Workshop in Warsaw university of technology, April 1,2008

Title :

Research activities of Biofunctional materials group

- **Nanofiber scaffold for early neovascularization**
  - ***Nanofiber sponge scaffold**
    collaborate with Tokyo University
  - ***Quantitative analysis of cell adhesion on aligned micro- and nanofibers**
- **Challenge to develop the reliable artificial corneal stroma**
  - ***Study on PVA based artificial cornea**
    Collaborate with Keio University, Tokyo dental university
  - ***Decellularization of cornea using ultra-high hydrostatic pressure treatment for corneal scaffold**
    collaborate with Tokyo Medical & Dental University

ワルシャワ工科大学でのワークショップ

演題：高次機能生体材料グループの研究活動

早期血管化のためのナノファイバー足場

＊ナノファイバースポンジ （東京大学との共同研究）

＊配向したマイクローナノファイバーに対する細胞接着の定量的評価

信頼性の高い人工角膜実質の開発を目指して

＊PVA を基材とした人工角膜の研究

＊超高静水圧を利用した脱細胞化角膜足場材料

に関して報告した。

Top down and bottom up approaches to develop reliable artificial cornea

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In the past 60 years, several groups have attempted to develop reliable artificial cornea, but still not yet developed completely satisfactory level. . In general, clinically available synthetic devices do not support an intact epithelium, which poses a risk of microbial infection or protrusion of the prosthesis. Previously we have found that the immobilization of Type I collagen on the poly(vinyl alcohol)(PVA) hydrogel disc was effective in supporting adhesion and growth of the corneal epithelium and stroma cell in vitro. And this was very stable and highly compatible in the corneal stroma. But the durability of the produced corneal epithelium layer on the PVA-disc in vivo has some problem. The epithelium on the PVA hydrogel discs could not produce basement membrane in vivo. We concluded the permeability of nutrition and some biological factors are not achieving the enough level in the shape of hydrogel disc structure. It is thought that stroma of cornea forms a clear frame because fibril of collagen forms standardized cancelli. The ideal structure seems to produce the sufficient permeability as well as the transparency. Currently, we try to make the mimic structure of the natural corneal stroma by utilizing the nanomaterials and nanotechnology(bottom up approach) and decellularization from porcine cornea by using ultra high pressure treatment(top down approach). In this presentation, recent outcomes from both approaches will be presented.

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Novel Decellularization Technique of the Cornea using Ultra-high Hydrostatic Pressure

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Introduction

Corneal transplantation is an established transplant surgery to reverse blindness due to corneal injuries and diseases, such as corneal dystrophy, bullous keratopathy and corneal scarring. The success rate is high compared to other forms of tissue transplantation, but many complications, such as infection, immune rejection and graft failure, still occur. Also, an absolute shortage of donor corneas has been a major problem in many countries. To overcome these problems, artificial corneas have been studied as alternatives [1]. Various biocompatible polymer materials have been investigated, some of which were recently approved by the FDA and clinical test data is accumulating. However, it has often been reported that such implants fail to significantly connect to corneal tissue and the recipient corneal cells do not expand on or in these materials, resulting in the extrusion of them through melting around the prosthetic rim [2].

We investigated the possibility of decellularized cornea as a novel artificial bio-cornea, which the cells and antigens are removed to diminish the host immune reaction but the biological structure is remained. In this study, we prepared some acellular cornea using ultra-high hydrostatic pressure (UHP) method, and then carried out the preliminary animal study of the decellularized cornea.

Materials and methods

Tissues.

Corneas were dissected out of oculars of freshly killed mature pigs and washed in phosphate-buffer saline (PBS) containing antibiotics and 3.5 % w/v dextran.

Decellularization of corneas by UHP method.

The corneas were pressurized at 10,000 atm at 10 °C for 10 min using a high-pressure machine (Kobe Steel Ltd.), washed by continuous shaking in an EGM-2 medium containing DNase I, antibiotics and 3.5 % w/v dextran at 37°C for 72hours, and then subjected to histological study (H-E staining).

Biochemistry.

Decellularized corneas were analyzed quantitatively by biochemical assays for residual DNA and GAG.

Characterization of decellularized cornea.

Decellularized corneas were investigated characteristics such as the transmittance, swelling ratio, strength.

Preliminary animal implantation study.

To examine the biocompatibility of decellularized cornea, they were implanted in rabbit corneal stroma. The animals were sacrificed at 8 weeks after implantation.

Results and Discussion

By H-E staining, the complete removal of epithelial and stromal cells was confirmed in all of the pressurized corneas. The super-structure of collagen fibrils were relatively maintained after the UHP decellularization. Also, when the decellularized porcine cornea was implanted in a rabbit cornea, vascularization and inflammatory reaction were not observed, suggesting the decellularized cornea obtained through the UHP method could be useful as a corneal scaffold for tissue regeneration. From these result, it suggested that this method could be used as one of decellularization method of the other tissues or organs.

Figure.1 H-E staining of native and decellularized cornea by UHP treatment.

Conclusion

We have successfully developed a corneal decellularization method that uses UHP technology. The superstructure of the acellular cornea was relatively preserved. This decellularization method appears to be a promising contribution with regard to corneal replacement and tissue engineering of the cornea.

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Ultra-structure analysis of decellularization of cornea using ultra-high hydrostatical pressurization treatment

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Introduction

In order to recover from corneal blindness, corneal transplant surgery is needed. But, tissue transplantation has many problems, such as infection and immune rejection. Moreover, donor shotage of corneas has been a major problem in many countries. So, artificial corneas were studied by various approaches. Synthetic biocompatible polymers, such as polymethyl methacrylate, poly(2-hydroxyethyl methacrylate), and poly(vinyl alcohol) were tested for artificial cornea. Unfortunately, implanted materials were finally excruded. Some reports suggest that corneal tissue was regenerated through a combination of cornea cells and collagen scaffold [1]. But reported collagen scaffold has low mechanical strength, so not adequate for clinical applications. Acellular cornea scafforld seems to natural cornea same structure without host cells and antigen molecules. Therefore, acellular cornea scaffold was expected to infiltrate the donor cells into the scaffold and regenerate the tissues. Generally decelluratization were undergone using detergents, such as Triton® X-100, sodium dodecyl sulfate (SDS) [2] and sodium deoxycholate. However, detergent decellurarization need wash out process because of its cytotoxicologies and washing process may be denatured biological propaties. Therefore, we made acellular cornea scaffold using ultra-high pressure (UHP) method without detergents [3]. Our objective of this study is to investigate the ultrastructual difference of acellular corneas between UHP method and detergents methods

Materials and Methods

Cornea preparation: Entire porcurine corneas were explanted from the oculars and washed with phosphate-buffer saline (PBS) containing penicillin (100 units/ml) and streptomycin (0.1 mg/ml). The corneas were then stored in PBS containing antibiotics and dextran (3.5% w/v) at 4°C until the experiments.

Decellularization of corneas by the chemical method: 1% w/v solutions of Triton X-100 and of SDS were prepared. The cornea was immersed in one of these at 37°C for 24 hours, washed with PBS containing penicillin (100 units/ml) and streptomycin (0.1 mg/ml) for 24 hours. **Decellularization of corneas by the pressurization method:** The corneas were pressurized at 4,000 or 10,000 atm at 10 or 30°C for 10 min using a high-pressure machine (Kobe Steel Ltd.), washed by continuous shaking in an EGM-2 medium containing DNase I (0.2 mg/ml), antibiotics and 3.5 % w/v dextran at 37°C under 5% CO2, 95% air for 72 hours.

Ultrastructure of decellularization corneas: After decellularization, each cornea was fixed with glutaraldehyde and osmium tetraoxide. After fixation, tissues were enbedded with epoxy resin. 80nm thickness sections were obtained. Cutting sections were observed using transmission electron microscopy (LEO922, LEO Electron Microscopy Ltd., Cambrige, England).

Results and Discussion

Ultrastructual properties of decellularized corneas made by UHP method were resembled with natural cornea (Fig.1). Collagen bundles were observed by TEM picture. In addition, there were no host cells in the UHP decellurized corea. On the other hand, microstructual properties of detergents decellularized corneas were far from natural cornea (Fig.1). No collagen bundles were in it. The results indicated that UHP decellurized cornea might be had a potential ability for the scaffold to regenerate cornea tissue. More detail informations are now collecting.

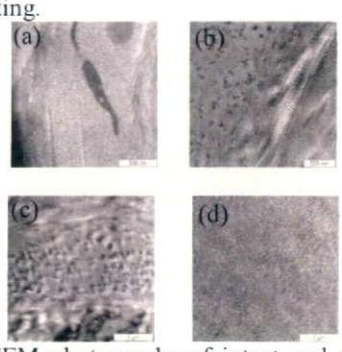

Figure.1 TEM photographs of intact and decellurized cornea. 10000 atm UHP corneas have collagen bundles: (a) intact cornea, (b) at 10000 atm for 10 min. (c) at 10000 atm for 30 min. (d) SDS detergents

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Preparation and Characterization of Collagen Gel Designed for Tissue Membrane

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Introduction: The construction of an extracellular matrix (ECM) using collagen has been performed by many researchers worldwide. Ever since Weinberg and Bell succeeded in preparing a blood vessel using collagen [1], diverse approaches using collagen gel to prepare an ECM had been executed. To use a collagen gel as a biomaterial, prevention of thrombus formation, and reinforcement of physical and biological properties is required. In this study, a novel cross-linking technique using ethanol/water co-solvent with *N*-(3-dimethyl-aminopropyl)-*N'*-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) is introduced. 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer, a well-known material for its blood compatibility [2], was immobilized on the collagen gel to develop a mechanically reinforced and biocompatible collagen hybrid gel [3]. With the EDC/NHS cross-linked collagen gel and the polymer-collagen hybrid gel, the physical and biological property was characterized so as to apply the collagen gel as a new type of tissue membrane .

Materials and Methods: Collagen film was fabricated (l=0.018mm) and was cross-linked with EDC and NHS in ethanol/water co-solvent series (0%→100%) for 24 hrs at 4°C to prepare an intrahelically cross-linked collagen gel (EN gel). Poly(MPC-*co*-methacrylic acid) (PMA) was cross-linked with collagen film using EDC and NHS in ethanol/water co-solvent series (0%→100%) ethanol:water=3:7 for 24 hours at 4°C to make a MPC-immobilized collagen gel (MiC gel) [4]. X-ray photoelectron spectroscopy (XPS) and scanning electron microscope (SEM) was used to characterize the surface of the hybrid gel. Swelling ratio, free amine group analysis, and mechanical test was used to characterize the cross-linking efficiency. Biological behavior was observed using cell adhesion test *in vitro* and tissue compatibility *in vivo*. Collagen gel that is intrahelically cross-linked with EDC and NHS (EN gel), and that is inter-microfibrillar cross-linked with glutaraldehyde (G-gel) was prepared by conventional method [5] to compare the physical and biological property of the collagen gel.

Results and discussion: In ethanol/water co-solvent, the triple helix of the collagen did not denature. We found out that the triple helix, indicating that the collagen gel would retain its original property after the cross-linking. ON the other hand, the polymer changed according to the ethanol concentration. The electronegativity of the polymer would shift toward neutral state as the ethanol concentration increases. This is because the carboxylate anoions would turn into carboxyl group, which is neutral. It is known that the ethanol would prevent the hydrolysis of EDC, but since the reactivity of the EDC is higher with carboxylate anion, it is very important to obtain the balance between the hydrolysis of EDC and the protonation of carboxylate anions.

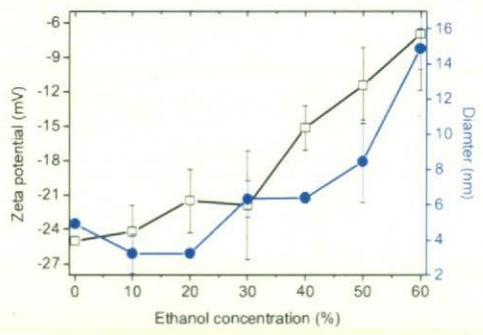

Figure 1. The zeta potential and the diameter change of the PMA according to the ethanol concentration.

Comparing the reacted amine group contents between EN gel and MiC gel, the cross-linking rate was slightly higher for EN gel. The highest cross-linking rate was shown when ethanol 30% co-solvent was used for EN gel and MiC gel. The strain-stress curve result showed that the viscoelastic behavior, or J-curve, appeared for the physically stabilized gel (Uc gel) and the EN gel, while the MiC gel showed brittle plastic behavior. This implies that the polymer-microfibril cross-linking is not a surface immobilization. The highest reacted amine group was recorded for G-gel. This is because the inter-microfibrillar cross-link could be achieved.

We chose EN gel and MiC gel which was prepared in ethanol 30% co-solvent to observe the bioloigical property of the collagen gels. The adhesion of the cell had decreased by the polymer cross-linking. Simple intrahelical cross-linking can also provide the decrease in the cell adhesion, but it did not suppress the proliferation of the cell. In the case of MiC gel, both cell adhesion and proliferation was suppressed. *In vivo* test, the chronic inflammatory reaction and capsulation for Uc gel and glutaraldehyde cross-linked collagen gel had occurred, but no significant inflammatory reaction for EN gel and MiC gel. In the short term and the long term, the capsule layer around EN gel and MiC gel shows the formation of the fibroblast and new collagen fibrils. This implies that the gel possesses inert property against the living tissue, prohibiting acute and chronic inflammatory reaction.

Conclusions: The high rate of MPC polymer was cross-linked with collagen microfibrils to make a collagen/polymer hybrid gel. It was stable in the harsh environment. The mechanical strength increased by the cross-linked network. Possession of MPC polymer on the surface suppressed the adhesion of the cell *in vitro* and inflammatory reaction *in vivo*. Using this technique, it is possible to prepare an tissue membrane which is stable and anti-inflammatory.

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Preparation of Decellularized Bone using Ultra-High Hydrostatic Pressure for Scaffold of Tissue Regeneration

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Introduction

One of key factors affecting the effective application of tissue engineering is the development of scaffolds, which provide the physical support for adhesion and expansion of cells that can regenerate the lost of diseased tissue. Recently, several scaffolds consisting of synthetic materials have been mainly studied [1-5]. However, it is difficult to actualize the same shape and structure as the biological tissue. As another approach for preparing resemble natural scaffold, there are decellularized tissues in which the cells and antigen molecules are removed to reduce the host immune response. The dcellularized tissue should be considered to have the same structure and composition as the natural tissue and the regeneration within the scaffold is expected to be modulated by newly cells. In the present study, we have demonstrated the preparation of decellularized bone using UHP technology[6] for tissue engineered bone.

Material and Method

The porcine costa was cut cylindrically (Φ6 x 5 mm). The femur was shaped cubically (4 x 4 x 4 mm). They were washed in PBS containing 5% (v/v) penicillin/streptomycin. They were transferred to sterile plastic package filled with PBS and then pressed hydrostatically at 10,000 atm, and 25 °C for 10 min using high pressure machine (Kobe steel Co. Ltd., Kobe, Japan) to disrupt the cells in them. The decellularization and alternation of micro-structure in bone during UHP processing were investigated by histological study and SEM observation. The adhesion and expansion of cells reseeded on the acellularized bone culture was examined in vitro. Also, the biocompatibility test of the dcellularized bone was carried out in vivo.

Result and discussion

The HE staining of dcellularized costa and femur prepared by UHP treatment showed cell free completely. Rat mesenchymal stem cells were reseeded on the decellularized bone in vitro. After cultivation for 3 days, SEM observation revealed that the reseeded cells were adhered on the surface of the decellularized bone in the outside and the inside of bone. In order to examine the biocompatibility of them, the dcellularized bone was implanted in rats. The animals were sacrificed at 6 months after implantation. Isolated tissue was examined by histological staining. Red blood cell and collagen fibers were observed in implanted bone tissue. But inflammatory cells were not observed in implanted bone tissue.

This study described the successful decellularization of bone using ultra high pressure technology. HE staining and SEM observation confirmed the complete removal of cells in bone treated by UHP processing. The significant adhesion and expansion of cells reseeded on the acellular bone was also confirmed without the cytotoxicity. It is expected to utilize the decellularized bone as culture matrices because the detergents, which are generally cytotoxic remaining in tissue scaffold, were not used at all in this procedure. The inhibition of immune reaction was exhibited for the decellularized bone by UHP treatment.

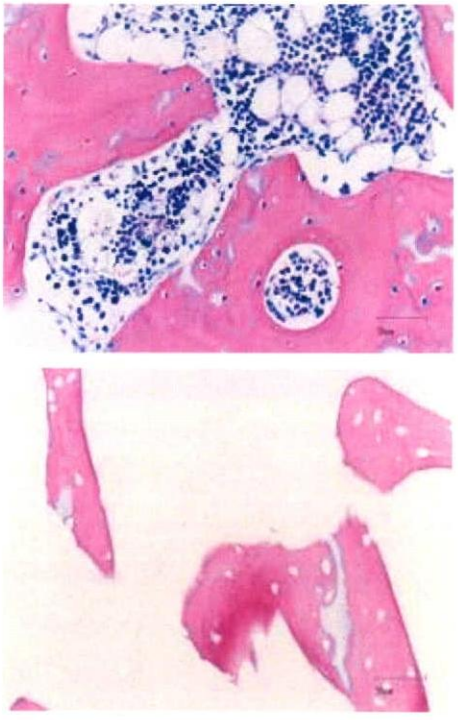

Figure 1. HE stainings of native and decellularized costa bone and bone marrow by UHP treatment

Conclusion

We have successfully developed a bone decellularization method that uses UHP technology. Histological observation confirmed the complete removal of cells in bone treated by UHP processing. Also Red blood cell and collagen fibers were observed in implanted bone tissue. But inflammatory cells were not observed in implanted bone tissue. These results suggest the utility of dcellularized bone for tissue regeneration

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Controlling Cross-linking Rate of Decellularized Blood Vessel using Collagen Coupling Reaction Technique

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Introduction: Constructing an engineered tissue for cell culture has been a major interest in bioscaffold field. Bioscaffold shows toxicity, and shrinkage when culturing the cell, and it is mechanically weak. Furthermore, calcification is also reported [1]. Recently, we found out that the cross-linking of collagen microfibrils using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) coupling reaction technique may alter the physical and biological property [2]. The cross-linking is significant when ethanol/water co-solvent is used as a coupling reaction solvent instead of aqueous solution. In this study, we report on physical and biological affect of cross-linking of decelluarized tissue, which was obtained from ultra high pressure method, to prepare a bioscaffold [3]. Furthermore, we cross-linked an anticoagulant polymer [2-methacryloyloxyethyl phosphorylcholine (MPC) polymer], which is known for its good hemocompatibility [4] with the decelluarized tissue, to obtain a polymer-cross-linked bioscaffold.

Methods: Decellularized blood vessel tissue was obtained by ultra high-pressure method [3]. EDC and NHS were added into the ethanol/water co-solvent (ethanol concentration 10, 30, 50%, and 70%) with the decellularized tissue. Then the cross-linking was continued for 24 hours at 4°C. The cross-linked tissue was cleansed for 24 hours in water and ethanol, respectively to obtain a cross-linked bioscaffod (E-scaf). The amount of cross-linked portion of each tissue was estimated by measuring the reacted amount of amine group and the carboxyl groups.

The decellularized tissue was cross-linked with MPC polymer [poly(MPC-co-methacrylic acid), PMA] at the ethanol 30% aqueous solution. MPC was pre-activated with EDC in 30% of ethanol aqueous solution and was cross-linked with decellularized tissue to obtain a polymer-cross-linked bioscaffold (P-scaf). With these bioscaffold, we investigated the structure and cross-linking structure of the bioscaffold. Furthermore, physical property and biological property of the bioscaffolds were characterized.

Results/Discussion: The cross-linking of the decellularized tissue showed that there is mild increase in the cross-linking rate when ethanol concentration increases. For the E-scaf, the highest cross-linking rate was shown when the ethanol concentration was 30%. The further increase in the ethanol concentration (≥40%) showed gradual decrease in the cross-linking rate. This is because the carboxyl group of aspartic and glutamic acid residues is less reactive in high ethanol concentration. After cross-linking, there still remains high amount of unreacted amine groups and carboxyl groups in the tissue. The further increases in the amide bond can be achieved when the tissue is re-cross-linked with EDC and NHS [2]. The mechanical strength showed that high or low cross-linking rate does not alter tissue's viscoelasticity significantly. However, when the blood vessel tissue is pulled longitudinally, the elongational modulus increased approximately 1.5~2 times of that of original blood vessel tissue. For P-scaf, it showed that the amine groups that took part in the cross-linking procedure was lower that that of E-scaf. However, its mechanical strength was increased, reaching 3.5 times higher elongational modulus compared to that of original tissue. This indicates that the network between the collagen and the polymer is very strong, but does not plasticize the tissue.

The cross-linking prohibited the tissue from shrinking in high temperature. When the tissue was inserted into the oven (80°C), the sudden shrinkage breaks out. In the case of E-scaf and P-scaf, no shrinkage had occurred. Morphology of the tissue observed with SEM showed that after cross-linking, the pores of the tissue are becoming smaller and forms much ordered layered-structure.

The strong network prohibits the degradability caused by the collagenase. The strong network is protecting the bioscaffold from degradation. After 2 weeks in the collagenase aqueous solutions, original tissue lost its original shape, while E-scaf and P-scaf maintained its shape.

We evaluated the toxicity of the tissue before and after cross-linking. It showed that the cross-linking of tissue does not induce any toxicity. This was same for the one that was cross-linked with PMA. The existence of methacrylic acid moiety was thought to cause toxicity, but it did not. This implies that it is safe to use the tissue that is cross-linked with PMA.

The tissue was inserted into the rate subcutaneously and was collected after 2 and 8 weeks. The decelluarized tissue was highly calcified, but the calcification was reduced for E-scaf. The P-scaf showed the lowest calcification, and the calcified location was located deep inside the tissue. This indicates that the polymer cross-linking had occurred not with only collagen, but also elastin, and the elastin is not damaged using the cross-linking process . Furthermore, there was almost no inflammatory reaction after implantation, indicating that the both E-scaf and P-scaf was compatible with the living tissue.

Conclusions: By controlling the cross-linking rate, we could obtain a bioscaffold that has stronger mechanical strength with safe biological property. Although some calcification was observed, it was very mimic, indicating that this bioscaffold is compatible with the living tissue.

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Effect of the Pressurizing Process on the Decellularized Aortic Tissue using Ultra High Pressurization

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Introduction: The decellularized tissues have been researched for utilization as a regenerative bio-scaffold. Generally, decellularization was executed by chemical and biological processes using detergents and enzymes. However, problems, such as ECM denaturation and the cell cytotoxicity caused by the residual reagents, have been reported. We have been developing a novel physical decellularization method using ultra high pressure (UHP) technology. This technique involves the hydrostatic ultra high pressuirization, which disrupts the cells inside and outside of the tissue. The cell debris can be eliminated by the simple washing process, producing a clean decellularized tissue. We already reported the excellent recellularization of the decellularized valve after the allotransplantaion. [1]

In this study, we investigated the affect of the UHP method to the conformation, and the structure of the collagen, and the mechanical property of the decellularized vessel.

Materials and Methods: At first, we examined the UHP treatment protocols among the selected candidates (the starting temperature; 5, 10, 15, 20, 25, 30 °C, and the pressurizing rate; 666, 1000, 2000, 5000 MPa/min.). We ascertained the temperature change during the treatments and the freezing points at each pressure so as to optimize the UHP protocol. Secondly, we compared our the UHP method with other researchers' methods [2]-[4] about the efficacy of decellularization and the influence on the biological tissues. The treated tissue sections were assessed following hematoxylin and eosin staining and TEM microscopy. The residual DNA was quantified within the tissues. The mechanical properties of the treated tissues were measured about the ultimate tensile strength, the failure strain, the elastic modulus of elastin, and the elastic modulus of collagen. Thirdly, the influence of temperature on ECM was investigated by the circular dichroism spectrum of collagens which were treated by the UHP method, and the incubations at the several temperature (5, 30, 37, 50 °C)

Results/Discussion: We evaluated the UHP method (the starting temperature; 30 °C, the pressurizing rate; 666 MPa/min.) as the optimized method for processing decellularized tissues. Hematoxylin and eosin staining of the UHP treated tissue sections demonstrated that no cell fragments were remained within the tissue. DNA assay test demonstrated that the UHP method could eliminate the residual DNA within the treated tissues under the detection limit. The elastic modulus of elastin was decreased, but other properties were well maintained. The CD spectrum of collagen treated by UHP was almost consistent with the spectrum of non-treated collagen.

We previously reported that the UHP method could certainly decellularized all of the cells and residual DNA from the arterial tissues. In this study, the optimization of UHP treatment could well soften the influence on the biological and biochemical properties of biological tissues by the temperature. Before optimization, the spaces between collagen fibrils in the center of tissues opened more significantly. The bundles and the triple-helical structure of collagen were often broken. After optimization, the influence was softened and the structure of collagen fibrils was well maintained. The triple-helical structure of collagen was also well sustained. The optimized UHP method also improved to keep the mechanical properties. The elastic modulus of elastin was a little weakened, but it is enough appropriate for the cardiovascular alternative.

Conclusion: This study has successfully developed the porcine arterial scaffold not to influence any biological property, which is decellularized using UHP. We showed that the optimized UHP method was able to not only decellularized completely from the elastic tissues such as aorta, but also maintain the structure and biological property of tissues. Future work will apply the assessment for recellularization on the UHP treated scaffold.

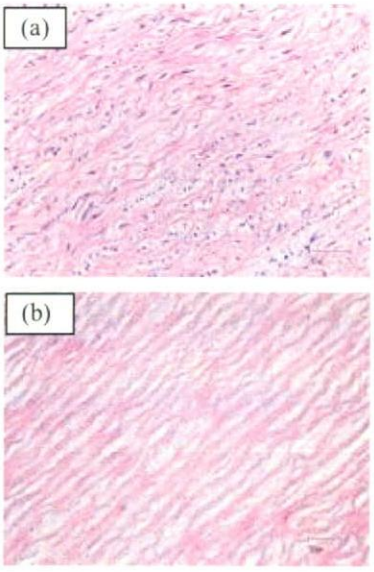

Fig. Hematoxylin and eosin staining paraffin-embedded sections of arterial scaffolds. (a)non-treated (b)optimized UHP treated.

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Surface modified Poly(vinyl alcohol) nanofibers for regeneration of corneal stroma

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Introduction

In the past 60 years, several groups have attempted to develop reliable artificial cornea, but still not yet developed completely satisfactory level. . In general, clinically available synthetic devices do not support an intact epithelium, which poses a risk of microbial infection or protrusion of the prosthesis. Previously we have found that the immobilization of Type I collagen on the poly(vinyl alcohol)(PVA) hydrogel disc was effective in supporting adhesion and growth of the corneal epithelium and stroma cell in vitro. And this was very stable and highly compatible in the corneal stroma. But the durability of the produced corneal epithelium layer on the PVA-disc in vivo has some problem [1-2]. The epithelium on the PVA hydrogel discs could not produce basement membrane in vivo. We concluded the permeability of nutrition and some biological factors are not achieving the enough level in the shape of hydrogel disc structure. It is thought that stroma of cornea forms a clear frame because fibril of collagen forms standardized cancelli. The ideal structure seems to produce the sufficient permeability as well as the transparency. Currently, we try to make the mimic structure of the natural corneal stroma by utilizing the nanomaterials and nanotechnology. In this paper, we prepared the one direction oriented PVA nanofibers by using the electro spinning method and modified the surface to give an surface affinity towards corneal stroma cells and checked the cell-nanofiber interactions.

Materials and Methods

PVA powder(Mw. 77,000, 99.9% saponification, purchased from Wako Pure Chemical Industries, LTD) was dissolved in water. **Electrospinning:** Electrospinning are performed based on previous reported condition by LL.Wu et al [3] with some modification. Applied potential is 25kV, the PVA solution was adjusted at 5wt % and its flow rate was controlled at 10mL/hour by syringe pump (Model '11' Plus, Harvard apparatus Inc. Massachusetts U.S.). The distance between nozzle and collector was 25cm. Aligned PVA nanofibers were produced between the specially prepared two-separated collectors. This material is dried overnight in a vacuum at room temperature. After drying, materials were used for further experiments. **Surface modification:** the PVA nanofibers were used to the modification reaction. Isocyanate groups were first introduced onto the surface by the reaction between the surface OH groups of the PVA and the isocyanate group of hexamethylene diisocyantate(HMDI). 10wt% HMDI/toluene with 0.07vol% di-n-butyl-tin dilauarate was prepared under the nitrogen purged condition and the prepared substrates were added in the solution and gently stirred for 40 minutes at room temperature.
After the reaction, the activated PVA was rinsed with acetone to remove the residual HMDI. All the process was done under the nitrogen gas blow condition. The surface activated PVA immersed in the type I collagen solution (0.5mg/ml) to immobilize the collagen on the surface of the PVA. **Scanning electron microscopy**: Scanning electron micrographs were obtained with scanning electron microscopy (JSM-5600LV, JEOL, Tokyo Japan). **Cell culture:** To evaluate the nanofibers-cells interaction and the cell behavior, primary rabbit corneal stroma cells was used. Cells are seeded to materials at a density of 4.77×10^4 cells/cm^2 (SEM observations) . To prepare the samples, cells are fixed by 4% glutaraldehyde solution and lyophilization with *t*-butanol. Cell morphology and nanofibers alignment were evaluated microscopically.

Results and Discussion

Average diameter of the PVA fiber is about 700nm at dry condition and about 1000nm at wet condition. The property was kept after the collagen modification process. The collagen immobilized PVA nanofibers can support primary rabbit corneal stroma cells adhesion. The PVA nanofibers we prepared have enough tenacity to support corneal stroma cells, and the cells aligned to the same direction of the nanofibers substrate as shown in Fig.1. The aligned cells were also proliferated well on the surface of nanofibers keeping the cell alignment. In this stage, we were able to control the stroma cell alignment, but still not confirmed the collagen synthesis of the aligned cells. In the future study, long term cell culture will be performed in the medium containing 5% ascorbic acid to enhance the collagen synthesis of the stroma cells and check the produced collagen alignment.

(a) SEM Image of aligned PVA nanofibers

(b) Stroma cell adhesion on the aligned(arrow direction) modified PVA nanofibers.

Fig.1 SEM and Light microscopic observation of (a) the Aligned modified PVA nanofibers and (b) corneal stroma cell adhesion; 2days after seeding on the nanofibers.

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14) 組織膜を目指したコラーゲンゲルの作製と生物学的特性検討 III

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【緒論】 従来より、コラーゲンを生体組織膜として利用するため、コラーゲンの架橋およびポリマー修飾による物性および生物学的特性の改善に関する研究が多く行われている。本研究グループでは 1-ethyl-3-(3-dimethylaminopropyl)-1-carbodiimide hydrochloride (EDC) と *N*-hydroxysuccinimide (NHS)を用いて優秀な血液適合性を示す 2-メタクリロイルオキシエチルホスホリルコリン(MPC) ポリマーをコラーゲンゲルに架橋したリン脂質ポリマー/コラーゲンハイブリッドゲルを作製した。しかしながら、EDC/NHS 架橋およびポリマー修飾は水溶液中で修飾させるため、EDC が加水分解され、低架橋率となる問題がある。[1)]

前回、エタノール濃度変化による EDC/NHS 架橋効果に関して発表した。コラーゲンはエタノール 30%水溶液条件で最適なトリプルヘリックス内部架橋条件を述べた。[2)] これは、MPC ポリマーコラーゲン間架橋条件でも同じ挙動を示すことから、EDC/NHS を架橋剤として使用してコラーゲンゲルを作製するときは、カルボキシル基の陰イオンと EDC の加水分解のバランスがもっとも重要であることを見出した。[3)] 本研究では、最大の架橋効率を示したエタノール 30%水溶液で架橋したコラーゲンゲルの機械的な物性と生物学的特性について報告する。

【実験】 コラーゲン 2wt%水溶液からコラーゲンフィルムを調製した。コラーゲンフィルムを EDC と NHS を含有するエタノール 30%水溶液中に浸漬し、24 時間架橋させ、コラーゲンゲルを得た(EN ゲル)[1)]。MPC ポリマーをコラーゲンゲルと修飾させるため、カルボキシル基を有する poly(MPC-*co*-methacrylic acid)(PMA)をエタノール 30%水溶液中で EDC と NHS と反応させ PMA のカルボキシル基を活性化させた。その後、コラーゲンゲルを加え 4°C で 24 時間反応させ MPC と架橋されたコラーゲンゲル(MPC-immobilized Collagen gel:MiC ゲル)を得た。さらに PMA の固定化過程を再度行い MPC の含有率の高いリン脂質/コラーゲンハイブリッドゲル(MPC-doubled Collagen gel:MdC ゲル)を作製した。また、架橋剤による影響を調べるため、グルタルアルデヒドを用いてコラーゲンゲル架橋を行い G-ゲルを得た。架橋されていないコラーゲンゲル(Uc-ゲル)、EN ゲル、MiC ゲル、MdC ゲル、G-ゲルの化学的特性と力学物性と表面分析等により検討し、トリプルヘリックス内、トリプルヘリックス間架橋、そして PMA との架橋によるコラーゲンゲルの特性を評価した。さらに、細胞接着実験と動物移植実験によりコラーゲンゲルの生物学特性を検討した。

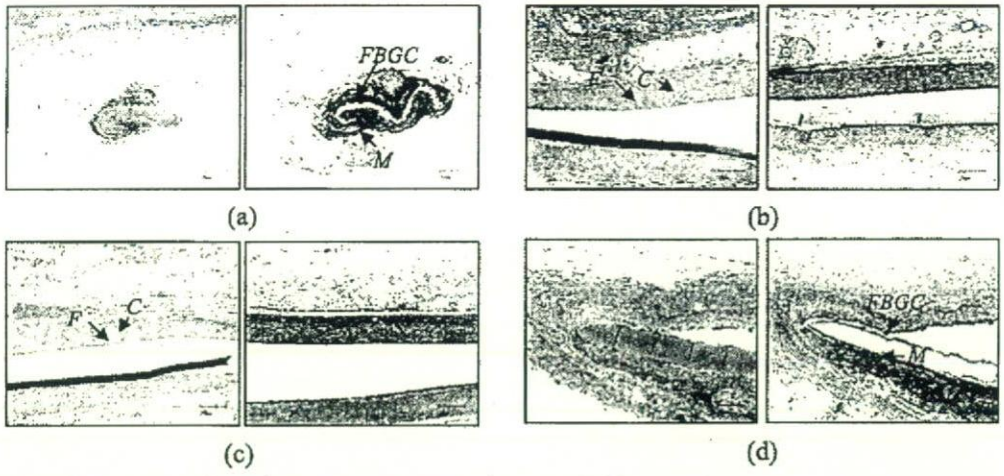
【結果と考察】 各コラーゲンゲルの自由アミン基を測定し、架橋率を計算した。その結果、EN ゲルの場合約 50%、MiC ゲルは約 60%、そして MdC ゲルは約 70%の架橋効率を示した。PMA を再架橋することにより、もっとも高い架橋率が得られることを確認した。また、3 度目の PMA 架橋は効果がなかったことから、PMA とコラーゲン間の最大架橋率は 70%であると考えられる。

コラーゲンゲルの生体分解性についてコラゲナーゼを用いて調べた。その結果、化学的架橋による安定性が確認された。Uc-ゲルは 24 時間以内に完全分解したものの、化学的に架橋されたコラーゲンゲルは遅い分解性を示した。特に、MiC ゲルと MdC ゲルの場合、コラゲナーゼ溶液中で 7 日間反応させても分解しなかった。これは、分子間架橋がコラゲナーゼによるトリプルヘリックス切断を防ぐ効果によるものであり、切断されたトリプルヘリックスがあっても、分子間架橋およびトリプルヘリックス間架橋によりコラーゲン構造は守られると言える。

Uc-ゲル、トリプルヘリックス内部架橋(EN ゲル)、トリプルヘリックス間架橋(G-ゲル)による機械的物性の変化を調べた結果、Uc-ゲルが最低物性を有することを確認した。化学的架橋の場合、線形粘弾性挙動を示した。これは、トリプルヘリックスの架橋による効果であると考えられる。また、G-ゲルと EN ゲルの機械的な物性を比べた結果、トリプルヘリックス間架橋がトリプルヘリックス内

部架橋より強いことが分かった。MiC ゲルと MdC ゲルの場合、人工ポリマーとの同様、線形粘弾性挙動を示したうえ、他のゲルより機械的物性が増加したことが明らかとなった。MiC ゲルと MdC ゲルの機械的な物性はほぼ同じであった。

表面での PMA の濃度の増加によりタンパク質の吸着と接着細胞数が減少した。これは、表面での MPC がタンパク質の吸着と細胞の接着を抑制したからである。MiC と MdC ゲルの表面に接着した細胞の形態は全部球形であった。これは PMA 表面と細胞間相互作用が弱いことを示しており、MPCポリマーの細胞接着抑制特性に起因するからである。[4] 動物移植実験結果、移植 2 週間後では、Uc-ゲルと G-ゲルの周辺組織には炎症反応によるマクロファージと異物巨細胞の生成が認められた。一方、EN ゲル、MiC ゲル、MdC ゲルの周辺には炎症反応が見られず、繊維細胞と新たなコラーゲン組織の生成が確認された。これは、生体が異物としてこのゲルの認識していないことを示しており、また、これらのゲルとの組織間相互作用が存在するので、細胞の非接着による死滅がなかったからであると考えられる。移植 8 週間後では、架橋されたコラーゲンゲルはほぼ分解せずに、体中で膜として残っていた(Figure 1)。この時、MiC ゲルと MdC ゲルの周辺のコラーゲン組織は 2 週間後と比べもっと厚くなっており、Uc ゲルと G-ゲルの場合炎症反応が続いていることが分かった。



F: fibroblast, C: new collagen layer, FBGC: foreign body giant cell, M: macrophage

Figure 1. H-E and RM-4 stained collagen gels after 8 weeks of subcutaneous implantation in rats. (a) Uc-gel, (b) EN gel, (c) MiC gel, and (d) G-gel. The images on the right side are H-E stained gels and those on the left side are RM-4 stained gels.

【結論】 コラーゲンゲルは化学的架橋により安定性が増加し、コラゲナーゼによる生体分解性が低下した。また、コラーゲン架橋法による機械的な物性の変化を確認した。コラーゲン架橋はタンパク質吸着性と細胞接着性に影響を与えると思われる。PMA との架橋は、ゲルの機械的な物性を大幅に増加されるとともに、細胞との弱い相互作用が起し、細胞の増殖を防ぐ効果を誘発する。生体内に移植した場合、マクロファージ生成などの炎症反応が抑制され、早く治癒段階に入ることを発見した。

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15　脱細胞化角膜を用いた眼科用足場材料の研究

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【緒言】

角膜移植は重篤な角膜疾患において有効な治療法の一つである。現在、角膜移植術を要する患者は、世界中で 1000 万人以上と推定されているが、実際に角膜移植を受けている患者数は年間 12 万人足らずであり、多くの国で提供眼球不足が大きな問題となっている。

このような問題の抜本的解決策として、高分子材料を用いた人工角膜の開発および再生医療技術による角膜再生が検討されている。前者は、従来より、ポリメチルメタクリレート (PMMA)、ポリヒドロキシエチルメタクリレート (PHEMA)、ポリビニルアルコール (PVA)などが試みられているが、生体組織と人工材料の接着性が悪いことや材料と生体組織との機械的コンプライアンスの不一致により発生するメカニカルストレスで、接合部での角膜実質融解による人工角膜の脱落が報告されている。一方、角膜再生においては、*in vitro* で角膜様組織の構築が報告され、上皮細胞シートによる角膜上皮再生も臨床応用されており、細胞工学からのアプローチによる再生医療技術の有用性が示されつつある。しかしながら、これら細胞の足場となる角膜実質部としては、コラーゲンゲルやフィブリンゲルなどが用いられており、これらは生体角膜に比べ脆弱かつ構造も大きく異なることが挙げられる。角膜は、コラーゲン線維が格子状に高次に配列することにより透明性を維持している組織であり、角膜実質部としては高次の組織構造が要求される。そこで、生体に類似した物性と構造を有する脱細胞化組織に着目した。脱細胞化組織は、生体組織から細胞を除去した後、残存する細胞外マトリックスを指す。本研究では、種々の手法による脱細胞化角膜の作製と眼科用足場材料としての可能性について検討した。

【実験】

成体ブタ眼球の角膜輪部に沿ってサージカルナイフで切開し、角膜を採取した。その後 3.5 % w/v Dextran を含む PBS (DEX/PBS)で洗浄した。界面活性剤による角膜の脱細胞化法は、1 % w/v TritonX-100 およびドデシル硫酸ナトリウム (SDS)溶液を調製した。角膜を浸漬し、37℃にて 24 時間の振盪処理を行った。続いて、DEX/PBS による振盪洗浄を 48 時間行った。超高圧処理による方法は、冷間等方加圧装置, Dr. CHEF ((株)神戸製鋼所)を用い、10℃または 30℃にて 10,000 気圧の超高圧印加処理 (UHP) を 10 分間行った後、直ちに 3.5 % w/v Dextran、0.2 mg/ml DNase I を含む洗浄培地 (DEX/EBM)による振盪洗浄を 72 時間行い、細胞残渣を除去した。それぞれの方法で得られた脱細胞化角膜組織に

対して組織学的評価と力学的評価を行ない、超高圧処理角膜では動物実験も検討した。

【結果と考察】

角膜の脱細胞化と組織学的評価において界面活性剤による脱細胞化処理では、不透明な角膜が得られた。H-E 染色では、膨潤によるコラーゲン線維間隙の拡大および配向の乱れが観察された。また、角膜上皮細胞、角膜実質細胞も残存していた。

超高圧処理による角膜の脱細胞化においては、白濁した角膜が得られた。H-E 染色においては、コラーゲン線維の配向は維持されており、角膜上皮細胞および角膜実質細胞が完全に除去された。

力学特性評価では、未処理角膜の応力-歪曲線は、生体組織に特有である J カーブであった。界面活性剤による脱細胞化角膜は歪率が高く、応力-歪曲線の変化が顕著であった。一方、超高圧処理による脱細胞化角膜では、歪率は未処理と同程度であり、J カーブに類似した応力-歪曲線が得られた。また、弾性率においても、未処理と意な差は示されず、力学強度に大きな変化は認められなかった。

動物実験においては、未処理角膜で移植後 1 週間経過した頃から血管の誘導が観察され、2 週間後では、移植角膜片が混濁し始めた。8 週間後、移植片部位に多数の新生血管が観察され、免疫反応の惹起が認められた。一方、脱細胞角膜移植では、移植後 1 週間経過した頃から、移植片が透明になり始め、2 週間後では、移植片は完全に透明化した。8 週間後においても、透明性は維持されており、新生血管の誘導は観察されなかった。

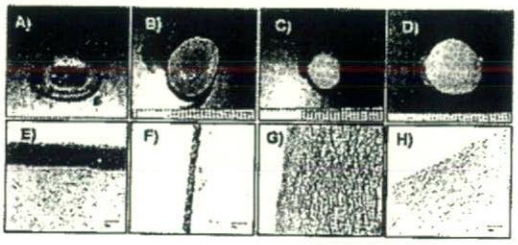

図 1　未処理角膜と脱細胞化角膜
A・E) 未処理、B・F) TritonX-100、C・G) SDS、D・H) 10℃/UHP

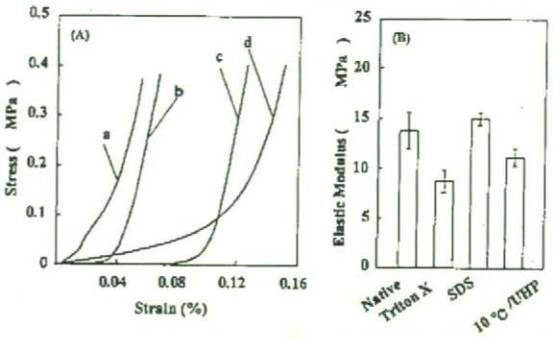

図 2　脱細胞化角膜の力学特性
a 10℃/UHP、b 未処理 c SDS、d TritonX-100

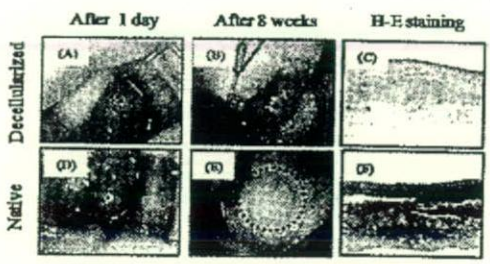

図 3　日本白色兎への移植

これらのことより細胞化角膜は、生体角膜に類似した構造を有しており、かつ、ドナー細胞を完全に除去することにより免疫反応の惹起を抑制することが可能であった。また、移植後、角膜組織の透明性が回復が観察された。これは角膜内皮細胞によるポンプ機能によるものと考えられる。以上より、脱細胞化角膜の眼科用足場材料としての可能性が示唆された。

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1V15

角膜再生用スキャフォールドの開発と機能評価

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【緒言】近年、角膜移植技術の向上によりその成功率は90%を超えるが、圧倒的なドナー不足が大きな問題である。このような問題の抜本的解決策として、合成高分子材料を用いた人工角膜の開発および再生医療技術による角膜再生が検討されている。前者ではポリメチルメタクリレート (PMMA)、ポリヒドロキシエチルメタクリレート (PHEMA)、ポリビニルアルコール (PVA)などの透明で生体不活性な素材を用いた試みがなされている。しかしながら、生体組織と人工材料の接着性、形状のミスマッチおよび組織との機械的コンプライアンスが一致していないために生じるメカニカルストレスなどによって、局所のタンパク質分解酵素が活性化し、縫合部での実質融解による人工角膜の脱落が報告されている。後者では角膜上皮疾患に由来する症例に対する治療として、角膜上皮シート移植や角膜輪部の幹細胞移植などが行われ、良好な成績を収めている。しかし、角膜実質部の疾患においては適応とならず、角膜の約 90%を占める角膜実質部の再生に関しては、未だ達成されていない。本研究では、界面活性剤法と超高圧処理法を用いた脱細胞化角膜の開発と移植用角膜としての可能性を検討した。

【実験】成体ブタの眼球から角膜を採取した。界面活性剤としては、TritonX-100、SDS を用いた。各々の 1w/v%溶液に角膜を浸漬し、24 時間振盪処理した。続いて、DEX/PBS による振盪洗浄を 48 時間行った。一方、冷間等方加圧装置(株)神戸製鋼所)を用い、10℃または 30℃にて 10,000 気圧の超高圧印加を 10 分間行い、細胞を破壊した。続いて、3.5% Dextran、0.2mg/mlDNaseI を含む洗浄培地(DEX/EBM)による振盪洗浄を行うことで組織内細胞を除去した。脱細胞化を組織学的観察、残存 DNA、残存 GAG の定量により評価した。電子顕微鏡観察により組織の微細構造変化について検討した。膨張率、透過率、力学強度測定により特性評価を行った。日本白色家兎を用いた動物実験により脱細胞化角膜の透明性、炎症反応について検討した。

Preparation and characterization of decellularized corneal scaffold

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Key Word: ultra-high pressure/ decellularization

Abstract: We investigated the decellularization of porcine cornea by two methods; detergent method and ultra-high pressure (UHP) method. The incomplete removal of cells was confirmed in detergent method. On the other hand, For H-E staining of the cornea decellularized with the UHP method, the complete removal of corneal cells and maintenance of the superstructure of collagen fibrils were confirmed. These results indicate that the decellularized cornea by UHP method would be useful as corneal scaffold for regeneration.

【結果・考察】

TritonX-100 による脱細胞化処理では、未処理の約 5 倍に膨潤した不透明な角膜が得られた。H-E 染色では、膨潤によるコラーゲン線維間隙の拡大および配向の乱れが観察された。また、角膜上皮細胞、角膜実質細胞も残存していた。SDS による脱細胞化処理では、TritonX-100 に比べて、膨潤は抑制されたが、白濁および角膜の融解によるサイズの縮小が観察された。H-E 染色では、コラーゲン線維間隙の拡大と配向の大きな乱れ、角膜実質細胞の残渣が確認された。超高圧処理による角膜の脱細胞化において、10、30℃にて 10,000 気圧の高圧印加を施した。処理温度に依らず、いずれも白濁した角膜が得られ、若干の膨潤が認められた。また、H-E 染色では、コラーゲン線維の配向は維持されており、角膜上皮細胞および角膜実質細胞が完全に除去された。しかしながら、30℃で超高圧処理した角膜では、コラーゲン線維間隙の拡大が観察された。

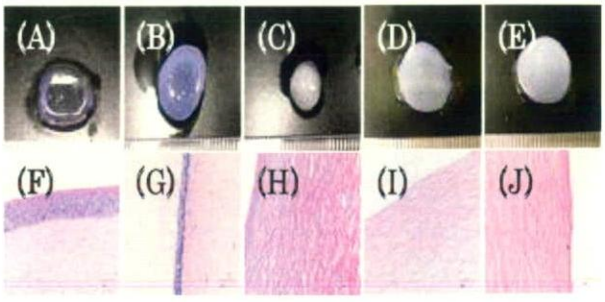

Fig 1 Photographs and H-E staining of (A), (F) non-treatment cornea, (B), (G) the cornea decellularized with Triton X-100, (C), (H) the cornea decellularized with SDS, (D), (I) the cornea decellularized by UHP at 30℃ and (E), (J) the cornea decellularized by UHP at 10℃.

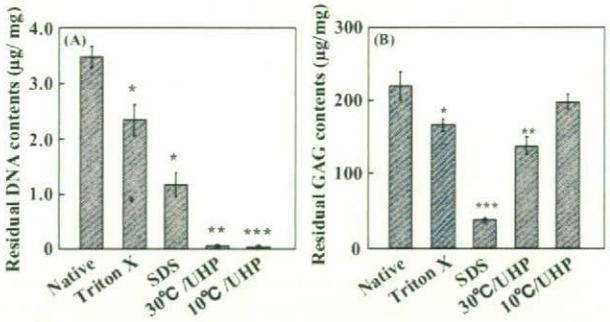

Fig. 2 Quantification analysis of (A) residual DNA contents, (B) residual GAG contents in the corneas treated under various conditions. (*p<0.05, **p<0.005, ***p<0.001).

脱細胞化処理後の角膜に残存する DNA と GAG の定量結果を Fig. 2A, B に示す。界面活性剤による脱細胞化角膜では、残存 DNA 量が減少した。一方、超高圧処理による脱細胞化角膜では、検出限界の残存 DNA 量であり、完全な細胞除去が示された。また、TritonX-100、SDS および 30℃/UHP による脱細胞化角膜では、未処理に比べ、残存 GAG 量が減少したが、10℃/UHP による脱細胞化角膜では、残存 GAG 量に変化は認められなかった。動物実験において、未処理角膜では移植後 1 週間経過した頃から血管の誘導が観察され、2 週間後では、移植片が混濁し始めた。8 週間後、移植片部位に多数の新生血管が観察され、免疫反応の惹起が認められた。一方、脱細胞化角膜では、移植後 1 週間経過した頃から移植片が透明になり始め、2 週間後では、移植片が完全に透明化した。8 週間後においても、透明性は維持されており、新生血管の誘導は観察されなかった。以上の結果より、脱細胞化角膜の移植用角膜としての可能性が示唆された。

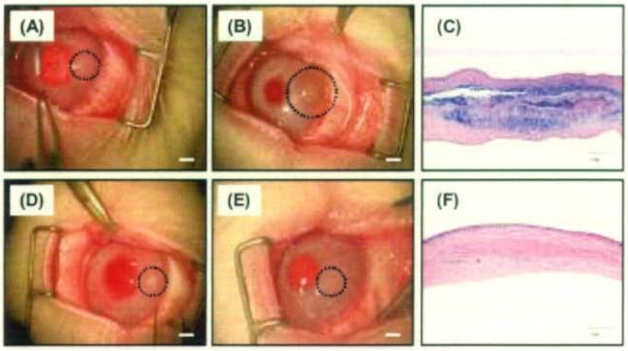

Fig. 3 Photographs and H-E staining of the non-treated (upper) and decellularized (lower) corneas implanted in rabbit eye after immediately (A), (D) and eight weeks (B, C, E, F). Scale bar 2mm (A, B, D, E), 50μm (C, F).

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2Pe149

脱細胞化処理による角膜実質の微細線維構造の変化に関する研究

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[緒言] 正常角膜実質の細胞外基質は配行したコラーゲン線維で構成されるため、角膜実質層の再生には、正常角膜実質同等の配行した構造が必要であると考えている。近年、脱細胞化の技術を用いて、組織再生の足場材料として用いる試みがなされている。一般的な脱細胞化は界面活性剤や薬剤を用いて行われるが、組織内への薬剤の残存などによる細胞毒性などの問題がある。そこで、脱細胞化の方法として超高静水圧印加（UHP）処理を採用した。UHP処理を施した組織は、界面活性剤による脱細胞化の際に問題となる細菌、ウイルスおよび免疫反応を引き起こすような抗原が除去され、細胞毒性はないと考えられる。脱細胞化後の角膜は主に実質部の細胞外基質であるコラーゲンで構成されており、微細線維構造が正常角膜実質部に近い状態のまま保持されていれば角膜実質再生足場材料となりえる可能性を保持している。そこでUHP法により作成した脱細胞化角膜実質の超微細線維構造の変化を調査し、角膜実質再生足場材料となりえるかを検討した。

[実験] ブタから採取した角膜を洗浄後、冷間等方加圧装置（(株) 神戸製鋼）を用いて10000 atmの高圧を10℃および30℃の条件化で10分印加し、0.2mg/mlのDNase I、3.5%デキストランおよび抗生物質を含むEGM-2培地に浸漬し、37℃、5% CO_2雰囲気下で72時間洗浄した。構造変化の比較対象物として界面活性剤による脱細胞化も行った。UHPと同じくブタから採取した角膜を洗浄後、1% Triton-Xおよびドデシル硫酸ナトリウム（SDS）に37℃で24時間浸漬後、抗生物質を含むPBSで洗浄した。脱細胞化処理後の検体は2.5%グルタールアルデヒドで固定後、1%オスミウム酸で後固定し、エポキシ樹脂に包埋し、透過電子顕微鏡にて観察した。

[結果と考察] 界面活性剤で処理した角膜はコラーゲン線維構造の乱れおよび細胞様構造物の残存が認められた。一方のUHP処理による脱細胞化角膜では、界面活性剤を用いた脱細胞化と比較するとより正常角膜に近い線維構造を保持していた。このことからUHPによる角膜脱細胞化では、処理に伴うコラーゲン線維の変性は界面活性剤を用いた処理より軽度であることが示唆され、UHPによる脱細胞化処理を施した角膜組織は角膜実質再生の足場材料となりうる構造を保持していることが明らかになった。

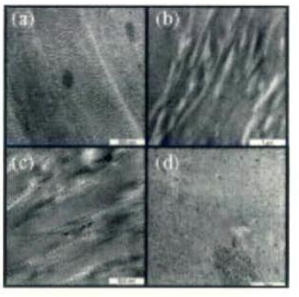

Figure 1 TEM images of decellularized cornea (a) Native cornea, (b) UHP at 10℃, (c) UHP at 30℃, (d) Triton-X

Ultrastructural changes of corneal stroma after decellularization

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Key Word: artificial cornea / decellularization / ultrastructure

Abstract: To recover from the corneal blindness, corneal transplantation is needed. However, donor shortage is the problem. Then, to fabricate artificial cornea is important. Recently, decellularized tissues are used for tissue regenerative scaffold. We performed ultra high hydrostatical pressurization (UHP) treatment to get decellularized cornea. After this treatment, aligned collagen structure of corneal stroma was maintained compared with detergents decellularization method. According to our results, UHP treated decellularized cornea can be a good scaffold for regeneration of corneal stroma.

1W07

1W07　コラーゲンゲルの構造特性による物理・生物学的評価

(東京医歯大生材研、JST-CREST)○南　広祐、木村　剛、岸田　晶夫

【緒論】 コラーゲンは人体組織を構成するポリペプチドであり、優れた生体適合性を有するため、種々なバイオマテリアル分野に広く応用されている。具体的には、コラーゲンをコーティングしたディッシュ、コラーゲンスキャフォード、およびコラーゲンゲルなどがある。コラーゲンは機械的物性が弱いため、一般的に化学的架橋法を利用し、機械的物性を調節することが多い。

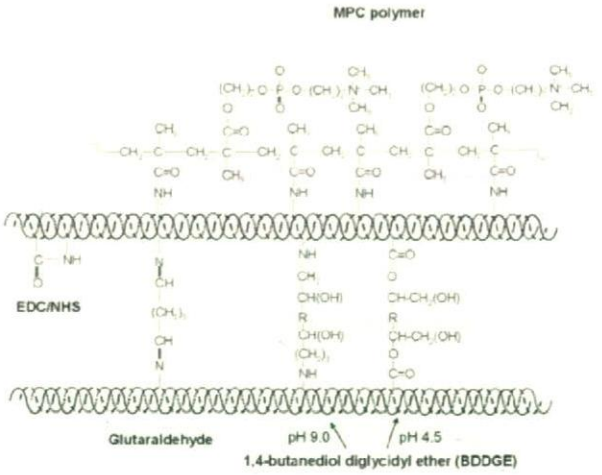

Fig 1. Schematic preparation scheme of the preparation of the collagen gels by respective cross-linkers and polymers.

今まで、本研究グループは 1-ethyl-3-(3-dimethylaminopropyl)-1-carbodiimide hydrochloride (EDC)と*N*-hydroxysuccinimide (NHS)を用いて優秀な血液適合性を示す 2-メタクリロイルオキシエチルホスホリルコリン(MPC)ポリマーをコラーゲンゲルに架橋したリン脂質ポリマー/コラーゲンハイブリッドゲルを作製した。[1,2] しかし、コラーゲン架橋効果に関する研究は、トリプルヘリックス内部架橋にとどまり、トリプルヘリックス間架橋が起こす架橋効果についての研究はまだ進めていない。本研究では、トリプルヘリックス内部架橋、トリプルヘリックス間架橋、ヘリックス-ポリマー間架橋によるコラーゲンゲルの物性と生物学的特性を調べ、各架橋がコラーゲンゲルに与える影響に関して発表する。

【実験】 コラーゲン 2wt%水溶液から透明なコラーゲンフィルムを調製した。トリプルヘリックス内部架橋の影響を調べるため、コラーゲンフィルムを EDC と NHS を含有するエタノール 30%水溶液中に浸漬し、24 時間架橋させ、コラーゲンゲルを得た(EN ゲル)[1]。また、トリプルヘリックス間架橋による影響を調べるため、グルタルアルデヒドを用いてコラーゲンゲルアミン基間架橋を行い、G-ゲルを得た。カルボキシル間架橋を行うため、1,4-butanediol diglycidyl ether (BDDGE)を pH4.5 で 120 時間架橋剤として使い B*a*-ゲルを得た。また、BDDGE を用いてコラーゲンを pH9.0 で 120 時間架橋し、B*b*-ゲルを作製した。MPC ポリマーをコラーゲンゲルと修飾させるため、カルボキシル基を有する poly(MPC-*co*-methacrylic acid)(PMA)をエタノール 30%水溶液中で EDC

How would collagen gel structure affect the physical and bioliogical properties?

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Key Word: collagen / viscoelsticity / in vivo test

Abstract: Collagen gel was prepared by immobilizing phospholipids polymer using various cross-linkers. 1-ethyl-3-(3-dimethylaminopropyl)-1-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) was sued for the amide intrahelical cross-linking, 1,4-butanediol diglycidyl ether (BDDGE) was used for carboxyl-carboxyl interhelical cross-linking, and glutaraldehyde was used for the amide-amine interhelical cross-linking. For the polymer-collagen cross-linking, [poly(2-methacryloyloxyethyl phosphorylcholine (MPC)-*co*-methacylic acid) (PMA)] was adopted. The mechanical cross-linking and the stability varied according to the cross-linking method. The collagen gel with polymer was the most stabilized while the physically cross-linked collagen gel was the weakest of all. The addition of PMA decreases the amount of adsorbed protein and adhered cells. The morphology of the cells on the PMA-immobilized collagen gel's surface was round indicating that the cell-surface interaction is weak.

とNHSと反応させPMAのカルボキシル基を活性化させた。その後、コラーゲンゲルを加え4°Cで24時間反応させMPCと架橋されたコラーゲンゲル(MPC-immobilized Collagen gel:MiC ゲル)を得た。コラーゲン架橋構造をFig. 1に示した。物理的に架橋されたコラーゲンゲル(Uc-ゲル)、トリプルヘリックス内部架橋ゲル(ENゲル)、トリプルヘリックス間架橋ゲル(G-ゲル、B*a*-ゲル、B*b*-ゲル)、ヘリックス-ポリマー間架橋ゲル(MiCゲル)の化学的特性と力学物性と表面分析等により検討し、トリプルヘリックス内、トリプルヘリックス間架橋、そしてPMAとの架橋によるコラーゲンゲルの特性を評価した。さらに、細胞接着実験と動物移植実験によりコラーゲンゲルの生物学特性を検討した。

【結果と考察】 走査電子顕微鏡でゲルの表面観察した結果、全てのゲルは非孔性であることを確認した。破断面は、Ba-ゲルとBb-ゲル以外は全て多孔性構造を有することが明らかとなった。また、架橋はコラーゲンゲルの内部形体を緻密化されることが分かった。各コラーゲンゲルの自由アミン基と自由カルボキシル基を測定し、架橋率を計算した(Fig. 2)。その結果、G-ゲルとB*a*-ゲルのトリプルヘリックス間架橋が最高架橋率を示した。

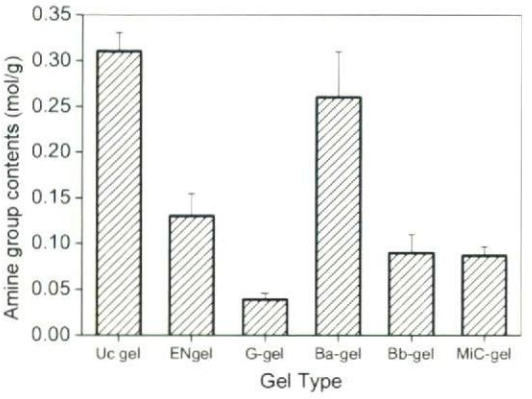

Fig 2. Amine group contents of the collagen gels prepared by respective methods.

コラーゲンゲルの生体分解性についてコラゲナーゼを用いて調べた。その結果、化学的架橋による安定性が確認された。Uc-ゲルは24時間以内に完全分解したものの、化学的に架橋されたコラーゲンゲルは遅い分解性を示した。トリプルヘリックス内およびトリプルヘリックス間架橋はトリプルヘリックス切断されても、架橋によりコラーゲンゲル構造は維持されると言える。Uc-ゲル、トリプルヘリックス内部架橋(ENゲル)、トリプルヘリックス間架橋(G-ゲル、B*a*-ゲル、B*b*-ゲル)によるゲルの機械的物性の変化を調べた。その結果、化学的架橋の場合、線形粘弾性挙動を示した。これは、トリプルヘリックスの架橋による効果であると考えられる。また、トリプルヘリックス内部と、トリプルヘリックス間架橋によるゲルの機械的な物性を比べた結果、トリプルヘリックス間架橋がトリプルヘリックス内部架橋より強いことが分かった。MiCゲルの場合、人工ポリマーとの同様、線形粘弾性挙動を示したうえ、他のゲルより機械的物性が増加することが明らかとなった。

トリプルヘリックス内部架橋によるタンパク質吸着性と細胞接着性は少し低下したものの、細胞の増殖には影響しなかった。一方、トリプルヘリックス間架橋はタンパク質吸着性と細胞接着性を急激に低下させることが分かった。これは、トリプルヘリックス間架橋の高い架橋率と架橋剤の毒性による影響であると考えられる。MiCゲルの場合、細胞が接着したものの、細胞の増殖は抑えられた。これは、架橋したMPCポリマーの機能性が発現することであると考えられる。[2,3] *In vivo*での実験データも発表当日行う予定である。

【結論】 架橋によるコラーゲンゲルの構造変化は、架橋法によって物性と生物学的特性に異なる影響を与えた。トリプルヘリックス内部架橋はコラーゲンゲルの毒性を抑える特徴を有する。トリプルヘリックス間架橋はコラーゲンゲルの機械的物性を増加させるものの、毒性が現れる。ポリマーとの架橋は物性の増加を誘発するとともに、ポリマーの機能性を発現させることが可能である。

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Poster

Structure evaluation of Porcine Cornea Decellularized by Ultra High Hydrostatical Pressurization

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Introduction: The success rate of a corneal transplantation is high compared to other forms of tissue transplantation, but many complications still occur. Also, an absolute shortage of donor corneas has been an important problem in many countries. Artificial corneas have been studied as alternatives in order to overcome these problems. Acellular cornea scafforld seems to natural cornea same structure without host cells and antigen molecules. Therefore, acellular cornea scaffold was expected to infiltrate the donor cells into the scaffold and regenerate the tissues. We made acellular cornea scaffold using ultra-high pressure (UHP) method without detergents. Our objective of this study is to investigate the structual difference of acellular corneas between UHP method and current methods. **Materials and Methods:Decellularization of corneas:** The corneas were pressurized at 4,000 or 10,000 atm at 10 or 30°C for

10 min using a high-pressure machine, washed by continuous shaking in an EGM-2 medium containing DNase I (0.2 mg/ml), antibiotics and 3.5 % w/v dextran at 37°C under 5% CO2, 95% air for 72 hours. **Ultrastructure of decellularization corneas:** After decellularization, each cornea was fixed with glutaraldehyde and osmium tetraoxide. After fixation, tissues were enbedded with epoxy resin. 80nm thickness sections were obtained. Cutting sections were observed using transmission electron microscopy. **Results and Discussion:** Ultrastructual properties of decellularized corneas made by UHP method were resembled with natural cornea. Collagen bundles were observed by TEM. In addition, there were no host cells in the UHP decellurized corea. On the other hand, microstructual properties of SDS decellularized corneas were far from natural cornea. No collagen bundles were in it. The results indicated that UHP decellurized cornea might be had a potential ability for the scaffold to regenerate cornea tissue. The results of 6 month implantation will be presented.