Furthermore, Leor et al. reported that bioengineered heart grafts using porous alginate scaffolds attenuated left ventricular dilatation and heart function deterioration in myocardial infarction model [14]. As the technique premixing cells and ECM alternatives instead of seeding cells into preformed scaffolds, Zimmermann et al. engineered 3-D heart tissue by gelling the mixture of cardiomyocytes and collagen solution [15,16]. The construct has allowed direct measurement of isometric contractile force as heart tissue model.

In spite of these desirable results, insufficient cell migration into scaffolds and inflammatory reaction due to scaffold biodegradation remain problems to be solved [13,14]. In native myocardial tissue, cells are considerably dense (Fig. 1A) in comparison with other tissues including cartilage, vascular, and heart valve, which are cell-sparse tissues and have been successfully engineered by using biodegradable scaffolds (Fig. 1B). Cardiomyocytes are also tightly interconnected with gap junctions, which mediated the reciprocal exchange of small molecules and ions resulting in electrically synchronous beating [17]. In myocardial tissue engineering, biodegradable scaffolds themselves attenuate cell-to-cell connections and scaffold biodegradation leads to fibrous tissues containing excessive amount of ECM, which is shown in pathological states including ischemic heart disease or dilated cardiomyopathy. Investigators are now trying to fabricate more porous structure of biodegradable scaffolds and to develop new techniques seeding more cells into the scaffolds. In particular, structural balance between cells and ECM should be controlled to fabricate native heart-like tissues.

By contrast, we now propose novel tissue engineering methodology that is to construct 3-D functional tissues by layering 2-D cell sheets without any biodegradable alternatives for ECM. To obtain viable cell sheets, we have exploited intelligent culture surfaces, from which cultured cells detach as a cell sheet simply by reducing temperature. In this paper, we present the new technology "cell sheet engineering" and its application to myocardial tissue reconstruction.

2. Temperature-responsive culture surfaces

Temperature-responsive culture surfaces were developed among the research to control cell adhesion to biomaterials. Cells adhere to culture surfaces via membrane receptors and cell adhesive proteins, including fibronectin, that reside in serum or are secreted from the cells in culture (Fig. 2A). The interaction between adhesive proteins and culture surfaces depends on the wettability of the surface. Normal tissue culture polystyrene (TCPS) dishes are hydrophobic and absorb ECM proteins resulting in cell attachment and proliferation. To harvest cells from the surfaces, enzymatic digestion including trypsin and dispase are usually utilized. In that case, both adhesive proteins and membrane receptors are disrupted, then cells detach with considerable damages (Fig. 2B). On the other hand, we graft temperature-responsive polymer, poly(*N*-isopropylacrylamide)(PIPAAm) to TCPS dishes covalently by electron beam. The surfaces are hydrophobic and cells adhere and proliferate under culture condition at 37°C. By lowering temperature below 32°C, the surfaces change reversibly to hydrophilic and not cell adhesive due to rapid hydration and swelling of the grafted PIPAAm. This unique surface change allows cultured cells to detach spontaneously from these grafted surfaces simply by lowering temperature [18]. As against using enzymatic digestion, only the interaction between adhesive proteins and material surfaces is released and cells detach together with intact membrane proteins and adhesive proteins (Fig. 2C) [19]. As a result, cells recovered by using PIPAAm-grafted surfaces maintain their differentiated functions more strongly than the cells recovered by protease digestion [20]. For example, trypsin-treated hepatocytes decrease albumin production, on the other hand, those cells harvested from PIPAAm-grafted surface preserve albumin secretion [21].

In addition to the passive mechanism of the surface change from hydrophobic to hydrophilic, cell-mediated active processes have been ascertained as cell detachment mechanisms [22]. Sodium azide, an ATP synthesis inhibitor, considerably retarded cell release from PIPAAm-grafted surfaces, indicating that energy-dependent metabolic process is one of major mechanisms. The active processes are also mediated by intracellular signal transduction, including tyrosine phosphorylation and cytoskeltal reorganization and lead to the cell morphological change from spread to round after surface property change [23].

3. Cell sheet engineering

When cells are cultured confluently, they connect to each other via cell-to-cell junction proteins and ECM (Fig. 3A). With enzymatic digestions, these proteins are disrupted and each cell is released separately (Fig. 3B). In the case using PIPAAm-grafted surfaces, cell-to-cell connections are not disrupted and cells are harvested as a contiguous cell sheet by decreasing temperature (Fig. 3C). Furthermore, adhesive proteins underneath cell sheets are also maintained and they play a desirable role as an adhesive agent in transferring cell sheets onto other culture materials or other cell sheets [24]. These viable cell sheets are composed of cells and biological ECM without any artificial scaffolds. Various types of cell sheets have been successfully lifted up and transferred on other surfaces [25–32].

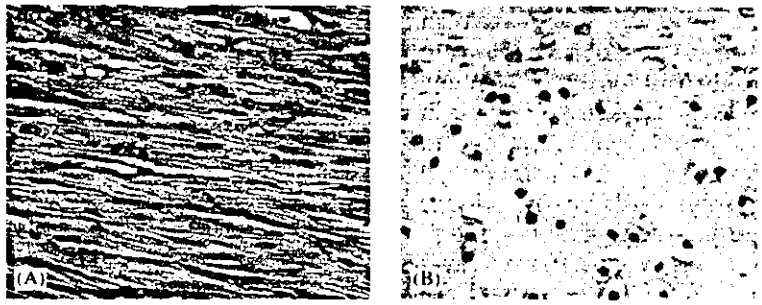

Fig. 1. Histological comparison between cell-dense and cell-sparse tissues. Hematoxilin and eosin staining shows that cells are dense and tightly connected in myocardial tissue (A) On the other hand, cartilage tissue includes sparse cells and large amount of ECM (B).

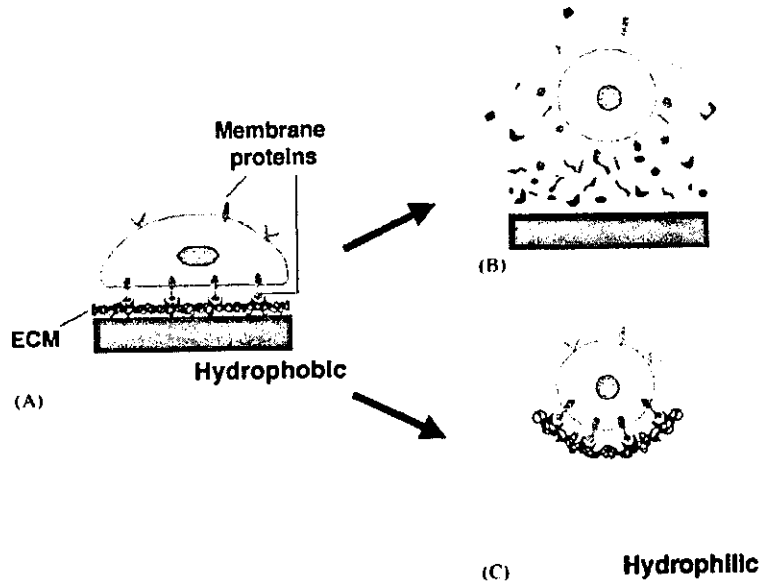

Fig. 2. Cell harvest mechanism by using temperature-responsive culture surfaces. (A) Cells attach to hydrophobic culture surfaces via cell membrane proteins and ECM, which reside in serum or are secreted from the cells. (B) When enzymatic digestion is used, both membrane and ECM proteins are disrupted, resulting in cell detachment. (C) When cells are cultured on temperature-responsive culture surfaces, the interconnection between ECM and hydrophilic culture surfaces is released only by lowering temperature. Then the cells detach together with intact proteins.

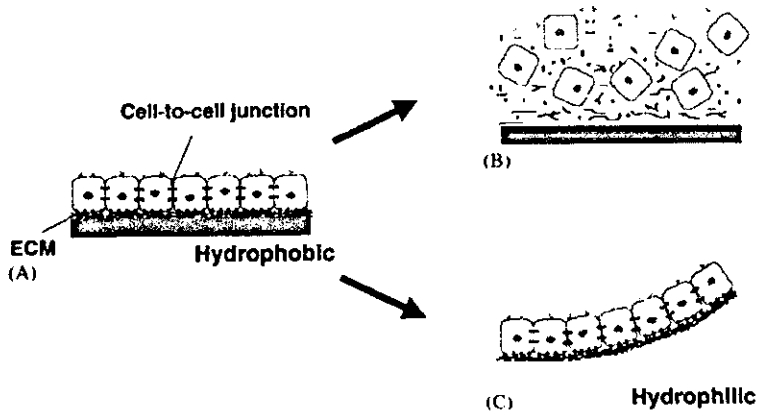

Fig. 3. Cell sheet release from temperature-responsive culture surfaces. (A) When cells are cultured confluently, the cells connect to each other via cell-to-cell junction proteins. (B) When harvested by protease treatments, cell-to-cell connections are disrupted and cells are released separately. (C) When PIPAAm-grafted surfaces are used, cell-to-cell connections are completely preserved and the cells are released as a contiguous cell sheet. ECM retained underneath the cell sheets play a role as a adhesive agent.

As cell sheet manipulation, two techniques have been performed according to cell types and objects. One is to manipulate cell sheets directly with forceps or pipetting after the sheets are completely harvested resulting in proportionally shrunk and thicker constructs due to active cytoskeletal reorganization. As indicated by synchronized beating of shrunk cardiomyocyte sheets, cell-to-cell connections are preserved after this procedure [31]. The other is to use support membranes including a hydrophilically modified poly(vinylidene difluoride)(PVDF) membrane for preserving cell sheet morphology without any shrinkage. Before cell sheets release, support membranes are placed over the confluent cells. Then the cell sheets physically attached to the support membranes are harvested from PIPAAm-grafted surfaces below 32°C and transferred onto other surfaces. Incubation at 37°C causes reattachment of the cell sheets to new surfaces via remaining adhesive proteins. Finally, only the support membranes are removed. The latter technique has realized the cell sheet manipulation preserving their structure and function [26–30].

These cell sheet manipulation techniques without using any biodegradable scaffolds have been applied to tissue engineering in three types of contexts (Fig. 4). First is transplanting single cell sheet for skin and cornea reconstruction. Advantages of skin epithelial cell sheets harvested by using PIPAAm-grafted surfaces have been confirmed in comparison with those harvested by dispase treatments. E-cadherin, which is an essential protein for skin cell-to-cell junctions, and laminin 5, which is a major component of epithelial basement membranes, were retained in skin cell sheets released from PIPAAm-grafted surfaces [27]. It should attenuate the risk of infection after artificial skin transplantation. Second is to layer same cell sheets for reconstructing homogeneous tissues including myocardium. Third is to layer several types of cell sheets for fabricating laminar structures including liver, kidney and vascular. Layered co-culture comprising a hepatocyte sheet and an endothelial cell sheet has revealed the differentiated cell shape and extensive albumin expression of hepatocytes, which have never been seen in hepatocyte mono-culture [32]. We have been now applied these technologies "cell sheet engineering" to reconstructing various types of tissues. Among them, myocardial tissue engineering based on the second context is described below.

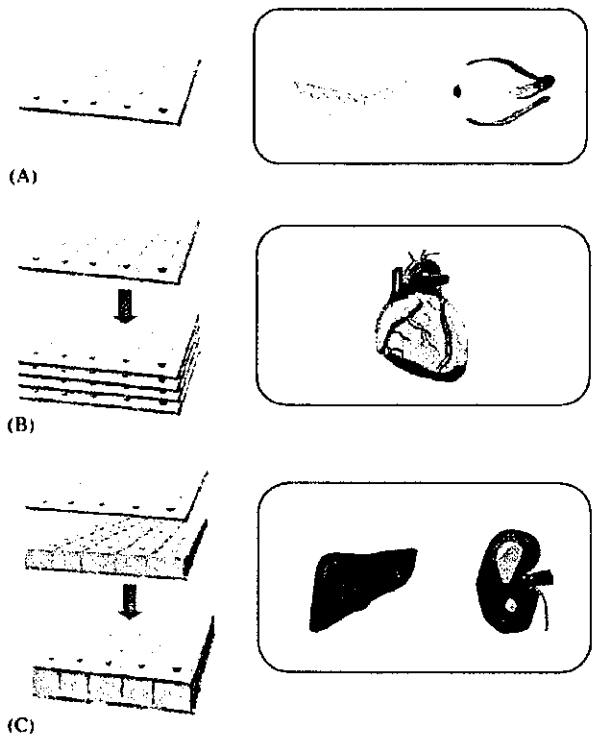

Fig. 4. Three contexts in cell sheet engineering. (A) Single cell sheet is useful for skin or cornea transplantation. (B) Same cell sheets are layered to reconstruct homogeneous 3-D tissues including myocardium. (C) Several types of cell sheets are co-layered to fabricate laminar structures including liver and kidney.

4. Myocardial tissue reconstruction by layering cardiomyocyte sheets [28,30,31]

Cardiomyocytes are tightly interconnected with gap junctions and pulsate simultaneously in native heart tissue. It is also well-known that confluent cultured cardiomyocytes on culture surfaces connect via gap junctions and beat simultaneously [33]. Therefore, in myocardial tissue engineering by layering cell sheets, it is a crucial point whether electrical and morphological communications are established between bilayer cell sheets. Chick embryo or neonatal rat cardiomyocyte sheets released from PIPAAm-grafted surfaces presented synchronized pulsation. To examine the electrical communication, two cardiomyocyte sheets were overlaid partially as schematically illustrated in Fig. 5. Two

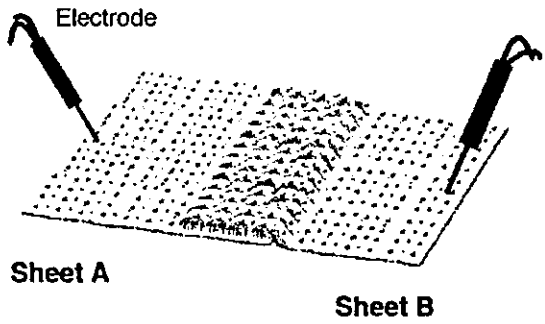

Fig. 5. Schematic illustration of electrical analysis of layered cardiomyocyte sheets. To examine the electrical synchronization, two cardiomyocyte sheets (A, B) are overlaid partially. Two electrodes are set over monolayer parts of both cell sheets to detect the electrical potentials separately.

electrodes were set over monolayer parts of both cell sheets. Detected electrical potentials of the two sheets completely synchronized (Fig. 6). Furthermore, electrical stimulation to the single-layer region of one sheet was transmitted to the other cell sheet and the two cell sheets pulsated simultaneously. Histological analysis showed that bilayer cardiomyocyte sheets contacted intimately resulting in homogeneous tissue. Cell-to-cell connections including desmosomes and intercalated disks were confirmed by transmission electron microscopic images. These data indicate that electrical and morphological communications are established between layered cardiomyocyte sheets.

Under conventional culture conditions, cardiac myocytes are fixed to rigid material surfaces and their motion is highly limited. To minimize the interaction between cell sheets and culture materials, the sheets were overlaid on several types of materials including polyethylene meshes, elastic polyurethane meshes or frame-like collagen membranes. In any cases, the constructs pulsated simultaneously with higher amplitude than the cells fixed on rigid culture surfaces. When cardiomyocyte sheets were layered on frame-like collagen membranes, the center part of them is free from any culture materials. In result, 4-layer cardiac constructs on the frame-like collagen membranes pulsated spontaneously in macroscopic view.

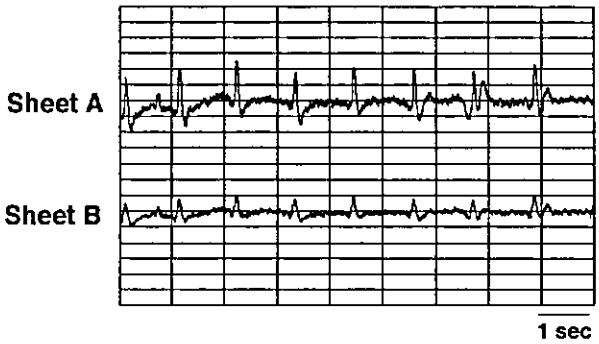

Fig. 6. Synchronization of layered cardiomyocyte sheets. Representative tracings of electrical potentials of sheet A and sheet B show complete synchronization.

To examine in vivo survival and function of layered cardiomyocyte sheets, the constructs were transplanted into dorsal subcutaneous tissues of nude rats. Surface electrograms originating from transplanted constructs were detected independently from host electrocardiograms, in the earliest case, at 2 weeks after the operation (Fig. 7). When transplantation sites were opened, macroscopic simultaneous graft beatings were observed at the earliest period, 3 days after the transplantation. Furthermore, graft survival was confirmed at least up to 1 year. Morphological analysis demonstrated that neovascularizations occurred in a few days and that vascular network was organized within a week (Fig. 8A). Cross-sectional views revealed stratified cell-dense myocardial tissues (Fig. 8B), well-differentiated sarcomeres and diffuse formation of gap junctions. In comparison between 2-layer and 4-layer cardiac tissue grafts, fractional shortening increased depending on the number of layered cell sheets.

Thus, the basic technology has been established to fabricate electrically communicative, pulsatile myocardial tissues by using cell sheets both in vitro and in vivo.

5. Future perspectives

Recently, research on myocardial tissue engineering has been accelerated to develop further advanced therapy for severe heart failure. Transplantation of layered cardiomyocyte sheets on the myocardial scar may be more beneficial than that of bioengineered heart tissue including biodegradable scaffolds in the point of scaffold-mediated disadvantages. However, there are several common problems in myocardial tissue

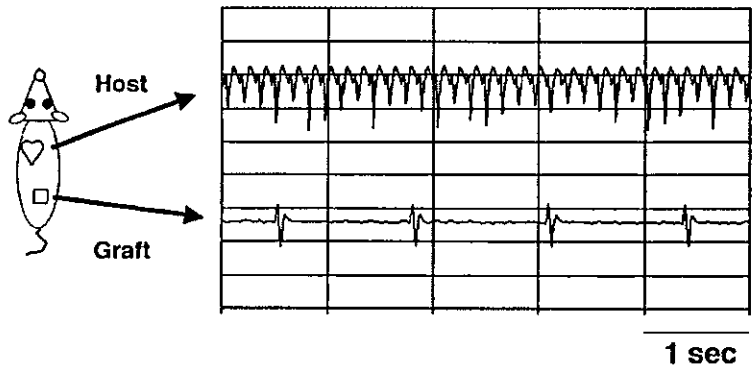

Fig. 7. Skin surface electrogram of transplanted cardiomyocyte sheets. Representative tracings of the host electrocardiogram (upper) and the electrical potential detected via the electrode set at the skin just above the transplanted heart graft (lower) are shown. Skin surface electrogram originating from the graft is detected independently from host electrocardiogram.

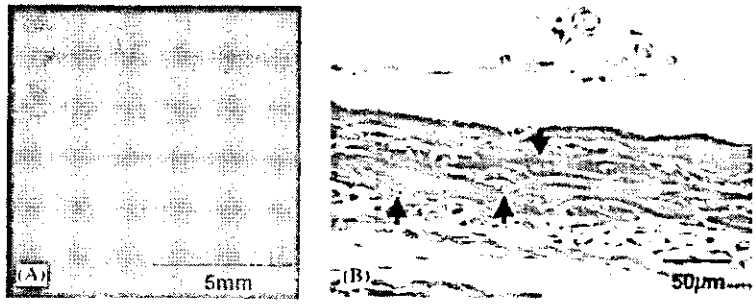

Fig. 8. (A) Macroscopic view of the transplanted cardiac graft. Multiple neovascularization is shown in the square-designed cardiac graft transplanted into dorsal subcutaneous tissue. (B) Azan staining shows a stratified cardiac tissue graft including elongated cardiomyocytes and microvasculars (arrows).

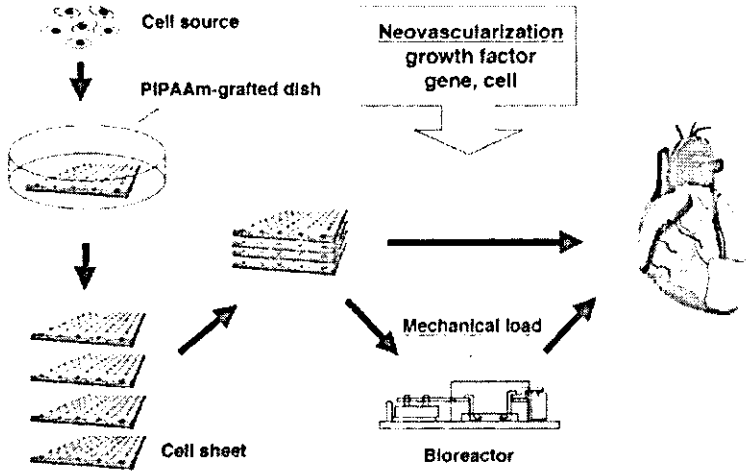

Fig. 9. Schematic illustration of myocardial reconstruction based on cell sheet engineering. We now propose the application of "cell sheet engineering" to myocardial tissue reconstruction. Cell sourcing remains a crucial problem. Neovascularization for oxygen and nutrition supply is also critical to fabricate human applicable myocardial tissue. Growth factors, gene delivery and the utility of gene-modified cells or endothelial cells may be helpful. Mechanical load by using bioreactors should strengthen the engineered myocardial tissues. Transplantation of engineered tissue into myocardial infarction model is now in progress.

engineering. As described in Section 1, myocardial cell sourcing remains a crucial problem. Further advance in stem cell biology for cardiomyocytes will be needed to realize clinical application of bioengineered myocardial tissues.

Vascular reconstruction is also one of the most critical issues in myocardial tissue engineering. Sufficient supply of oxygen and nutrition is required for functionally beating heart tissue. It has been reported that cells are dense in the graft periphery, but sparse in the interior part due to insufficient oxygen perfusion in scaffold-based heart tissue grafts [34]. Although, in our studies, multiple neovascularization arose in transplanted cardiac grafts in a few days, primary insufficient oxygen and nutrition permeation also limit the number of transplanted cardiomyocyte sheets. Hence, new methods to accelerate blood vessel formation are now requested to engineer larger or thicker constructs for heart tissue repair. As examined in isolated cell injection, gene-modified cells may be also applicable for engineering more vascularized heart tissues [35]. Using cell sheet technology, it has been reported that a single layer of endothelial cell sheet enhances the capillary formation in vivo [36]. Therefore, heterogenous layering of endothelial cell sheets between cardiomyocyte sheets may promote neovascularization. Further research and development will be needed to engineer vascular networks sufficient for fabricating clinically applicable heart tissues.

In native heart, cardiomyocytes are gradually elongated and hypertrophied by mechanical load increase in accordance with the growth of the body. Therefore, some investigators have attempted to strengthen bioengineered heart tissues by using mechanical devices. Carrier et al. used a rotating bioreactor for culturing cardiomyocytes on PGA scaffolds [37]. Fink et al.

clearly demonstrated that application of stretch devices to engineering heart tissues strengthened the contraction power and oriented the cells unidirectionally [38]. We are now trying to stretch layered cardiomyocyte sheets to fabricate more powerful cardiac constructs in vitro.

Finally, our concept of myocardial tissue engineering is schematically illustrated in Fig. 9. Although further interdisciplinary research will be needed to clear the existing several problems, cell sheet engineering should have enormous potential for constructing clinically applicable heart grafts and should promote tissue engineering research fields.

Acknowledgements

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細胞シートを用いた心筋の組織工学

Myocardial tissue engineering by cell sheet technology

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Tatsuya SHIMIZU

◎重症心不全患者に対する次世代の再生医療として注目されているのが，組織工学(tissue enigineering)の技術により細胞から再構築した心筋組織の不全心筋部への移植である．世界的には，生体吸収性の支持体に細胞を播種する方法により心筋組織を再構築する研究が行われている．一方，著者らの研究室では，シート状の細胞を積層化することで三次元組織を再構築するという新規手法(細胞シート工学)により心筋組織の再生を試みている．すでに肉眼レベルで同期して拍動する心筋組織の作製が可能となっている．組織工学による心筋再生は，細胞ソース，三次元組織構築法，血管新生法，移植法と多分野にまたがって解決すべき課題も多いが，今後フィールドを越えた研究者・臨床医の連携，技術開発により臨床応用可能になると予測され，重症心不全患者に対する医療に大きく貢献することが期待されている．

Key word：心筋再生，組織工学，細胞シート

再生医療(regenerative medicine)あるいは組織工学(tissue engineering)の近年の急速な進歩により，不全・欠損組織の補完を目的とした細胞単独あるいは再生組織の移植が種々の臓器において現実的なものとなっている．重症心不全の患者に対しても自己の筋芽細胞や骨髄単核球細胞を用いた細胞移植療法がすでに臨床応用されており，その成果に期待がかかっている．一方，次世代の再生医療として注目されているのが，細胞を使ってengineerした心筋組織(心筋パッチ)の不全心筋部への移植である．アメリカではこの心筋パッチの開発を目的とした大型のプロジェクトが進行中であり，世界的な研究者人口も増加している．

本稿では，tissue engineeringによる心筋組織再構築に関する研究の現状と将来展望について概説するとともに，著者らが提唱している独自の組織工学的手法“細胞シート工学”による心筋組織再構築の研究について紹介する．

Tissue engineeringによる心筋組織再構築の現状

Tissue engineeringは1993年，工学者であるLangerおよび医師であるVacantiが共同で提唱した概念であり，医学と工学の融合により生まれたあらたな学問である[1]．実際には細胞から組織を再構築するという研究は以前より行われていたが，マウスの背中で耳を再生させたことにより彼らはtissue engineeringを世界に知らしめた．彼らのコンセプトは，組織の再生には，①細胞，②細胞の足場となる細胞外マトリックス(extracellular matrix：ECM)，③細胞の分化・増殖のためのサイトカインが必要であるとし，ECMの代替としてポリグリコール酸やポリL乳酸およびその共重合体からなる生体吸収性の高分子を用いた．この支持体に細胞を培養し生体内に移植することにより支持体が徐々に分解し，細胞がつくりだすECMと置換され，生体類似の組織が再構築されるというものである．これまでに，骨，軟骨，血管をはじ

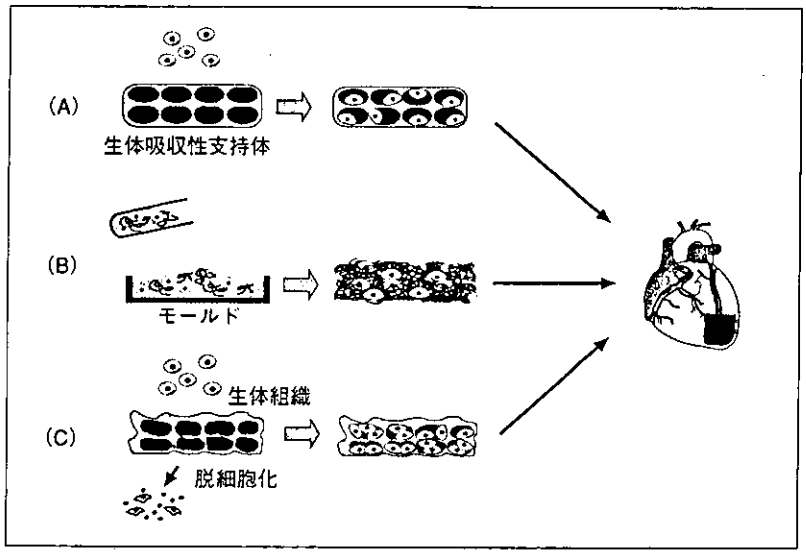

図 1　支持体を用いた心筋組織の再構築

A：生体吸収性高分子からなる多孔性の三次元支持体を作製，それを足場として細胞を播種する．支持体は徐々に分解し，細胞あるいは生体が産生するECM と置換されて組織が再生される．

B：溶液状の生体吸収性材料と細胞を混合した後，モールドに流し込み，重合させることで細胞を三次元化する．

C：生体組織を脱細胞化した結合組織を支持体として，細胞を播種し培養する．

めあらゆる組織に関して，細胞の足場として生体吸収性の三次元高分子材料を用いた組織再生の研究が行われている．

心筋組織に関しても Langer および Vacanti の提唱したコンセプトに基づいた研究が報告されている[2]．これらの研究は細胞の足場の作製法の観点から大きく 3 つの手法に分類できる．①生体吸収性の高分子材料からなる支持体を作製し，それを足場として細胞を播種する(図 1-A)，②溶液状の支持材料と細胞を混合したうえで重合し三次元化する(図 1-B)，③生体由来組織を脱細胞化して支持体とし細胞を播種する(図 1-C)．

①の手法としては生体吸収性高分子としてポリグリコール酸，コラーゲン，ゼラチン，アルギン酸を用いた研究が報告されている．Bursac らは，ポリグリコール酸からなる生体吸収性の支持体に心筋細胞を播種し，回転式のバイオリアクターを用いることにより心筋様組織を構築，その電気的特性を解析している[3]．また Li らのグループは，スポンジ状のゼラチンを使った心筋グラフトを作製し，心筋梗塞モデルに移植したところ，心機能の改善を認め，さらに右心系心筋欠損部に移植したところ，ゼラチンが完全に分解された後も組織として残存し欠損部を補完したと報告している[4,5]．生体吸収性高分子としてアルギン酸を用いた研究では，心筋梗塞モデルへの移植により収縮能の改善は認めなかったものの心拡大が回避されたと報告されている[6]．

つぎに，②の手法として Eschenhagen らは，ECM の主成分であるコラーゲンの溶液と心筋細胞を混和しシリコーン製のモールド内で培養することにより三次元の心筋組織を作成その張力の測定を可能とし，反復伸展刺激により細胞が配向性を獲得し肥大することを報告している[7]．

③の手法は食道や弁の組織再生にも用いられている手法であるが，心筋に関しても膀胱組織を脱細胞化したものに心筋細胞を播種しグラフト化する研究が行われている．

以上のように，培養心筋細胞と支持体となる生体材料を用いた心筋組織再構築の研究が世界的に行われている．

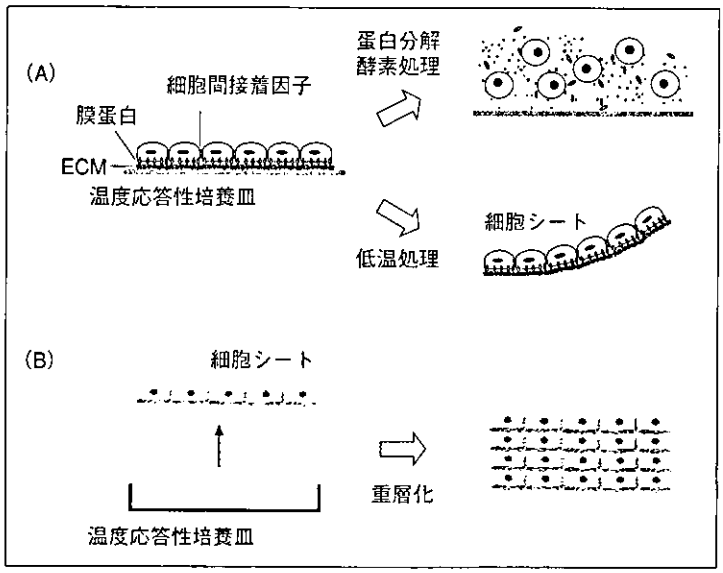

図 2　温度応答性培養皿からの細胞シートの脱着と細胞シートの積層化

A：細胞を密に培養した場合は細胞と細胞が細胞間接着分子によりたがいに接着する．蛋白分解酵素を用いた場合は細胞と培養皿の接着が解離するとともに，細胞間接着も破壊されるため，それぞれの細胞は解離して浮遊することになる．これに対し温度応答性培養皿を使った低温処理においては，この細胞間接着はまったく影響を受けずに，シート下面の ECM と培養皿表面の接着のみ解離するため，細胞がシート状に脱着する．

B：細胞シートの積層化により，三次元組織を再構築できる．

細胞シート工学による心筋組織再構築

Tissue engineering は組織再構築の足場として生体吸収性高分子を使うというコンセプトで展開してきた．しかし，支持体内へ十分な細胞を播種することが困難なため，肝や心筋組織のような細胞の密な組織の再構築が困難であることが課題となっている．また，支持体の分解に伴い炎症反応が生じることも問題となっている．そこで著者らは，支持体を用いずにシート状の細胞を回収し積層化することで三次元組織を再構築する“細胞シート工学”とよばれる手法により組織再構築の研究を行っている．シート状の細胞の回収には，通常の培養皿上に温度応答性高分子であるポリ(*N*-イソプロピルアクリルアミド)を電子線を用いてグラフトした温度応答性培養皿を用いる．培養温度である 37℃ では疎水性表面となり細胞接着性であるが，32℃ 以下の低温処理で親水性表面に可逆的に変化し細胞非接着性となる．これによって，接着した細胞をトリプシンなどの蛋白分解酵素を用いることなく温度変化のみで脱着させることが可能で，細胞を密に培養した場合は，低温処理により細胞と培養表面の接着が解離し細胞がその下面の ECM とともに培養皿から脱着するものの，細胞間の結合はまったく解離せず維持されるため，トリプシンを用いたときのように細胞をばらばらにすることなく，シート状に回収できる(図 2-A)．また，細胞シート下面の ECM があらたな細胞シート上への移動時に糊の役目を果たすため，速やかな積層化が可能である(図 2-B)．

著者らはこの技術を心筋組織再構築に応用した[8,9]．新生仔ラットから単離した心筋細胞を温度応答性培養皿上に培養し，電気的に結合した心筋細胞を低温処理を行いシート状に回収，重層化した．重層化後数日で 2 枚の心筋細胞シートは同期して拍動し，一方の心筋細胞シートへの電気刺激が他方のシートへ伝達することが確認された．さらに，形態的にも 2 枚の心筋細胞シートは密に接着し，gap junction が形成されることが示された．心筋細胞シートを *in vitro* でコラーゲン膜上に 4 層まで積層化したところ，全体が同期して拍動し，

肉眼レベルでその動きが確認された．つぎに重層化した心筋細胞シートをヌードラットの背部皮下組織に移植したとろ，ホスト心臓の心電図とは異なる心筋移植グラフトに固有の電位が測定された．移植部を切開したところ，心筋グラフトが肉眼レベルで拍動するのが確認された．移植組織内には毛細血管網が新生しており，円柱状に伸びた心筋細胞，gap junction，デスモゾームなど生体心筋組織類似の組織像を示した(図3)．移植後，最長1年まで，心筋グラフトが拍動を維持したまま生着することが明らかとなっている．また，時間の経過とともに移植組織のサイズ，電気伝達速度，収縮力が増大することが示されており，移植した心筋組織がホストの月齢に合わせて成長しうることも示されている．

図3　重層化心筋細胞シートの組織切片像
重層化心筋細胞シートの移植後3週間の時点での組織切片像(Azan染色)を示す．皮下組織内の層状の心筋組織内には円柱状に伸展した心筋細胞および多数の毛細血管(矢印)を認める．

重層化心筋細胞シートの心不全治療への応用に関しては大阪大学臓器機能制御外科と共同研究を行っているが，ラット心筋梗塞モデルへの移植実験により心機能の改善が確認されている(詳細は澤の稿「心筋細胞シート移植による心不全治療」参照)．

将来の課題と展望

心筋組織の再構築(myocardial tissue engneering)における当面の課題としては，①細胞ソース，②組織内血管網の再構築によるスケールアップ，③移植法，があげられる．

①に関しては代替として筋芽細胞を使うことにより臨床への応用が可能かもしれない．一方，胚性幹細胞(ES細胞)や骨髄由来の幹細胞から心筋細胞を分化誘導する試みが世界中で行われており，近い将来，臨床で使用可能な心筋細胞が開発されればそれらの細胞を用いた心筋組織の構築が可能であろう．

②はtissue engineeringの研究フィールドに共通の課題である．心筋組織などのように細胞が密

サイドメモ

細胞シート工学

細胞は培養皿とインテグリンなどの膜蛋白およびフィブロネクチンなどのECMを介して接着するが，通常細胞の脱着にはトリプシンなどの蛋白分解酵素が用いられ，膜蛋白やECMを破壊することにより培養皿からの解離を起こす．これに対し，著者らは低温処理のみで細胞を膜蛋白およびECMとともにに損傷を与えることなく脱着させることのできる温度応答性培養皿を開発した．この培養皿は，温度応答性高分子ポリN-イソプロピルアクリルアミド(PIPAAm)を，電子線を用いて培養皿表面に共有結合させたものである．PIPAAmは温度に応答して，親水・疎水の可逆的な変化を起こす．ECMを介した細胞の接着は培養基材表面の親水・疎水度に依存しており，通常の培養温度である37℃では軽度疎水性となり，細胞接着性であるのに対し，32℃以下の低温では高度に親水化するため細胞非接着性となり，培養皿表面のPIPAAm分子とECMの結合が解離し，細胞が膜蛋白やECMとともに損傷を受けることなく脱着する．細胞を密な状態に培養した場合は，細胞と細胞が細胞接着分子を介して直接あるいはECMにより接着する．蛋白分解酵素を使用した場合は，細胞と培養皿の接着が解離するとともに，細胞どうしの接着も破壊されるため，細胞は解離して浮遊する．これに対し温度応答性培養皿を使った場合は低温処理により細胞間接着はまったく影響を受けずに，シート下面のECMと培養皿の接着のみ解離するためシート状に細胞が脱着する．また，細胞シート下面に維持されているECMは他の培養皿への移動時や細胞シート積層時に"糊"として働き再接着を促進する．

この細胞シートを用いた組織工学的手法を細胞シート工学とよび，単層シート移植(皮膚・角膜)，同一細胞シートの積層化による均一な組織構築(心筋)，数種の細胞シートの積層化による層状構造の組織構築(肝・腎・血管)の研究が行われている．

な組織の場合，酸素・栄養の透過性の問題から再構築可能な組織の厚みは100 μm程度と考えられている．これを解決する方法として組織を灌流したり，血管となりうる細胞を一緒に播種したり，血管新生を促進する遺伝子を導入する方法などが追究されている．また，三次元の支持体の内部に毛細血管網となるような管腔を微細加工し，内部に血管内皮細胞を播種したうえで目的の細胞を播種するという試みもある．著者らの研究室でも灌流培養装置や内皮細胞との共培養シートを使用し，より厚い組織の作製を追究している．また別のアプローチとして，すくなくとも *in vivo* においては重層化心筋細胞シート(3枚)の移植を血管新生に必要な時間をおいて繰り返すことにより，より厚い(～1 mm)心筋組織の構築に成功している．

③の移植法に関しては，パッチ状のものをつくり不全部にはりつけるか欠損部と置換する研究が行われているが，細胞移植でも問題となっているようにホストと移植グラフトの電気的・機能的なつながりがどうなるのかを十分に検証する必要がある．また，臓器そのものの再構築を目標にチューブ状の心筋組織を作製しようという試みもある．

おわりに

組織工学による心筋再生は，細胞ソース，三次元組織構築法，血管新生法，移植法と多分野にまたがって解決すべき課題も多いが，今後既存のコンセプトにこだわらないフィールドを越えた研究者・臨床医の連携，技術開発により臨床応用可能になると予測され，重症心不全患者に対する医療に大きく貢献するものと考える．

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*　　*　　*

心　筋

清　水　達　也*

JJSB

Myocardial tissue engineering

心筋組織に対する再生医療としては，単離細胞を心筋組織内に直接注入する細胞移植療法がすでに臨床応用されている．また，組織工学的手法により体外で心筋組織を再構築し移植する方法が次世代の治療法として注目され，細胞の足場として生体吸収性高分子を用いた研究が行われている．筆者らは温度応答性培養皿を用いシート状の心筋細胞を回収，その積層化により支持体を用いずに3次元心筋組織を再構築することを可能とした．

*Tatsuya Shimizu**

Key words：心筋再生，細胞シート，温度応答性培養皿

心筋組織に対する再生医療

これまで，虚血性心疾患や拡張型心筋症に伴う重症心不全に対しては脳死患者からの心臓移植が最終的な治療法と考えられてきたが，近年の幹細胞生物学や組織工学の発達により細胞を使った再生医療が新たな治療法として注目を集めている．治療法としては，① 単離細胞を心筋組織内に注入することにより血管新生あるいは心筋組織を再生させる方法，② 骨髄内の幹細胞を体外からのサイトカイン投与により不全心筋組織に動員し組織を再生させる方法，③ 組織工学の技術を用い3次元的な心筋組織を再構築して移植グラフトを作製し移植する方法がある．すでに自己の筋芽細胞や骨髄由来細胞の注入による治療は臨床応用され，サイトカインであるG-CSFの皮下注射も臨床応用が近い．一方，組織工学的な手法による3次元的な移植心筋グラフトの作製はより有効な次世代型の移植療法として期待されているが，その研究開発にはバイオマテリアルが必要不可欠である．

組織工学的手法による心筋組織の再構築[1)]

単離細胞の移植に関しては，開胸下に注射針を用いて心外膜側から注入する方法，電位と位置情報を同時に取得できるカテーテルを用いた心腔内側からの注入法，冠動脈からの注入法がある．しかしながら，細胞浮遊液としてたがいに解離した状態の細胞を不全心筋組織内に注入するため，移植場所の制御が困難なことや流出・壊死により細胞が損失することが課題となっている．また，これらの細胞移植では先天性心疾患など欠損組織に対する治療は困難である．そこで心筋に対しても組織工学的手法による組織再構築の研究が加速している．他の組織と同様，細胞外マトリクス(ECM)の代わりに生体吸収性の支持体を用いた心筋組織の再構築が報告されている．

方法としては，① 生体吸収性高分子からなる支持体を作製し，それを足場として細胞を播種する手法(図1A)，② 溶液状の支持材料と細胞を混合したのち重合する手法(図1B)，③ 生体由来の脱細胞化組織を支持体として細胞を播種する方法がある(図1C)．①の手法としては，生体吸収性高分子としてポリグリコール酸，コラーゲン，ゼラチン，アルギン酸を用いた研究が報告されている．これらの研究ではメッシュあるいはスポンジ状の多孔性のバイオマテリアルが用いられている．ゼラチンあるいはアルギン酸を使った心筋移植グラフトに関しては不全心筋モデルへの移植実験が行われ，その生着と心機能の改善が報告されている．②の手法としてコラーゲン溶液と心筋細胞を混和し，シリコーンモールド内で培養することにより3次元心筋組織の構築が試みられている．この研究では *in vitro* での張力測定ならびに伸展負荷により，組織に配向性を付与することに成功している．また③としては膀胱組織を脱細胞化したものに心筋細胞を播種し，グラフト化するといった研究が行われている．

細胞シート工学による心筋組織の再構築

以上のように，心筋組織の再構築にはECMの代わりとなる生体吸収性のバイオマテリアルを用いる手法が世界的な主流である．しかしながら，支持体内へ充分な細胞を播種することが困難なため，生体内のような細胞の密な心筋組織の再構築が困難であることが課題となっている．また，移植後支持体の分解に伴い炎症反応が生じることも問題となっている．そこで筆者らは，支持体を用いずにシート状の細胞を回収し積層化することで3次

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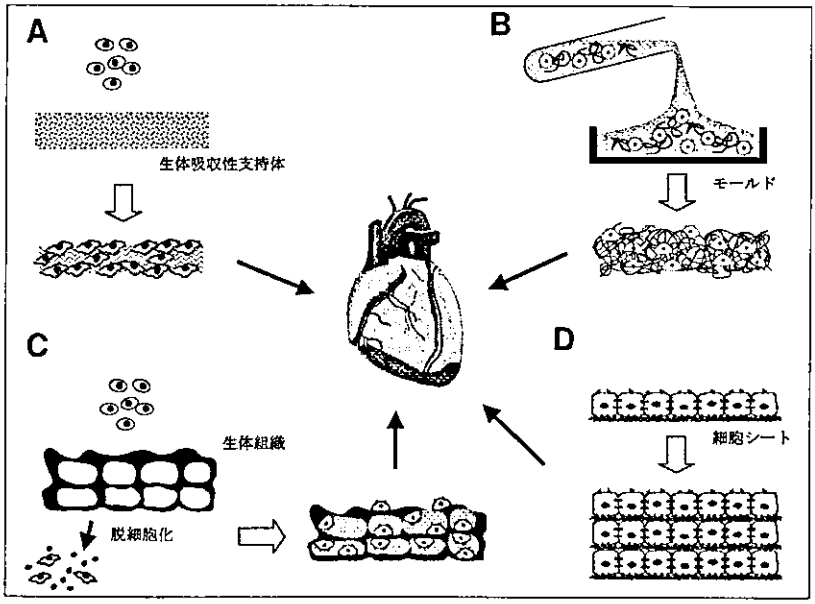

図1　組織工学的手法による心筋組織の再構築
A：生体吸収性高分子からなる多孔性の3次元支持体を作製，それを足場として細胞を播種する．支持体は徐々に分解し細胞あるいは生体が産生するECMと置換されて組織が再生される．
B：溶液状の生体吸収性材料と細胞を混合したのちモールドに流し込み，重合させることで細胞を3次元化する．
C：生体組織を脱細胞化した結合組織を支持体として細胞を播種し培養する．
D：温度応答性培養皿から低温処理のみで脱着した細胞シートを積層化することにより生体吸収性支持体を用いずに3次元的な組織を再構築する．

元組織を再構築する新規手法“細胞シート工学”により心筋組織再生の研究を行っている(図1D)．

細胞シートの回収には温度応答性培養皿を用いる．これは温度応答性高分子ポリ*N*-イソプロピルアクリルアミド(PIPAAm)を電子線を用い培養皿表面に共有結合させたものである．PIPAAmは温度に応答して親水・疎水の可逆的な変化を起こす．通常の培養温度である37℃では軽度疎水性となるため，細胞が接着するのに対し，32℃以下の低温では高度に親水化するため培養皿表面とECMの結合が解離，細胞が培養皿から脱着する．細胞を密な状態に培養した場合は細胞と細胞が細胞接着因子を介して，直接あるいはECMにより接着する．蛋白分解酵素を使用した場合，細胞同士の接着が破壊されるため細胞は解離して浮遊するが，温度応答性培養皿の場合は，低温処理により細胞間接着はまったく影響を受けずに，シート底面のECMと培養皿の接着のみ解離するためシート状に細胞が脱着する．また細胞シートの底面に維持されているECMは細胞シート積層時に“糊”として働き再接着を促進する．

この温度応答性培養皿を用い，新生仔ラット心筋細胞シートを回収・重層化した．数日中に重層化した2枚の心筋細胞シートは同期して拍動するようになり，形態的にも心筋細胞シート間に密な接着が形成されることが示された[2]．さらに心筋細胞シートを積層化したところ，*in vitro* および *in vivo* において肉眼レベルで同期して拍動する心筋組織の作製が可能となった[3]．皮下での移植組織には周囲から血管が侵入，毛細血管網が発達し，生体心筋と同様の組織再構築が確認された．すでに移植後1年まで，心筋グラフトが拍動を維持したまま生着しうることを確認している．また，ラット心筋梗塞モデルへの移植実験による心機能の改善も確認している(大阪大学大学院医学系研究科機能制御外科学教室との共同研究による)[4]．

心筋再生医療におけるバイオマテリアル

以上のように，次世代の心筋再生医療として期待されている組織工学的手法による移植心筋組織の再構築に関しては，さまざまなバイオマテリアルを用いた研究が行われている．しかしながら，細胞同士が電気的・形態的に密着し，組織全体が同期して拍動し生体と同様の機能を発揮する心筋組織を再構築するには，既存の手法にとらわれない心筋組織に最適なバイオマテリアルの開発や技術革新が必要となっている．そのなかで細胞シート工学は，シート状の細胞の回収という既存の技術では不可能であったことを可能としており，生体吸収性材料を用いない新たな組織工学的手法として心筋組織の再生に貢献するものと考える．

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GENE DELIVERY

PEGylation enhances tumor targeting of plasmid DNA by an artificial cationized protein with repeated RGD sequences, Pronectin®

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Abstract

The objective of this study is to investigate feasibility of a non-viral gene carrier with repeated RGD sequences (Pronectin F^+) in tumor targeting for gene expression. The Pronectin F^+ was cationized by introducing spermine (Sm) to the hydroxyl groups to allow to polyionically complex with plasmid DNA. The cationized Pronectin F^+ prepared was additionally modified with poly(ethylene glycol) (PEG) molecules which have active ester and methoxy groups at the terminal, to form various PEG-introduced cationized Pronectin F^+. The cationized Pronectin F^+ with or without PEGylation at different extents was mixed with a plasmid DNA of LacZ to form respective cationized Pronectin F^+–plasmid DNA complexes. The plasmid DNA was electrophoretically complexed with cationized Pronectin F^+ and PEG-introduced cationized Pronectin F^+, irrespective of the PEGylation extent, although the higher N/P ratio of complexes was needed for complexation with the latter Pronectin F^+. The molecular size and zeta potential measurements revealed that the plasmid DNA was reduced in size to about 250 nm and the charge was changed to be positive by the complexation with cationized Pronectin F^+. For the complexation with PEG-introduced cationized Pronectin F^+, the charge of complex became neutral being almost 0 mV with the increasing PEGylation extents, while the molecular size was similar to that of cationized Pronectin F^+. When cationized Pronectin F^+–plasmid DNA complexes with or without PEGylation were intravenously injected to mice carrying a subcutaneous Meth-AR-1 fibrosarcoma mass, the PEG-introduced cationized Pronectin F^+–plasmid DNA complex specifically enhanced the level of gene expression in the tumor, to a significantly high extent compared with the cationized Pronectin F^+–plasmid DNA complexes and free plasmid DNA. The enhanced level of gene expression depended on the percentage of PEG introduced, the N/P ratio, and the plasmid DNA dose. A fluorescent microscopic study revealed that the localization of plasmid DNA in the tumor tissue was observed only for the PEG-introduced cationized Pronectin F^+–plasmid DNA complex injected. We conclude that the PEGylation of cationized Pronectin F^+ is a promising way to enable the plasmid DNA to target to the tumor for gene expression.

Keywords: Tumor targeting; Enhanced gene expression; Pronectin; Cationization; PEGylation

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1. Introduction

One of the major goals for in vivo gene therapy is to achieve the specific delivery of gene to a target tissue. For such successful gene delivery, there are many issues to be overcome. The in vivo gene expression depends on the body distribution of DNA intravenously injected which is greatly influenced by anatomical feature of tissue/organ and the interaction manners with biological fluids, extracellular matrix (ECM), and cells.

The simple injection of plasmid DNA solution only shows a very low efficiency of gene transfection. When a plasmid DNA is intravenously injected without any combination of the carrier vector, 60–70% of plasmid DNA injected is cleared up from the blood circulation by Kupffer cells [1,2]. In addition, the plasmid DNA is enzymatically degraded in the blood circulation [3]. On the other hand, even if a plasmid DNA is complexed with the vector, it is well recognized that the molecular size and surface charge of complex obtained affect the body fate of plasmid DNA. For example, the complex of plasmid DNA with cationic liposomes is mainly accumulated in the lung, liver, and spleen [4–8]. As one trial to change the body fate, the liposome surface has been modified by polymers, such as poly(ethylene glycol) (PEG) and other hydrophilic polymers [9–12]. This modification enabled the plasmid DNA to prolong the half-life in the blood circulation. For the gene delivery study, various cationic polymers and lipids have been used to form the complex with plasmid DNA. The size, charge, and surface characteristics of complexes have great influence on the fate of plasmid DNA in the body as well as the property to protect the plasmid DNA from enzymatic degradation [3,5,13–19].

There are several researches on the gene therapy for tumor. A promising therapeutic result was initially reported for melanoma by the intratumoral injection of a plasmid DNA–liposome complex [20]. Based on research results by various tumor-bearing animal models, the in vivo tumor gene therapy by the plasmid DNA complexed with non-viral vectors was not really successful [21,22]. As one trial, the targeting of plasmid DNA to the tumor has been experimentally performed [23,24]. The ligands for tumor cell receptors have been incorporated into the vector materials of plasmid DNA to expect the natural receptor-mediated endocytosis. One of the practically available ligands is transferrin, an iron-transporting serum glycoprotein which binds to a receptor expressed strongly on the surface of most proliferating cells, such as erythroblasts and tumor cells [25]. For example, tumor targeting of a plasmid DNA was achieved through complexation with low-molecular-weight polyethylenimine (PEI) covalently coupled to transferrin [26]. Chemical modification of liposomes surface by monoclonal antibodies or other targeting moieties specific to tumor cells has been proposed for the tumor-specific delivery of plasmid DNA [27–33].

The newly formed vasculature in the tumor tissue has a high substance permeability compared with that of normal tissues, while lymphatic systems are immature in the tumor tissue. These anatomical features enable macromolecules and liposomes to accumulate and remain in the perivascular regions of solid tumors for a longer time period than in the normal tissue, which is referred as the enhanced permeability and retention (EPR) effect [34–36]. It has been demonstrated that conjugation with water-soluble polymers, such as poly(vinyl alcohol), poly(ethylene glycol), and dextran, increases the accumulation of drugs in the tumor tissue as well as prolongs their life-time in the blood circulation [37–45]. This passive drug targeting is supposed not only to facilitate the tumor therapeutic effect, but also to attenuate the adverse effects, because the amount of drugs associated with non-targeted tissues is acceptably reduced. It has been demonstrated that the PEGylation of drugs and plasmid DNA–vector complexes suppressed their interaction with plasma components and erythrocytes and prolonged the circulated period in the blood, resulting in the enhanced therapeutic effects and gene expression in the tumor tissue even after systemic administration [46–54].

The basic idea of research approach with non-viral vectors is not only to neutralize the negative charge of plasmid DNA, but also to condense the DNA size by polyion complexation with various polymers and liposomes of positive charge [55–58]. It is likely that the plasmid DNA complex condensed in size and having a positive charge effectively interacts with cells to accelerate the internalization, resulting in the enhanced efficiency of gene transfection. However, since this complex–cell interaction is based on the simple and nonspecific electrostatic force, a more efficient and cell-specific cell transfection cannot be

always expected. One of practically possible ways to improve this limited situation is to take advantage of cell receptor systems which play an important role in the specific cellular uptake of several substances. In this study, we selected and used a ligand specific to the cell receptor as the non-viral vector of plasmid DNA. The sequence of RGD (arginine-glycine-aspartic acid) has been discovered as a cell attachment moiety in various adhesive proteins present in the extracellular matrix (ECM), and found in many proteins, such as fibronectin, collagen type I, vitronectin, fibrin, and Von Willebrand Factor [59]. It has been well recognized that the sequence interacts with various types of integrin receptors. There are several ports on the synthesis of polymers incorporating the RGD sequence [60]. Some polymers comprised of repeated blocks of RGD sequence have been genetically synthesized to assess their therapeutic effects [61,62]. 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 is 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 [63,64]. 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 [65]. 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 explored as a coating reagent of cell culture dishes. 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 [62]. It is possible that Pronectin F is readily adsorbed onto the polymer surface based on the hydrophobic interaction force. 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 make Pronectin F water-soluble (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. The integrin is a membrane protein which is apparently always present on angiogenic, or newly growing, blood vessels but rarely on established ones. Various studies in recent years have shown that $\alpha_v\beta_3$ integrin is up-regulated on angiogenic endothelial cells, and can therefore be considered as a target molecule [66]. The integrin, $\alpha_v\beta_3$, has another quality that would turn out to be convenient; it can propel viruses or other small particles into cells. Their crucial role in tumor growth-related angiogenesis and their location in the body make angiogenic endothelial cells to be an important candidate of target cells for therapeutic intervention [67]. Many molecules specifically expressed by tumor endothelial cells have been proposed as target molecules for vascular targeting strategies [68]. The integrin $\alpha_v\beta_3$ was previously identified as a target molecule on angiogenic endothelium [69]. It can interact with various RGD sequence-containing extracellular matrix components [70]. Peptides containing this RGD sequence in a constrained configuration inhibited angiogenesis based on induction of endothelial cell apoptosis in tumors and inflammatory sites [71,72]. Tumor vasculature can be targeted by non-viral vector containing an RGD sequence, which binds to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins on angiogenic endothelial cells.

This study was undertaken to investigate feasibility of Pronectin F^+ with RGD sequences in targeting a plasmid DNA to the tumor tissue for gene expression. To give the Pronectin F^+ cationized charges necessary for the formation of polyion complexation with the plasmid DNA, spermine (Sm) was introduced into the hydroxyl group of serine residues in Pronectin F^+. Cationized Pronectin F^+ with different extents of aminization was prepared by changing the conditions of amine introduction. The cationized Pronectin F^+ was then reacted with PEG and mixed with a plasmid DNA encoding LacZ in aqueous solution to assess the zeta potential and molecular size of cationized Pronectin F^+–plasmid DNA complexes with or without PEGylation. Following intravenous injection of the complexes of cationized Pronectin F^+ with or without

PEGylation and plasmid DNA to mice, the gene expression in the tumor was compared with that in other tissues to assess the tumor targetability from the viewpoint of gene expression level and histological examination.

2. Materials and methods

2.1. Materials

Pronectin F^+ (molecular weight (Mw) = 110,000) was kindly supplied from Sanyo Chemical Industries, Ltd., Kyoto, Japan. Succinimidyl succinate-methoxy PEG (MEC-PEG, molecular weight (Mw) = 5250) was kindly supplied from Nihon Oil Fat, Tokyo, Japan and was used as obtained. Spermine (Sm) was purchased from from Wako Pure Chemical Industries, Ltd., Osaka, Japan and was used as obtained. 2,4,6-Trinitrobezenesulfonic acid (TNBS), and β-alanine were purchased from Nacalai Tesque, Kyoto, Japan and was used without further purification. As 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 and Takara Shuzo, Co. Ltd., Shiga, Japan, respectively. Rhodamine B isothiocyanate (RITC) was obtained from Sigma-Aldrich Japan K.K., Tokyo, Japan.

2.2. Preparation of plasmid DNA

The plasmid DNA used was the expression vector consisting of the coding sequence of LacZ and a SV40 promoter inserted at the upstream (pSV-LacZ, 7931 bp). The plasmid DNA was amplified in a transformant of *E. coli* bacteria and isolated from the bacteria by Qiagen Maxi kit-25 (Qiagen K.K., Tokyo, Japan). The absorbance ratio at the wavelength of 260 to 280 nm for purity assessment of plasmid DNA obtained was measured to be between 1.8 and 2.0.

2.3. Preparation of cationized Pronectin F^+

Cationized Pronectin F^+ with different extents of aminization was prepared by introduction of Sm into the hydroxyl group of serine residues in Pronectin F^+ based on the conventional CDI method [73]. Briefly, varied amounts of 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 Sm residue to the hydroxyl groups of Pronectin F^+, followed by dialysis against double-distilled water (DDW) for 2 days and freeze-dry to obtain the Sm-derivatives of Pronectin F^+. The molar percentage of Sm introduced into the hydroxyl groups was quantitated by the conventional TNBS method [74] based on the calibration curve prepared by using β-alanine.

2.4. Preparation of PEG-introduced cationized Pronectin F^+ and the complexation with plasmid DNA

PEG-introduced cationized Pronectin F^+ with different extents of PEGylation was prepared by introduction of succinimidyl succinate-methoxy PEG (MEC-PEG) into the amine group of Sm residue in Pronectin F^+. Briefly, varied amounts of MEC-PEG were added to 10 ml of dehydrated dimethyl sulfoxide containing 10 mg of Pronectin F^+. The reaction solution was agitated at 25 °C for 16 h to introduce MEC-PEG to the terminal amine group of Sm residue of Pronectin F^+, followed by dialysis against DDW for 2 days and freeze-drying to obtain PEG-introduced cationized Pronectin F^+. The molar percentage of PEG introduced into the amine groups was quantitated by the conventional TNBS method [74] based on the calibration curve prepared by using β-alanine.

Complexation of cationized Pronectin F^+ with or without PEGylation with the plasmid DNA was performed by simple mixing the two materials at various charge 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, 400, and 500 μg of cationized Pronectin F^+ with or without PEGylation 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 cationized Pronectin F^+–plasmid DNA complexes with or without PEGylation. The charge ratio (N/P) was presented as the molar ratio of cationized Pronectin F^+ nitrogen to plasmid DNA phosphate. Therefore, the N/P ratios of 0.25,

0.5, 0.75, 1, 1.5, 2, and 2.3 were chosen for the following experiments of cationized Pronectin F^+–plasmid DNA complex.

2.5. Electrophoresis of cationized Pronectin F^+–plasmid DNA complexes with or without PEGylation

Cationized Pronectin F^+–plasmid DNA complexes with or without PEGylation of different extents were prepared at various N/P ratios 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).

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

The complexes of cationized Pronectin F^+ with or without PEGylation and plasmid DNA at various N/P ratios were prepared by the similar procedure described above. Every solution was filtered through a 0.45 μm filter (Millex-HV, Millipore) prior to mixing. The mixed plasmid DNA and cationized Pronectin F^+ with or without PEGylation was placed in a DLS cell and DLS measurement was carried out using a DLS-DPA-60HD instrument (Otsuka Electronic Co. Ltd., 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 cationized Pronectin F^+ with or without PEGylation 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 electrophoretic mobility measured u:$\zeta = 4\pi\eta u/\varepsilon$, where η and ε are the viscosity and the dielectric constant of the solvent, respectively.

2.7. In vivo experiments

Meth-AR-1 fibrosarcoma cells (6×10^7 cells/ml RPMI-1640 culture medium) maintained by the in vivo passage in the intraperitoneal cavity of CDF_1 male mice, aged 5–6 weeks (Oriental Bioservice, Kyoto, Japan), were subcutaneously inoculated at a volume of 0.02 ml into back of male CDF_1 mice (6 weeks). All the experimental procedures were performed according to the specifications of Guideline for Animal Experiments of Kyoto University.

CDF_1 mice carrying a tumor mass of 5 mm in the average diameter on the back subcutis received an intravenous injection of PBS or that containing free plasmid DNA, cationized Pronectin F^+, PEG-introduced cationized Pronectin F^+, and cationized Pronectin F^+–plasmid DNA complexes with or without PEGylation in a volume of 200 μl. After intravenous injection of the complexes or other agents to mice, mice were sacrificed with cervical dislocation, and the blood sample was taken out directly from the heart by syringe aspiration and the organs of mice were taken 2 days later, washed with PBS, frozen in liquid nitrogen, and stored at −85 °C. The organ samples were subjected to the gene expression assay.

2.8. Evaluation of gene expression

For evaluation of gene expression, β-galactosidase activity was measured by use of Invitrogen kit (Invitrogen Co., CA, USA). Briefly, the tumor and organ samples were immersed and homogenized in the lysis buffer (0.1 M Tris–HCl, 2 mM EDTA, 0.1% Triton X-100) at the lysis buffer volume (ml)/sample weight (mg) ratio of 4 to 1 in order to normalize the influence of weight variance on the β-galactosidase assay. The sample lysate (2 ml) was transferred to a centrifuge tube, followed by freeze-and-thaw three times and centrifugation at 14,000 rpm at 4 °C for 5

min. The supernatant (30 μl) was mixed with 70 μl of 4 mg/ml *o*-nitrophenyl β-D-galactopyranoside (ONPG) aqueous solution and 200 μl of cleavage buffer (60 mM Na_2HPO_4-$7H_2O$, 40 mM NaH_2PO_4-H_2O, 10 mM KCl, and 1 mM $MgSO_4$-$7H_2O$, pH 7) in a fresh microcentrifuge tube. After incubation at 37 °C for 30 min, 500 μl of 1 M sodium carbonate solution was added to the solution mixture. The solution absorbance was measured at the wavelength of 420 nm for β-galactosidase activity. Each experiment was done for three mice independently unless mentioned otherwise.

2.9. Fluorescent microscopic observation

For the fluorescent labeling of plasmid DNA, the pSV-LacZ 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. Then, the reaction mixture was applied to gel filtration of a PD 10 column (Amersham Pharmacia Biotech K.K., Tokyo, Japan) to separate the non-reacted RITC from the RITC-labeled pSV-LacZ, followed by ethanol precipitation for collection.

Aqueous solution of 2 mg/ml RITC-labeled plasmid DNA (50 μl) was mixed with 40 μl of cationized Pronectin F^+ with or without PEGylation at the N/P molar ratio of 2.3 for the complex of PEG-introduced cationized Pronectin F^+–plasmid DNA. After intravenous injection of the complexes, the tumor was taken 2 days later and embedded in Tissue-Tek (OCT Compound, Miles Inc., IN, USA). The cryosections (5-μm thickness) of the tumor samples were prepared to view the fluorescent localization of plasmid DNA on Olympus AX-80 fluorescence microscope equipped with Olympus

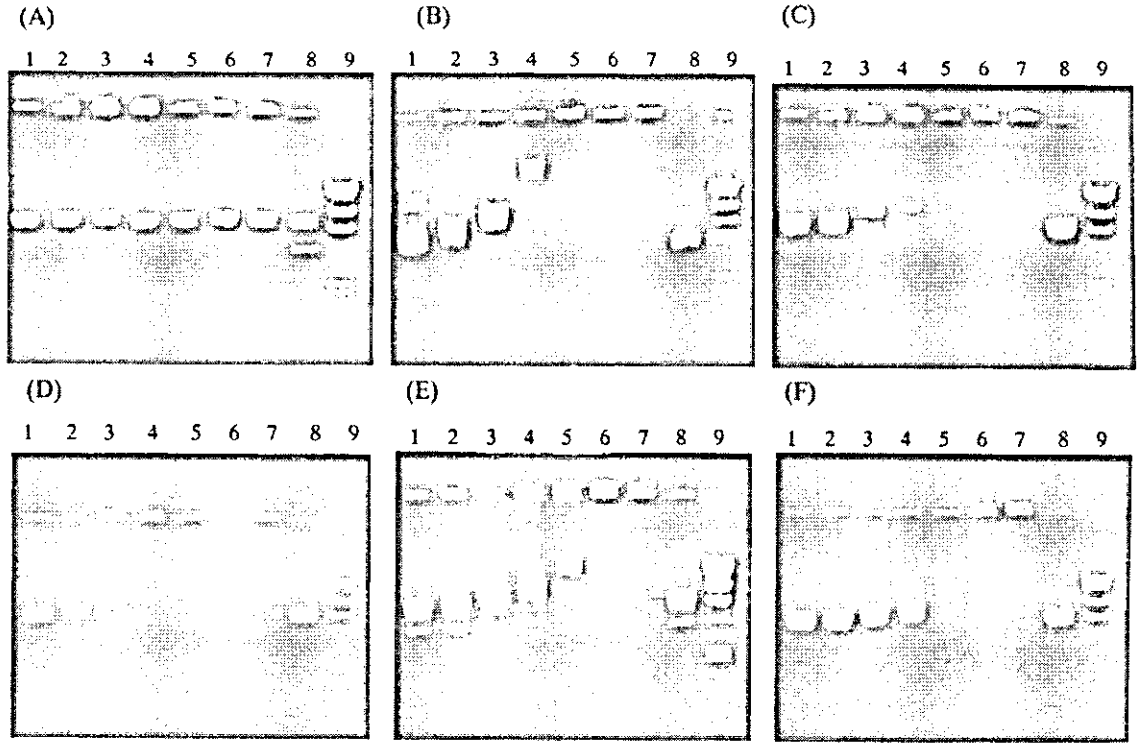

Fig. 1. Electrophoretic patterns of plasmid DNA complexes with Pronectin F^+, cationized Pronectin F^+, and PEG-introduced cationized Pronectin F^+ with different extents of PEGylation and different charge ratios (N/P) of cationized Pronectin F^+ to plasmid DNA: (A) original Pronectin F^+, (B) cationized Pronectin F^+, (C) PEG-introduced cationized Pronectin F^+ (5.1 mol% introduced), (D) PEG-introduced cationized Pronectin F^+ (10.1 mol% introduced), (E) PEG-introduced cationized Pronectin F^+ (20.1 mol% introduced), and (F) PEG-introduced cationized Pronectin F^+ (50.1 mol% introduced). Lanes 1–7: the N/P molar ratios of 0.25, 0.5, 0.75, 1, 1.5, 2, and 2.3. Lane 8: free plasmid DNA. Lane 9: DNA marker.