[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.

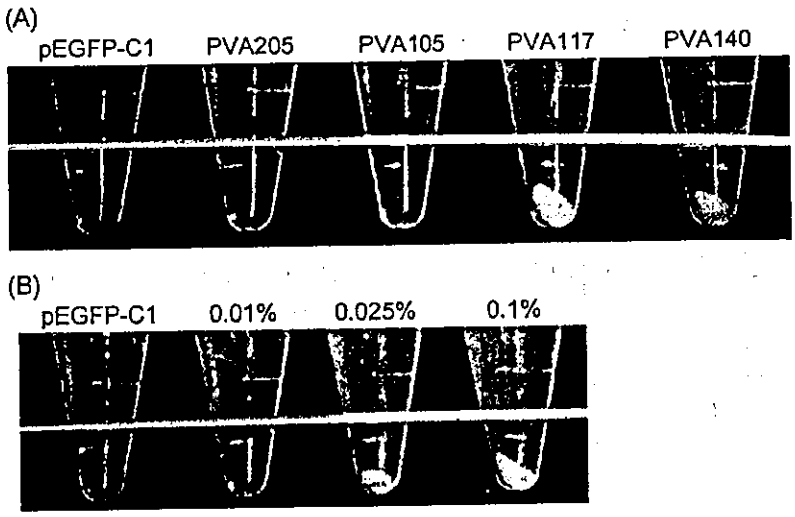

Fig. 1. Microphotographs of mixture solutions of DNA and (A) various PVAs of 0.1% concentration and (B) PVA117 of different concentration treated by UHP.

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

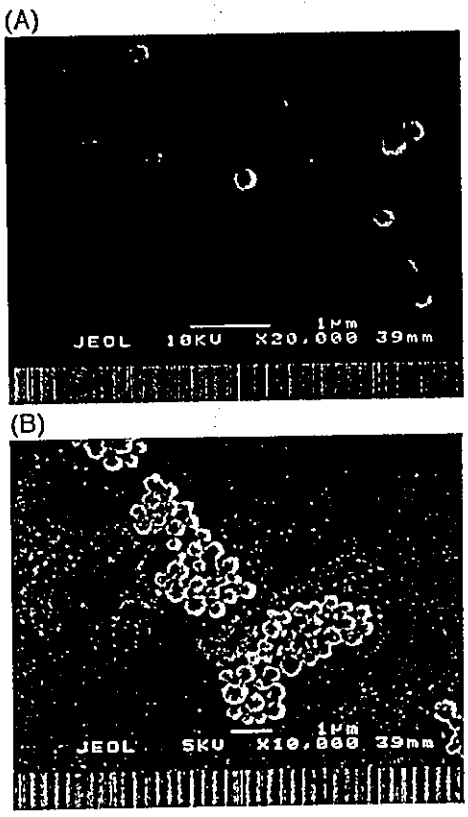

Fig. 2. SEM images of PVA–DNA nanoparticles. PVA117 was used.

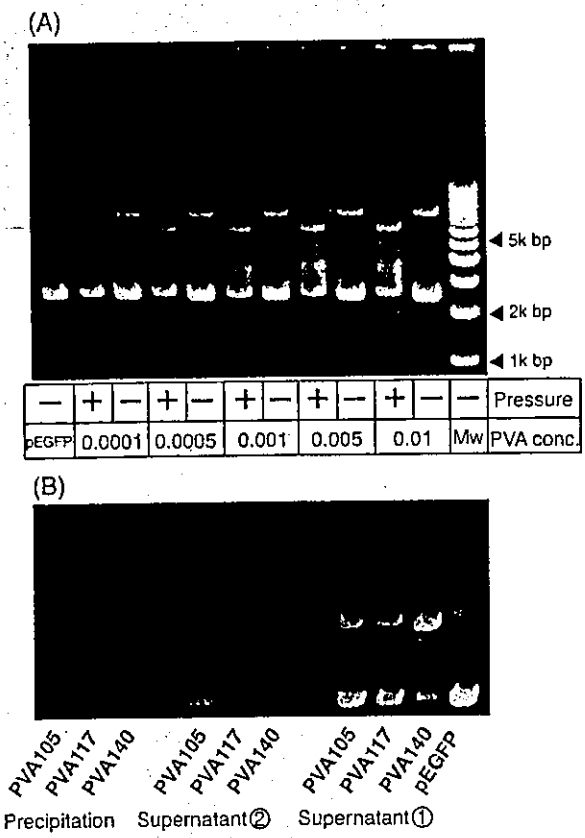

Fig. 3. Agarose gel electrophoresis of (A) PVA117-DNA nanoparticles prepared at 0.01% concentration and (B) the aggregates of PVA–DNA nanoparticles at 0.1% concentration after heat treatment.

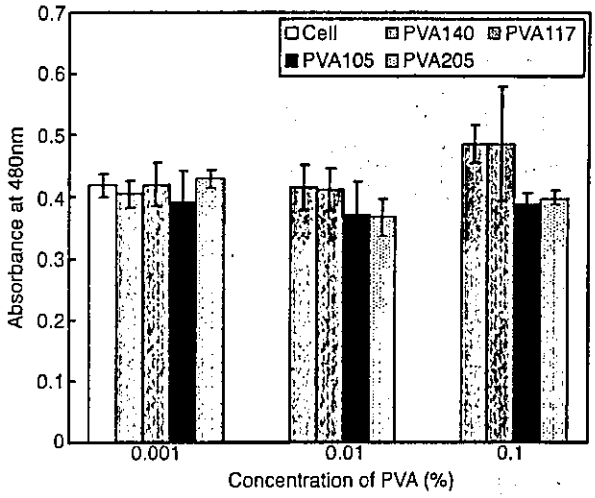

Fig. 4. Cytotoxicity of PVA–DNA nanoparticles.

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

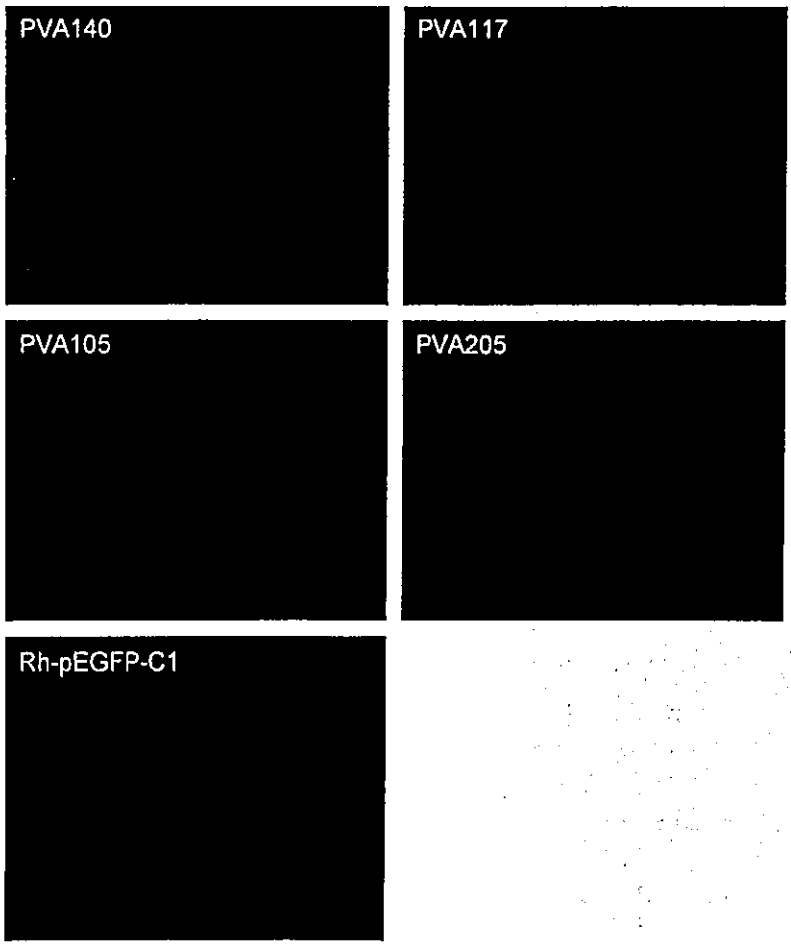

Fig. 5. Fluorescent images of Raw264 incubated with the nanoparticles of PVA and rhodamine-labeled pEGFP-C1 for 24 h.

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

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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.

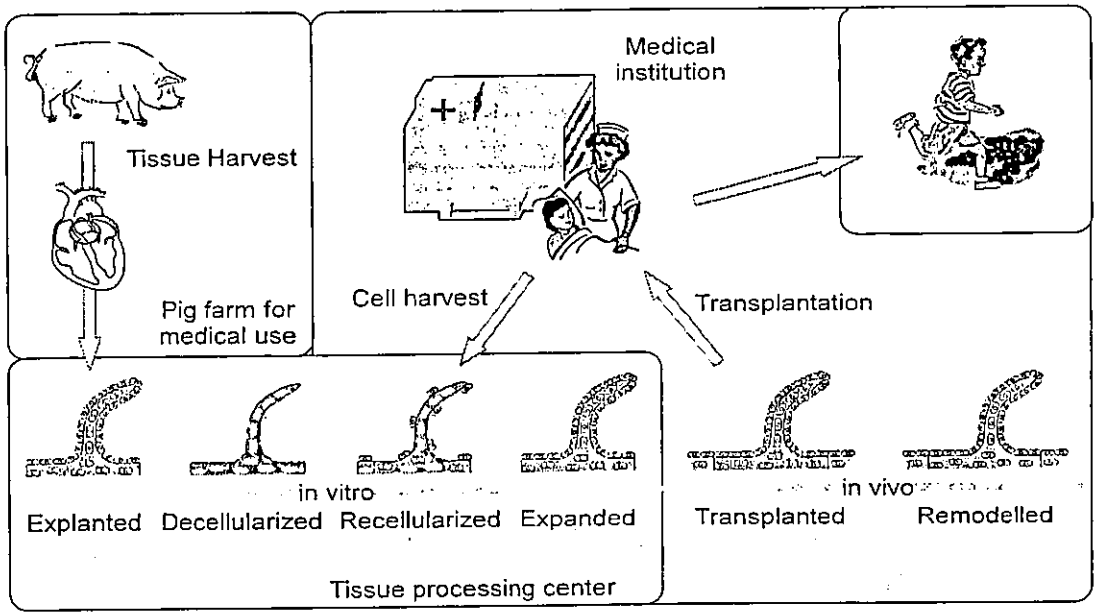


Fig. 1. Custom-made tissue transplantation.

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.

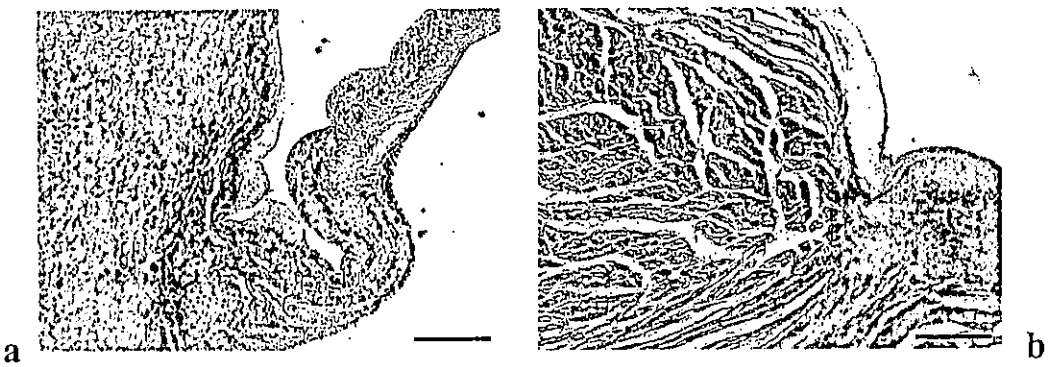


Fig. 2a. b. Decellularization of porcine heart valve basal tissue by detergent. **a** Native. **b** Treated by 1% Triton® X-100 for 24 hrs. Scale bars are 500 μm.

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.

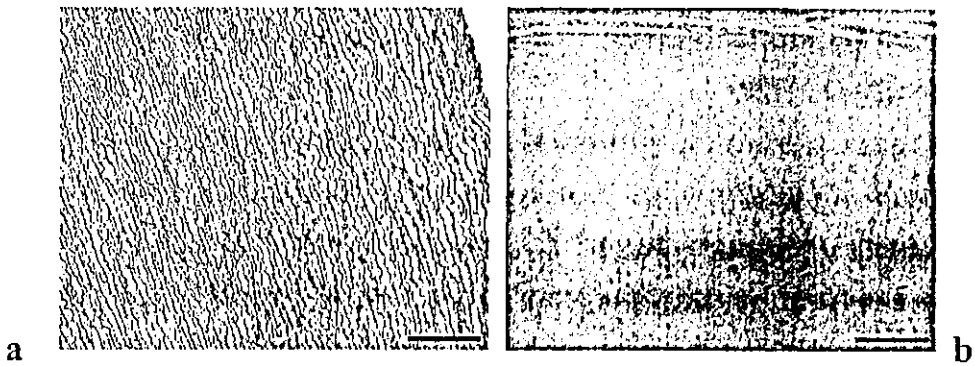


Fig. 3a. b. Decellularization by Power Graft technology using CIP at 980 MPa for 10 min. **a** Aorta. **b** Trachea. Scale bars are 100 μm.

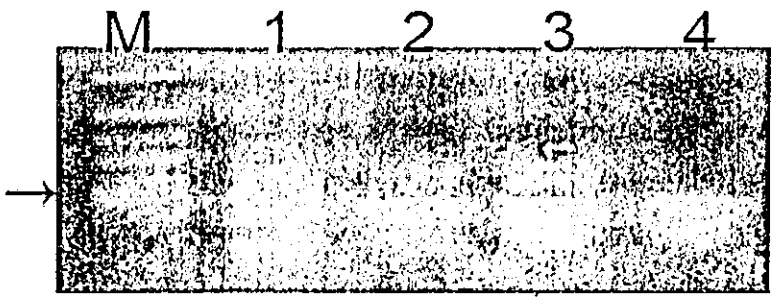
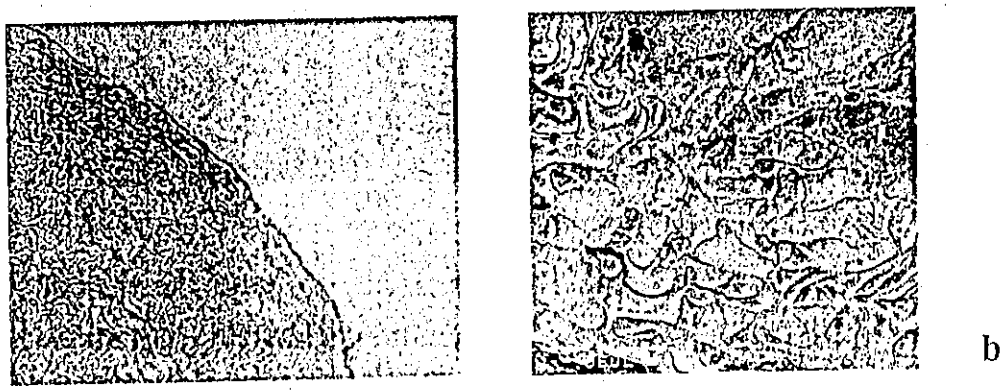


Fig. 4. PERV assay on the decellularized heart valves. (M: Marker, 1: Native, 2,3: 1% Triton® X-100 for 24 hrs, 4: PowerGraft of 980 MPa for 10 min)

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).



a b

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.

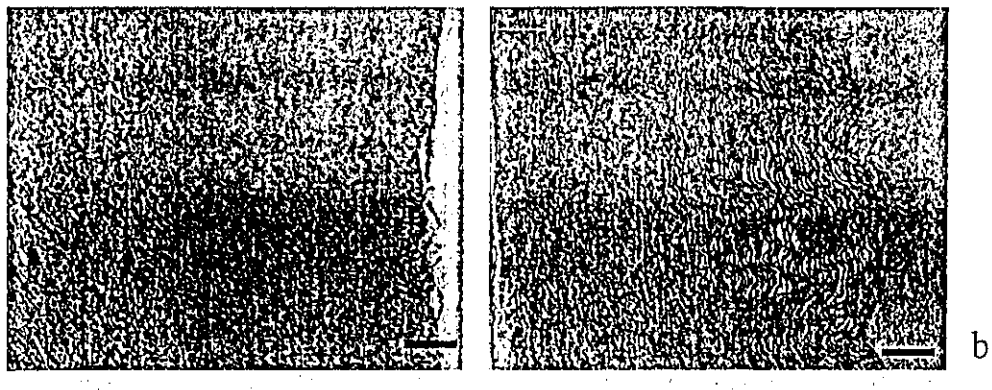


Fig. 6a. b. Explanted aortas decellularized by PowerGraft technology without cell seeding after the allogeneic transplantation in the miniature pig model. a 4 weeks and b 12 weeks after the transplantation. Left side is the outer side of the implanted graft. Scale bars are 200 μm.

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.

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

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

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

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

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

Materials and methods

Cryopreservation

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

Decellularization

Four of the six harvested grafts were decellularized. After thawing, the grafts were immediately stored in Hank's balanced solution (HBSS; Biochrom, Berlin, Germany), and maintained at 4°C. The graft was then placed in a solution of 1% tetra-octylphenyl-polyoxyethylene (Triton X; BioRad, Germany) with 0.02% EDTA in phosphate-buffered saline (PBS; Bichrom, Germany), for 24 h, with RNase (20 μg/ml; Boehringer, Mannheim, Germany) and DNase (0.2 mg/ml). The graft was washed several times with PBS and then stored in a 5% CO_2 incubator, at 37°C, with continuous shaking.

Cell harvesting and seeding

Autologous endothelial cells were seeded onto two of the four decellularized grafts before implantation. At 21 days before implantation, the femoral artery was harvested from a recipient pig under local anesthesia. A 23 Fr cannula was inserted into each side of the artery. EBM2 (Clonetics, MD, USA) was injected, and both cannulas were affixed to a Petri dish filled with HBSS and incubated in a 5% CO_2 incubator, at 37°C for 20 min. The artery was washed through with 50 ml 10% fetal bovine serum (FBS; Life Technologies, Germany). Endothelial cells were then pelleted by centrifugation for 5 min at 3,000 rpm (190 × *g*). The endothelial cells were subsequently resuspended in 5 ml of culture medium containing endothelial cell growth factor. During endothelial cell culture, the medium was changed every three days. After 14 days of cell expansion, the cells were detached with 0.05% trypsin (Biochrom) and 0.02% EDTA (Sigma) in PBS. Following resuspension of the endothelial cells in culture medium (EBM2), a 20 ml volume of culture medium, containing ~5×10^6 endothelial cells, was placed into a centrifuge tube. The decellularized valve was immersed in this tube and, for 48 h, static reseeding of the valve surface was performed in a 5% CO_2 incubator, maintained at 37°C. Following this procedure, the valve was removed from the tube and soaked in EBM2 for 48 h.

Surgical technique

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

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

Postoperative evaluation

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

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

Macroscopic findings

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

Echocardiography

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

Direct pressure measurement

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

Histology

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

Immunohistochemistry

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

Results

Postoperative course

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

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

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

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

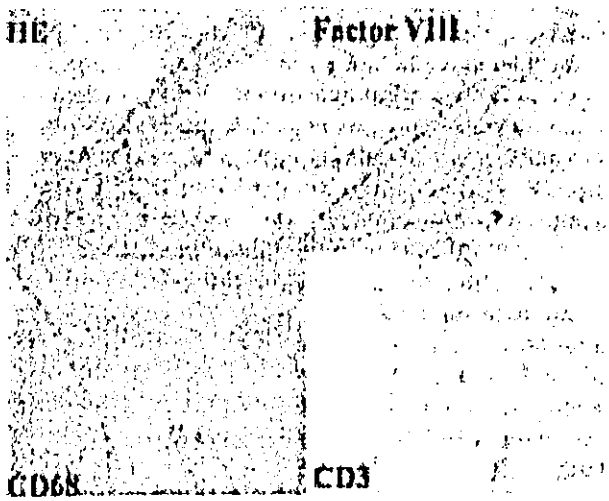

Figure 1: Histological and immunohistochemical findings of pig #3 (cryopreserved and decellularized graft; not cell-seeded). The cusp was covered with confluent endothelial cell lining. These cells were positive for von Willebrand factor, as a marker of endothelial cells. At the valve interstitium there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

Macroscopic findings

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

Echocardiography

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

Direct pressure measurement

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

Histology

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

Immunohistochemistry

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

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

Table II: Histological evaluation of the pigs.

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

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