researchers point out the possibility that stem cells may fuse only with native cardiomyocytes. Further studies will be needed to clarify this controversial point.

As also mentioned previously, it is difficult to control the shape, size, and location of injected cells in isolated cell transplantation. Moreover, spilling out of transplanted cells into microvessels has occurred. Bioengineered cardiac grafts using 3-D scaffolds may solve these problems. However, several difficulties have also been raised in engineering 3-D heart tissue grafts. One is heterogeneity of the tissues due to insufficient migration of the cells into biodegradable scaffolds. Another is the inflammatory reaction accompanying scaffold biodegradation. To prevent these problems, newly designed biodegradable scaffolds and culture systems will be needed. Elasticity of the scaffolds is also important to allow following of the pulsatile native heart. In light of these concerns, layering cardiomyocyte sheets without any artificial scaffolds may be a feasible technology for myocardial tissue engineering.

Vascular reconstruction is also one of the most critical issues in myocardial tissue engineering. Although multiple sites of neovascularization originating from host tissues into implanted cardiac grafts have been demonstrated, primary insufficient oxygen and nutrition permeation limit the size of transplanted myocardial tissues. Zhang et al. reported that many injected cells die in rat infarction models.[48] It has also been noted in tissue-engineered heart grafts that cells are dense in the graft periphery, but sparse in the interior part due to insufficient oxygen perfusion.[28] New techniques to accelerate blood vessel formation are needed to engineer larger or thicker constructs for repair of heart tissues. Gene-modified cell transplantation is one possible way of improving neovasculogenesis.[49,50] In vitro microvascular engineering may be one approach to the construction of larger tissues. Recently, Kaihara et al. reported an attempt to fabricate branched microvascular structures on silicon and pyrex surfaces by micromachining technology.[51] Further research and development will be needed to engineer preformed vascular networks for clinically applicable heart tissues.

Several bioreactors have been used to strengthen heart tissue grafts, as described above. Akins et al. originally used a rotating bioreactor for culturing cardiomyocytes.[52] The flow condition significantly affects cell migration and differentiation. Improved bioreactors and appropriate culture conditions may augment engineered heart function. It is well known that mechanical stretch causes cardiomyocyte hypertrophy. Eschenhagen et al. clearly demonstrated that application of stretch devices to engineering heart tissues strengthened the tissues and oriented the cells.[36] Mechanical stretch seems to be very useful in myocardial tissue engineering. Further development of these devices will be accomplished by participation of mechanical engineers in myocardial tissue engineering research.

In native hearts, cardiomyocytes are elongated and aligned, resulting in unidirectional contraction. Imitating this cell alignment may improve the contraction power of engineered tissues. One method of orienting randomly cultured cells is to stretch the constructs unidirectionally, as Escenhagen et al. demonstrated. Another approach is to align cardiomyocytes primarily by controlling cell adhesion. Stuart et al. demonstrated that cardiomyocytes attached to stripe-patterned cell-adhesive culture surfaces fabricated by a photolithographic technique, resulting in cell elongation and unidirectional contraction.[53] They found a faster electrical conduction velocity in aligned cells than in randomly cultured cells. Application of microfabrication technology has become popular in tissue engineering research. Aligned 3-D scaffolds for heart tissue reconstruction may be realized in the near future.

In the clinical setting, patients may wait for several days until autologous cells are expanded and engineered into 3-D heart tissues. In addition, it may take a long time until graft cells reconstruct and perform functionally after the transplantation. In such cases, combined therapy with mechanical support may be beneficial. A combination of

several therapeutic strategies should be pursued to rescue patients suffering from severe heart failure.

This review has focused on heart tissue repair, but engineered 3-D heart tissues should be applicable to various types of studies as in vitro heart models. Bursac et al. demonstrated the feasibility of engineered heart constructs as in vitro impulse propagation study models.[28] Eschenhagen et al. first directly measured the isometric contractile force of engineered 3-D heart tissues.[34] These bioengineered heart tissues should contribute to biological, physiological, and pharmacological studies.

Myocardial tissue engineering has rapidly progressed by the development of stem cell biology and tissue engineering. Further interdisciplinary research will be needed to solve several problems. We expect that transplantation of cells or engineered heart tissue grafts will become a major therapeutic strategy for severe heart failure in the near future.

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GENE DELIVERY

In vitro gene expression by cationized derivatives of an artificial protein with repeated RGD sequences, Pronectin®

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Abstract

The objective of this study is to investigate the efficiency of a non-viral gene carrier with RGD sequences, Pronectin F^+ for gene transfection. The Pronectin F^+ was cationized by introducing ethylenediamine (Ed), spermidine (Sd), and spermine (Sm) to the hydroxyl groups while the corresponding gelatin derivative was prepared similarly because gelatin also has one RGD sequence per molecule. The ζ potential and molecular size of Pronectin F^+ and gelatin derivatives were examined before and after polyion complexation with a plasmid DNA of luciferase. When complexed with the plasmid DNA at the Pronectin F^+/plasmid DNA mixing ratio of 50, the complex exhibited a ζ potential of about 10 mV, which is similar to that of the gelatin derivative–plasmid DNA complex. Irrespective of the type of Pronectin F^+ and gelatin derivatives, their complexation enabled the apparent molecular size of plasmid DNA to reduce to about 200 nm, the size decreasing with the increased derivative/plasmid DNA weight mixing ratio. The rat gastric mucosal (RGM)-1 cells treated with both complexes exhibited significantly stronger luciferase activities than free plasmid DNA although the enhanced extent was significant for the Sm derivative compared with the corresponding Ed and Sd derivatives. Cell attachment was enhanced by the Pronectin F^+ derivative to a significant high extent compared with the gelatin derivative. The amount of plasmid DNA internalized into the cells was enhanced by the complexation with every Pronectin F^+ derivative compared with the gelatin derivative. For both of Pronectin F^+ and gelatin carriers, the buffering capacity of Sm derivatives was higher than that of Ed and Sd derivatives and comparable to that of polyethyleneimine. It is likely that the high efficiency of gene transfection for the Sm derivative is due to the superior buffering effect. We conclude that the Sm derivative of Pronectin F^+ is promising as a non-viral vector of gene transfection.

Keywords: Pronectin; RGD sequences; Cationization; In vitro transfection; Buffering capacity

1. Introduction

Gene transfection is a powerful and promising technique that involves the in vitro or in vivo introduction of exogenous genes into cells for experimental and therapeutic purposes. Whichever is the final goal for experimental biology and gene therapy, the first key issue to be dealt is to enable the gene to internalize into the cell as efficiently as possible and to facilitate the expression for a long or

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short time period. Recently, a number of DNA delivery systems have been investigated targeting the improved the efficacy of gene transfection [1–3]. There have been two major approaches proposed for gene delivery: the viral-mediated and non-viral-mediated gene transfection [4]. However, considering the immunological and safety issues of viral vectors, necessity in the development of non-viral vector systems has been increasingly magnified. There are several advantages of chemically based, non-viral vectors. Besides their lower toxicity and inducivity of immune responses than the viral vector, no integration into the genome is indicated.

The basic research approach of non-viral vectors is to neutralize the negative charge of plasmid DNA and to condense the DNA size by polyion complexation with various polymers and liposomes of positive charge [5–8]. It is likely that the condensed plasmid DNA complex with a positive charge effectively interacts with cells to internalize, enhancing the efficiency of gene transfection. However, since this complex-cell interaction is based on the simple and non-specific electrostatic force, the more enhanced and cell-specific cell transfection cannot be always expected. One of practically possible ways to improve this situation is to take advantage of cell receptor systems which play an important role in the cellular uptake of several substances. In this study, we selected and use a ligand specific to the cell receptor as the non-viral vector of plasmid DNA. The sequences of RGD (arginine–glycine–aspartic acid) has been discovered as a cell attachment sequence in various adhesive proteins present in the extracellular matrix (ECM), and found in many proteins, such as fibronectin, collagen type 1, vitronectin, fibrin, and Von Willebrand Factor [9]. It has been well recognized that the sequence interacts with various types of integrin receptors. There are several receptors on the synthesis of polymers incorporating the RGD sequence [10]. Some polymers comprised of repeated blocks of RGD sequence have been genetically synthetized to assess their therapeutic effects [11,12]. Pronectin® is an artificially synthesized protein which has a silk-like protein (SLP) backbone into which the amino acid sequence with an inherent ability for biological recognition are introduced. Among them, Pronectin F consists of two types of oligopeptide blocks, a SLP sequence of six amino acids and a human fibronectin (FN) sequence of 17 amino acids including RGD [13,14]. The SLP sequence gives Pronectin F structural stability, thermal and chemical resistance, and the nature susceptible to the adsorption to hydrophobic surfaces, while the FN sequence contributes to the activity of biologically specific cell adhesion [15]. One RGD sequence is configured into nine times of repeating SLP sequence and localized on the surface of Pronectin F molecules. This is because the Pronectin F possesses the nature to enhance the cell attachment through interaction of the repeated RGD sequence with the integrin receptor of cells. Pronectin F has been widely used as a coating reagent of cell cultureware. The Pronectin F coating is found to promote the adhesion of more than 50 types of animal cells onto the surface of polymer substrates, like polystyrene, polyester, and Teflon because of the RGD sequence [12]. It is possible that Pronectin F readily adsorbs onto the polymer surfaces through the hydrophobic interaction. Pronectin F is not water-soluble since the SLP sequence forms strong hydrogen bonds intermolecularly. To break the bonds, the hydroxyl groups of Pronectin F serine residues are chemically modified by introducing dimethylaminoethyl groups to prepare a water-soluble form of Pronectin F (Pronectin F^+). This Pronectin F^+ is water-soluble and has 13 of RGD sequences in one molecule which contribute to the strong cell adhesion via the integrin receptors.

This study is undertaken to investigate feasibility of the Pronectin F^+ with RGD sequences as the non-viral vector of plasmid DNA. To give the Pronectin F^+ cationized charges necessary for the formation of polyion complexation with the plasmid DNA, ethylenediamine (Ed), spermidine (Sd), and spermine (Sm) were introduced into the hydroxyl group of serine residues in Pronectin F^+. The Pronectin F^+ derivatives with different extents of aminization were prepared by changing the conditions of amine introduction, while for comparison, the similar cationized derivatives of gelatin which has one RGD sequence per molecule were prepared. The two cationized derivatives were mixed with a plasmid DNA encoding luciferase in aqueous solution to assess the ζ potential and molecular size of

Pronectin F^+ derivatives–plasmid DNA and gelatin derivatives–plasmid DNA complexes. After rat gastric mucosal cells were incubated with the complexes, their gene expression was compared between the types of Pronectin F^+ derivatives or Pronectin F^+ and gelatin derivatives. We also examine the internalization of the derivatives–plasmid DNA complexes into the cells as well as the buffering effect of every derivative.

2. Materials and methods

2.1. Materials

Pronectin F^+ (molecular weight (M_w)=110 000) was kindly supplied by Sanyo Chemical Industries (Kyoto, Japan). Gelatin, prepared through an acid process of type I collagen (pig skin) was kindly supplied by Nitta Gelatin (Osaka, Japan). Ethylenediamine (Ed), 2,4,6-trinitrobezenesulfonic acid (TNBS), β-alanine, and protein assay kit (Lot No. L8900) were purchased from Nacalai Tesque (Kyoto, Japan) and was used as obtained. As a coupling agents, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt (EDC) and *N,N'*-carbonyldiimidazole (CDI), and DNA MW Standard Marker (1-kb DNA Ladder) were obtained from Dojindo Laboratories, (Kumamoto, Japan) and Takara Shuzo (Shiga, Japan), respectively. Spermidine (Sd) and spermine (Sm) were purchased from Wako Pure Chemical Industries (Osaka, Japan) and was used as obtained. Rhodamine B isothiocyanate (RITC) was obtained from Sigma–Aldrich (Tokyo, Japan).

2.2. Preparation of plasmid DNA

The plasmid DNA used is the DNA construct (12.5-kb DNA) which contains a cytomegalovirus (CMV) promoter inserted at the upstream region of sequence coding the firefly (*Photinus pyralis*) luciferase. The plasmid DNA was amplified in an *E. coli* bacteria transformant and isolated from the bacteria by Qiagen Maxi kit-25 (Qiagen, Tokyo, Japan). The absorbance ratio at the wavelength of 260–280 nm for purity assessment of plasmid DNA obtained was measured to be between 1.8 and 2.0.

2.3. Preparation of Pronectin F^+ and gelatin derivatives of Ed, Sd, and Sm and their complexation with plasmid DNA

Pronectin F^+ derivatives with different extents of aminization were prepared by introduction of Ed, Sd, and Sm into the hydroxyl group of serine residues in Pronectin F^+ based on the conventional CDI method [16]. Briefly, varied amounts of Ed, Sd, and Sm together with varied amounts of CDI were added to 5 ml of dehydrated dimethyl sulfoxide containing 5 mg of Pronectin F^+. The reaction solution was agitated at 25 °C for 20 h to introduce Ed, Sd, and Sm residues to the hydroxyl groups of Pronectin F^+, followed by dialysis against double-distilled water (DDW) for 2 days and freeze–dry to obtain Ed-, Sd- and Sm-derivatives of Pronectin F^+, respectively. The molar percentage of Ed, Sd, and Sm introduced into the hydroxyl groups was quantitated by the conventional TNBS method [17] based on the calibration curve prepared by using β-alanine.

Gelatin derivatives with different extents of aminization were prepared by introduction of Ed, Sd, and Sm into the carboxyl groups of gelatin based on the conventional EDC method [16]. Briefly, Ed, Sd, and Sm together with 21.39 mg of EDC were added into 2200 ml of DDW containing 44 mg of gelatin at different molar ratios of the amine compounds to the carboxyl groups of gelatin, followed by immediate adjustment of the solution pH at 5.0 by HCl addition. The reaction mixture was agitated at 37 °C for 18 h and then dialyzed against DDW for 48 h at 25 °C. The dialyzed solutions were freeze-dried to obtain powdered different gelatin derivatives. The percentage of amino groups introduced into the carboxyl groups of gelatin (the aminization of gelatin) was determined by the TNBS method similarly. Complexation of Pronectin F^+ and gelatin derivatives with the plasmid DNA was performed by simple mixing the two materials at various mixing weight ratios in aqueous solution. Briefly, 150 μl of 0.1 M phosphate-buffered saline solution (PBS, pH 7.4) containing 10, 50, 100, 200, 300, and 500 μg of Pronectin F^+ derivatives and 2.5, 5, 10, 25, 50, and

100 μg of gelatin derivatives was added to the same volume of PBS containing 10 μg of plasmid DNA. The solution was gently agitated at 37 °C for 30 min to form Pronectin F^+ derivatives–plasmid DNA and gelatin derivatives–plasmid DNA complexes.

2.4. Electrophoresis of Pronectin F^+ derivatives–plasmid DNA and gelatin derivatives–plasmid DNA complexes

Several Pronectin F^+ derivatives–plasmid DNA and gelatin derivatives–plasmid DNA complexes were prepared at various mixing weight ratios of Pronectin F^+ and gelatin derivatives to plasmid DNA (0.1 μg) according to the same procedure as described above. The complex samples were electrophoresed for 40 min at 100 V in 0.75 wt% of agarose gel by 45 mM Tris–Borate and 1 mM EDTA buffer (pH 8.0). The gel was stained with 0.5 mg/ml ethidium bromide solution for 30 min to visualize the localization of plasmid DNA with a Gel Doc 2000 (Bio-Rad Laboratories, Tokyo, Japan).

2.5. Measurement of dynamic light scattering (DLS) and electrophoretic light scattering (ELS)

The complexes of Pronectin F^+ derivatives and plasmid DNA were prepared by the similar procedure described above. Briefly, 2.5 ml of PBS containing 5 mg of Pronectin F^+ derivatives was mixed with 2.5 ml of PBS containing 0.1, 0.17, 0.25, 0.5, 1, and 5 mg of plasmid DNA. The complexes of gelatin derivatives and plasmid DNA was prepared by mixing of 2.5 ml of PBS containing 1.25, 2.5, 5, 12.5, 25, and 50 mg cationized gelatin with 2.5 ml of PBS containing 5 mg of plasmid DNA. Every solution was filtered through a 0.45-μm filter (Millex-HV, Millipore) prior to mixing. The mixed plasmid DNA and Pronectin F^+ derivatives and plasmid DNA and gelatin derivatives solution were placed in a DLS cell and DLS measurement was carried out using a DLS-DPA-60HD instrument (Otsuka Electronic, Osaka, Japan) equipped with an Ar^+ laser at a detection angle of 90° at 37 °C for 30 min and performed three times for every sample. The corresponding hydrodynamic radius, R_s, can be calculated from Einstein-Stokes' equation: $R_s=kT/3\pi\eta D$, where k is the Boltzman constant, T is the absolute temperature, η is the solvent viscosity, and D is translational diffusion coefficient obtained from the DLS measurements. In the present study, the autocorrelation function of samples was analyzed based on the cumulants method and the R_s value was automatically calculated by the equipped computer software and expressed as the apparent molecular size of samples. ELS measurement was carried on an ELS-7000AS instrument (Otsuka Electronic Co. Ltd., Osaka, Japan) for mixed plasmid DNA and Pronectin F^+ and plasmid DNA and gelatin derivatives aqueous solution at 37 °C and an electric field strength of 100 V/cm. The ELS measurement was done three times for every sample.

The zeta potential (ζ) was automatically calculated using the Smoluchouski equation based on the elecrtrophoretic mobility measured u: $\zeta=4\pi\eta u/\varepsilon$, where η and ε are the viscosity and the dielectric constant of the solvent, respectively.

2.6. Transfection experiment by Pronectin F^+ derivatives–plasmid DNA and gelatin derivatives–plasmid DNA complexes

Rat gastric mucosal (RGM)-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Lot No. 1073750, Gibco-BRL, Life Technology, NY, USA) supplemented with 10 wt% fetal calf serum, 0.12 wt% sodium bicarbonate, and 100 units/ml mixed penicillin–streptomycin solution. The cell suspension (1×10^5 cells/2 ml) was plated into each well of six-well multi-well culture plates (Code 3800-6100, Iwaki brand, Scitech Div. Asahi Techno Glass, Chiba, Japan) and cultured at 37 °C for 1 day in a 95% air–5% CO_2 atmosphere to reach the cell confluency of about 70%. After the Pronectin F^+ derivatives–plasmid DNA and gelatin-derivatives–plasmid DNA complexes were added to each well, followed by incubation for 4 h, the culture medium was exchanged to exclude the complexes added. Then, the cells were incubated for additional 48 h, washed twice with 1 ml of PBS, and lysed by 100 μl of a lysis buffer (Luciferase Assay System, Cat# E 1500, Promega, WI, USA). The cell lysate was centrifuged at 12 000 rpm for 5 s at 4 °C, and the supernatant was carefully collected and kept in ice. The supernatant sample (16 μl) was mixed with 80 μl of a reconstituted luciferase assay solution

(Luciferase Assay System, Cat# E 1500) and the relative light unit (RLU) of the solution mixture was determined by a luminometer (Lumat Lb 9507, Wallac-Berthold, Germany). Each experimental group was carried out for three wells. As controls, the cells were incubated with the cultured medium alone or that containing free plasmid DNA or the original Pronectin F^+ and gelatin–plasmid DNA complexes. The protein concentration of the lysate was also assayed by the Lowry kit (Lot. No. L8900, Nacalai Tesque, Kyoto, Japan). Briefly, 50 μl of lysate were mixed with the 1 ml of the copper solution, followed by leaving for 10 min at 25 °C. After addition 0.1 ml of 1 N phenol aqueous solution, the solution mixture was incubated for 30 min at 25 °C and the absorbance was determined at the wavelength of 750 nm. The protein concentration was calculated based on the calibration curve prepared by use of a standard albumin solution.

2.7. Cell attachment

The original Pronectin F^+ and gelatin, Pronectin F^+ and gelatin derivatives were dissolved in PBS to obtain the coating solution. The concentration of Pronectin F^+ and Pronectin F^+ derivatives used was 10 μg/ml [18] while for the gelatin and gelatin derivatives used was 0.1 mg/ml [19]. The solution (1 ml) was poured into each well of six-well multi-well culture plates (Code 3800-6100, Iwaki brand, Scitech Div. Asahi Techno Glass), followed by leaving for 5 min at 25 °C. After the excess solution was removed, the plate was left for air drying for 2 h at 25 °C and then rinsed twice with PBS.

RGM-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Lot No. 1073750, Gibco-BRL) supplemented with 10 wt% fetal calf serum, 0.12 wt% sodium bicarbonate, and 100 units/ml mixed penicillin–streptomycin solution. The RGM-1 cell suspension (1×10^5 cells/2 ml) was added into non-coated or the coated wells and cultured at 37 °C for 6 h in a 95% air–5% CO_2 atmosphere. Non-adherent cells were thoroughly washed twice with PBS.

The number of cells attached was determined by the fluorometric assay of cell DNA [20]. Briefly, the adherent cells were lysed by 1 ml of a lysis buffer (2% trypsin–EDTA in PBS). The cell lysate was digested in a buffer solution (pH 7.4) containing 0.5 mg/ml proteinase K, 0.2 mg/ml sodium dodecylsulfate (SDS), and 30 mM saline–sodium citrate (SSC) at 55 °C for 12 h with occasional mixing. The enzyme-digested samples (100 μl) was mixed with SSC buffer (400 μl) in a glass tube. After mixing with a dye solution (500 μl; composition: 30 mM SSC, 1 μg/ml Hoechst 33258 dye) the fluorescent intensity of mixed solution was read in a fluorescence spectrometer (F-2000 Fluorescent Spectrophotometer, Hitachi, Tokyo, Japan, Ex 355 nm/Em 460 nm). The calibration curve between the DNA and cell number was prepared by use of cell suspensions with different cell densities. The DNA assay was done three times independently for every experimental sample unless otherwise mentioned.

2.8. Internalization assay of Pronectin F^+ derivatives–plasmid DNA and gelatin derivatives–plasmid DNA complexes into cells

For the fluorescent labeling of plasmid DNA, the pCMV-Luciferase and RITC were mixed in 0.2 M sodium carbonate-buffered solution (pH 9.7) at 4 °C for 12 h at both the concentrations of 1 mg/ml. The residual RITC was separated by gel filtration of a PD 10 column (Amersham–Pharmacia Biotech, Tokyo, Japan) and the RITC-labeled pCMV-luciferase was obtained by ethanol precipitation. Then, different Pronectin F^+ and gelatin derivatives were mixed with the RITC-labeled pCMV-luciferase in PBS at the Pronectin F^+–plasmid DNA and gelatin–plasmid DNA weight mixing ratio of 50 and 5 to prepare respective complexes. The Pronectin F^+ derivative–RITC-labeled plasmid DNA and gelatin derivative–RITC-labeled plasmid DNA complexes were added to each well with RGM-1 cells grown at the 70% confluency. As controls, the cells incubated with the RITC-labeled-plasmid DNA alone or that containing the original Pronectin F^+–RITC-labeled-plasmid DNA complexes and the original gelatin–RITC-labeled-plasmid DNA complexes. Following incubation further for 48 h, the cells were washed carefully three times with 1 ml of PBS to exclude the fluorescent agents added, and lysed by 500 μl of a lysis buffer (Luciferase Assay System, Cat# E 1500, Promega). The fluorescent intensity of cell lysates was measured by a fluorescent spectrophotometer

Table 1
Preparation and characterization of Pronectin F^+ derivatives with different extents of aminization

Pronectin F^+ concentration (mg/ml)	CDI (mg/ml)	Ed (mg/ml)	Sd (mg/ml)	Sm (mg/ml)	Introduction percentage[a]
1	0.87	2.84			1.23±0.23[b]
1	1.75	6.94			14.1±0.17
1	9.74	19.2			26.0±0.27
1	0.49		4.07		5.13±0.17
1	1.63		8.24		13.1±0.42
1	4.89		29.8		26.0±1.18
1	1.63			6.54	1.86±0.78
1	4.89			13.9	12.9±0.38
1	9.78			37.7	26.0±0.45

[a] Calculated according to the formula: $\frac{\text{The number of amino groups introduced}}{\text{The number of OH groups}} \times 100(\%)$.

[b] Mean±S.D.

(F-2000 Fluorescent Spectrophotometer, Hitachi, Tokyo, Japan, Ex 570 nm/Em 595 nm) and divided by that initially added to obtain the percent internalized. Each experiment was carried out independently for six wells.

2.9. Evaluation of buffering capacity of Pronectin F^+ and gelatin derivatives

An aqueous solution containing Pronectin F^+ and gelatin derivatives or branched polyethylenimine (PEI) with the molecular weight of 25 000 as a control sample was prepared and the solution pH was adjusted around 8.0 by 0.1 M NaOH. The amount of each materials used for buffering experiments was the concentration of amine groups of 1 mM. The resulting solution was titrated by stepwise addition of 0.01 N HCl solution (0.2 ml). The solution pH was measured by Horiba D-22 pH meter (Horiba, Kyoto, Japan). The experiment was performed independently three times to obtain the average pH value.

2.10. Statistical analysis

All the data were statistically analyzed to express the mean±the standard deviation (S.D.) of the mean. Student's t-test was performed and $P<0.05$ was accepted to be significance.

3. Results

3.1. Preparation and characterization of Pronectin F^+ and gelatin derivatives

Tables 1 and 2 show the aminization of Pronectin F^+ and gelatin prepared under different reaction conditions. It is apparent that the percentage of

Table 2
Preparation and characterization of gelatin derivatives with different extents of aminization

Gelatin concentration (mg/ml)	Concentration of amino compounds (mg/ml)			Molar ratio[a]	Amino residues introduced[b] (mol/mol%)		
	Ed	Sd	Sm		Ed	Sd	Sm
2×10^{-2}	1.1×10^{-2}	2.7×10^{-2}	3.8×10^{-2}	10	33.0	42.6	44.0
2×10^{-2}	2.8×10^{-2}	6.7×10^{-2}	9.5×10^{-2}	25	42.6	47.6	47.0
2×10^{-2}	5.6×10^{-2}	1.3×10^{-1}	1.9×10^{-1}	50	47.8	48.1	49

[a] The ratio of ethylenediamine, spermidine, and spermine to the carboxyl groups of gelatin.
[b] The percentage of amino residues introduced to the carboxyl groups of gelatin.

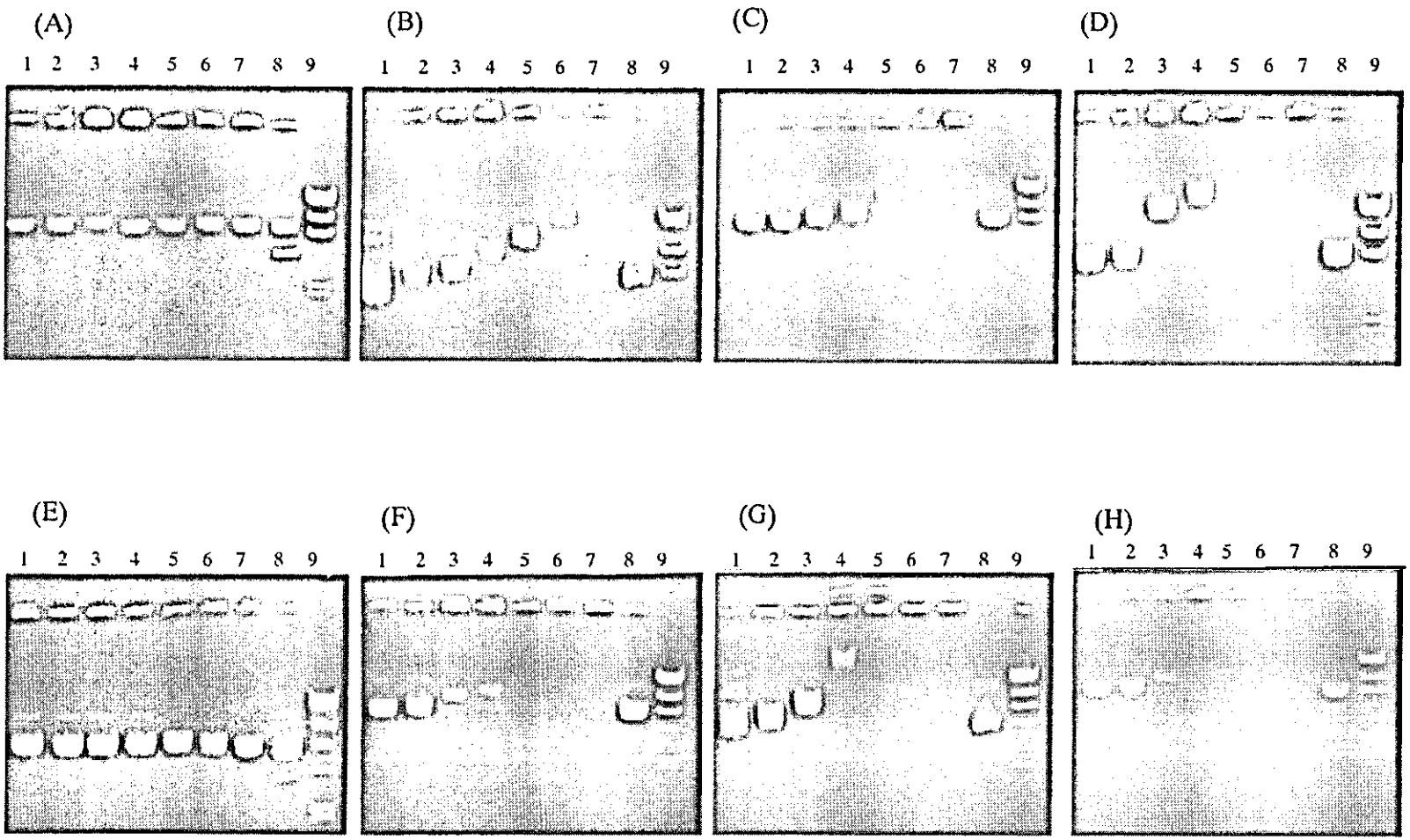

Fig. 1. Electrophoretic patterns of plasmid DNA complexes with Pronectin F^+, gelatin, and Pronectin F^+ or gelatin derivatives prepared at different mixing weight ratios of Pronectin F^+ or gelatin to plasmid DNA: (A) original Pronectin F^+, (B) Pronectin F^+–Ed, (C) Pronectin F^+–Sd, (D) Pronectin F^+–Sm, (E) original gelatin, (F) Ed-50, (G) Sd-50, and (H) Sm-50. Lanes 1–7: at Pronectin F^+/plasmid DNA mixing ratios of 1, 2.5, 5, 10, 20, 30, and 50, and at gelatin–plasmid DNA mixing ratios of 0.25, 0.5, 1.0, 1.25, 2.5, 5.0, and 10.0, Lane 8, free plasmid DNA; lane 9, DNA marker. The molar percentage of amino groups introduced to the hydroxyl groups of Pronectin F^+ is 26%.