胞層に覆われていることと，血管壁深部までの細胞化を確認している[27]．彼らのグループでは，脱細胞化ブタ肺動脈弁のヒツジへの異種移植で，石灰化することなく再細胞化し，レシピエントの成長に伴って血管径も増すことを証明している[28,29]．また，内皮細胞播種の必要性についても検討を行っており，移植に先立つ *in vitro* での内皮化は必要ないとの結論を示している[30]．現時点でヒトへの適用は右室流出路再建に限られているが，同種，異種ともに移植組織の成長を窺わせる素晴らしい研究である．

Haverich A(ハノーバー大学，ドイツ)らのグループも，脱細胞化心臓弁のヒトへの移植を行っている．肺動脈弁に先天的疾患を持つ小児患者2例(13歳と11歳)に対して，自己末梢血より単離した単核球細胞を播種した脱細胞化ヒト肺動脈弁を移植し，移植3年後の成績を報告している[31]．小児患者の成長に追従した移植組織の成長が確認されていることは，非常に興味深い．この臨床試験に先立ち，基礎研究や前臨床試験についての結果も多数報告されており[32~35]，今後の研究成果も大いに期待される．

心臓弁再生を目的としたバイオスキャフォールドでは，Fisher J と Ingham E(リーズ大学，イギリス)らのグループも前臨床試験で良好な成績を報告しており[36~38]，Simon P(ヴィエナ医大，オーストリア)らのグループの研究成果も多数報告されている[39~41]．心臓弁は形状が複雑なうえ，絶えず動的な血流負荷に抗しなければならないため，組織再生にはスキャフォールドが必要不可欠である．心臓弁再生にバイオスキャフォールドを利用する研究はほかにも多数見受けられる[26,42]．

Badylack SF(ピッツバーグ大学，アメリカ)らのグループは，脱細胞化したブタ由来小腸粘膜下組織や膀胱組織に関して精力的に研究を行っている[43~45]．先述のHaverichらの研究はレシピエント細胞を播種したテーラーメイド型であるのに対して，Badylackらの研究は汎用的な利用を目的として，異所組織への応用，保存，滅菌方法に関する検討[46,47]などが多い．今年2月にフェニックス(アメリカ)で開催された"Biologic Scaffolds for Regenerative Medicine, 5th Symposium"では，全48件の研究発表中，8件が彼らのグループによるものであった．また，同氏はバイオスキャフォールドに関する執筆も多い[48~50]．

わが国のバイオスキャフォールドに関する研究では，澤(大阪大学)らのグループがすぐれた成果を多数報告している[51,52]．生体吸収性ポリマーを用いた再生型人工血管は，ポリマーの分解吸収に伴う強度低下により左心系への適用が懸念されてきたが，同グループはバイオスキャフォールドを左心系へ移植しており，移植後6カ月での良好な成績を報告している[53]．ブタ組織から作製したバイオスキャフォールドをイヌに対して移植しており，異種移植で成果を上げている点でも興味深い．

また，梅津(早稲田大学)らのグループもバイオスキャフォールドを用いた心臓弁再生に取り組んでいる[54]．

バイオスキャフォールドの作製方法

臨床で用いられているブタ由来の生体弁は脱細胞化されておらず，通常，ブタ細胞の抗原性を抑制するためにグルタールアルデヒド(GA)で固定化処理が施されている．しかしながら，GA処理によって抗原性を抑えられる一方で，生体弁が体内で分解吸収されることもないため，自己弁の再生を期待することは不可能である．

バイオスキャフォールドは，それ自体の分解吸収性を損なうことなく，ドナー細胞の抗原性を除去しなければならない．そのため，界面活性剤を用いて組織内部からドナー細胞を洗い流すのが一般的である．SDS(sodium dodecylsulfate)，Triton X-100 あるいはSD(sodium deoxycholate)といった界面活性剤を用いた脱細胞化処理が主流であり，トリプシンやDNase，RNase などの酵素と併用する場合もある．各種の脱細胞化法の効果を比較検討する研究も多い[55]．

Haverichらによると，酵素法は基底膜に損傷を与え，組織の引張り特性を低下させるが，界面活性剤はそれらをよく保存する[56]．また，Taylor DAらは，ラット心臓の冠状動脈に SDS を灌流することにより，PEG(polyethylene glycol)，Triton X-100あるいは酵素法よりもすぐれた脱細胞化効果を得られることを示している[57]．一方，Simon Pらは，SDS はマト

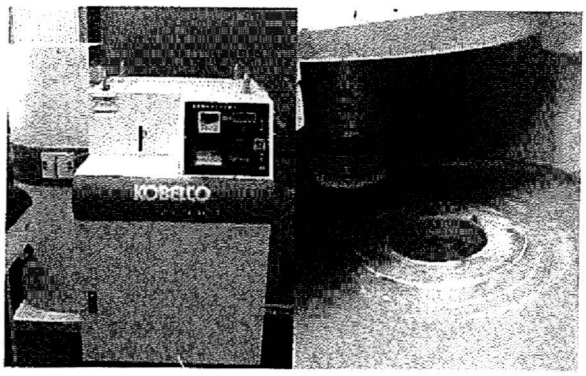

図5　冷間等方圧印加装置(神戸製鋼所製 Dr.CHEF)

図6　凍結保存ヒト心臓弁組織

リクス構造を変性させるという理由から，Triton X-100 と SD がよいと結論付けている[58]．

界面活性剤は対象組織によって脱細胞化効果が異なることが知られている．また，研究者によって処理条件や評価方法は異なる．脱細胞化度の評価は，一般的に組織染色とDNA定量によってなされるが，今後，臨床での安全性を確保するための統一的な評価項目，評価方法，評価基準の確立が必要であり，ISO への提案も検討されている．

筆者らの研究

筆者らのグループでは，界面活性剤を用いない脱細胞化法として，超高静水圧印加法を提案してきた(図5)．対象組織に静水圧(980 MPa＝約10,000 atm)を印加してドナー細胞を破壊したのち，界面活性剤を含まない洗浄液で細胞残渣を洗い流す方法である．

図7　移植6カ月後の肺動脈弁バイオスキャフォールド

超高圧を印加することでレトロウイルスを不賦化でき，ドナー組織に感染している細菌類を死滅できる効果もあり，バイオスキャフォールドの安全性を高める技術として研究を行ってきた．超高圧印加処理を施したブタ組織からは，細菌とPERVが検出されなくなることを確認している．

本人あるいは家族の同意により死体から提供されるホモグラフト(移植用ヒト組織)には，摘出時の細菌検査で感染が認められ，ヒトへの移植に供されずに凍結保存処置される組織が少なからずある．そのような感染ヒト組織(国立循環器病センター組織保存バンクより提供)に対しても，超高静水圧印加法による滅菌効果と脱細胞化効果が得られることを確認している(図6)．貴重な提供組織を有効に利用できる技術として研究を進めている．

1．心臓弁バイオスキャフォールド

超高圧印加法によってミニブタ肺動脈弁組織から作製したバイオスキャフォールドを，同種ミニブタの肺動脈位へ置換移植して6カ月後の様子を図7に示す．肉眼所見で異常は認められず，ネイティブ組織と同様の平滑な内腔面を有している様子がわかる．また，組織染色像において，バイオスキャフォールド内に無数のレシピエント細胞が浸潤している様子と，内腔表面が内皮細胞に覆われている様子を確認

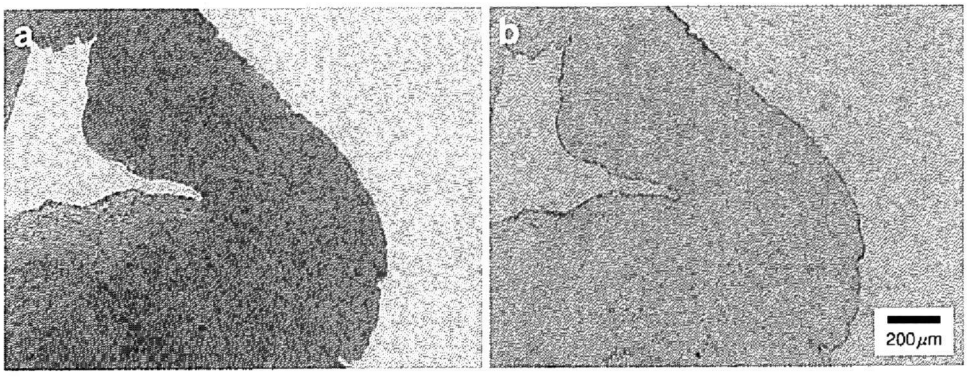

図8　肺動脈弁バイオスキャフォールドの移植6カ月後の組織染色写真
a：HE染色，b：vWF染色

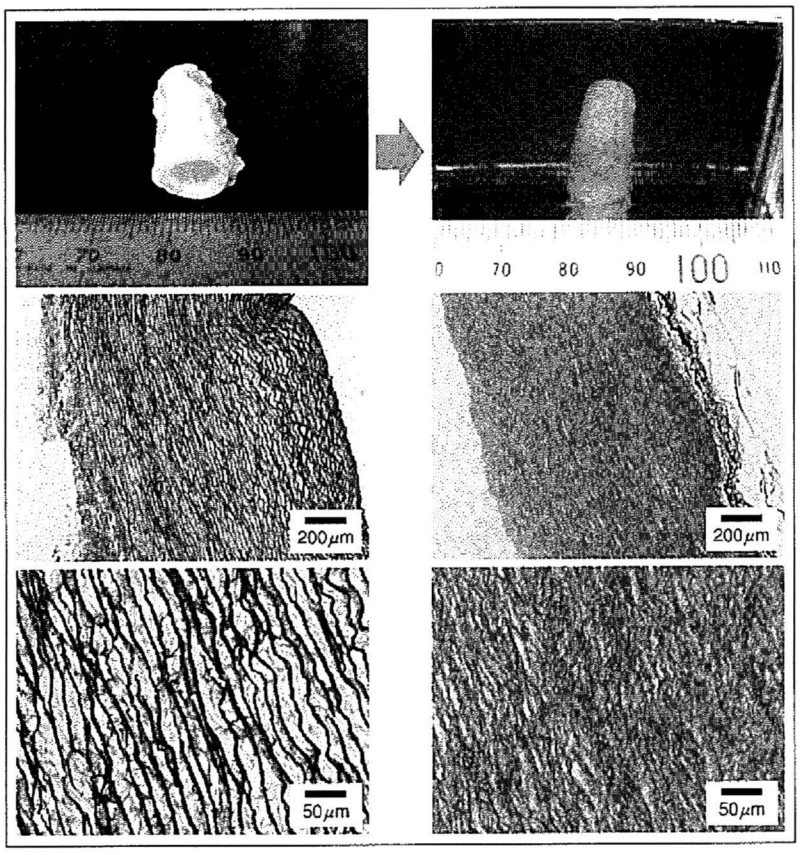

図9
ミニブタ動脈組織から作製した
バイオスキャフォールド
酵素法によって動脈組織から弾性線維を分解除去し，コラーゲン線維のみからなるバイオスキャフォールドを作製した．EVG染色によって弾性線維の消失を確認することが出来る．

することが出来る(図8)．さらに，移植後12カ月の時点において，エコー検査によって正常な弁機能を確認している．

筆者らの心臓弁バイオスキャフォールドは，肺動脈弁置換で非常に良好な成績を得ることが出来るが，大動脈弁位では石灰化を呈するレシピエント固体がある．臨床でバイオスキャフォールド心臓弁の移植を行っているKonertzやHaverichらのグループにおいても，肺動脈弁置換に限られており，大動脈弁置換に関する研究は報告されていない．大動脈弁置換では，おそらく筆者らと同様の問題に直面しているのではないだろうか．右心系と左心系とでは，血流の成分や血圧など，バイオスキャフォールドの晒される環境にいくつかの違いがある．現在，国立循環器病センターと共同で改善方法を検討中である．

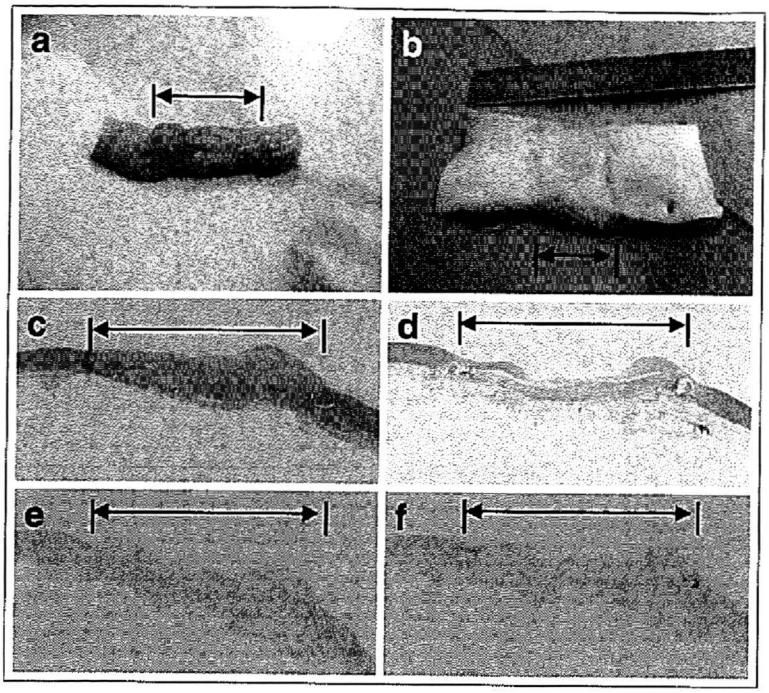

図10
移植3カ月後に取り出したバイオスキャフォールドの外観と組織染色写真
a：外植組織の外観
b：血管内腔面
c：HE染色
d：α-SMA染色
e：CD3染色
f：Kossa染色

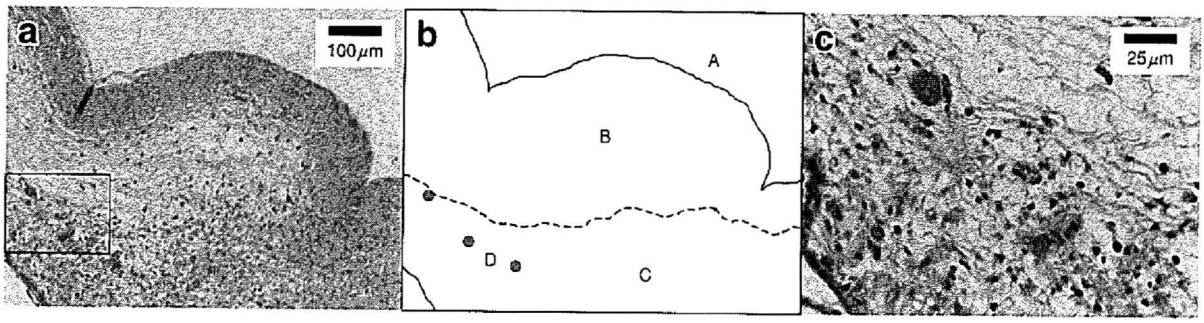

図11　動脈バイオスキャフォールドの移植1カ月後のHE染色写真
a：血管軸方向断面
b：a解説図．A：血管内腔面，B：細胞が達していない領域，C：再細胞化した領域，D：微小血管
c：a中枠内の強拡大写真

2. 角膜バイオスキャフォールド

角膜実質はコラーゲン線維が格子状に配列した比較的堅固な組織である．長時間の界面活性剤処理はコラーゲン線維の配向を乱し，組織自体を溶解してしまうため，通常の界面活性剤法による脱細胞化は難しい．しかし，超高静水圧印加法を適用することによって，線維配向を乱すことなく脱細胞化を達成する技術を開発した．東京医科歯科大学で，脱細胞化ブタ角膜のラビット角膜位への置換移植実験を行った結果，拒絶反応はなく，透明な再生角膜を獲得できるという結果を得ている．

3. 動脈バイオスキャフォールド

さらに筆者らは，バイオスキャフォールドの新たな作製方法についても検討を行ってきた．弾性線維の豊富な動脈組織から，ドナー由来細胞のみならず，酵素法によって弾性線維をも除去することによって，コラーゲンのみからなるバイオスキャフォールドを作製することが出来る(図9)．熱架橋によってコラーゲンの力学強度を向上させることで，動脈位移植に必要な強度と縫糸耐性を付与している．動脈組織の大部分を占める弾性線維を分解除去したことで，非常にポーラスな構造体が得られており，そのこと

が移植後早期の再細胞化を可能にしている．

同方法によってミニブタ動脈組織から動脈再生用バイオスキャフォールドを作製し，同種ミニブタ大動脈位への置換移植を行った．移植3カ月後組織の外観と組織染色写真を図10に示した．スキャフォールド内は無数の平滑筋細胞で再細胞化されている様子が確認できる．

別法で作製した動脈バイオスキャフォールドをミニブタ動脈位へ移植し，1カ月後に外植したものの組織染色写真を図11に示した．レシピエント細胞の迅速な浸潤と並行して，再細胞化領域に血管が新生されている様子を見ることが出来る．バイオスキャフォールド内のドナー血管跡が再血管化されたのか，あるいはレシピエント血管が新生されたのかは，現時点では確認できていない．しかしながら，血管跡や神経跡が細胞へのシグナルとなって再組織化を促進する可能性も指摘されている．血管網を有する組織を*in vitro*で作製するために，バイオスキャフォールド内に残存する血管跡を積極的に利用することは出来ないだろうか．

先述のとおり，バイオスキャフォールドの問題点の一つとして，石灰化があげられる．この動脈バイオスキャフォールドは"石灰化の可能性因子を可能なかぎり除去する"という発想で開発を行い，最終的に多孔質コラーゲン構造体のみが残ったものである．この多孔性は，結果的に細胞浸潤を加速し，移植後早期の再細胞化を可能にした．大動物への移植実験により，効果的に石灰化を抑制できることも確認している．

しかしながら，少数ではあるが偶発的に不全を呈する場合がある．現在のところ，これらの不全が動脈バイオスキャフォールド自体に起因するものなのか，ドナー組織に内在するものなのか，あるいはレシピエント固体の性質によるものなのかは解明できていない．異種動物由来のコラーゲン自体がレシピエント免疫細胞を引き付けるとする研究[59]や，石灰化を生じるかどうかは異種によるとする研究が[60]あるため，今後の異種移植実験へ向けて改良していかなければならない．

おわりに

世界の研究動向で述べたとおり，ドイツではバイオスキャフォールドをヒトへ移植して，レシピエントの成長に追従した移植弁の成長が報告されている．また，日本や欧米の大学でも前臨床試験が盛んに行われている．それにもかかわらず，国内外の再生医療，組織工学に関する書籍にバイオスキャフォールドに関する詳しい記述は皆無である．このような現状の背景には，バイオスキャフォールドには生体由来ゆえの未解明要素や不確定要素が含まれており，統一的な見解や方法論が確立していないという事情があるように思われる．

しかしながら，適切なバイオスキャフォールドによって，組織再生を促進する適切な足場を提供できることは確かである．今後，バイオスキャフォールドを利用した再生医療の研究が増えることを期待したい．また，再生医療に関する現行の法規制のなかにあって，バイオスキャフォールドは最も臨床応用に近い位置にあるマテリアルの一つである．バイオスキャフォールドのポテンシャルをいち早く臨床の現場で発揮させられるよう，筆者らも努力をつづけていきたい．

本稿の当初の目的は，筆者らの研究成果をアピールすることであったが，バイオスキャフォールドというマテリアル自体の潜在能力をアピールする機会と捉えなおし，そのことに紙面の多くを割いてしまったことをお詫びしなければならない．今後，筆者らの研究グループをはじめ，国内外のバイオスキャフォールド研究の成果にご期待いただければ幸甚である．

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Controlling coupling reaction of EDC and NHS for preparation of collagen gels using ethanol/water co-solvents

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To control the cross-linking rate of the collagen gel, ethanol/water co-solvent was adopted for the reaction solvent for the collagen microfibril cross-linking. Collagen gel was prepared by using 1-ethyl-3-(3-dimethyl aminopropyl)-1-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) as coupling agents. Ethanol did not denature the helical structure of the collagen and showed the prevention of the hydrolysis of EDC, but showed the protonation of carboxylate anions. In order to control the intra- and interhelical cross-link of the collagen triple helix, mole ratio of carboxyl group:EDC:NHS, and the ethanol mole concentration was altered. Increase in the EDC ratio against the carboxyl group increased the cross-linking rate. Furthermore, the increase in the ethanol mole concentration against the water brought increase in the cross-linking rate until ethanol mole concentration is 0.12 (ethanol concentration 30%, v/v), but showed gradual decrease as the ethanol mole concentration increased further. This is because the adsorption of solvent by the collagen gel, protonation of carboxylate anion, and hydrolysis of EDC is at its most optimum condition for the coupling reaction when the ethanol mole concentration is 0.12.

Keyword collagen, cross-linking, ethanol,

Based on the fact that an ECM mainly composed of collagen and elastin, many researchers have attempted to prepare a collagen- or elastin-based material to construct an ECM. However, the critical aspect in using collagen gel is that its mechanical strength is too small and easily deforms its triple-helix structure into a random coil structure when heated. The low mechanical strength and easy deformability make collagen shrink easily due to external stimuli. These aspects make it difficult to use collagen as an ECM. The use of cross-linkers to overcome these problems was investigated and is well reviewed by Khor.[1] By cross-linking collagen triple-helices, it is possible to maintain its mechanical strength and suppress any deformation caused by external stimuli. However, it is very important to consider biological responses in the designing stage of a cross-linking process because of the possibilities of severe problems such as toxicity, inflammatory response or the alteration of protein structure.

A intrahelical cross-linking method using 1-ethyl-3-(3-dimethylaminopropyl)-1-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) in aqueous condition is a one of the best method to produce a non-toxic collagen product (Figure 1). However, it is regarded as an inappropriate method, especially in tissue engineering, owing to its extremely low coupling efficiency. This is because EDC tends to hydrolyze in aqueous conditions within a short time span.[2-6] The use of NHS to suppress the hydrolysis does not function to the desired extent. Furthermore, since collagen consists of triple helices, the efficiency of the coupling reaction is lower than that of cross-linkers such as diol-related cross-linkers or glutaraldehyde because the only possible reactions are the intra- and interhelical coupling reactions. Hence, the question of whether it is possible to control the coupling reaction rate of EDC for collagen cross-linking was brought up.

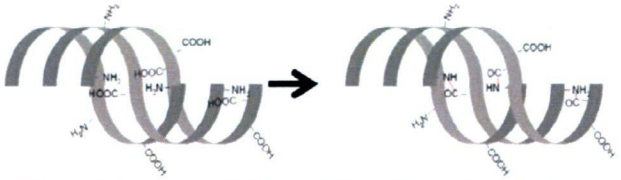

Figure 1. Intracross-linking of the collagen triple helix by EDC.

We attempted to control the EDC/NHS coupling reaction rate by making the reaction environment highly hydrophobic. To achieve the hydrophobic environment, we used ethanol, which is miscible with water. Ethanol/water mixed solvents were prepared in different mole concentrations to control the hydrophobicity of the solvent. However, we are not yet sure on how ethanol would affect the collagen molecules. The structure of collagen is known to depend on the concentration of the alcohols, because an increase in the hydrophobic interactions between the solvent and collagen stabilized the structure of collagen.[7] The hydrophobic interactions between the non-polar amino acid side chains are also very important factors that contribute to the stabilization of the helices. Figure 2 shows how the solubility of the collagen molecules would differ according to the solvent. The collagen solution would dissolve completely until ethanol mole concentration is 0.32 (ethanol/water = 6/4 v/v). Then the aggregation of the collagen molecules starts to appear when the $N_A \approx 0.42$ (ethanol/water = 7/3 v/v). The collagen precipitate as the ethanol concentration increases further. It should be noted that this is different from fibrillogenesis which is the regular alignment of the collagen molecules. It was assumed that this is because of the dehydration, but how is dehydration related to the aggregation and precipitation?

We checked this phenomenon by characterizing the triple helix of the collagen. The exposure of the collagen triple-helices to ethanol induces hydrophobic interactions, which may lead to a change in the conformation of the

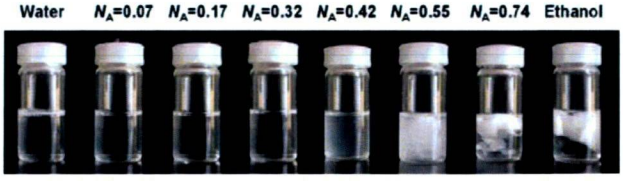

Figure 2. Change in the solubility of collagen according to the ethanol mole concentration.

collagen microfibrils. Using a circular dichroism (CD)

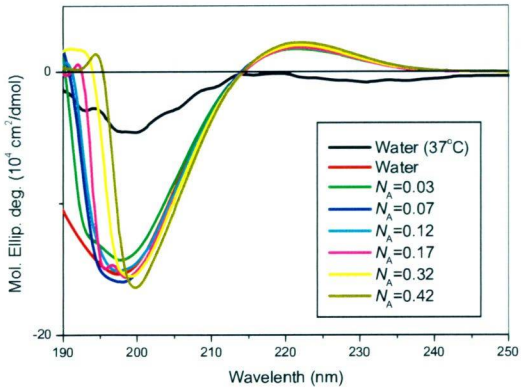

Figure 3. CD spectra of the collagen microfibrils under various ethanol mole concentrations.

spectrometer, we observed the conformation structure of collagen in the range of $N_A \approx 0$~0.42. The increase in ethanol concentration against water did not bring about any distinguishable change in the triple helical structure (Figure 3). The positive band and the cross-band seen in the CD spectra were the same for all the tested samples ($N_A \approx 0$~0.42). No signs of denaturation, such as decrease in the peak intensity of positive and negative band, were detected.[8-9] Hence, it is assumed that ethanol does not change the triple helices into random coils.[10] The main forces that hold the helical structure of collagen are hydrogen bonds, electrostatic interactions, and hydrophobic interactions. In water, the hydrogen bonds and electrostatic interactions within collagen contribute to the stabilization of the helices, but they are not the dominant factors. Exposure of the non-polar amino acid side chains to the outer side would induce hydrophobic interactions, which were not observed under aqueous conditions. This causes a hydrophobic shielding effect.[10] In the case of ethanol, the secondary and the quaternary structures of collagen would be affected.[10,11] As result, it is assumed that the 'triple helix→random coil' does not occur, and the use of ethanol for the amide coupling reaction for collagen cross-linking is preferable. This can be observed much clear when the collagen molecules incubated in the 37°C is compared.

However, it should be noted that the use of ethanol is not a solution for the control of the coupling reaction. The surface of collagen is too hydrophobic and rigid, in which the fibrillar structure disappears. The solubility test showed that the ethanol mole concentration should be at least 0.42 to dissolve collagen. The same phenomenon was observed for the collagen film. The collagen film, which is uncross-linked, could be dissolved at $N_A \approx 0.42$, but would remain undissolved in higher hydrophobic conditions. Expectedly, the time required for complete dissolution was different, where high-hydrophobic conditions delayed the dissolution time. This suggests that for the collagen film, the adsorption of ethanol by the collagen gel would be extremely low. To prove this, we have calculated the diffusion coefficients D for various mole concentrations of ethanol, as shown in Figure 3, using the collagen gel cross-linked with EDC/NHS in a 2-morpholinoethane sulfonic acid (MES) buffer that was prepared by the method reported previously.[4] This shows that the D of the solvent decreases rapidly when $N_A \geq 0.55$ (ethanol/water = 8/2, v/v); furthermore, the D value of pure ethanol (1.2×10^{-10} cm^2/min) is approximately 1400 times lower than that of pure water. This directly affects the cross-linking ability. The solvent adsorption ability in pure ethanol and at $N_A \approx 0.74$ (ethanol/water = 9/1, v/v) is about 50% of that of pure water and 80% at $N_A \approx 0.55$ after 24 h of solvent adsorption. This implies that ethanol could not completely reach the interior of the collagen gel throughout the cross-linking procedure.

Using EDC and NHS, we obtained cross-linked collagen gels under various ethanol concentrations (Figure 4). When EDC and NHS are used for the cross-linking process, the hihest the lowest value of the free amine group content was approximately 45% (60% when cross-linked in MES buffer). This can be achieved when the cross-linking was executed for 24 h at N_A~0.07~0.17 (ethanol/water = 2/8~4/6, v/v) with 51 mmol of EDC. This range is assumed to be the most proficient range for the coupling reaction, where the suppression of hydrolysis and fast solvent absorption has occurred. The addition of ethanol is thought to have prevented the hydrolysis of EDC. On the other hand, when $N_A \approx 0.24$ (ethanol/water = 5/5, v/v), the free amine group content increases again, and from $N_A \approx 0.42$ to higher, the free amine group content increases to higher than that of pure water. This is because of the decrease in the number carboxyl groups reacting with EDC.[12,13] The reactivity of the carboxyl groups decreases as the ethanol concentration increases because EDC reacts with the carboxylate anions.

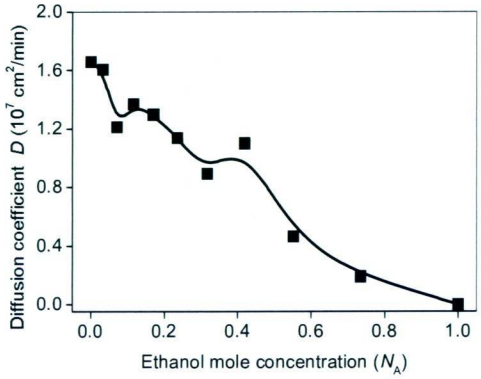

Figure 4. Change in the diffusion coefficient D of collagen gel according to ethanol mole concentrations.

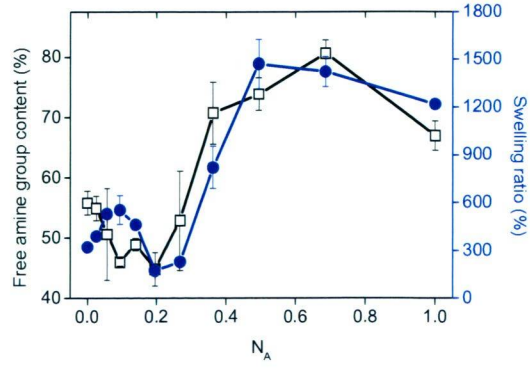

Figure 5. Change in the reacted amine group contents and swelling ratio of collagen gel according to ethanol mole concentrations.

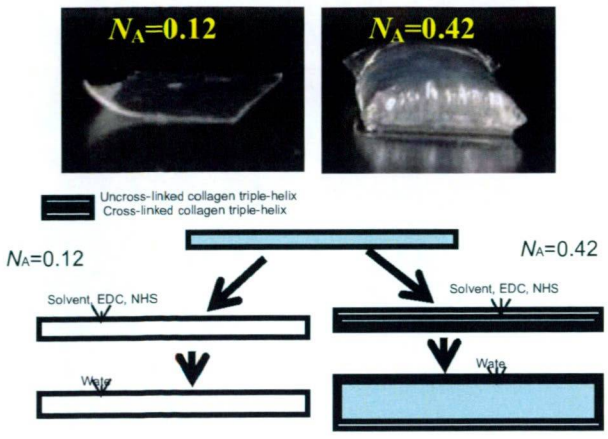

Figure 6. Swelling of the collagen gels in pure water after the coupling reaction in different ethanol mole concentrations (above) and the schematic picture of the swelling by the high ethanol concnentration (below).

The increase in the number of neutral carboxyl groups would lead to relatively low *o*-isoacylurea formation.[5] Furthermore, when $N_A \geq 0.42$, the cross-linking is assumed to be mainly concentrated on the surface of the collagen gel. That is, the partly cross-linked network of the collagen gel could be mainly located on the surface of the gel. This can be confirmed when the collagen gels prepared at $N_A \geq 0.42$ are placed in pure water. As shown in Figure 5 (blue line) and Figure 6, the sudden change in the environment causes the gel to adsorb a large amount of water, which makes the uncross-linked collagen microfibrils dissolve and expand to the maximum extent by an increase in the free energy. The expansion of the collagen microfibrils is obstructed by the cross-linked part, which is mainly located on the surface. For the collagen gel prepared at $N_A \approx 0.42$, D is approximately the same as that of the gel prepared at $N_A \approx 0.32$, but it is thought that the protonation of the carboxyl groups prevents the formation of *o*-isoacylurea. The reactivity between the carboxyl groups and alters the formation of the collagen gel. When the morphology of the razor-cut surface was observed, the monolithic morphology of the collagen gel was found to form a layered structure as the hydrophobicity increased, which eventually collapses. The collapse of the inner part of the collagen gel is due to the dissolution of the uncross-linked collagen microfibrils (Figure 5 (below). This implies that the cross-linking of the collagen gel would start from the surface and then occur inside the collagen gel. Furthermore, it is possible to cross-link only the surface of the collagen gel to obtain a phase-separated collagen gel when the ethanol concentration is controlled.

In conclusion, we have proposed a new method for controlling the coupling reaction rate using EDC and NHS for collagen cross-linking. The collagen triple-helix was stable in ethanol/water mixed solvent, but the properties of the collagen gel prepared in the above solvent could be altered by the ethanol mole concentration. We also discovered that the coupling reaction begins from the surface of the collagen gel. The coupling reaction was limited to the surface of the collagen when NA > 0.55; this was because of the slow penetration of EDC and NHS caused by the high-ethanol environment and the decrease in the number of carboxylate anions. It is thought that the same procedure could be repeated not only in collagen but also in collagen-based materials such as body tissue and proteins.

Experimental

The preparation of the collagen film was executed using 0.5 wt% of collagen type I solution (I-AC, KOKEN, Tokyo, Japan) was made into a 2 wt% collagen type I solution and used for the film preparation. The collagen solution was dropped onto the polyethylene film and dried at room temperature. A transparent film with a thickness of 56 ± 3 μm was obtained. The films were stored in a dry environment. The preparation method of the collagen gel using EDC and NHS is same as reported elsewhere.[4,6,14]

A solubility test was executed in the ethanol/water mixed solvents. The collagen films and collagen chunks obtained from lyophilization were immersed in ethanol/water mixed solvents. The collagen solutions were left at room temperature until complete dissolution occurred. The triple-helix structure was characterized using a circular dichroism (CD) spectrometer (J-720W, Jasco, Tokyo, Japan). Collagen solution was prepared at a concentration of 1×10–7 M and characterized 5 times for each sample to obtain the average spectra. Surface analysis was performed by scanning electron microscopy (SEM, SM-200, Topcon, Tokyo, Japan). The same solubility test was repeated using the collagen film. The diffusion coefficient D was calculated using a collagen gel that was prepared in an MES buffer. The collagen gels were immersed in the ethanol/water mixed solvents at pH 9.0. The gels were then removed at 10, 60, 120, 240, 360, 1440, and 4320 min (3 d) and the adsorbed amounts of the solvent were measured.

The primary amine group concentrations in the tissue samples were determined using a colorimetric assay.[14] 2–4 mg of each sample was prepared. These samples were immersed in a 4 wt% $NaHCO_3$ aqueous solution (Kanto Chemicals, Tokyo, Japan) and 2,4,6-trinitrobenzene sulphonic acid (TNBS). Then, 0.5 wt% TNBS aqueous solution was added (Wako chemicals, Osaka, Japan). The reaction was continued for 2 h at 40 °C, after which the samples were rinsed in saline solution using a vortex mixer to remove the unreacted TNBS. The samples were freeze-dried overnight, after which the dry mass was determined. The dry samples were immersed in 2 mL of 6 M aqueous HCl until fully dissolved. The obtained solution was then diluted with distilled water (8 ml) and the absorbance was measured at 345 nm (V-560, Jasco, Tokyo, Japan). The experiment was repeated five times and the average along with the standard deviation was calculated.

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In vivo evaluation of a novel scaffold for artificial corneas prepared by using ultrahigh hydrostatic pressure to decellularize porcine corneas

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Purpose: To evaluate the stability and biocompatibility of artificial corneal stroma that was prepared by using ultrahigh hydrostatic pressurization treatment to decellularize corneas.

Methods: The porcine cornea was decellularized by two methods, a detergent method and an ultrahigh hydrostatic pressure (UHP) method. Either 1% w/v Triton® X-100 or sodium dodecyl sulfate (SDS) was used for the detergent method, and 10,000 atmospheres (atm; 7.6×10^6 mmHg) was applied to the cornea for 10 min at 10 °C by a high-pressure machine for the UHP method. Hematoxylin-eosin staining was performed to confirm the removal of the corneal cells, and then decellularized porcine corneal stroma was implanted into rabbit corneal pockets. After eight weeks, the rabbit eyes were enucleated to examine the tissue compatibility of the implanted stroma.

Results: Complete decellularization was confirmed only in corneas treated by the UHP method, and little inflammation was seen when they were implanted into the rabbit corneal pockets.

Conclusions: Porcine corneal stroma completely decellularized by the UHP method has extremely high biocompatibility and is a possible corneal scaffold for an artificial cornea.

Injury or corneal diseases can lead to corneal opacification for which currently the only effective therapy is corneal transplantation [1]. Conditions such as corneal dystrophy, bullous keratopathy, and corneal scarring are treated by replacing the defective cornea with a clear donor cornea. Since the first human corneal transplant in 1905, corneal transplantation has been one of the most successful forms of tissue transplantation [2]. However, complications such as infection, immune rejection, and graft failure are possible, and allograft reaction has been reported to be seen in 31% of penetration keratoplasty patients. Furthermore, there is a worldwide shortage of donor corneas, due in part to many donated corneas not being able to be used because of infection.

One way to overcome these difficulties is to develop artificial corneas [3], and among the various synthetic polymers investigated for this purpose are poly(methyl methacrylate) [4], poly(2-hydroxyethyl methacrylate) [5], and poly(vinyl alcohol) [6]. Alphacor™ was the first synthetic artificial cornea available commercially [7-9], but no artificial cornea has been fully successful yet. Their failure to be accepted by recipient tissue and to be invaded by the recipient's corneal cells results in their extrusion through melting around the prosthetic rim [10] or other adverse effects such as protein adsorption, rejection with down-growth, and infection.

The engineering of cornea tissue has recently been presented as a promising solution to the limited corneal replacement with allografts. Pellegrini et al. [11] reported that the ocular surface can be reconstituted using epithelial cells cultured in vitro on a contact lens. Furthermore, Minami et al. [12] attempted to reconstitute a cornea (including the epithelium, stroma, and endothelium) in vitro by using a collagen gel culture system under an air-liquid interface. Orwin et al. [13] reported that corneal tissue could also be reconstituted in vivo by combining corneal cells and a collagen sponge. While these reports indicate the feasibility of corneal regeneration using corneal cells and collagen scaffolds, the structure and mechanical properties of their collagen gel and sponge were inadequate for an artificial cornea that can be used clinically. The mechanical properties and structure of the scaffold for corneal reconstruction must be similar to those of the natural cornea. The ideal scaffold for corneal tissue engineering would allow epithelization, endothelialization, and repopulation with autologous interstitial cells.

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One strategy for preparing a scaffold is the use of decellularized tissue in which the donor cells and antigen molecules are completely removed to diminish the host immune reaction. Some groups have attempted to use porcine cornea for xenografting because it would be available in amounts sufficient to meet clinical demand [14]. Many decellularizing methods for preparing acellular tissues have been reported, and most use detergents to remove cells from tissues. Acellular tissues of the vessel, heart valve, dermis, and ligament have been successfully prepared by using Triton® X-100 [15,16], sodium dodecyl sulfate (SDS) [17,18], sodium deoxycholate [19,20], and polyethylene glycol [21] to remove the donor cells and their components. However, detergents are generally toxic and need to be washed out. Sometime, detergent treatment and the following wash-out process may lead to the denaturation of the tissue and destroy their structures.

For ideal xenografting, the cellular immune reaction should be decreased by removing donor cells from the cornea, but the corneal superstructure should be maintained to keep the cornea transparent. In general, the transparency of the cornea is explained by a lattice theory of the corneal materials in which the corneal superstructure is an optically clear lattice of regularly aligned collagen fibrils. Thus, the ideal decellularization process would be one that removes all the cell components without destroying the corneal superstructure.

Several methods have been reported to be effective for decellularizing corneas [22-26], and decellularizing corneal tissues have been shown to be biocompatible. However, their mechanical characteristics still need to be improved. We recently developed a novel physical process that uses ultrahigh hydrostatic pressure (UHP) technology to decellularize tissue without using detergents [27] (Figure 1), and in the work reported here, we compared its use with that of a detergent method in the decellularization of porcine cornea. Using decellularized porcine cornea, an implantation experiment into the rabbit eye was performed to see if we could apply the decellularized porcine cornea for xenografting as an artificial cornea.

METHODS

Materials: Porcine eyes were purchased from Shibaura Zoki Co., Ltd. (Tokyo, Japan). Japanese white rabbits were purchased from Kitayama Labes Co., Ltd. (Nagano, Japan). Dextran (molecular weight=70,000 g/mol) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). SDS was purchased from Wako Co., Ltd. (Osaka, Japan). Triton® X-100 was purchased from Sigma-Aldrich Co., Ltd (Tokyo, Japan). Phosphate buffer saline (PBS) was purchased from Invitrogen Co., Ltd. (Tokyo, Japan). Endothelial growth medium (EGM-2) was purchased from Sanko Junyaku Co., Ltd. (Tokyo, Japan).

Preparation of porcine cornea: The entire cornea was removed from the eye, washed with PBS containing penicillin (100 units/ml) and streptomycin (0.1 mg/ml), and stored at 4 °C in PBS containing these antibiotics and dextran (3.5% w/v) until the experiments were performed.

Chemical decellularization: Corneas were immersed in a 1% w/v solution of either Triton® X-100 or SDS at 37 °C for 24 h, washed with PBS containing penicillin (100 units/ml) and streptomycin (0.1 mg/ml) for another 24 h, and then subjected to hematoxylin-eosin (H-E) staining for histological study.

UHP decellularization: Corneas were pressurized at 10,000 atm for 10 min at 10 °C by using a high-pressure machine (Kobe Steel, Ltd., Kobe, Japan), washed under air containing 5% CO_2 by continuous shaking for 72 h at 37 °C in an EGM-2 medium containing DNase I (0.2 mg/ml), antibiotics, and 3.5% w/v dextran, and then subjected to hematoxylin-eosin (H-E) staining for histological study before they were used for transplantation.

Histological study: Native and decellularized corneas (five of each) were fixed for 24 h in a 10% neutral buffered formalin solution at room temperature. They were then cut, stained with H-E, and observed with an optical microscope.

Measurement of residual DNA content: After 20 mg of each freeze-dried decellularized cornea was digested at 55 °C for 12 h in 0.5 ml of a tissue lysis buffer containing 50 mM Tris-HCl, 50 µg/ml proteinase K, 1% w/v SDS, 100 mM NaCl, and 20 mM disodium EDTA, the DNA content was calculated from the difference in the absorbance at 260 nm measured

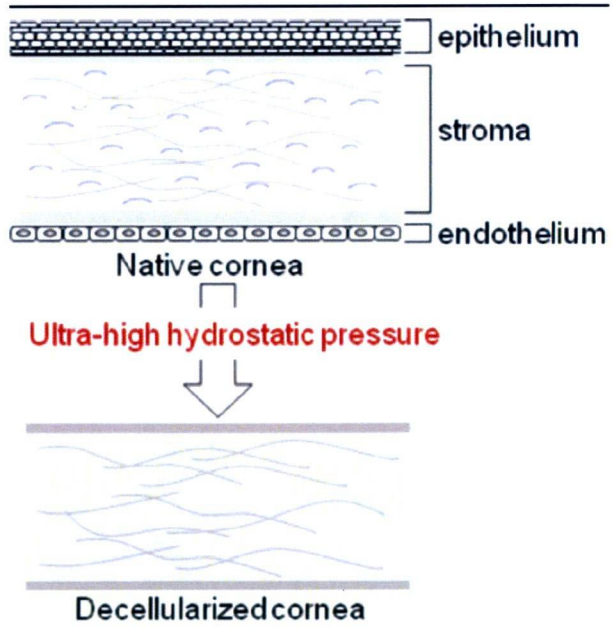

Figure 1. Concept of decellularization using ultrahigh hydrostatic pressure. Ultrahigh hydrostatic pressure (UHP) treatment removes all the cells of the native cornea (epithelial cells, keratocytes, and endothelial cells).

before and after extracting DNA with phenol and chloroform and precipitating it with ethanol. Five corneas were used for each group.

Measurement of residual GAG content: Glycosaminoglycans (GAGs) or mucopolysaccharides such as hyaluronic acid and chondroitin sulfate help maintain the structure of connective tissues, so a decreased GAG content would indicate destruction of tissue structure. We therefore used an Alcian blue assay to measure the residual GAG in the tissue. After 20 mg of each freeze-dried decellularized cornea was digested at 65 °C for 24 h in a papain solution (100 mM sodium acetate buffer, 0.5 mg/ml papain, 0.5 mM disodium EDTA), Alcian blue was added and a microplate reader was used to measure the absorbance at 600 nm. The GAG was calculated from the absorbance by using chondroitin sulfate standard solutions. Five corneas were used for each group.

Statistical analysis: Measurements of residual DNA and GAG content were performed three times. Mean±SD values were calculated. Data were analyzed statistically by Student's *t*-test. A $p<0.05$ was regarded as significant.

Preparation of decellularized porcine cornea: After physiologic saline was injected into the vitreous humor of a porcine eyeball to raise the intraocular pressure, a microkeratome was used to prepare a corneal flap 160 µm thick. The corneal flap was treated with ultrahigh hydrostatic pressure three days before the transplantation, and it was stored in an EGM-2 medium at 4 °C until transplantation. Corneal discs 2 mm in diameter were prepared with a corneal punch.

Transplantation of decellularized porcine cornea into rabbit corneal stroma: Decellularized porcine cornea was transplanted into the left eye of Japanese white rabbits (female, 3 kg and 12 weeks old) according to the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research. All animal experiments were approved by the ethical committees for animal welfare of Tokyo Medical and Dental University (Tokyo, Japan) and National Institutes for Materials Science (Tsukuba, Japan). The corneas of the recipient animals (n=11) anesthetized with intravenous Nembutal™ (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) and topical Xylocaine™ (AstraZeneca, Osaka, Japan) were incised with a surgical knife to about half the depth of the corneal stroma, tangent to the pupil at four positions 90° apart around the edge of the pupil (3, 6, 9, and 12 o'clock). Stromal pockets were then formed by inserting a spatula into each incision, directing the inserted edge of the spatula toward the corneal limbus, and using it to separate the stromal layers. A decellularized porcine corneal disc was put into three of the pockets, and a non-decellularized one was put into the other pocket as a positive control (Figure 2). Eight weeks after the transplantation, the left eye was enucleated and the cornea was stained with hematoxylin and eosin for histological study.

RESULTS

Decellularization: After a porcine cornea was immersed in a 1% w/v solution of Triton® X-100 for 24 h and then washed with PBS for 24 h, it was cloudy and more than five times thicker (Figure 3C) than it was before treatment (Figure 3A). Comparing an H-E stained section of the native cornea (Figure 3B) with that of a cornea treated with Triton® X-100 (Figure 3D), one sees loosening of the collagen fibrils in the corneal stroma and shrinkage of the epithelial layer in the treated cornea and also that few cells of the cornea were removed by the treatment. The cornea treated with SDS (Figure 3E) was not swollen as much as the one treated with Triton® X-100 (Figure 3C), but it was smaller, its surface was melted, and its interior was extremely cloudy because the nuclear materials of the disrupted cells remained (Figure 3F). Thinning of the epithelial layer and disordering of the superstructure of collagen fibrils in the stroma were also observed in the corneas treated with the detergents.

Although the porcine cornea treated with UHP was also extremely cloudy and slightly swollen after washing in an EGM-2 medium (Figure 3G), H-E staining showed the absence of cells in the epithelium and stroma and the maintenance of the superstructure of collagen fibrils in the stroma (Figure 3H). These results indicate that the UHP method is useful for decellularizing the porcine cornea without destroying its structure.

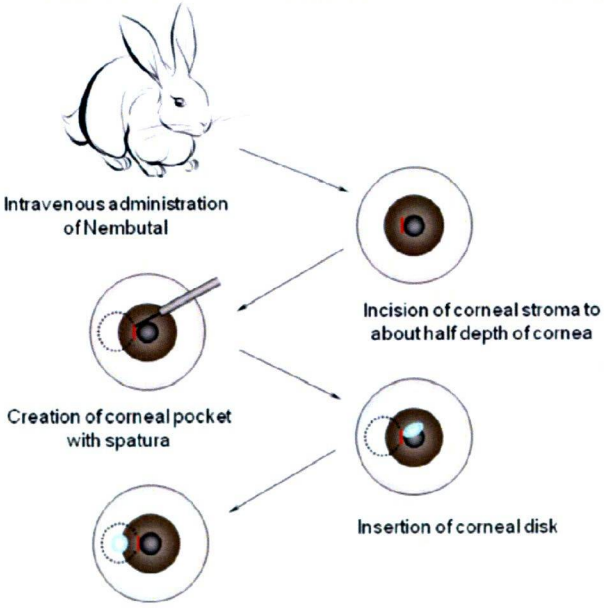

Figure 2. Procedure of transplantation of decellularized porcine corneal disc into a rabbit corneal pocket. The recipient rabbit is anesthetized with intravenous Nembutal and topical Xylocaine, a corneal pocket is made with a spatula, and a decellularized or native porcine corneal disc is inserted into the corneal pocket.

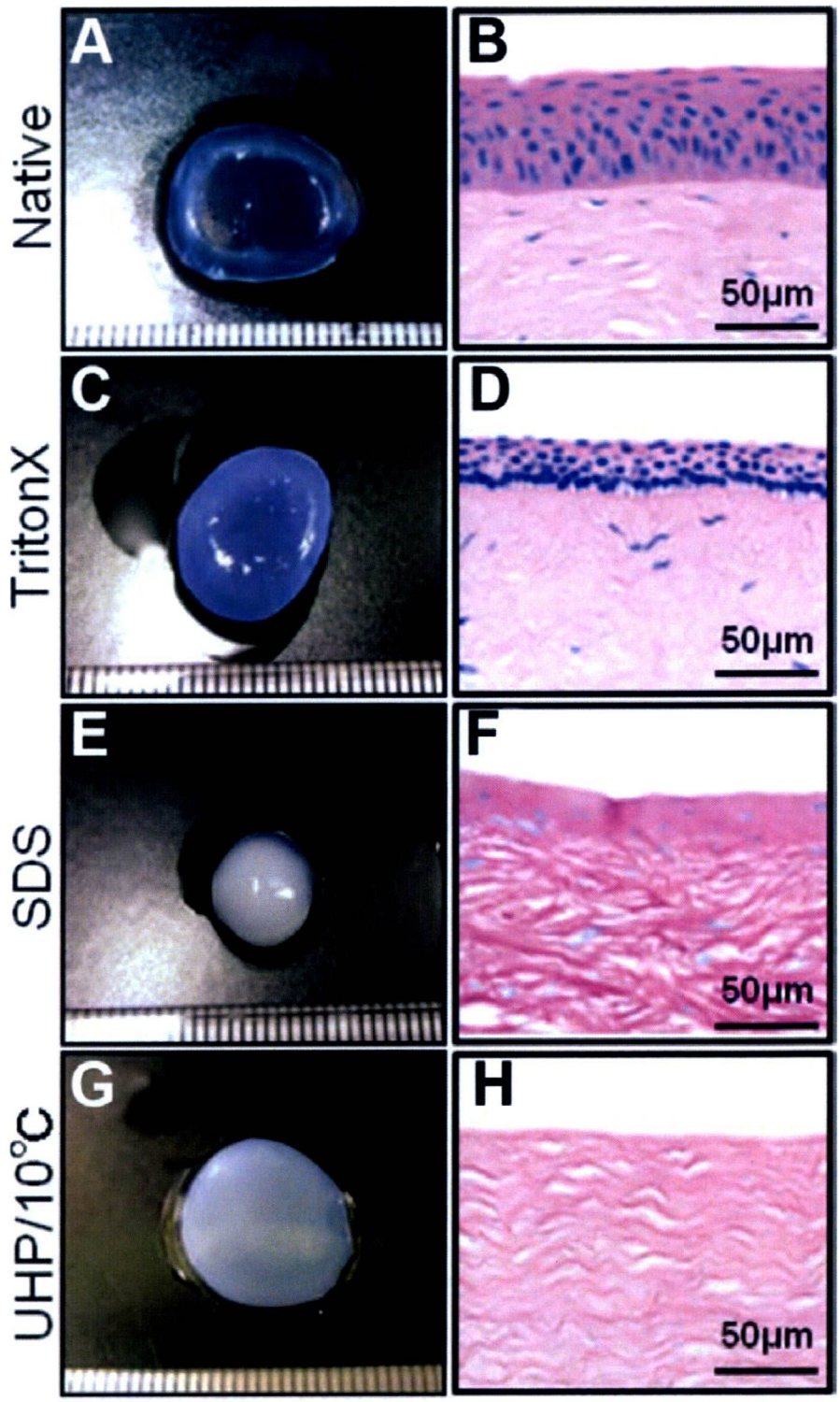

Figure 3. Representative photographs and H-E stained sections of the porcine corneas decellularized by various methods. The left column shows photograph of native cornea (**A**), cornea treated with Triton® X-100 (**C**), cornea treated with SDS (**E**) and cornea decellularized by UHP (**G**). The right column shows H&E stained section of native cornea (**B**), cornea treated with Triton® X-100 (**D**), cornea treated with SDS (**F**) and cornea decellularized by UHP (**H**). Epithelial cells and keratocytes are seen in the corneas treated with Triton® X-100 or SDS but not in the cornea treated with UHP. Scale bar, 50 µm.

Table 1. Confirmation of decellularization.

Sample	DNA content mean±SD (μg/mg dry wt)	GAG content mean±SD (μg/mg dry wt)
Native cornea	3.46±0.18	223.0±19.1
Triton® X-100	2.32±0.28*	169.9±8.33*
SDS	1.16±0.21*	38.6±3.01*
UHP/10 °C	0.12±0.02*	201.3±10.1

The DNA and GAG content of the corneas decellularized by various methods were measured. These results indicate that only the corneas treated with UHP lost cells without losing GAG-collagen interactions. Data are expressed as the mean±SD; n=5 in each group. The asterisk indicates a $p<0.05$.

Confirmation of decellularization: The DNA content of the corneal tissues treated with Triton® X-100 and SDS were 2.32±0.28 and 1.16±0.21 μg/mg, respectively. They were significantly lower than the DNA concentration of the native cornea (3.46±0.18 μg/mg) but much greater than zero. On the other hand, the DNA concentration of the porcine corneal tissues treated with UHP is almost zero (0.12±0.02 μg/mg), which indicates complete removal of cell components from the corneal tissue. (Table 1)

GAG content: The GAG content of the corneal tissues treated with Triton® X-100 and SDS were 169.9±8.33 and 38.6±3.01 μg/mg, respectively, significantly lower than the GAG content of native cornea (223.0±19.1 μg/mg), indicating that the structure of the connective tissues is not maintained in detergent-treated corneas. The GAG content of the corneal tissues treated with UHP, on the other hand, is almost the same as that of native cornea, which means the connective tissue structure was not destroyed by the UHP treatment. (Table 1)

Transplantation of decellularized porcine cornea into rabbit corneal stroma: To evaluate the possibility of using porcine corneas decellularized by the UHP method as a substitute for corneal stroma, we implanted them in corneal stromal pockets in rabbits and implanted native porcine corneas as positive controls. The native implanted corneal discs were fairly clear just after the operation (Figure 4D), but one week later, blood vessels were seen in them and they began to become cloudy (data not shown). The native donor tissue was extremely cloudy four weeks after implantation, and eight weeks after implantation, many vessels were observed around it, indicating the occurrence of an immune reaction (Figure 4E). The histological section showed the infiltration of neutrophils and macrophages in and around the donor tissue and also showed the formation of a cell layer (Figure 4F). On the other hand, in the decellularized cornea group, the donor tissue appeared very cloudy just after implantation (Figure 4A) but began to become transparent one week later (data not shown). Two weeks later, the donor tissue in the decellularized cornea group was completely transparent and could not be recognized without a microscope. The transparency was kept until eight weeks after the implantation, and no vessels were observed (Figure 4B). The histological section showed minimal inflammation around the donor tissue, which indicated that no immune rejection occurred (Figure 4C).

DISCUSSION

In this study, we compared two methods of tissue decellularization and demonstrated the extremely high biocompatibility of porcine corneal discs decellularized by the UHP method.

Corneal decellularization was performed by chemical and physical methods. Triton® X-100 and SDS, which have often been used in decellularization protocols [28], were used for chemical decellularization. These detergents caused the corneas to swell and become irreversibly cloudy. They did not become transparent again even when they were treated with glycerol (data not shown). The obvious decrease of GAG content also indicates the disruption of the corneal superstructure. These results suggest that the superstructure of the cornea was strongly denatured by Triton® X-100 and SDS. The UHP method, on the other hand, removed all corneal cells. Although corneas treated with UHP were extremely cloudy just after they were treated, they became transparent again when treated with glycerol (data not shown). The glycerol treatment dehydrated the UHP-treated cornea, playing the role normally played by the Na^+-K^+ pump of the endothelial cells. The results of the histological study and the measurement of GAG content also indicate the maintenance of the corneal superstructure. The swelling seems to be caused not by the disruption of corneal superstructure but by the lack of the pumping function of the endothelial cells.

In the transplantation study, only native corneas and corneas decellularized by the UHP method were used. Triton® X-100 and SDS did not remove all the cells from the corneas. It was obvious that corneas with residual cells would cause a severe immune reaction to the recipients like native corneas. Moreover, SDS decreased the mechanical strength of the corneas so much that they could not be inserted into stromal pockets. Therefore, we used native corneas and corneas decellularized by the UHP method from the viewpoint of animal protection.

The transplantation of decellularized porcine corneas into rabbit corneal pockets induced little immune reaction whereas

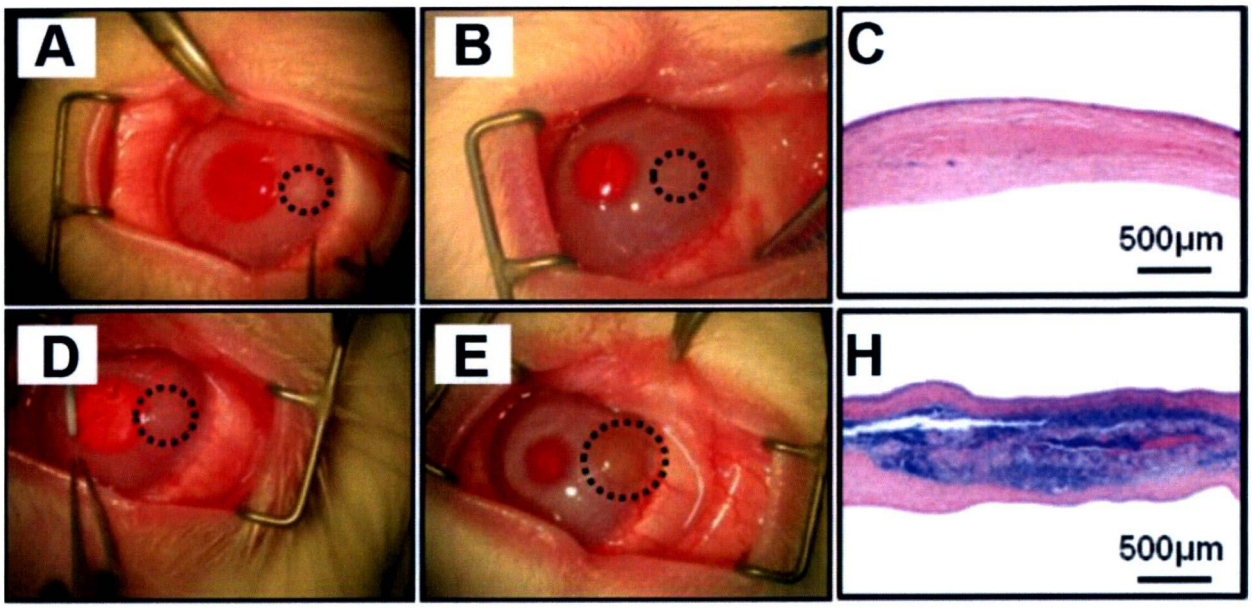

Figure 4. Representative photographs and H-E stained sections of transplanted porcine corneal discs. The left column shows photograph of decellularized (**A**) and native (**D**) porcine corneal disc just after transplantation into rabbit corneal pockets. The middle column shows photograph of decellularized (**B**) and native (**E**) porcine corneal disc eight weeks after transplantation. The right column shows H&E stained section of decellularized (**C**) and native (**F**) porcine corneal disc eight weeks after transplantation. Decellularized corneal discs caused slight inflammation and recovered transparency whereas native corneal discs caused severe immune reactions and remained cloudy. Scale bar, 500 μm.

native corneas caused severe inflammation. This decellularization method involves two processes, disruption of the cells, bacteria, and viruses by ultrahigh pressurization [29,30] and removal of the residues of cellular components by washing in a culture medium. We previously reported the successful decellularization of porcine heart valve and trachea by this method [27]. In the transplantation experiment, the decellularization process removed all the corneal cell components including bacteria and viruses. The native cornea tissue may have been rejected not only of xenografting but also because of infectious pathogens. Although many countries are running short of donated corneas, some are rejected because of infection. The UHP method may reproduce unusable corneas from infected corneas by removing the pathogens.

In conclusion, we have developed a corneal decellularization method using UHP technology. The decellularized corneal tissue did not cause immunological rejection in a xenograft transplantation model. These results indicate that decellularized cornea stroma that was prepared using UHP technology is a possible scaffold for an artificial cornea.

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ORIGINAL ARTICLE

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Beating behavior of primary neonatal cardiomyocytes and cardiac-differentiated P19.CL6 cells on different extracellular matrix components

Abstract Stem cell-based therapy in cardiac tissue engineering is an emerging field that shows great potential for treating heart diseases. However, even preliminary issues, such as the ideal niche for cardiomyocytes, have not been clarified yet. In the present study, the effects of extracellular matrix (ECM) components on the beating duration of neonatal rat cardiomyocytes (RCMs) and on the cardiac differentiation of P19.CL6 carcinoma stem cells were studied. RCMs were cultured on gelatin-, fibronectin-, and collagen type I-coated dishes and on noncoated polystyrene dishes, and their beating rate, beating duration, and cardiac gene expression were evaluated. The beating period and the expression of troponin T type-2 (TNNT2) and troponin C type-1 (TNNC1) of cardiomyocytes cultured on gelatin-coated dishes were longer and higher than for those on dishes with other coatings. For the cardiac differentiation of P19.CL6 cells, troponin T type-2 expression on gelatin- and fibronectin-coated dishes was five times that on collagen type I-coated dishes or polystyrene dishes 11days after induction. These results indicate that a gelatin-coated surface has a high ability not only to maintain the cardiac phenotype but also to enhance cardiac differentiation.

Key words Extracellular matrix · Cardiomyocyte · Beating · Differentiation

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Introduction

Cardiac tissue engineering, such as cardiomyocyte transplantation for patients with ischemic heart disease or dilated cardiomyopathies, is of great potential therapeutic value to enhance the contractile function of the failing heart. Recently, fetal or neonatal rat cardiomyocytes were reported to form mature cardiac tissue in syngeneic hearts, acutely injured myocardium, and granulation tissue in the heart.[1] However, the best cell sources for clinical cardiomyocyte transplantation are still under debate. In general, three types of potential cell sources have been proposed.[2] One is the allogeneic source, including human embryonic stem cells or fetal allogeneic cardiomyocytes, but there still remain ethical issues. Another is the transgenic source. Genetically engineered animal cardiomyocytes have been studied in an attempt to reduce the rejection reaction in vivo, which is still a long-term problem in recipients.

The most promising cell source is the autogeneic one. Isolating cardiomyocytes from patients' hearts is unrealistic at present, and autologous skeletal muscle precursors, fibroblasts, or mesenchymal stem cells have been studied so far.[3] However, since beating cardiomyocytes are more promising,[4] we have been trying to differentiate bone marrow mesenchymal stem cells (BMSCs) into "beating" cardiomyocytes. There is no certain induction method for BMSC differentiation into beating cardiomyocytes. Many researchers have observed cardiac gene expression in MSCs treated with various inducers[5–7] or passage numbers,[8] but they do not beat spontaneously. Wakitani et al. and Makino et al. reported that murine BMSCs were differentiated to beating cardiomyocyte-like cells in vitro by exposing them to DNA-demethylating agent 5-azacytidine.[5,6] This is in contrast with a report stating that functional cardiac cells and gene expression were not obtained by treatment with 5-azacytidine.[9]

Producing autologous beating cardiomyocytes is thus an attractive goal for cell-based therapy. The crucial part is how to differentiate cells to cardiomyocytes in vitro and how to maintain the beating. Various microenvironments surrounding the cells (niches) play important roles not only

in cell proliferation but also in cell differentiation. The effect of extracellular matrix (ECM) proteins such as collagen type I, collagen type IV, gelatin, laminin, fibronectin, Matrigel (a mixture of laminin, collagen type IV, heparan sulfate proteoglycans, and entactin), and Cardiogel (a mixture of collagen types I and III, glycoproteins, laminin, fibronectin, and proteoglycans) on cell viability, proliferation rate, and cardiomyocyte gene expression have been reported;[10,11] however, the cardiomyocyte beating behavior has not fully been discussed.

In the present study, differentiation to beating cardiomyocytes and the beating duration of the cardiomyocytes were studied using two types of model cells. Murine embryonal carcinoma (EC) stem cells (P19.CL6),[12] which are widely used for investigating cardiac differentiation, were treated with differentiation medium containing 1% dimethyl sulfoxide (DMSO) on various ECM proteins (collagen, type I gelatin, and fibronectin), and their differentiation efficiency was evaluated. The effect of these substrates on the beating duration of rat neonatal cardiomyocytes was also investigated, along with intracellular cardiac marker genes [troponin T type-2 (TNNT2) and troponin C type-1 (TNNC1)][13] and skeletal muscle marker gene [troponin C type-2 (TNNC2)], which is reported to be expressed in the early developing heart.[14] Any fundamental information obtained would be important for the cardiac differentiation of various stem cells, including autologous BMSCs.

Materials and methods

Cardiomyocytes

Cardiomyocytes were isolated from neonatal Sprague–Dawley rat heart (1 to 2 days old) by the collagenase digestion method with modifications.[15,16] Institutional guidelines for the care and use of laboratory animals were observed. The hearts were removed and carefully minced with a scalpel blade into fragments and were rinsed several times with Hanks' balanced salt solution (Sigma-Aldrich, St. Louis, MO, USA) to remove blood and cellular debris. The minced hearts were gently stirred in 50 ml collagenase solution [0.15 M Sodium Chloride (NaCl), 5.63 mM Potassium Chloride (KCl), 0.02 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.02 M Sodium Hydrogen Carbonate ($NaHCO_3$), 3.74 mM Calcium Chloride Dihydrate ($CaCl_2{\cdot}2H_2O$), and 6.5×10^4 U collagenase (Wako, Osaka, Japan, Lot no: 06032W)] at 37°C for 30 min. The resulting cell suspension was filtered through a nylon cell strainer (BD Falcon, BD Biosciences, Bedford, MA, USA) with a 40-μm pore size and centrifuged at 78 g for 3 min.

Isolated cardiomyocytes (1.0×10^5) were cultured in minimum essential medium alpha medium (α-MEM, Gibco, Invitrogen, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, MP Biomedicals, Eschwege, Germany, lot no: 7297H), and 100 IU/l penicillin–streptomycin (Wako, Osaka, Japan) on 60-mm gelatin-(IWAKI, Asahi Glass, Tokyo, Japan), fibronectin-(BD Falcon, BD BioCoat, BD Biosciences), or collagen type I-coated dishes and noncoated polystyrene dishes (IWAKI).

Differentiation of P19.CL6 cells

Differentiation of P19.CL6 cells was performed as described by Ohkubo with modifications.[12] Briefly, P19.CL6 cells were plated at a density of 3.7×10^5 cells on 60-mm gelatin-, fibronectin-, or collagen type I-coated dishes or noncoated polystyrene dishes with a α-MEM supplemented with 10% (v/v) FBS containing 1% DMSO (Wako). As a control experiment, P19.CL6 cells were cultured with α-MEM supplemented with 10% (v/v) FBS without 1% DMSO. The medium was changed every 2 days.

Measurement of action potential

Cultured plates on which beating colonies appeared were placed on the stage of an inverted phase-contrast optical microscope (ZEISS, Axiovert 135, Munich, Germany) and action potentials were measured immediately by a conventional microelectrode. The measurements were conducted after 1, 2, and 3 weeks of cultivation. Silicon-coated Ag wire (A-M System, Carlsborg, WA, USA, 250 μm bare, 330 μm coated) was used as the microelectrode. The microelectrode was set in a micromanipulator system (MON-202D, Nikon Narishige, Tokyo, Japan) and connected to a bioelectric amplifier (AB-621G, Nihon Kohde, Osaka, Japan). The sensitivity and time constant of the bioelectric amplifier were set at 0.1 mV/div and 0.003 s. For the measurements, the microelectrode was adjusted using the micromanipulator until it was attached to the membrane of beating cells. The voltage difference was amplified by the bioelectric amplifier and was displayed and recorded using Chart 5 software (AD Instrument, Bella Vista, Australia).

Total RNA isolation and reverse transcription

Total RNAs of cardiomyocytes and DMSO-treated P19.CL6 cells cultured on various dishes were extracted by QuickGene RNA cultured cell kit S (Fujifilm Life Science, Tokyo, Japan) 4 weeks after culture and 11 days after culture, respectively.

First-strand cDNAs were synthesized using a mixture of $oligo(dT)_{18}$ primer. Total cellular RNAs (200 ng) were incubated with 2.5 μM $oligo(dT)_{18}$ primer at 70°C for 10 min to denature the RNA secondary structure and then incubated at 4°C to let the primer anneal to the RNA. A given amount of 5X RT buffer (Toyobo, Osaka, Japan) and 2.5 mM Deoxynucleotide Trisphasphate (dNTP) mixture (Takara Bio, Shiga, Japan) (4 μl) were added and incubated at 37°C for 5 min. The reverse transcriptase (100 Units, Toyobo) was added into the mixture and the reverse transcriptase (RT) reaction was extended at 37°C for 1 h. Then, the reac-