group was less than that in the CN group ($p = 0.019$). The TX group did not differ significantly from the SH group ($p = 0.3$, Figure 10).

ANP Concentration

The ANP concentration in the TX group (241.1 ± 19.8 pg/ml), which was not statistically different from that in the normal rats (209.3 ± 9.9 pg/ml, $p = 0.4$), was significantly less than that in the CN group (388.5 ± 41.8 pg/ml, $p = 0.0003$) and in the SH group (344.7 ± 20.4 pg/ml, $p = 0.0,052$, Figure 11).

Histologic Study

In the hematoxylin and eosin staining, the area of the injection was indistinguishable from other areas, but some rats were distinguished with an inflammatory change in the epicardium caused by the injections. We found no cartilage or bone formation at the transplantation sites.

Vessel Number

In the left ventricular free wall (transplant area), the number of vessels in the TX group (11.7 ± 0.98 vessels/HPF, at ×100 magnification) was larger than in the CN group (9.3 ± 0.8 vessels/HPF, $p = 0.039$) or in the SH group (7.5 ± 0.48 vessels/HPF, $p = 0.0007$, Figure 12). In the transplanted area, we saw much smaller vessels. The diameter of most vessels was <50 μm. In the septum (remote area), the number of vessels in the TX group (10.2 ± 0.47 vessels/HPF) was larger ($p < 0.0001$) than the number in the CN group (5.8 ± 0.34 vessels/HPF) or in the SH group (5.8 ± 0.43 vessels/HPF). In normal, CN, and SH groups, the number of vessels in the left ventricular free wall was larger than the number in the septum (normal, $p = 0.018$; CN, $p = 0.003$; and SH, $p = 0.047$; Figure 13). In the TX group, we observed more venules than in the CN and SH groups.

Electron Microscopic Study

Table 1 shows semi-quantitative scoring for electron microscopic findings in the 3 groups. We found a trend for the total score in the TX group to be the smallest of all.

In the TX group, myofibrils were almost well-organized. Proliferation of mitochondria was mild. Minor changes included dilatation of the endoplasmic reticulum and the T tubules (Figure 14a).

In contrast, in the SH and the CN groups, we recognized metamorphic myofibrils, and endoplasmic reticulum and T tubules were dilated moderately. Proliferation of mitochondria was severe in the SH group and moderate in the CN group. We found widened intercalated discs and irregular nuclei in the SH group (Figure 14, b and c).

In order, we observed many more vessel in the TX, in the CN, and then in the SH group. The infiltrating cell number was zero or 1 in all groups.

DISCUSSION

We used doxorubicin-induced cardiomyopathy as the model of IDCM. In electron microscopic study, cardiotoxicity was moderate in the hearts of the SH group. We succeeded in creating a heart failure model.

In this study, we showed that BMMNC transplantation had beneficial effects on non-ischemic heart failure, especially for systolic function. The function study, in which we used a Langendorff apparatus, demonstrated the greatest peak systolic pressure and developed pressure (the parameters of systolic function) in the TX group. An increase in systolic pressure without the parallel increase in end-diastolic pressure in the TX group, with increasing balloon volume, suggested that transplantation maintained elasticity instead of stiffness, which also could be changed by inflammation, changes in vasculature, or extracellular matrix. As demonstrated by LVPW/LVDs, transplantation prevented the left ventricular wall from remodeling and may support myocardial reserve for contraction. Although part of the cardiac function data (LVDd, LVDs, FS, and end-diastolic pressure) did not show a significant difference between the TX and the CN groups, it is obvious that results in the TX group were superior to those of the SH group, whereas results in the CN group were similar to those in the SH group.

In addition, secondary changes (decreased heart weight, developed ascites,[17] increased ANP concentration, and destruction of myocardium) caused by doxorubicin-induced heart failure were attenuated by BMMNC transplantation.

Regarding the possible underlying mechanism for improved non-ischemic heart failure after cell transplantation, several paracrine factors released from transplanted cells have been suggested.[4,5] In the ischemic heart model, BMMNC transplantation works as an enhancer for angiogenic ligands beta fibrogenic growth factor (bFGF), vascular endothelial growth factor (VEGF),[9] insulin-like growth factor 1,[18] and angiopoietin 1 and cytokines (interleukin-1β and tumor necrosis factor-α).[8]

In this study, BMMNC transplantation increased blood vessel density not only in the left ventricular free wall (transplant area) but also in the septum (remote area), and we found no significant difference between the 2 areas. In the normal and SH groups, we showed significant difference between the left ventricular free wall and the septum, suggesting that vascular density was originally greater in the left ventricular free wall than in the septum, and the difference was greater in the CN group, suggesting

that the injection itself induced angiogenesis at the injection site.[19] Electron microscopic study showed that the structure of myocardium in the TX group clearly was maintained, with many more vessels than in the SH or CN groups.

Considering previous reports[4,5,8,9,18] and our observations, the possible mechanism by which BMMNC transplantation was beneficial in doxorubicin-induced cardiomyopathy may have been the following: The BMMNC transplantation induced angiogenesis in the whole heart. Microcirculation improved by angiogenesis could contribute to preserving myocardium. Preserved myocardium might have contributed to preventing deterioration of cardiac function.

This study had several limitations. We did not label transplanted BMMNC for identification, because labeling technique may compromise cell function.[20] Our main aim was to verify the efficacy of BMMNC transplantation. Further studies of dose response, fate of transplanted cells, and long-term effect should be conducted.

In conclusion, BMMNC transplantation had beneficial effects in non-ischemic heart failure: doxorubicin-induced cardiomyopathy in rats.

The authors thank Dr. Ohgushi (Tissue Engineering Research Center, National Institute of Advanced Industrial Science of Technology, Japan) for investigating bone formation and tumorigenic formation, K. Hattori for her help in feeding the rats, and Mr. Masuda for his technical assistance in the histologic study.

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

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Received 10 June 2004; received in revised form 30 June 2004; accepted 11 August 2004
Available online 3 October 2004

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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doi:10.1016/j.msec.2004.08.046

[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.

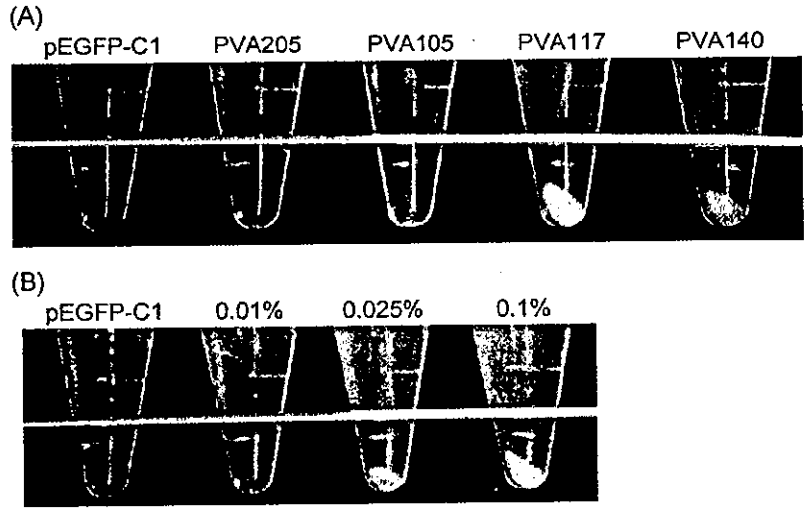

Fig. 1. Microphotographs of mixture solutions of DNA and (A) various PVAs of 0.1% concentration and (B) PVA117 of different concentration treated by UHP.

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

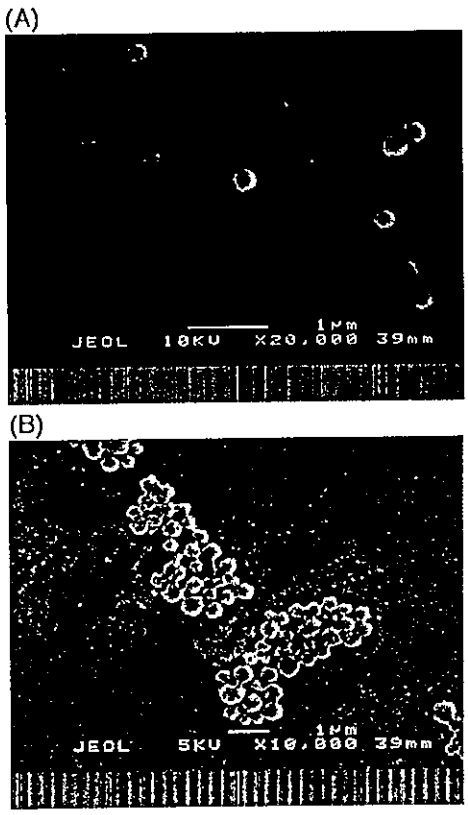

Fig. 2. SEM images of PVA–DNA nanoparticles. PVA117 was used.

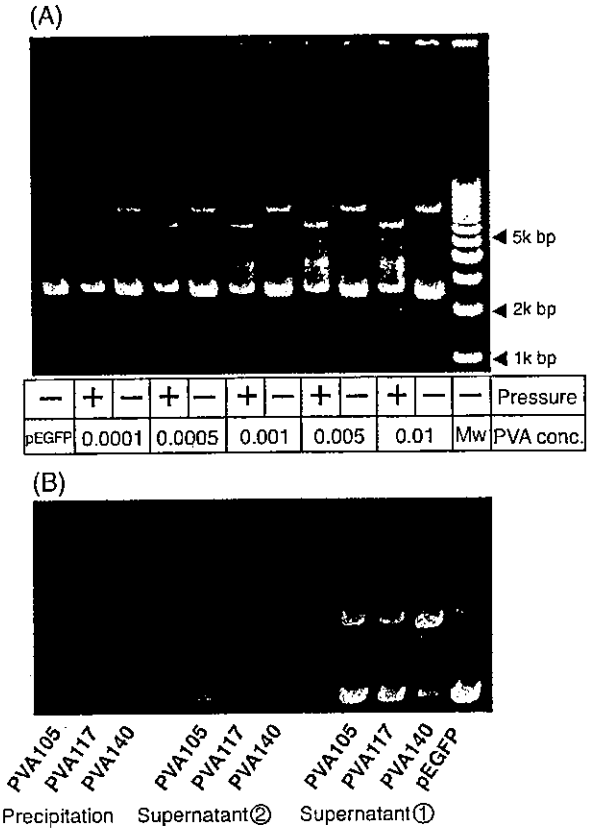

Fig. 3. Agarose gel electrophoresis of (A) PVA117-DNA nanoparticles prepared at 0.01% concentration and (B) the aggregates of PVA–DNA nanoparticles at 0.1% concentration after heat treatment.

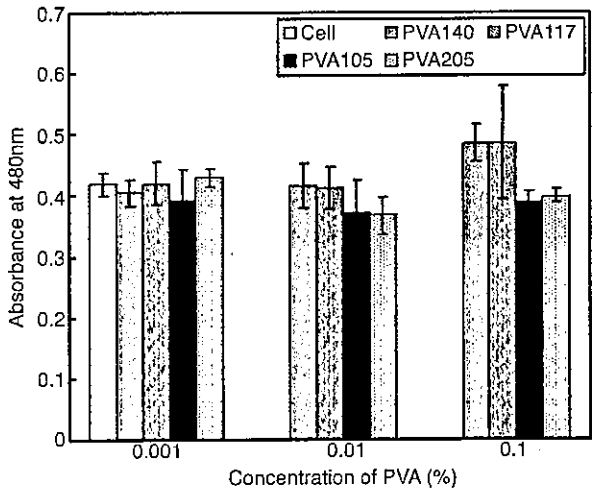

Fig. 4. Cytotoxicity of PVA–DNA nanoparticles.

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

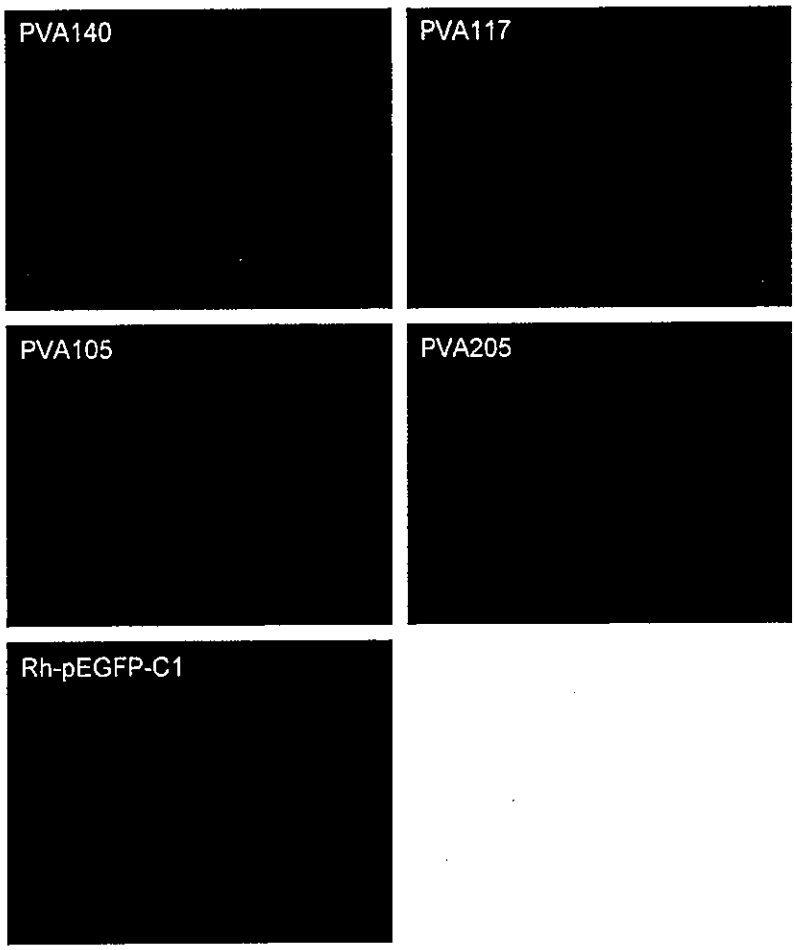

Fig. 5. Fluorescent images of Raw264 incubated with the nanoparticles of PVA and rhodamine-labeled pEGFP-C1 for 24 h.

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.

Acknowledgements

This work was supported by a grant from the Ministry of Health, Labour, and Welfare (MHLW). We thank Kuraray, for supply of polyvinyl alcohols.

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Preparation and Recellularization of Tissue Engineered Bioscaffold for Heart Valve Replacement

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Summary. Tissue engineered grafts based on polymeric or acellular xenogeneric matrices have been widely studied, and found to have greater durability and functionality with growth potential and less immunogenicity than current bioprostheses. On the other hand, there are still several problems to be solved such as degradation control of biodegradable polymeric scaffolds and unwanted transfer of unknown animal related infectious diseases. In this chapter, our novel tissue processing of decellularization named PowerGraft by ultrahigh pressure treatment for safe tissue transplantation is reported. Porcine heart valves were isolated under sterile conditions and treated by cold isostatic pressing (CIP) at 4°C for disruption of donor cells. The cell debris was then washed out in PBS under microwave irradiation at 4°C. The tissues were completely cell free when they were treated by a CIP of 980 MPa (10,000 atm) for 10 min. There was no porcine endogeneous retrovirus (PERV) detected in the treated tissue. There were no significant changes in biomechanical properties of breaking strength and elastic modulus. From the in vitro incubation test, the tissues were disinfected when CIP was applied to the tissues contaminated by normal bacteria floras. The endothelial cells were well seeded on the acellular bioscaffold by the roller and circulation culture systems sequentially. This PowerGraft processing may provide a more durable and safe bioscaffold for tissue transplantation.

Key words. Scaffold, Acellular tissue, PERV, High pressure, Microwave

Introduction

The artificial heart valve is one of the most clinically used artificial devices applied to about 300,000 patients per year worldwide, whereas it has still several shortcomings should be solved. The mechanical valve made of the pyrolytic carbon has good durability that might be longer than the patient's life time, however it has poor biocompatibility due to blood coagulation and patients must take an anti-coagulant drug under strict regulations throughout the rest of their lives. This drug, warfarin, is teratogenic and the female patient who wants to have a baby can not receive a mechanical valve. The xenograft valve made of the chemically crosslinked porcine valve or bovine pericardium in order to minimize the host's immune reaction has good biocompatibility, and hemodynamics, and is resistant to infections. The use of the xenograft valve is on the increase since it is superior to the mechanical valve in the quality of life, because it does not require any administration of an anti-coagulant drug. However, the durability of the xenograft is shorter than the mechanical valve, being about 15 to 20 years in elderly and 5 to 10 years in younger patients, due to calcification of the glutaraldehyde-fixed animal tissue. It is recommended that the xenograft should be used for the elderly patient over 65 years old in the guidelines of the American Heart Association and the American Association of Thoracic Surgeons.

Thanks to the establishment of tissue banks in this decade, some patients have had their defective tissues (heart valve, blood vessel, skin, and bone) replaced with cryopreserved donated tissue from a cadaver, rather than the current imperfect artificial devices.

The cryopreserved allograft valve, referred as the homograft valve, is clinically available in many countries and has been reported to have good clinical results. The homograft valve has the advantages of better biocompatibility compared to the mechanical valve, in durability to the xenograft valve, and in resistance to infections the both valves. However, the limitation on homograft valve availability might never be improved even in the

future. The Ross operation, in which the dysfunctional aortic valve is replaced by the patient's own autologous pulmonary valve and the homograft valve is implanted at the compromised pulmonary position, has been reported to have good clinical results especially in pediatric patients. The autologous tissue does not evoke an immune rejection and becomes bigger in size depending on the patient's growth. Since the other mechanical, xenograft, and homograft valve remains as an exogenous material in the patient's body and never grows, the pediatric recipients must have multiple operations through their lives.

To overcome these shortcomings in the current mechanical and biological heart valves, many research groups have been developing tissue engineered (TE) heart valves with properties similar to autologous valve tissue. Since the TE valves might be substituted by the host cells and tissues after the transplantation, the recipients can enjoy their good biocompatibility, durability, and growth potential.

TE heart valve

For the recovery of defected tissues, substitutional scaffolds must be implanted for tissue regeneration. There are two approaches that allow the scaffold materials to realize the TE tissues. One approach is using artificial biodegradable polymeric materials such as polylactic acid, polyglycolic acid, and polycaprolacton. Prof. Shin-oka and his group have reported successful clinical experiences of about 50 patients implanted with TE blood vessels made of the polyglycolic acid seeded with the patients' autologous bone marrow cells (see the chapter by Shin-oka T, this volume). However, the biodegradable polymeric materials are generally stiffer than the native biological tissues and do not easily take the same shape and structure as the biological tissues. Especially for aortic heart valve replacement, the scaffold requires flexible mechanical properties and strict degradation control for sufficient strength against the blood pressure.

The other approach is using acellular tissues for the scaffold as described in this chapter in which the cells and antigen molecules are removed to diminish the host tissue reaction. The acellular scaffold may have the same structure and composition as the natural tissue and be regu-

lated by interaction with the host tissue cells. CryoLife, Inc. (Kennesaw, GA) is the first company that provided acellular heart valves and blood vessels both from allogeneic and xenogeneic tissues. This company obtained a patent for the decellularization process using the gentle enzymatic treatment named SynerGraft® technology in 1994 and put the decellularized porcine heart valves (SynerGraft® Heart Valve, Model 700) on the market of Europe in 2001. It was reported in 2001 that they were successfully repopulated in a few months after the transplantation (Elkins et al. 2001). Whereas the multicenter clinical outcomes in Europe and Australia for the reconstruction of the right ventricular outflow tract in pediatric patients from 2001 to 2002 showed that only 7 of 19 valves remained implanted and clinically functional at the last follow-up, with 4 deaths (Simon et al. 2003 and R. Chard et al. 2004). On the other hand, the multicenter registry of the decellularized allograft both of pulmonary and aortic valves (CryoValve® SG) in USA from 2000 to 2003 demonstrated excellent clinical performance with more than 92% patient survival after 2 years transplantation (Clarke et al. 2004). Factors on the failure in the xenogeneic decellularized valves were not clear but were presumed to be mainly the result of the processing methods and remaining xenogeneic cell debris inside the tissue. Prof. Konertz and his group in Germany have also started clinical trials of the porcine pulmonary heart valves decellularized by the sodium-deoxycholate named AutoTissue technology in Ross procedure since 2002 (Dohmen PM, et al. 2002). They have reported that the pulmonary grafts named Matrix P showed excellent postoperative results with only 1 death in more than 120 patients and no functional failure and calcification in the grafts (Konertz WF. 2004). Prof. Haverich and his group in Germany have started clinical study too on decellularized allograft valves seeded by the patients' endothelial progenitor cells from 2002 and reported successful results (Teebken OE, et al. 2003 and S. Cebotari et al. 2004). They have been using the detergent Triton® X-100 (Bader A, et al. 1998) or the enzyme trypsin as the agent for the decellularization. In addition, there are several research groups developing acellular heart valves such as Prof. Ingham (Booth C, et al. 2002) in England and Prof. Stock (Schenke-Layland K, et al. 2003) in Germany.

We have been developing acelluar scaffolds for heart valve, blood vessel and trachea made of porcine tissue and their patients' autologous recellularization in vitro for the custom-made tissue transplantation since 2000 (Fig. 1). The scaffold with autologous cells may be replaced by the host tissue by the remodeling process regulated by the surrounding cells through digestion of the scaffold matrices and production of the autologous extracellular matrices. After the remodeling had been completed, the implanted tissue may be identical with an original autologous tissue and may have growth potential. Also, the recellularized grafts may enhance the functional performance such as anti-coagulation and anti-calcification in the early stage of the postoperation.

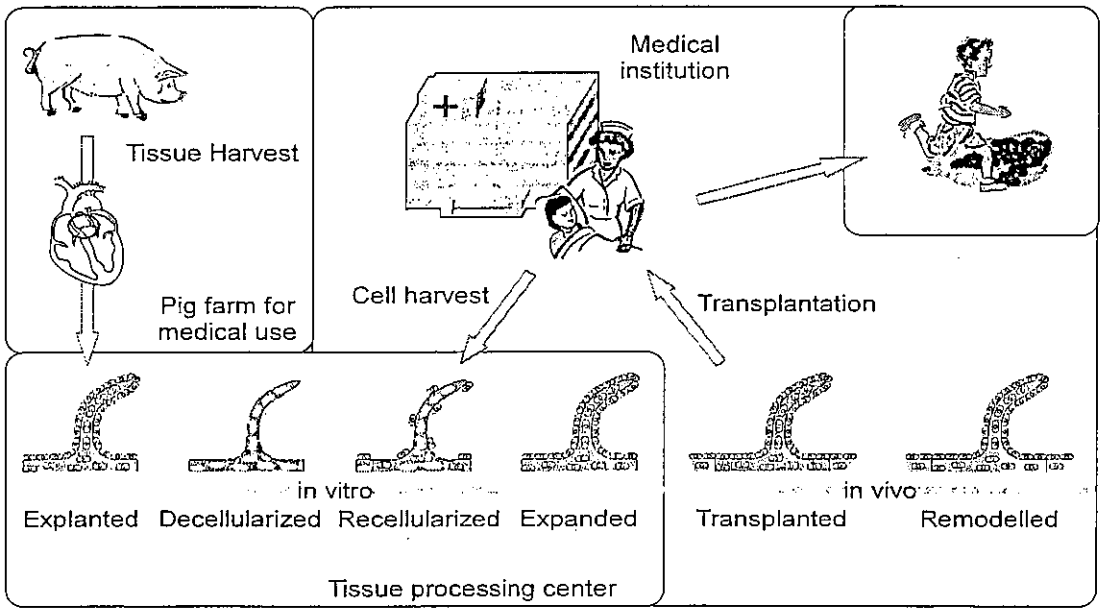

Fig. 1. Custom-made tissue transplantation.

Power Graft technology

All of the above groups are using detergents and/or enzymes as decellularization media such as Triton® X-100, sodium dodecyl sulfate, deoxycholate, trypsine, DNase, and RNase. We have started to investigate decellularization of porcine heart valves using Triton® X-100 and found that the cells in the cusps were unstained by H-E staining after 6 hrs treatment,

however cells still stained deep inside the basal tissue of cusps even after 24 hrs incubation regardless of the detergent concentrations of 0.5%, 1% and 2.5% (Fig.2). Under scanning electron microscopy, gaps between endothelial cells were observed after more than 3 hrs of treatment, however residues of the endothelial cells on the basal membrane were still attached. Since the detergents are generally cytotoxic and it takes time for their removal before the transplantation and cell seeding, it may lead to denature of biological properties and contamination in the process. Recent BSE (Bovine Spongiform Encephalopathy) and vCJD (variant Creutzfeldt-Jakob disease) issues have been affecting tissue transplantation from the point of view of safety. Especially if the scaffolds are prepared from animal tissues, the animal cell components must be removed completely for the prevention of unknown transfer of animal related infectious diseases. In addition, if the tissue source is porcine, the removal of porcine endogenous retroviruses (PERVs) that have ability of infection to the human cells in vitro must be validated (Magre S, et al. 2003). However, it is not easy to remove the cell components completely in the decellularization process by the detergent and proteinase as described above because of the limited permeability of the agents.

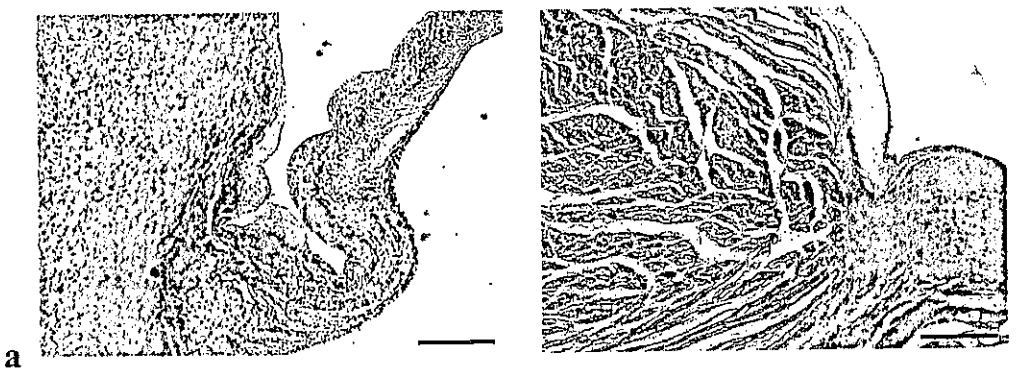

Fig. 2a. b. Decellularization of porcine heart valve basal tissue by detergent. **a** Native. **b** Treated by 1% Triton® X-100 for 24 hrs. Scale bars are 500 μm.

We have introduced a novel decellularization process to create a safe tissue scaffold by the combination of ultrahigh pressure treatment of the cold isostatic pressing (CIP) and washing under the microwave irradiation named PowerGraft technology. During CIP, when fluid pressure is added to the material enclosed in a pressure vessel, the whole surface of the ma-

terial will receive the pressure evenly which is equal to the fluid pressure and then compressed without flattening. This CIP technology has already been established in the food industry and Meijiya Food Co., LTD, Japanese Jam factory, has already commercialized the world first food processed by the CIP in 1990. It has been reported that the functional proteins are denatured by pressing at about 300 MPa and the most of the viruses like Human Immunodeficiency Virus are inactivated at more than 600 MPa (Hayashi R. 2002).

The porcine aortic and pulmonary valves, aorta, and trachea were isolated from 6 month-old Clawn miniature pigs (Japan Farm Co. Ltd, Kagoshima, Japan) weighing about 15 kg under sterile conditions. The harvested tissues were washed and packed in sterile bags filled with PBS. The packed tissues were treated by ultrahigh pressure at 4 °C using a CIP apparatus (Kobe steel LTD, Kobe, Japan). They were then washed by PBS under microwave irradiation at 4 °C (Azumaya Medical Devices Inc., Tokyo, Japan) for accelerated removal of the residues of the broken cells from the CIP treated tissues. H-E staining of the cusps of porcine aortic heart valve showed that the tissues were completely cell free when the CIP of 970 MPa was applied for 10 min and washed under microwave irradiation for 5 days. The pulmonary valve, aortic tissue, and trachea were also completely cell free even in the cartilage tissue of the trachea (Fig.3). We have chosen the Clawn miniature pig as a donor animal since its size adapts to human tissues well and its genome has been well studied in order to develop a human gene induced transgenic animal for organ transplantation. There was no PERV products detected in a PCR assay from the aortic and tracheal tissues processed by the CIP, whereas it was still detected in the tissue treated by Triton® X-100 after 24-hr incubation (Fig. 4). Tissues pre-contaminated by the normal bacteria floras were decontaminated when treated at more than 485 MPa. There were no significant changes in biomechanical properties in terms of the breaking strength and elastic modulus of the leaflets treated at 970 MPa for 10 min. This was supported by elastica-van Gieson staining, which showed collagen and elastin fibers were well maintained in the bioscaffold tissue decellularized by the CIP. The effect of microwave irradiation is the same as the appliance of conventional microwave oven using the vibration of water molecules at 2,450

MHz. The principle of accelerating the washing time by microwave irradiation is still unclear, but it is presumed that the high-speed motion of water molecules and enhances the permeation of the tissues. It is not necessary to use the microwave for washing after the CIP treatment, however it makes washing time about one tenth of that compared to conventional incubation.

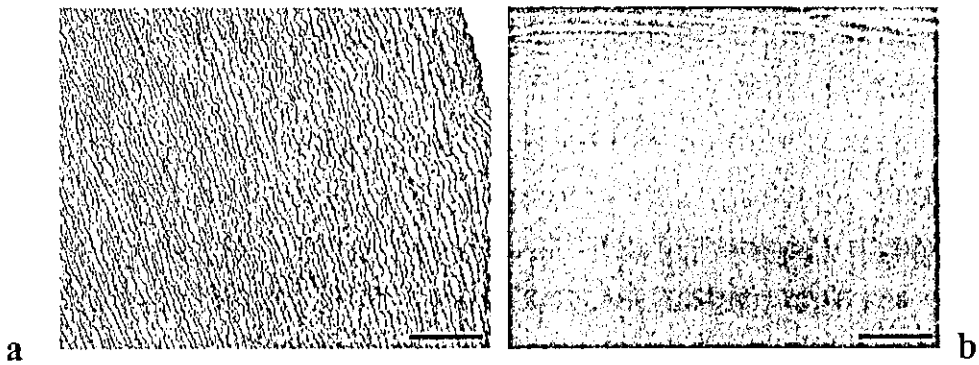

Fig. 3a. b. Decellularization by Power Graft technology using CIP at 980 MPa for 10 min. **a** Aorta. **b** Trachea. Scale bars are 100 μm.

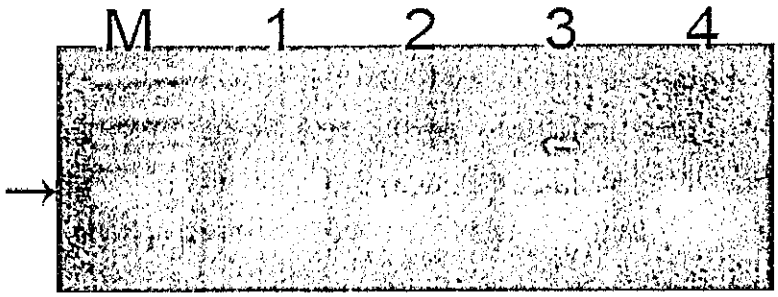

Fig. 4. PERV assay on the decellularized heart valves. (M: Marker, 1: Native, 2,3: 1% Triton® X-100 for 24 hrs, 4: PowerGraft of 980 MPa for 10 min)

Recellularization of Power Graft in vitro

The native heart valve tissue is fully covered with endothelial cells and is occupied mainly by smooth muscle cells. As described above, the incorporation of autologous cells to the acellular scaffold may maintain physiological activity and prevent calcification at the graft site during the early

stage. Endothelial cell seeding on the acellular scaffold was studied. Generally, the cell seeding onto the culture dish is achieved by a static incubation of the cell suspension in about a few hours. It is not difficult to seed the cells on the inner surface of a tubular body like blood vessels by the rolling of the body at a low speed continuously or intermittently in the cell suspension. Since the heart valve has relatively complicated three dimensional cusp surfaces, it is not easy to seed the cells uniformly and completely on them by a simple roller culture. Some research groups have reported pulsatile circulatory bioreactors and/or multi-rotation axes bioreactors for cell seeding and expansion on the heart valve scaffolds (Zeltinger J, et al. 2001, Laube and Matthaus 2001, and Hildebrand DK, et al. 2004). Prof. Umezu and his group have reported a combined bioreactor with multi-axes and a circulation culture for decellularization and endothelial cell seeding sequentially (Iwasaki K. 2004). We have developed a simple double axes roller culture system using a bottle roller for the cell seeding on the valve scaffold and a circulation culture system using a blood pump and gas exchanger for their expansion. Porcine endothelial cells were isolated from the femoral artery of a future recipient by collagenase digestion. After 3 weeks in vitro expansion of the cells, the endothelial cells were suspended and seeded by the roller culture system for 4 hrs. The cells were then expanded in the circulation culture system for 5 days. The autologous endothelial cells were well seeded onto the three dimensional surfaces of the PowerGraft heart valve by the culture systems (Fig. 5).

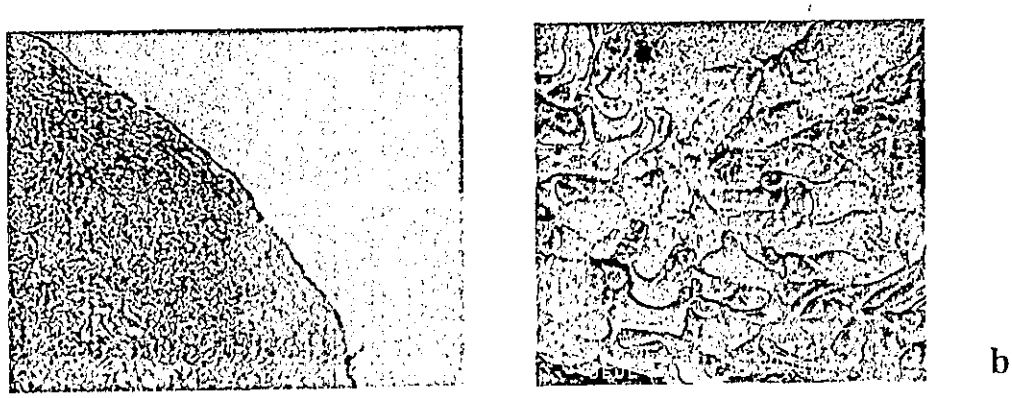

Fig. 5a b. Endothelial cells seeded on the PowerGraft heart valve by the roller and circulation culture systems. a H-E. b SEM.

Preliminary Animal study of Power Graft

Some research groups have already put their TE heart valves to clinical trials. The allogeneic transplantation study of the PowerGraft aorta and pulmonary heart valve was continued in a porcine model as a pre-clinical study. The morphological and histological changes after the implantation were evaluated in the aorta model without cell seeding, because it seemed the strength and calcification of the acellular tissue in the artery was more critical than the pulmonary tissue. The decellularized porcine aorta was implanted at the descending aorta in the Clawn miniature pig through a left thoracotomy. Surgery was carried out with single clamp technique and the animals were sacrificed at 4 weeks and 12 weeks after the implantation. The explanted grafts were examined histologically and immunohistologically. There was no dilatation and no aneurysmal change and the explanted grafts showed no macroscopic abnormality including their anastomoses. The inner surface was smooth and had no thrombus formation. Cell infiltration was identified at 4 weeks dominantly on the outer side and intimal thickening was observed at 12 weeks. The luminal surfaces of the aorta were completely covered with endothelial cells at 4 weeks after the implantation (Fig. 6). These results are very encouraging to produce durable and safe TE heart valves.

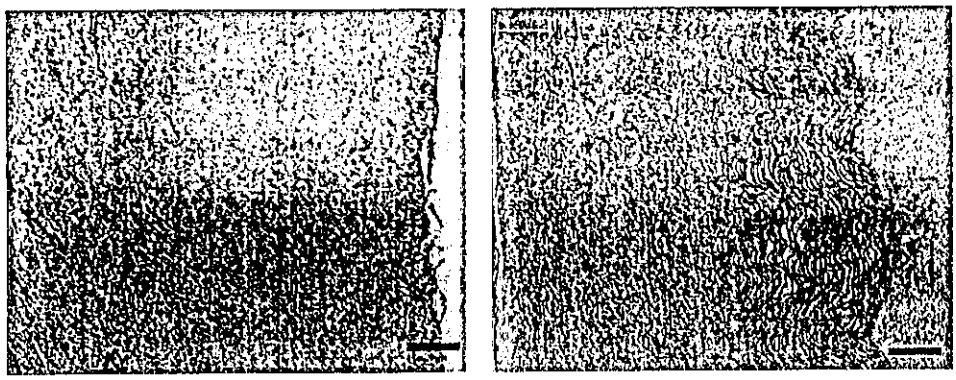

Fig. 6a. b. Explanted aortas decellularized by PowerGraft technology without cell seeding after the allogeneic transplantation in the miniature pig model. **a** 4 weeks and **b** 12 weeks after the transplantation. Left side is the outer side of the implanted graft. Scale bars are 200 μm.

Conclusion

There have been a lot of medical devices developed that still require innovation in many areas and are unable to give growth activity to the current artificial devices. In the heart valves, limitations on homograft valve availability require the need for a better clinical option for the patient and surgeon especially with respect to the pediatric patients because of the limited outcomes of current artificial heart valves. We are developing custom-made tissue transplantation in which patient's autologous cells are seeded on and in the appropriate scaffold for defective tissues of heart valves, blood vessels, pericardium, trachea, esophagus, and dura mater. Our novel decellularization method of PowerGraft was developed to produce a safe bioscaffold by ultrahigh pressure treatment of the CIP and washing under microwave irradiation. Porcine cells and PERV were removed completely from the animal tissues in a short period by the CIP of 980 MPa without changing the biomechanical properties. These findings suggest the tissues treated with CIP can be used as a safe bioscaffold, even if based on xenogenic tissues that have risks of unknown animal related diseases. We are currently studying autologous cell seeding on and in the scaffold prepared by PowerGraft technology and their applications in animal experiments. These acellular tissues are going to be put into clinical study in the near future. The TE heart valves might be substituted for the current artificial heart valves in the future.

Acknowledgement

This study was supported by the Research Grants from Ministry of Health, Labour and Welfare and Ministry of Education, Culture, Sports, Science and Technology of Japan.

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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.

The Journal of Heart Valve Disease 2004;13:984-990

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

Presented as a poster at the Second Biennial Meeting of the Society for Heart Valve Disease, 28th June-1st July 2003, Palais des Congrès, Paris, France

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