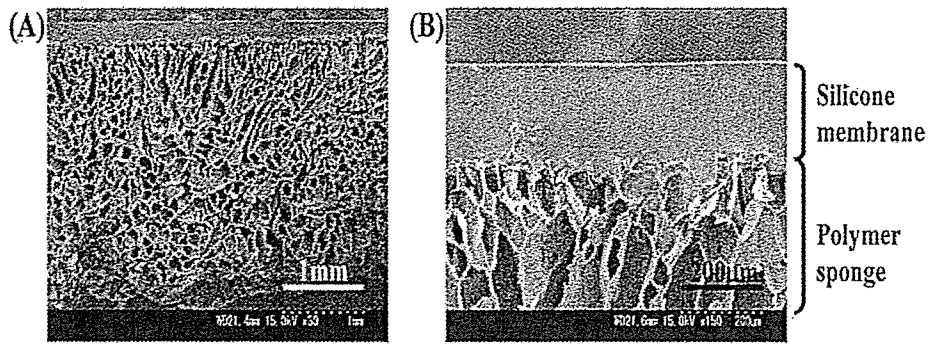

図6　マルチブロック共重合体スポンジとシリコン薄膜からなる，
完全合成型人工皮膚のSEM写真

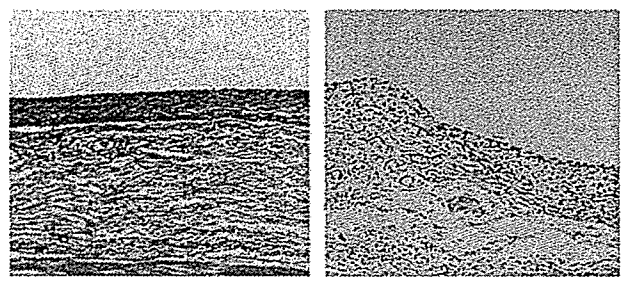

図7　ラット皮下2週埋入後の組織反応

の複合材料をしのぐ特性であり，このマルチブロック共重合体ハイドロゲルスポンジ／HAp複合体は，その柔軟な特性とマイルドな炎症反応を併せもつ皮膚組織修復材料として期待される。

3.2　細胞移植用インジェクタブルスキャホールド

近年，心筋梗塞部位への細胞移植などによる著効が報告されているが，移植した細胞を患部へ効率的に送達（固定）することは容易ではなく，細胞生着率は10％以下との報告もある。そこで，移植細胞を懸濁させるマトリックスとしてインジェクタブルスキャホールドが注目されている。インジェクタブルスキャホールドとは，生体外では溶液であり，患部に適応された後に何らかの刺激によりゲル化（固化）する相転移材料である。我々のグループでは，ポリ-L-乳酸（PLLA），あるいはポリ-D-乳酸（PDLA）と，PEGとのABA型トリブロックコポリマーを利用して，低温では液状で32℃以上でゲル状に相転移するインジェクタブルスキャホールドの開発に成功した[8]。まず，PLLA-PEG-PLLAトリブロック共重合体を水系中に分散してPLLAコアとPEGコロナからなる高分子ミセル（L体ミセル）を作製する。同様にPDLA-PEG-PDLAからD体ミセルを調製した。両者の10％懸濁液を混合し（図8，d），37℃で処理すると透明なゲル状に変化した（図8，e)。このような相転移現象は，L体ミセルのみの懸濁液では観察されない

こと（図８，a･c），および，X線散乱解析の結果から，この相転移現象がPLLAとPDLAとのステレオコンプレックス形成に基づいてミセル間に架橋が生じるためであることが明かとなっている。このインジェクタブルゲルは，生体内で分解されるPLAと生体内非蓄積性であるPEGのみからなる，含水率90％の完全生体吸収性のインジェクタブルスキャホールドである。

このゲルが細胞毒性を有さないこと，および，細胞の生存と増殖を許容するかを見当するために，緑色蛍光（GFP）発現マウス繊維芽細胞の移植実験を行った。L体・D体ミセル混合液に所定数のGFP発現細胞を添加した懸濁液を，GFP(－）マウスの大腿部に注入し，所定時間後に移植細胞の様子を蛍光顕微鏡下にて確認した（図９）。その結果，ゲル中で細胞は正常に蛍光を発し，その毒性の低さと，細胞移植用インジェクタブル材料として機能することが実証された。

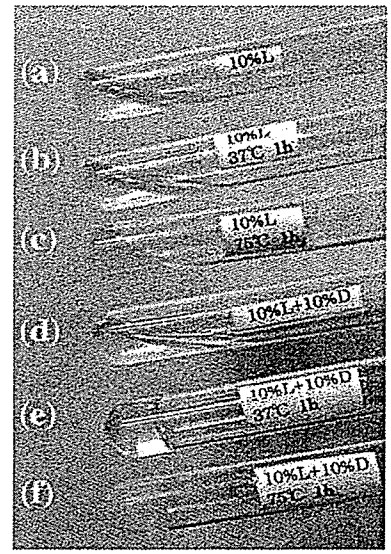

図８　L体ミセル懸濁液（a）は37度ではゲル化しない（b）が，L体･D体混合ミセル溶液（d）は，ステレオコンプレックス形成に基づいて，37度でゲル化する（e）

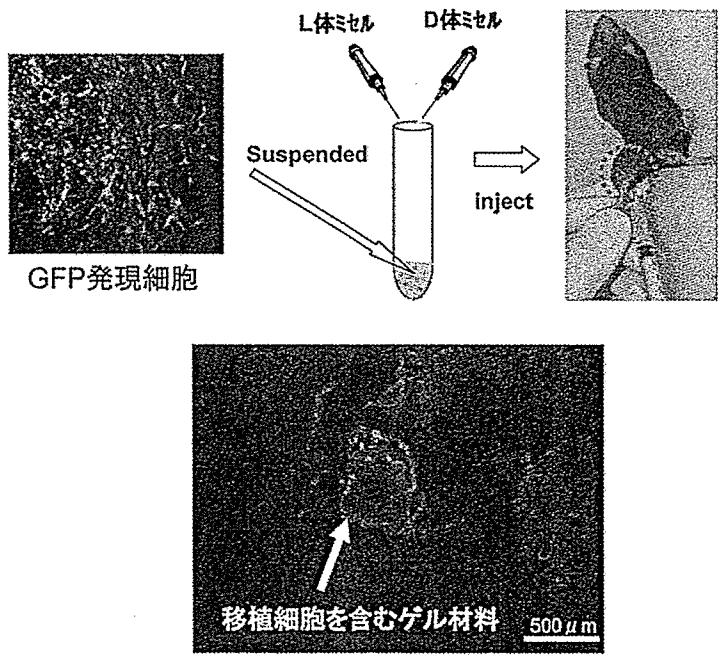

図９　緑色蛍光タンパクを発現する細胞の移植実験

4　生体組織の利用

医療分野におけるバイオベース材料の究極の利用は臓器移植ではないだろうか。現代の技術では，完全な臓器を作製することは不可能であり，多孔質スキャホールドを利用した再生医工学では，3次元構造を有する組織や器官への応用は容易ではない。そこで，我々は，さらに機能性に富んだスキャホールドとして，生体組織から細胞を除去して生体スキャフォールドとして利用するアプローチを試みている。ヒトあるいは動物から採取した心臓弁から，放射線照射及び洗浄処理によって細胞成分や細菌，ウイルス，DNAを完全に除去した脱細胞化組織は，移植後に自己細胞が侵入することでリモデリングされ，自己組織化されると期待される。さらに，この脱細胞化スキャフォールドに，*in vitro* において患者の自己細胞を播種するテーラーメード移植によって，より早期の自己化を獲得できると考えられる（図10）。

生後4ヶ月，体重約10 kgのクラウン系ミニブタから清潔下にて下行大動脈を採取し，PBSによる洗浄後，PBSを満たした滅菌容器に封入して，10，30，100，300，あるいは1000 Gyのガンマ線を照射して約2週間洗浄した。ガンマ線未照射の組織では洗浄後も組織内に核の残存が見られたが，照射線量が増えるにつれて洗浄後組織内の核の残存が減少し，さらに，残存DNAを測定したところ，100あるいは300 Gy以上の照射では大幅に減少した（図11）。種々のガンマ射線量による組織脱細胞化の基礎的検討を残存DNA定量試験，力学試験にて行った。

一方，作製した脱細胞組織の破断強度並びに弾性率は，もとの組織とほぼ同程度であった。すなわち，300 Gy以上のガンマ線を照射後，洗浄処理することによって，細胞外マトリックスの

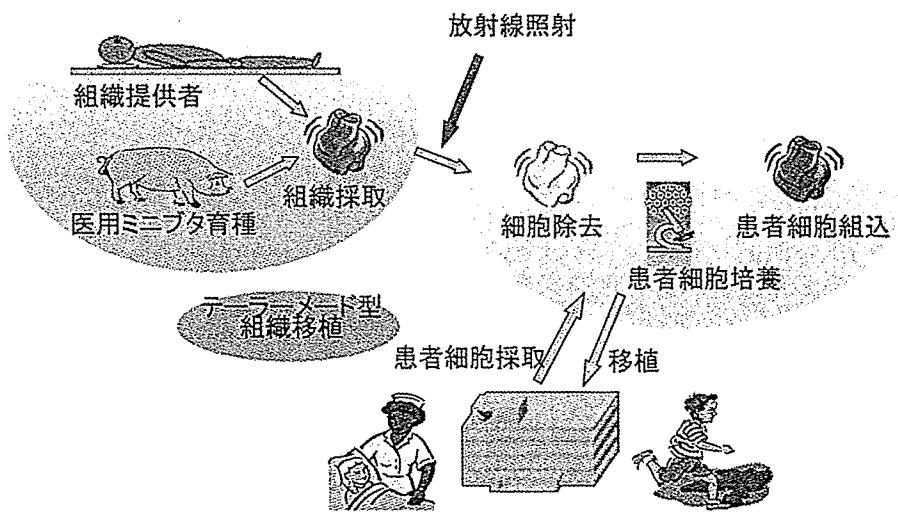

図10　テーラーメード型組織移

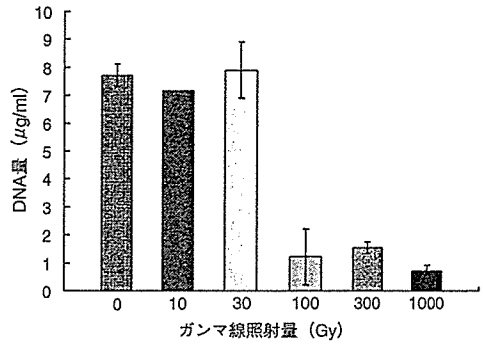

図11　ガンマ線照射及び洗浄処理によって細胞除去したブタ血管組織の残存 DNA 量

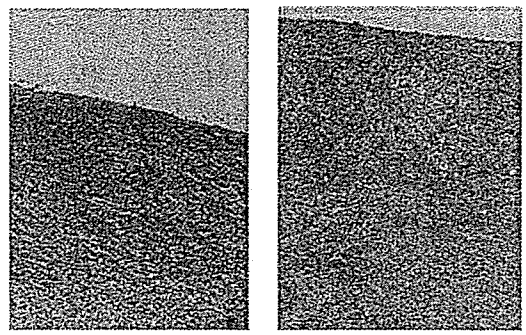

図12　ガンマ線照射（10kGy）及び洗浄処理によって脱細胞化したブタ心臓弁組織
（左：処理前、右：処理後）

特性を保持したままで，循環器系組織内の細胞はほぼ完全に除去できる。

Wister ラット（7週令）の皮下部位に上記脱細胞化ミニブタ大動脈を埋入し，2週間後に取り出して組織学的検討を行った。脱細胞化ミニブタ大動脈の場合では，血管新生が認められず，さらに，マクロファージ陽性を示す CD 68 陽性細胞数が優位に抑制されており，脱細胞組織のマイルドな炎症反応が証明された。上述したように，生体由来であるが故に懸念されるウイルスや感染性物質も，放射線処理により回避できる可能性も高く，今後，安全かつ優れた組織親和性を有するスキャホールド材料として期待できる。

5　おわりに

これまでに生分解性と分解生成物の安全性が確認されてきた PLA や PGA のみならず，生体由来の物質の高い機能性は計り知れない。今後も，様々な合成手技や化学修飾法を開発することで，その機能性はさらに向上するであろう。生物学的に優れた細胞外マトリックスの働きを少し

でも再現できる機能性マトリックスにより，今後の組織再生医工学は新たなステージを迎えることとなる。

謝辞

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4　心臓弁

藤里俊哉[*1]，北村惣一郎[*2]

4.1　はじめに

日本人工臓器学会の調査によると，我が国で心臓弁膜症によって置換術を受けた患者は大動脈弁と僧帽弁あわせて1999年において年間８千人以上にのぼり，80％が機械弁，残り20％が異種生体弁である。また，米国胸部外科学会の調査によると，大動脈弁置換術は1997年において年間約９千人であり，判明しているもののうち約50％が機械弁，45％が異種生体弁，３％が同種弁，残りが自己弁である。術前診断や術中の体外循環技術の向上などもあり，大動脈弁置換術における死亡率は1998年において約４％とされている。このように，最も臨床的に使用される人工臓器の一つとして確立した感のある心臓弁置換術ではあるが，現在，どのような問題があり，これらを解決するために再生医療心臓弁にはどのような特性が要求されるのであろうか。まず，心臓弁置換術の現状について述べた後，現在，開発されつつある再生医療心臓弁の動向，そしてその将来展望について述べる。

4.2　心臓弁置換術の現状

現在用いられている機械弁は，主にパイロライトカーボン製の２枚の半月板弁葉をもった二葉弁である。従来，弁座部分の構造上の問題から弁前後の圧較差が無視できない大きさで，心機能や予後に影響を与えるとされてきたが，最近，弁座部分の改良によって有効弁口面積を広くしたものが開発され，弁輪が狭小の症例においても通常の弁置換術で対応可能である。また，独特の弁葉作動音を減少させたものも開発されている。機械弁はすでに十分な耐久性と血行動態を得ているが，依然として抗血栓性の問題は解決されていない。抗凝固のため，術後は生涯にわたり厳重なワーファリン服用のコントロールが必要であり，機械弁に血栓が付着した場合には急速な弁機能不全を招くとともに，脳塞栓症をきたす頻度も高くなる。また，ワーファリンが催奇形性を有することから，妊娠を希望する若年女性には使用できないという問題もある。

異種生体弁は，ブタ心臓弁あるいはウシ心膜を免疫原性の低下のためにグルタルアルデヒドで固定化したものである。従来，ステントへの固定のために有効弁口面積が減少するとともに，固定に伴うストレスが弁葉の石灰化や変成を促進するとされていたが，近年，後述の同種弁の成功をきっかけにステントを用いないステントレス異種生体弁が導入され，耐久性の向上が期待されている。異種生体弁は抗凝固性に優れてはいるが，若年者では５～10年程度の耐久性しかなく，

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通常は60歳以上の高齢者への適用とされる。また，BSE問題をきっかけに，ウシ心膜の使用は控えられる傾向にある。

欧米では1985年頃から，我が国でも，近年，凍結保存による組織バンクが整備されたことにより，死体から提供された凍結保存同種弁が臨床で使用されつつある。これは，機械弁に比べて抗血栓性で，異種生体弁に比べて耐久性で，そして両者に対して抗感染性で優れているとされる。しかしながら，提供数が絶対的に不足しているのが大きな問題である。また，若年者では比較的早期に機能不全をきたす症例も報告されており，免疫反応の関与が強く示唆されている。若年者に有効とされるRoss手術では，自己肺動脈弁を大動脈弁位に置換移植し，欠損した肺動脈弁を凍結保存同種弁によって再建するが，大動脈弁位に移植された自己肺動脈弁は患者の成長とともに増大するという特徴がある。これに対して，機械弁や異種生体弁はもとより，凍結保存同種弁でも成長性を有しないため，小児患者の場合では再移植となる場合が少なくない。以上のように，再生医療心臓弁に求められる特性として，抗凝固性，耐久性，成長性などが挙げられよう。

4.3 再生医療心臓弁の世界的動向

マサチューセッツ工科大学のLangerやVacantiらによって提唱された組織工学の手法は，すでに米国で細胞を組み込んだ人工皮膚として製品化されている。同様の手法を用いた再生医療人工弁が，1995年以降，彼らのグループから報告されている[1]。新岡らはヒツジを用いた実験で，末梢血管壁の細切によって血管内皮細胞，平滑筋細胞，および線維芽細胞を分離した後に8〜10週間培養し，ポリグリコール酸製のシート状メッシュ上にまず線維芽細胞と平滑筋細胞を，続けて1週間後に血管内皮細胞を播種することによって，再生医療心臓弁葉を作成した。ヒツジの肺動脈弁の一葉を再生医療心臓弁葉で置換したところ，6週後には正常組織と同様の組織が再生し，9週以降は力学特性も正常組織と同等であったと報告している[2]。最近，彼らは弁葉だけでなく，三葉を有するバルサルバ洞付きの心臓弁組織scaffoldを開発し，細胞を播種することで*in vitro*で弁全体を組織工学的に作成し，臨床応用を開始する計画である。このような生体吸収性scaffoldを用いた再生医療心臓弁は，韓国ソウル大学などからも報告されている[3]。

一方，米国のCryoLife社は1992年から米国政府の補助を受けて動物組織から細胞を除去した異種組織移植法の研究開発に取り組み，詳細を明らかにはしていないが，SynerGraftと称する細胞除去方法を発表している。同社は1999年から脱細胞化ブタ大動脈弁の臨床使用を開始し，2001年には世界初の再生医療心臓弁と称して欧州で市販を開始した。移植後数ヶ月間で自己細胞が組織内に浸潤し，自己組織化すると報告している[4]。

ドイツ・ハノーバー医科大学のHaverichらのグループは，1998年からCryoLife社と同様に異種生体弁から動物由来細胞を除去し，さらにレシピエントの自己血管内皮細胞を播種している。

彼らは界面活性剤であるTriron X-100やタンパク分解酵素であるトリプシン溶液を細胞除去に用いている[5]。一方，英国リーズ大学のInghamらのグループは種々の薬液で細胞除去効果を検討し，SDSが最も細胞除去に適していると報告している[6]。また，ドイツ・フンボルト大学のKonertzらのグループはヒツジを用いた6ヶ月間の動物実験で，脱細胞化ブタ肺動脈弁に自己内皮細胞を播種すると，弁の変形も石灰化も見られなかったと報告しており[7]，臨床使用を開始している。

4.4 我々の最新成果の紹介

我々は2000年から，Haverichらの方法を対照として，脱細胞化した異種生体弁を用いた再生医療研究を開始した。我々が生体組織を選んだのは，以前から凍結保存同種弁の臨床使用に取り組んできたことと，心臓弁のような複雑な形状を吸収性人工材料で造形することが容易でないこと，およびポリ乳酸などの生体吸収性人工材料は生体よりも硬いために生体と同等の力学特性をもたせるのが難しいと考えたためである。ミニブタあるいは食用ブタ肺動脈弁を採取し，Triton X-100溶液に浸漬して脱細胞化処理した。脱細胞化処理による生体力学特性への影響は力学試験機で測定した。ミニブタの大腿動脈から酵素処理によって採取した自己内皮細胞を2週間培養増殖後に播種し，2日後に右心バイパス下にて肺動脈弁置換術を施行した。心エコーと圧測定による血行動態測定後に移植弁組織を摘出し，免疫染色などによって組織学的所見を検討した。

24時間の無細胞化処理によって表面から1mm以内の組織内細胞を除去できた（図1）。組織表面の血管内皮細胞は破壊されていたが，完全に脱落することはなく，他の物理的方法の併用が必要であった（図2）。また，Triton X-100は細胞毒性を示すため，組織から除去して細胞を播種するためには2週間以上の洗浄を要した。脱細胞化処理によって強度，弾性率ともに増加したが，コラーゲン線維および弾性繊維の層内含有量と配列状態はほとんど変化なく，弁葉の厚さに

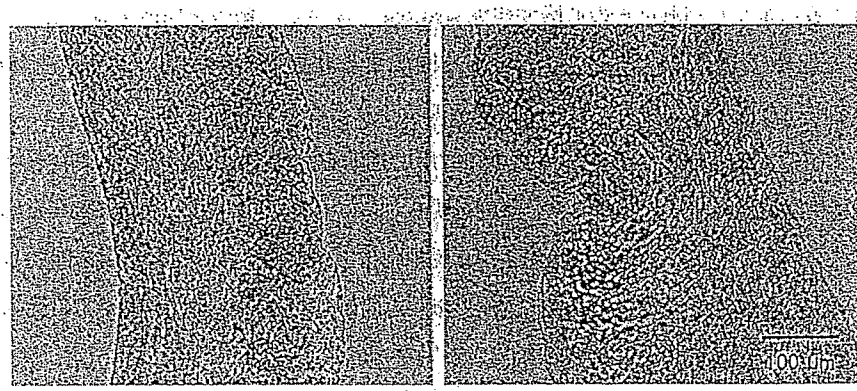
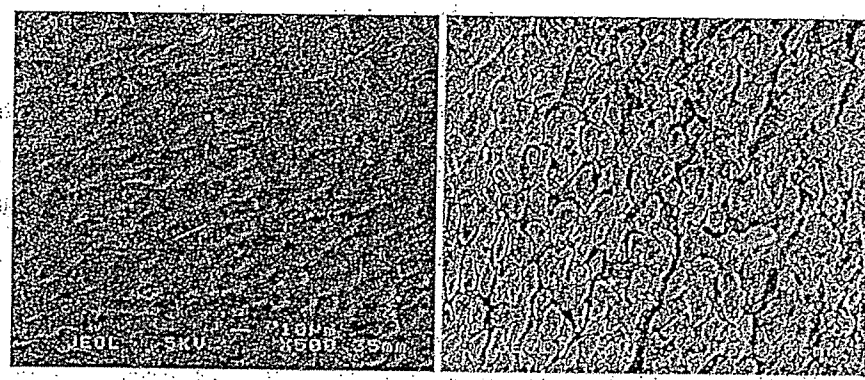


図1 Triton X-100によって脱細胞化された心臓弁葉の組織断面



未処理　　24時間の脱細胞化処理

図2 Triton X-100によって脱細胞化された心臓弁葉の表面

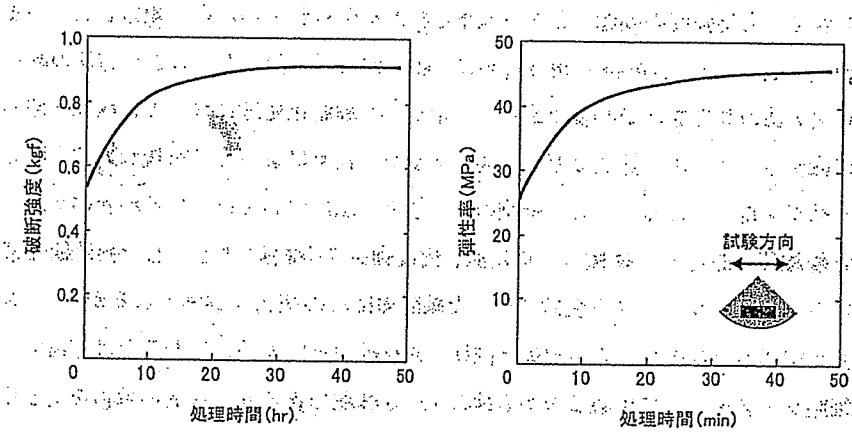
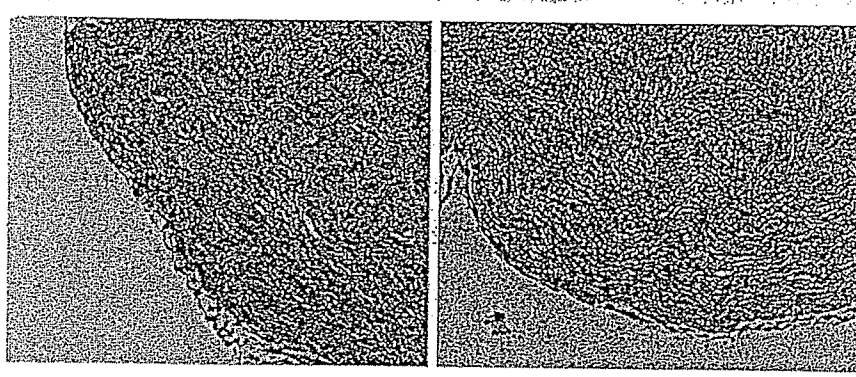


図3 Triton X-100によって脱細胞化された心臓弁葉の生体力学特性



自己細胞を播種した心臓弁　　細胞を播種しない心臓弁

図4 ミニブタに1ヶ月間移植された再生医療心臓弁

も変化は見られなかったため，置換術への影響はないと考えている（図3）。ミニブタ血管内皮細胞は分離も容易で，内皮細胞用培地で平易に増殖させることができ，静置下での培養で弁葉表面に内皮細胞を播種できた。ミニブタへの移植実験では術後1ヶ月においても良好な弁機能を示しており，自己内皮細胞を播種した再生医療弁では表面が完全に血管内皮細胞で覆われるとともに，組織内部への細胞浸潤も見られたのに対し，細胞を播種しないものでは，血管内皮細胞でほぼ覆われたものの，組織内への細胞浸潤はわずかであった（図4）。

4.5 問題点と将来展望

以上のように，現在，再生医療心臓弁のscaffoldには生体吸収性材料と脱細胞化生体組織とが研究されているが，現時点ではどちらが優れているかを見極めることは困難である。新岡らの再生医療心臓弁およびCryoLife社のSynerGraftともに，肺動脈弁では良好な結果が得られているが，大動脈弁では力学強度の問題などから満足な結果が得られていないと報告されている。大動脈位での血圧に耐えうるscaffoldを得るために，吸収性材料の材質および造形方法の改良，あるいは細胞除去方法の改良などが必要であろう。また，脱細胞化処理については組織深部の細胞除去，動物組織からのウイルス除去などが課題であるが，我々はまったく新規な方法を開発しており，有望な結果を得つつある。一方，細胞の組み込み方法については，いくつかのグループは平滑筋細胞と線維芽細胞を先に播種し，後に血管内皮細胞を（播種）することで複数種の細胞を組み込んでいる。バイオリアクター装置を用いた細胞播種法の報告が参考となるが[8]，弁葉部，弁葉基部，血管壁部のそれぞれに正常組織と同様に複数種の細胞を組み込むことは容易でないと思われる。細胞ソースをどこに求めるのかも検討すべき課題であるが，患者の負担をできるだけ下げるためには，骨髄細胞あるいは末梢血幹細胞などの利用が有効であろう。さらに臨床応用に際しては，GMP基準に適合した細胞プロセシング設備の設置も必要となる。

すでにいくつかの研究グループは臨床応用を始めつつある。いずれ，再生医療心臓弁が機械弁や異種生体弁に取って代わる日も近いと信ずる。

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Novel PVA–DNA nanoparticles prepared by ultra high pressure technology for gene delivery

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Abstract

Polyvinyl alcohol (PVA)–DNA nanoparticles have been developed by ultra high pressure (UHP) technology. Mixture solutions of DNA and PVA having various molecular weights (Mw) and degree of saponifications (DS) were treated under 10,000 atmospheres (981 MPa) condition at 40 °C for 10 min. Agarose gel electrophoresis and scanning electron microscope observation revealed that the PVA–DNA nanoparticles with average diameter of about 200 nm were formed. Using PVA of higher Mw and degree of saponifications, the amount of nanoparticles formed increased. The driving force of nanoparticle formation was the hydrogen bonding between DNA and PVA. In order to apply the PVA–DNA nanoparticles for gene delivery, the cytotoxicity and the cellular uptake of them were investigated using Raw264 cell lines. The cell viability was not influenced whether the presence of the PVA–DNA nanoparticles. Further, the nanoparticles internalized into cells were observed by fluorescent microscope. These results indicates that the PVA–DNA nanoparticles prepared by UHP technology showed be useful as drug carrier, especially for gene delivery.

Keywords: Ultra high pressure; Hydrogen bond; Nanoparticles; Biocompatibility; Gene delivery; Polyvinyl alcohol

1. Introduction

Pressure processing technology has been used in many fields. The range of pressure is varied in each method from 1 to 100,000 atmosphere (atm) (9810 MPa). In the field of chemistry and biology, the pressure of over 6000 atm is thought as ultra high pressure (UHP). It is well known that the hydrogen bond is strengthens than electrostatic and hydrophobic interactions under the UHP condition [1–3]. From this fact, we recently reported that UHP is one of powerful tools for manipulatory inter- or intra-molecular interaction triggered by hydrogen bond [4]. We have shown some evidence of this hypothesis by using polyvinyl alcohol (PVA), which is synthetic hydrogen bonding polymers having simple hydrogen bonding structure, associated each other to form nanoparticles via hydrogen bond by UHP processing [5]. Among various fields of application, we focused on the usage of the nanoparticle as drug and gene delivery system.

Nanoparticles as gene carrier are able to enhance intracellular gene delivery in vitro and in vivo due to protection of DNA from nuclease cleavage [6–10]. Many types of them, such as cationic compounds [6–8], biodegradable polymers

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[9,10] have been developed. Nanoparticles containing DNA have been formed by electrostatic interaction between negative charge of phosphate groups of DNA and positive charge of cationic compounds or encapsulation. However, it was reported that such cationic substances has the essential problem of the cytotoxicity, and the difficulty of controlling of DNA release from nanoparticles.

In the present study, we report the preparation of novel nanoparticles of plasmid DNA and PVA via hydrogen bond using UHP technology and their application for gene delivery. The interaction force of nanoparticle formation is hydrogen bond between PVA and DNA, because DNA is one of typical hydrogen bonding polymer as well as PVA. Further, the biocompatibility and neutral charge nature of PVA allows the low cytotoxicity. The cellular uptake of them was investigated in order to evaluate the nanoparticles as biocompatible gene carriers.

2. Materials and methods

2.1. Preparation of PVA–DNA nanoparticles by UHP method

PVAs having different molecular weights and degree of saponifications were supplied from Kuraray (Osaka, Japan) (Table 1). Plasmid DNA encoding green fluorescent protein under cytomegalovirus promoter (pEGFP-C1) was obtained from BD Biosciences Clontech (Tokyo, Japan). PVA solutions (0.0001–0.1 w/v%) and pEGFP-C1 solution (0.02 w/v%) were mixed in water and treated under 10,000 atm at 40 °C for 10 min (UHP method) using high-pressure machine (Dr. Chef, Kobe Steel, Kobe, Japan).

2.2. Characteristics of PVA–DNA nanoparticles

At 0.0001–0.01 w/v% of PVA concentration, PVA and pEGFP-C1 mixture solutions treated with UHP were analyzed by agarose gel electrophoresis (1.0 w/v%, 100 V, 1 h). At 0.025–0.1 w/v% of PVA concentration, after centrifugation of the UHP-treated mixture solutions at 5000 rpm for 5 min, the supernatant was collected and the precipitation was washed by water. This procedure was carried out twice. The precipitation was melted by heat treatment for 10 min. They were electrophoresed though 1.0 w/v% agarose gels at 100 V for 1 h. The gels were stained with ethidium bromide. The shape and size of structures were observed by scanning electron microscope (JSM-6301F, JEOL, Tokyo, Japan).

Table 1
Various polyvinyl alcohols used

PVA	DP[a]	DS[b]	Mw
PVA205	500	88	22,000
PVA105	500	98.5	22,000
PVA117	1700	99.3	74,800
PVA140	4000	99.8	176,000

[a] Degree of polymerization.
[b] Degree of saponification.

2.3. Cytotoxicity of PVA–DNA nanoparticles

Mouse macrophage cell lines of Raw264 cells were cultured in a complete modified eagle medium (DMEM, Invitrogen, Tokyo, Japan), supplemented with non-inactivated 10% fetal calf serum (FCS), 50 IU/ml of penicillin, 50 μg/ml of streptomycin (ICN Biomaterials, Ohio, USA). To evaluate the cytotoxicity of PVA–DNA nanoparticles, 2.0×10^4 cells incubated with PVA–DNA nanoparticles at 37 °C for 20 h in the present of FCS and the number of viable cells was assessed using a Cell Counting Kit-8 (Dojindo Laboratory, Tokyo, Japan) according to the manufacturer's instruction.

2.4. Cellular uptake of PVA–DNA nanoparticles

To investigate the cellular uptake of PVA–DNA nanoparticles, pEGFP-C1 labeled with rhodamine by Label It kit (Panvera, WI, USA) was added on 2.5×10^5 cells of Raw264 cells cultured in the present of non-inactivated FCS and incubated at 37 °C for 20 h. The cells were observed under fluorescent microscope.

3. Results and discussion

Fig. 1 shows the microscopic observation of the mixture solutions of pEGFP-C1 and various PVAs at 0.1 w/v% concentration treated with UHP after centrifugation at 5000 rpm for 5 min. The mixture solution of PVA205 remained as clear solution as well as pEGFP-C1. However, a little precipitation was observed in PVA105 and the white precipitation was observed in the case of PVA117 and PVA 140 (Fig. 1(A)). When DNA solution mixed with PVA117 at different concentration were pressurized under UHP condition, the amount of white precipitation was decreased with decreasing PVA concentration, and the precipitation was not observed at 0.01 w/v% of PVA117 (Fig. 1(B)). These results indicate that the size of particle obtained varied in each molecular weight and concentration of PVAs used, and that the higher molecular weights of PVA tended to form particles. This phenomena was observed even when the PVA solution without DNA was treated with UHP (data not shown). Fig. 2 shows SEM images of the UHP treated mixture solutions of DNA in the presence of (A) 0.01 w/v% or (B) 0.025% of PVA. Nanoparticles having average diameter of about 200 nm were observed in 0.01 w/v% of PVA concentration. At 0.025 w/v% concentration, the nanoparticles aggregated each other. It became clear that the precipitation formation at higher PVA concentration under UHP condition due to the aggregation of nanoparticles of PVA or PVA/DNA mixture.

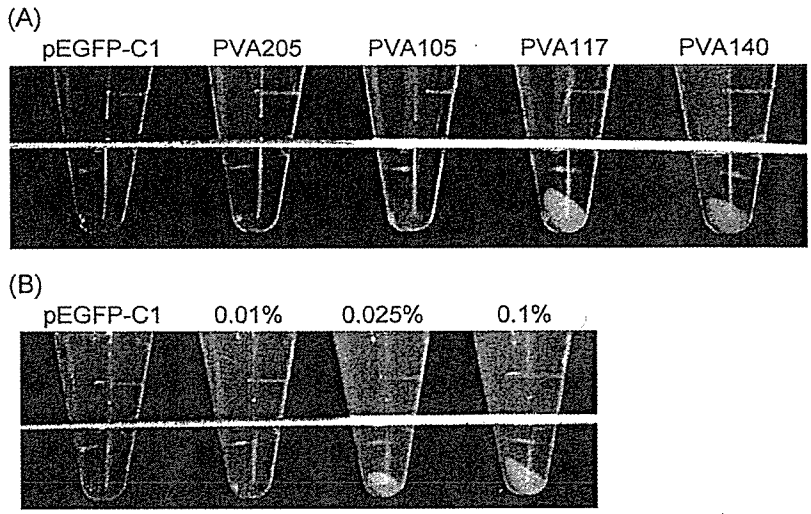

Fig. 1. Microphotographs of mixture solutions of DNA and (A) various PVAs of 0.1% concentration and (B) PVA117 of different concentration treated by UHP.

To confirm whether the nanoparticles contain DNA, the mixture solutions of DNA and PVA140 at less than 0.01% concentration treated with UHP were analyzed by agarose gel electrophoresis (Fig. 3(A)). The DNA bands in the non-treated mixture solutions were observed at the same pattern of pEGFP-C1, which contains circular, linear and super coiled form, irrespective of that concentration. On the other hand, the smear bands of DNA–PVA nanoparticles appeared at each concentration, indicating the nanoparticles consisting of DNA and PVA, but not PVA only. The heat melted aggregates of nanoparticles were

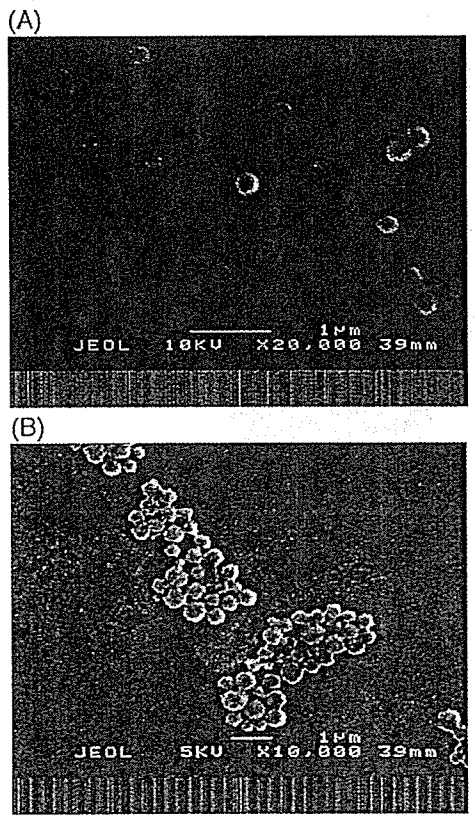

Fig. 2. SEM images of PVA–DNA nanoparticles. PVA117 was used.

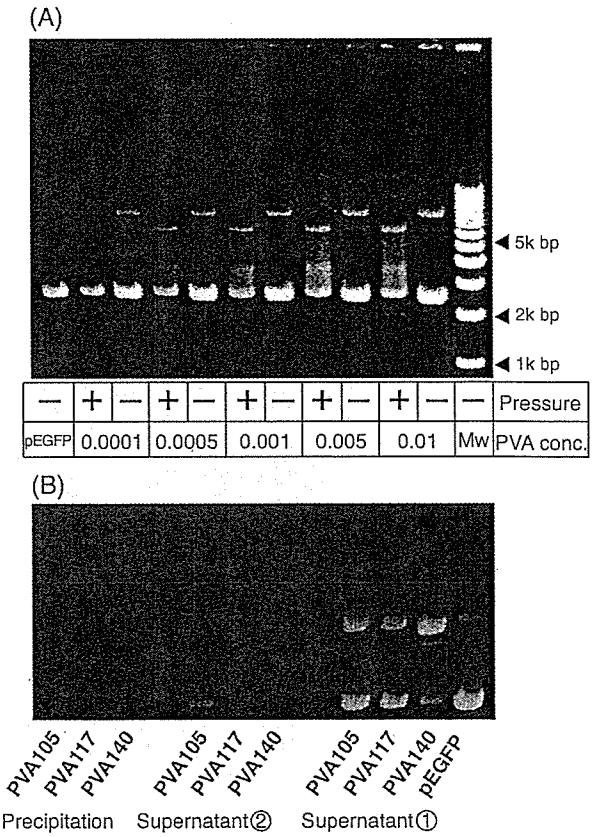

Fig. 3. Agarose gel electrophoresis of (A) PVA117-DNA nanoparticles prepared at 0.01% concentration and (B) the aggregates of PVA–DNA nanoparticles at 0.1% concentration after heat treatment.

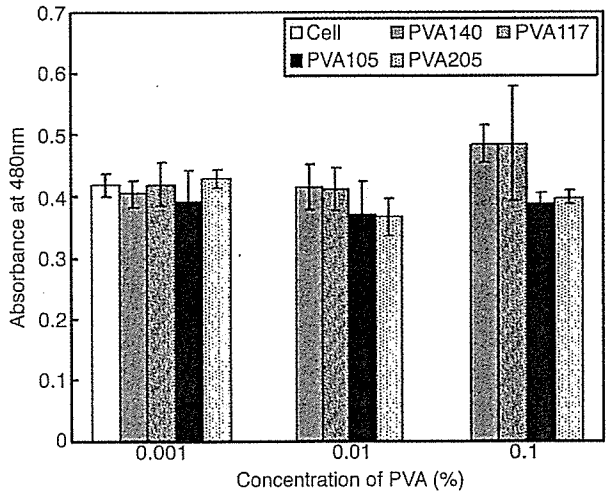

Fig. 4. Cytotoxicity of PVA–DNA nanoparticles.

also electrophoresed with agarose gel after twice washing procedure (Fig. 3(B)). The bands of DNA were observed not only in first and second supernatants but also in the collected precipitation. It was clear that the nanoparticles consisting of PVA and DNA.

Fig. 4 shows the result of the toxicity test of PVA–DNA nanoparticles. The result of high viability of Raw264 cells incubated with PVA–DNA nanoparticles was obtained irrespective of the molecular weights of PVA used. This result indicates that PVA–DNA nanoparticle is non-toxic. Conventionally, cationic polymers were widely used for gene delivery due to complex formation with DNA by electrostatic interaction, however, the cell damage for cationic nature of them was pointed out. Yamaoka et al. [11] reported that the cytotoxicity decreased with decreasing the charge density of polycations. Fischer et al. [12] suggests the necessity of optimizing the balance between the cytotoxicity and the biocompatibility of cationic polymers used as gene carrier. Therefore, it is considered that non-charged PVA permitted the low cytotoxicity of PVA–DNA nanoparticles formed by hydrogen bond.

In order to investigate cellular uptake of the PVA–DNA nanoparticles, the nanoparticles of PVA and pEGFP-C1 labeled with rhodamine were added to Raw264 cells in the present of FCS. In Fig. 5, a lot of red fluorescence spots

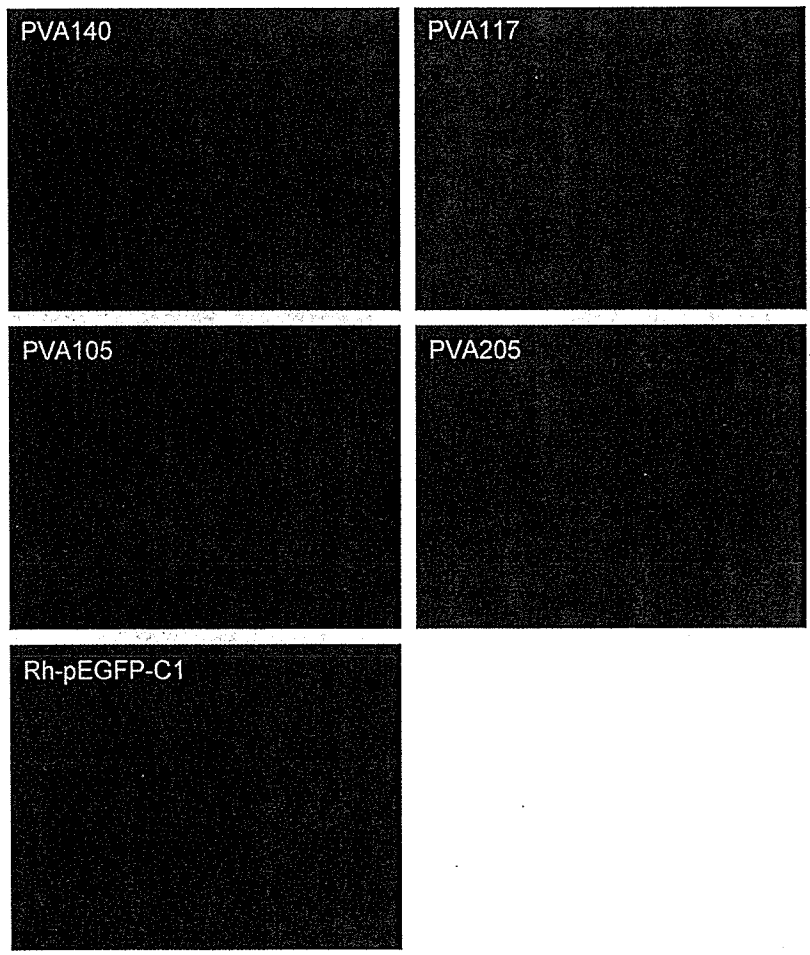

Fig. 5. Fluorescent images of Raw264 incubated with the nanoparticles of PVA and rhodamine-labeled pEGFP-C1 for 24 h.

in many cells were brightly observed in the case of PVA105, PVA117 and PVA140 except for pEGFP-C1 and PVA205. This result suggests that the significant internalization of DNA which means that the PVA–DNA nanoparticle was incorporated into cells. In the case of PVA205, as PVA–DNA particles formation was insufficient, low incorporation result was obtained. These results suggest that PVA–DNA nanoparticles have favorable characteristics for gene delivery system, are non-cytotoxic and high gene transfer into cell. The uptake of PVA–DNA nanoparticles by cells is probably achieved by complement activation because it is well-known fact that PVA activates complement system.

4. Conclusion

We have developed nanoparticles consisting DNA and PVA via hydrogen bonds using UHP technology. The average nanoparticle diameter was 200 nm. The nanoparticle formation could be controlled by the molecular weight of PVA used. Cell viability studies following incubation with the nanoparticles confirmed the lack of toxicity of PVA. The ability of the nanoparticles to delivery DNA into cells was also shown, and PVA–DNA nanoparticles are considered as a potential candidate for a gene carrier.

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Immunological and Histological Evaluation of Decellularized Allograft in a Pig Model: Comparison with Cryopreserved Allograft

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Background and aim of the study: The remodeling process of the decellularized allograft after implantation remains unclear. Herein, the hemodynamics, recellularization and immunological response of the decellularized allograft were evaluated at four weeks after implantation in a mini-pig model, and compared with a cryopreserved allograft.
Methods: Six porcine pulmonary allografts were harvested from mini-pigs, and cryopreserved for four weeks. In two pigs, the grafts were decellularized with Triton X solution, after which static reseeding of the valve surface was performed for 48 h with autologous endothelial cells harvested from a leg artery. Decellularization, but not reseeding, was carried out in two mini-pigs, and cryopreservation alone in two mini-pigs. Whilst under right heart bypass, the right ventricular outflow tract was replaced in six mini-pigs. The grafts were explanted after four weeks; analysis included direct pressure measurement, echocardiography, macroscopy, light microscopy with hematoxylin and eosin staining, and immunohistochemical studies to identify macrophages, T lymphocytes, and endothelial cells.
Results: Hemodynamically and macroscopically, there were no major differences between the three groups. In the cryopreservation-only group, immunohistochemistry showed an influx of macrophages, and T lymphocytes at the cusps. Endothelial cell coverage was found in the decellularized and decellularized + cell-seeded groups, but no macrophages and T lymphocytes were found at the cusps.
Conclusion: Decellularization of the cryopreserved allograft may reduce the inflammatory response and improve its long-term durability.

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Cryopreserved homografts have been shown to have superior hemodynamics and anti-infection potential, but their availability is limited by a shortage of donors. Furthermore, none of these valves is able to grow with the patient, and therefore they exhibit a limited durability over the longer term. This limitation would appear to be caused by the immune response against the donor cells, or by apoptosis of the donor cells. In consideration of these facts, the ideal alternative prosthetic valve should be made from autologous tissue.

Among recent reports of the tissue engineering of valves, two types of valve have been identified, namely autologous cells seeded onto a scaffold made from a biodegradable polymer (1-3), and decellularized allografts or xenografts (4-7). Transplantation of a tissue-engineered pulmonary artery constructed from a biodegradable polymer has been successfully carried out in humans (8). In this situation, the biodegradable polymer is absorbed, so that eventually the graft would be composed completely of autologous tissue and would have potential for growth. However, it is very difficult to construct an ideally shaped scaffold with biodegradable tissue. Decellularized allografts or xenografts have an ideal shape and physiological hemodynamics, and have already been marketed as SynerGraft® and CryovalveSG® (4,5). A recent experimental study reported good recellularization and hemodynamics after implantation in the pulmonary position (7). After implantation, an influx of the recipient's cells has been demonstrated at the decellularized valve. Over the longer term, the decellularized valve will eventually be reconstructed with abundant autologous cells. Furthermore, the decellularized valve only

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consisted of a small amount of donor cells. It is also expected that the immunological responses may be reduced after implantation. These factors may improve the subsequent durability of the graft. In the present study, the function and recellularization process of a decellularized allograft was evaluated in a mini-pig model, and compared with that in a cryopreserved allograft.

Materials and methods

Cryopreservation

Six pulmonary allografts from NIBS mini-pigs (body weight 25-30 kg) were obtained from Nihonnasan, Kanagawa. While under general anesthesia, the chest of each pig was opened though a median sternotomy and the heart exposed. At 1 min after intravenous infusion of heparin, potassium chloride (1 mEq/kg) was infused to evoke cardiac arrest. After euthanasia, the heart was excised and the main pulmonary artery divided at the bifurcation. The right ventricular outflow tract was then incised and the pulmonary autograft separated from the right ventricle. After harvesting, the grafts were placed in 10% dimethyl sulfoxide (DMSO; Fisher Scientific Co., Pittsburgh, PA, USA). The graft was then cryopreserved with a computer-controlled programmable freezer (Cryomed Program Controller; Forma Scientific Co., Marietta, OH, USA) that lowered the temperature to -90°C. The graft was then placed in the vapor phase of a liquid nitrogen freezer (-196°C). After four weeks of storage, the grafts were thawed in a 37°C shaking water bath, and then washed twice with lactate Ringer's solution.

Decellularization

Four of the six harvested grafts were decellularized. After thawing, the grafts were immediately stored in Hank's balanced solution (HBSS; Biochrom, Berlin, Germany), and maintained at 4°C. The graft was then placed in a solution of 1% tetra-octylphenyl-polyoxyethylene (Triton X; BioRad, Germany) with 0.02% EDTA in phosphate-buffered saline (PBS; Bichrom, Germany), for 24 h, with RNase (20 μg/ml; Boehringer, Mannheim, Germany) and DNase (0.2 mg/ml). The graft was washed several times with PBS and then stored in a 5% CO2 incubator, at 37°C, with continuous shaking.

Cell harvesting and seeding

Autologous endothelial cells were seeded onto two of the four decellularized grafts before implantation. At 21 days before implantation, the femoral artery was harvested from a recipient pig under local anesthesia. A 23 Fr cannula was inserted into each side of the artery. EBM2 (Clonetics, MD, USA) was injected, and both cannulas were affixed to a Petri dish filled with HBSS and incubated in a 5% CO2 incubator, at 37°C for 20 min. The artery was washed through with 50 ml 10% fetal bovine serum (FBS; Life Technologies, Germany). Endothelial cells were then pelleted by centrifugation for 5 min at 3,000 rpm (190 × *g*). The endothelial cells were subsequently resuspended in 5 ml of culture medium containing endothelial cell growth factor. During endothelial cell culture, the medium was changed every three days. After 14 days of cell expansion, the cells were detached with 0.05% trypsin (Biochrom) and 0.02% EDTA (Sigma) in PBS. Following resuspension of the endothelial cells in culture medium (EBM2), a 20 ml volume of culture medium, containing ~5×10^6 endothelial cells, was placed into a centrifuge tube. The decellularized valve was immersed in this tube and, for 48 h, static reseeding of the valve surface was performed in a 5% CO2 incubator, maintained at 37°C. Following this procedure, the valve was removed from the tube and soaked in EBM2 for 48 h.

Surgical technique

Pulmonary allografts were implanted into six NIBS mini-pigs (Nihonnasan, Kanagawa). Recipient pigs (n = 6) of similar body weight were selected. First, ketamine hydrochloride (4 mg/kg) and atropine sulfate (0.04 mg/kg) were injected intramuscularly, and a tracheal tube (6.5 Fr) was inserted. Intraoperative anesthesia was maintained with nitrous oxide, isoflurane, and muscle relaxant. The operation was performed through a median sternotomy. Bicaval and main pulmonary artery cannulations were established. Caval tapes were placed and extracorporeal circulation was initiated, without blood oxygenation. The main pulmonary artery was clamped proximal to the cannulation site. The main pulmonary trunk and pulmonary valve cusps were removed from the recipient pig. The pulmonary allograft was placed using running sutures with 5-0 polypropylene. After weaning from the extracorporeal circulation, the chest was closed. The animals were extubated in the operating theater. Postoperative anticoagulation or anti-platelet therapy was not instigated.

The animals were treated in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institute of Health (NIH publication No.85-23, revised in 1985).

Postoperative evaluation

The animals were euthanized at four weeks after implantation. During the euthanasia procedure, animals underwent a general anesthesia and the femoral arterial pressure was monitored. Following tracheal intubation, the fourth intercostal space was opened

and the heart exposed with the animal in the left recumbent position. Direct echocardiography and direct pressure measurements were performed. Heparin (1000 units/kg) was subsequently infused intravenously. At 1 min after the heparin infusion, potassium chloride (1 mEq/kg) was infused and cardiac arrest evoked. After sacrifice of the animal, the graft was excised and prepared for histology and immunohistochemistry.

Macroscopic findings

Immediately after euthanasia, the pulmonary graft was removed and opened transversely. The primary criterion for evaluation related to the presence or absence of pulmonary thrombus, and was categorized as: no thrombus at any of the cusps (0); one cusp with a thrombus (I); two cusps with thrombi (II); and three cusps with thrombi (III).

Echocardiography

Echocardiographic examination was performed using Power Version SSA-380A (Toshiba) with a 3-MHz probe positioned epicardially via a left thoracotomy. The criteria for the evaluation was pulmonary valve regurgitation, which was categorized as either none (0), mild (I), moderate (II), or severe (III).

Direct pressure measurement

Direct pressure measurement was performed though a left thoracotomy. The pressure was measured by puncturing with a 23 Fr needle at the right ventricle, distal pulmonary artery, left atrium, left ventricle and descending aorta.

Histology

The specimens were fixed by immersion with 2.5% glutaraldehyde in cacodylate buffer, pH 7.4, and post-fixed with 2% osmium tetroxide in the same buffer. The specimens were dehydrated in graded alcohol, embedded in epoxy resin (Serva), and stained with standard hematoxylin and eosin (H&E). One criterion for evaluation was recellularization of the valve surface, and this was categorized as: no apparent cells (0); a few cells (I); patchy cell lining (II); and confluent cell lining (III). Additionally, recellularization of the valve interstitium was evaluated, and categorized as: no apparent cells (0); patchy cell proliferation (I); focal cell proliferation (II); and complete recellularization (III).

Immunohistochemistry

Endothelial cells were characterized by the presence of factor VIII (von Willebrand factor)-related antigen (Zymed, California, USA). The criterion for evaluation was recellularization of the valve surface, which was categorized as: no apparent positive cells (0); a few positive cells (I); patchy positive cell lining (II); and confluent positive cell lining (III). Monoclonal antibodies (KP-1, UCHL-1; Dako, Hamburg, Germany) were used for the detection of infiltrating inflammatory cells. Monocytes/macrophages and T-lymphocytes were also detected by CD68 (Dako) and CD3 (Dako), respectively. The criterion for evaluation was infiltration of the inflammatory cells into the cusp, which was categorized as: no positive cells (0); a few positive cells (I); proliferated positive cells (II); and multi-layered positive cells (III).

Results

Postoperative course

All pigs survived the operation, and the body weight of each animal was not significantly changed. The mean preoperative body weight was 31.5 ± 4.5 kg (range: 26 to 38 kg), and at one month after implantation was 31.7 ± 5.6 kg (range: 26 to 38 kg). Operation durations and extracorporeal circulation times are listed in Table I.

Table I: Operative data and results of direct pressure measurement, echocardiography and macroscopic findings.

Pig no.	Cryo.	Decell.	Seed.	Time (min)		Echo PR	Pressure measurement*			Macroscopic findings
				Operation	ECC		RVP	Distal PAP	PG	
1	+	+	+	110	30	0	44/3 (21)	32/12 (20)	12	I
2	+	+	+	160	34	0	37/2 (20)	37/12 (22)	0	0
3	+	+	-	135	40	0	25/0 (13)	21/14 (19)	4	I
4	+	+	-	150	33	0	31/8 (18)	22/14 (18)	11	II
5	+	-	-	167	37	0	36/4 (14)	31/13 (15)	5	I
6	+	-	-	160	35	0	35/5 (13)	29/10 (14)	6	I

*Values are systolic/diastolic pressures; value in parentheses is mean pressure.
Cryo.: Cryopreservation; Decell.: Decellularization; ECC: Extracorporeal circulation; PAP: Pulmonary artery pressure; PG: Pressure gradient; PR: Pulmonary valve regurgitation; RVP: Right ventricular pressure; Seed.: Autologous cell-seeded.

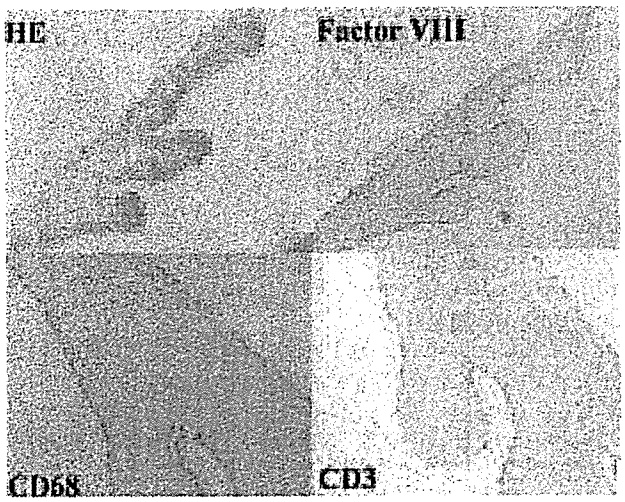

Figure 1: Histological and immunohistochemical findings of pig #3 (cryopreserved and decellularized graft; not cell-seeded). The cusp was covered with confluent endothelial cell lining. These cells were positive for von Willebrand factor, as a marker of endothelial cells. At the valve interstitium there were no cell components. Immunohistochemistry indicated an absence of positive cells on both the valve surface and the interstitium. HE: Hematoxylin and eosin staining.

Macroscopic findings

The gross morphology of the explanted valves was examined. Macroscopic thrombi were found in six pigs (Table I). All thrombi were red in color and fresh, and located at the bottom of the cusps. No relationship was observed between thrombogenesis and previous cell seeding. The cusps of the cryopreserved group were relatively thickened.

Echocardiography

No pulmonary valve regurgitation was observed for any of the implanted allografts. However, a small thrombus echo was present (Table I).

Direct pressure measurement

No significant pressure gradient was observed across the graft (mean 4.8 ± 5.6 mmHg; range: 0 to 11 mmHg) (Table I).

Histology

Histological examination of both the cell-seeded group and the non-cell-seeded group, with H&E staining, revealed a patchy-confluent monolayer of cells lining the cusps (Table II; Fig. 1). However, the interstitial valve tissue component cells were not repopulated in either group (Table II; Fig. 1). In one of two pigs with only-cryopreserved allograft valves (pig #6), near-complete depletion of cells was observed at the surface, and the trilaminar tissue architecture of the valves disappeared. No cell lining was observed at the valve surface. There were two to three regions of focal spindle or round cell proliferation in each cusp (Table II; Fig. 2). Few cells were observed at the valve interstitium. In the other pig in this group (pig #5), the cells were localized predominantly at the cusp surface with a multi-layer cell lining (Table II; Fig. 3). The tissue architecture was relatively preserved, and spindle or round cells were present at the interstitium.

Immunohistochemistry

In both the cell-seeded and non-cell-seeded groups, a patchy-confluent cell lining was observed at the surface of the cusps. These cells were positive for von Willebrand factor, as a marker of endothelial cells (Table II; Fig. 1). In the cryopreserved-only group, no positive cells were found at the valve surface.

Clear infiltration of inflammatory cells into the cusp of the cryopreserved-only valve was demonstrated. In one of two pigs (#6), CD68-positive cells were observed focally at the interstitium of the cusps, and CD3-positive cells were observed around these cells (Table II; Fig. 2). In the other pig that had multi-layer cell lining on the cusp, CD68-positive cells were observed at the multi-layer cells, and CD3-positive

Table II: Histological evaluation of the pigs.

Pig no.	H&E		Factor VIII	CD68	CD3
	Surface	Interstitium			
1	II	0	II	0	0
2	III	0	III	I	0
3	III	0	III	0	0
4	III	0	III	I	0
5	III	III	I	III	III
6	I	II	I	II	II

CD3: Immunohistochemical staining with anti-CD3 antibody; CD68: Immunohistochemical staining with anti-CD68 antibody; Factor VIII: Immunohistochemical staining with anti-Factor VIII antibody; H&E: hematoxylin and eosin staining.