学会 7

Development of vascular grafts having an in situ repopulationaility

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Introduction

Although an artificial blood vessel is in general use, the development of regenerative vascular grafts is strongly desired especially for the pediatric patients. To reconstruct the tissues mainly composed of extra cellular matrix such as arterial tissues or heart valves, an appropriate structural matrix as the scaffold for cell growth is needed [1]. There are many research works related to artificial grafts made of biodegradable synthetic materials. However, it is still difficult to control the biodegradability due to their hydrolysis, adapt the mechanical properties required in the artery, and reproduce complex shape such as an aortic arch. In this study, regenerative collagenic vascular grafts were developed from porcine aorta by removing cells and structural proteins except collagen from the tissue. They were transplanted to miniature pigs and in situ repopulation was studied.

Materials and Methods

Porcine aorta was isolated from the Clawn miniature pig (Japan Farm, Co. Ltd.). The tissue was placed in a vacuum oven at 120°C to cross-link collagen fibers. Elastin fibers were then taken away form the tissue by enzymatic digestion using elastase of 0.56 u/ml in tris buffer solution including $CaCl_2$ of 10 mM and NaN_3 of 0.02% at 37°C with gentle stir. The tissues were incubated in 80% ethanol solution for 3 days at 37°C to remove phospholipids from the inside. The obtained tissues were subjected to histological and biomechanical studies. The vascular grafts made of miniature pig descending aorta were transplanted allogenically. After 3 months of the implantation, the grafts were explanted and examined histologically.

Results and Discussion

The elastic fibers were digested enzymatically even after the cross-link and it was confirmed histologically that the tissue has no elastic fiber and cellular component inside (Fig. 1). The collagen fibers remaining in the tissue were also degraded completely by collagenase and it means that the biodegradability of the tissue was not affected by the cross-linking treatment. Biodegradability is one of the most important property as a regenerative graft. The tensile strength certainly decreased after the enzymatic treatment, however an appropriate cross-linking could reduce the decline in tensile strength. The graft may be applicable not only to the pulmonary artery but to the other arteries. There was no thrombus on the intimal surface and aneurysm formation even after 3 months of the implantation (Fig. 2).

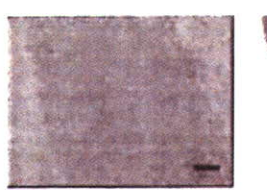

Fig.1 Histological staining of the cross-section of vascular grafts. Left, H.E. staining; Right, EVG staining. The bars in the pictures are corresponding to 200 μm.

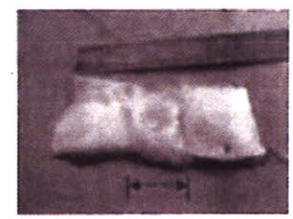

Fig.2 The intimal surface of the explanted graft. Implantation period was 3M. Scaffold part is shown in the figure.

A large amount of the cell migration into the graft was observed (Fig. 3). These cells were identified immunohistologically as smooth muscle cells and fibroblasts. And no calcific deposition was seen in the explanted graft after 3 months of the implantation.

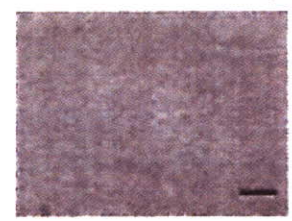

Fig.3 H.E. staining of the cross-section of the explanted graft. Implantation period was 3M. The bar in the figure is corresponding to 200 μm.

The processed graft may have better ability to promote cell infiltration and tissue remodeling compared with the acellular tissue without elastin digestion since the tissue may have more porous structure. We conclude that the collagenic vascular graft developed in this study may be adapted to the vascular tissue regeneration.

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学会 8

Effect of the stretch culture of mesenchymal stem cells on their differentiation into skeletal muscle cells

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Introduction

Replacing lost soft tissue due to trauma, diseases, or congenital anomalies is still challenging issue in plastic and reconstructive surgeries. In vitro tissue engineering of functional skeletal muscle would be an important subject because to provide physiological functions and physical shape by transplantation of tissue-engineered muscle would increase quality of patients' life.
Recently, it is reported that mesenchymal stem cells have ability to differentiate into skeletal muscle cells in conventional monolayer culture by addition of 5-azacytidine to their medium.[1] On the other hand, it is well known that dynamic stretch culture induces differentiation of the satellite cells and myoblasts into the skeletal muscle cells.[2]
In this study, mesenchymal stem cells were cultured in an acellular tissue scaffold, and the effects of the stretching of the scaffold on cultured cell differentiation were investigated.

Materials and Methods

The acellular miniature pig skeletal muscles as the scaffolds for cell culture were prepared by cold isostatic ultra-high pressuring of 980 MPa for 10 min followed by washing process. Mesenchymal stem cells were isolated from femoral bone of SD rats weighing 200 g and cultured on collagen-coated dish.
Two or three times subcultured cells were harvested from dishes and inoculated in the scaffolds by centrifugal force (100 *g* x 1 min. x 6 times), and the scaffolds with cells were placed in chambers (Fig. 1A). After 4 h, medium was added in each chamber, and cells were cultured for 3 days.
After 3 days of culture, scaffolds were transferred into the silicone chamber and were clipped at both edges of oblong scaffold. The scaffolds were then statically stretched up to 110% length of the scaffold and kept that length (Fig. 1B). As a control, cells were cultured in the original length of the scaffold.

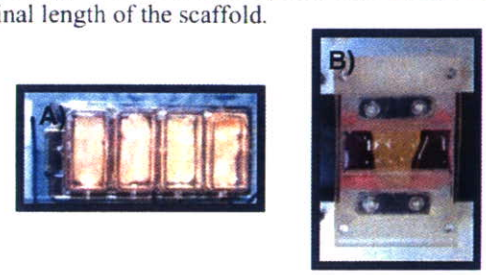

Fig. 1. Cells cultured in the scaffold. A) After cell inoculation into the scaffold, cells were cultured in the chamber for 3 days. B) On the 3rd day of pre-culture, scaffold were clipped at both edges and stretched

The effect of the elongating stimulation on the cells was evaluated by the cell shape in the scaffold and skeletal muscle specific marker expression rate by RT-PCR.

Results and Discussion

No nucleus was observed in the acellular skeletal muscle and the scaffold maintained original elastic modulus. In some area, small vessel paths were remained in the scaffold. This means the scaffold may have a possibility to be vascularized and easily reconstructed to skeletal muscle after transplantation.
During first 3 days of culture, cells were well grown in the scaffold. All scaffolds were shrinked following cell proliferation. Histological observation showed that cells were existed not only the edge of scaffold but in the collogenous region of the inner part of the scaffold.
After 3 days of static stretch stimulation, cells were elongated along the stretched direction (Fig. 2A). In some area, cells were likely to be fused as known in the myoblasts differentiation into skeletal muscle cells. On the other hand, all cells in the control group showed round shape (Fig. 2B).

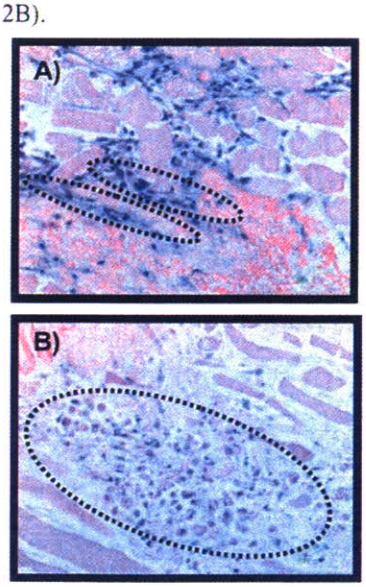

Fig. 2. Histological evaluation of the cells after stimulation. A) Statically stretched cells were elongated in the scaffold (circled area). B) Cells that were not stretched showed still round shape (circled area).

Conclusions

In conclusion, statically stretch stimulation in the mesenchymal stem cells cultured in the acellular scaffold may have possibility to effect on induction of cell differentiation into the skeletal muscle cells.

References

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Acknowledgements

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P97. Regenerative Vascular Graft For Aortic Root Reconstruction In Porcine Model

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OBJECTIVES: Tissue-engineered grafts may have the advantage of growth potential and anti-infection compared with current artificial devices. Biodegradable materials such as polylactide and/or polyglycolide are commonly used for the scaffolds. However, since they are degraded by a simple hydrolysis, it is not easy to have enough mechanical strength in the aortic tissue. Regenerative grafts made of collagenous tissue have been developed by an elimination of elastin and cellular components from porcine aortas.
METHODS: Porcine aortas were cross-linked in a vacuum oven followed by elastase digestion. They were implanted at descending aorta through left thoracotomy in the surgery carried out with single clamp technique. Postoperative anticoagulation or anti-platelet therapy was not instigated. They were explanted 4, 12, and 24 weeks after the implantation and examined histologically and immunohistologically.
RESULTS: There were no cells and elastin fibers observed in the tissues treated. The amounts of DNA and phospholipids were lower than 5% of the native. The breaking strength was lower than that of the native aorta but higher than of the native pulmonary artery. The explanted grafts showed no macroscopical abnormality and no dilatation and aneurysmal changes. The inner surface was completely covered with endothelial cells and the inside was infiltrated by smooth muscle cells and fibroblasts after 12 weeks. There was no calcium deposits observed in the graft.
CONCLUSIONS: The residual phospholipids and denatured elastin fibers may cause the calcification after the graft implantation. This process eliminates these substances and may be useful for having regenerative scaffolds for the vascular tissue regeneration.

3D12

移植用生体弁の力学評価

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緒　言

我が国では、現在年間1万件（機械弁，70%；異種生体弁，30%；同種生体弁，〈1%〉を越える心臓弁置換術が行われているが、いずれの置換弁も、不全弁の機能を代行することのみを目的としているため、患者の体内では永久的に異物として存在し続ける。また、置換弁には患者の成長に合わせた成長能が備わっていないため、小児患者への適応には問題が残る。近年、これらの問題を解決するために、組織工学的手法を用いた再生型移植用生体弁の研究が盛んに行われている。患者自身の自己弁組織を再構築し、その機能を回復させることを目的としており、再生弁組織には成長性の獲得も期待されている。一般的には、脱細胞化したブタ弁組織、あるいはポリマー製人工弁組織を用いた様々な研究が行われている。しかしながら、再生型移植弁に関する材料規格や試験方法は各研究室間で統一されておらず、臨床応用へ向けた規格の策定や、標準化された材料試験方法の確立が必要となってくる。そこで本研究では、ネイティブブタ弁組織や、再生型移植弁の開発を目的として作製された脱細胞化ブタ弁組織の力学評価を基に、材料試験法の標準化のための基礎的な知見を報告する。

実　験

試料準備

屠殺直後（㈱ジャパンファーム）に採取した食用ブタの心臓を、保冷下で輸送し、解体して大動脈弁尖組織を分離した。生理食塩水で洗浄した後、試験実施までの間、4 ℃のPBS（5%ペニシリンストレプトマイシン含有）中で保存した。試験実施直前に弁尖を基底部から切除し、引張り試験片形状に切り出して試験に供した。

引張試験

各弁尖の円周方向および半径方向に対して引張試験（オリエンテック万能試験機）を行った（図1参照）。試験片幅は3 mm、クロスヘッドスピードは20 mm/min、チャック圧は150 g/mm^{2}とし、試験片 - チャック間には、滑り防止のために耐水ペーパーを使用した。断面積は、試験片長さ、幅および比重より算出し、図2中の応力は、真応力を示している。

Fig.1　(a) appearance of aortic valve leaflet and (b) circumferential and (c) radial strips for tensile test. Arrows in the figure point specimens of each tensile directions.

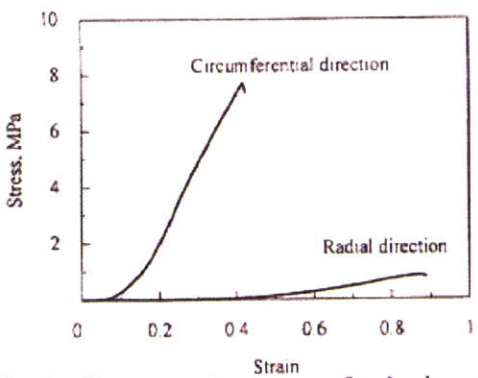

Fig.2　Stress-strain curves of valvular strip in circumferential and radial direction.

結果と考察

図2に、円周方向と半径方向の代表的な応力歪み曲線を示した。図のように、円周方向と半径方向とで大きな異方性が存在していることがわかる。この異方性は、図1に見られるように太いコラーゲン線維が円周方向に多く配向しているのに対し、半径方向には連続した線維が見られないことに起因している。この異方性は、大動脈弁の3尖弁（右冠尖、左冠尖、無冠尖）のいずれにも同様に認められ、弁尖の力学特性を評価する上で試験方向の重要性を示唆する結果である。また、3尖弁間の比較においてもその引張り挙動に差が認められた。これは、バルサルバ洞に右冠上動脈と左冠状動脈が存在し、それぞれの弁尖に対する血流負荷が異なることに起因すると考えられる。すなわち、移植用生体弁を評価する上で、いずれの弁尖を対象とするかも考慮しなければならない要素の1つである。なお、この研究は科学技術振興調整費によって行われたものである。

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2P-60　放射線照射による脱細胞バイオスキャホールドの調製

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1. はじめに

現在、我が国において人工心臓弁は年間1万個、人工血管は5万本が使用されている。屍体から提供されるヒト心臓弁や血管も組織バンクを通じて臨床使用されているが、年間数十件程度に過ぎない。米国では商業利用によって、年間数千件のヒト組織が使用されている。人工素材から作製される移植用組織は生体にとっては異物であり、自己組織と置き換わることはない。また、小児患者においては体の生育に伴った成長性が欠如しているという欠点もある。近年、移植後の拒絶反応を避けるために、自己組織と置換される素材を用いた組織再建が臨床応用され始め、東京女子医大グループによる生体内分解吸収性材料を用いた再生型血管や、ドイツ・フンボルト大学グループによるブタ脱細胞化組織を用いた再生型心臓弁が報告されている。我々は、放射線照射によって細胞を除去した生体組織をバイオスキャホールド（細胞足場材料）として用いた再生型移植用組織の開発を行っている。

2. 実験方法

細胞の除去処理：生後4ヶ月、体重約10 kgのクラウン系ミニブタ（㈱ジャパンファーム、鹿児島）から清潔下にて下行大動脈を採取した。PBSによる洗浄後、PBSを満たした滅菌容器に封入し、10, 30, 100, 300, あるいは1000 Gyのガンマ線を高崎量子応用研究所のCo-60照射施設を用いて照射した。吸収線量率は、それぞれ100, 300, 100, 300, 1000 Gy/hである。照射後、PBSをベースとする洗浄液にて2週間洗浄した。洗浄液は、適宜交換した。

皮下埋入試験：Wisterラット（7週令）の皮下部位に上記脱細胞化ミニブタ大動脈を埋入し、2週間後に取り出し、ヘマトキシリン－エオジン（HE）染色、マクロファージに対するCD68免疫染色にて組織学的検討を行った。

3. 結果及び考察

種々のガンマ線量による組織脱細胞化の基礎的検討を残存DNA定量試験、力学試験にて行った。その結果、残存DNA定量試験では、100あるいは300 Gy以上の照射では大幅なDNA減少傾向が見られた。また、力学特性は、破断強度並びに弾性率とも大きな影響は見られなかった。すなわち、300 Gy以上のガンマ線を照射後、洗浄処理することによって、循環器系組織内の細胞はほぼ完全に除去できると思われた。

次に、ガンマ線照射によって作製した脱細胞化ミニブタ大動脈の有効性を、ラット皮下への埋入試験により、組織学的、免疫組織学的検討を行った。図1には、マクロ観察及びHE染色の結果を示した。ガンマ線未照射ミニブタ大動脈の場合、ラット由来の血管の流入が認められた。一方、1000 Gyのガンマ線照射脱細胞化ミニブタ大動脈では、血管の流入は確認されなかった。HE組織染色結果からは、ガンマ線未照射ミニブタ大動脈では、組織反応による細胞の浸潤が見られたが、1000 Gyのガンマ線照射脱細胞化ミニブタ大動脈では、細胞の浸潤は認められなかった。

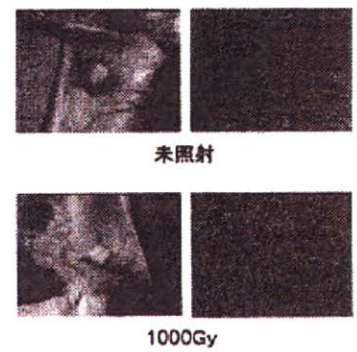

図1　脱細胞化ブタ血管組織のラット皮下埋殖（2週間、左：マクロ、右：HE）

また、移植片を免疫染色によって評価した結果、未処理血管では、マクロファージ陽性を示すCD68陽性部位が多く見られるのに対して、ガンマ線照射脱細胞化血管では、炎症部位の減少が示された。これら染色画像について画像解析ソフトを用いてCD68陽性細胞面積を計測した。その結果、未処理血管では、炎症細胞が多く存在していたが、ガンマ線照射の組織では炎症細胞が有意に減少していた。

演題 31.　再生型組織移植用の脱細胞化スキャフォールドの開発

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脱細胞化スキャフォールドを用いた再生型組織移植では、移植後に患者細胞が浸潤することで、生物学的な自己修復の機転や患者の成長に伴う組織の成長が期待できる。既に、脱細胞化同種皮膚や血管が米国や韓国で商品化されている他、脱細胞化異種心臓弁はドイツで臨床例が報告されている。これらは全て、界面活性剤あるいは酵素処理によって脱細胞化されているが、組織によっては完全な脱細胞化が容易でなく、細胞毒性を有する処理液の除去も必要である。我々は、これらの薬液を使用しない脱細胞化処理法（パワーグラフト法）を開発し、動物実験によってその有効性を検討している。血管、心臓弁の他、角膜や筋肉、神経、皮膚等の脱細胞化について報告する。

ミニブタやラットから清潔下にて各種組織を採取した。洗浄後、冷間等方圧加圧装置を用いた 980MPa（1 万気圧）の超高圧印加による細胞破壊処理、及び残渣成分の洗浄除去処理を行うことで組織内細胞を除去した。脱細胞化した各組織を、同種あるいは異種動物に同所性に移植し、所定期間経過後に摘出して組織学的に評価した。

下行大動脈の同種移植では、破断や膨化は見られず、内腔面は平滑で、血栓の付着も認めなかった。移植 12 ヶ月後では、移植時の約 1.5 ～ 2 倍の大きさとなり、前後の血管と完全に一体化していた。内腔面は血管内皮細胞によって完全に覆われており、組織内は大部分の領域で平滑筋及び線維芽細胞の浸潤を認めた。内膜付近にカルシウムの沈着を認めたが、石灰化は軽微であった。角膜実質部ミニブタ組織のウサギへの異種移植では、軽微な炎症反応を認めたものの、細胞浸潤は良好であり、透明性を維持しつつ周囲組織に定着していた。

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(42) Cell Culture on Nano-Vibrating Surface for Controlling Cell Function

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Introduction: Handling cell function is one of important factors for preparing functional cell groups. Recently, physical stress, such as hydrostatic pressure and shear stress, has been studied for controlling cell function. These systems are inspired by physiological stress. To examine the influence of non-physiological stress on cell function, we developed nano-vibration system. In this study, we report the effect of nano-vibration stimuli on cell function–cell adhesion, proliferation and differentiation.

Methods: To investigate the influence of nano-vibration on cell adhesion and proliferation, L929 cell and MEF were used as typical cells. In the differentiation experiment, PC12 cell was used. All cells were vibrated at 10 kHz for 1 hour everyday for 4 days. Then, the alternation was studied by counting cell number, observation of cell shapes and gene expression analysis using real-time RT-PCR.

Results: In L929 cell, there was no effect of nano-vibration on adhesion and proliferation. On the other hand, MEF cell showed drastic

change in adhesion and proliferation by nano-vibration. PC12 cell was hardly differentiated without nerve growth factor (NGF) addition, irrespective of nano-vibration. On the other hand, with NGF, the cell differentiation was promoted by nano-vibration in early culture period. However, the level of integrin and neuritin gene expression was not different in both nano-vibration and static culture.

Conclusion: We found that nano-vibration was effective on cell adhesion, proliferation and differentiation. These findings may lead to novel cell function controlling systems for stem and progenitor cells.

(52) Cellular Delivery of DNA-Polymer Complex Encapsulating Inorganic Nanoparticles Prepared by Ultra High Pressurization

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We have developed gene delivery system using DNA complex with non-ionic, water soluble polymers via hydrogen bond through ultra high pressurization (UHP) because the inter-, intra-molecular weak hydrogen bonding interaction was strengthen under high pressure condition. Previously, polyvinyl alcohol (PVA) was used as a model hydrogen bonding polymer, and the PVA/DNA complexes were formed by UHP treatment. Although the PVA/DNA complexes were up-taken by cells, a little enhancement of gene expression was observed using them. Therefore, in this study, to promote the endosomal escape of transferred DNA, we performed the development of PVA/DNA complexes encapsulating inorganic particle, which are dissolved under low pH condition in endosome vesicles and then the rupture of endosome is induced by osmotic shock, using UHP technology. Plasmid DNAs encoding luciferase gene or enhanced green fluorescent protein (EGFP) gene under CMV promoter were used. Nano-scaled inorganic particles having the average diameter of 50–200 nm were synthesized by modified micro-emulsion method. Nano-inorganic particles were dispersed ultrasonically in PVA solution and then mixed with DNA solution. Their mixtures were treated under 10,000 atmospheric pressures at 40°C for 10 min. By SEM observation, the irregular surface of PVA/DNA complexes including inorganic particles was observed, indicating the encapsulation of inorganic particles in PVA/DNA particle. The PVA/DNA complexes encapsulating inorganic particles showed a higher transfection activity. These results indicate the utility of the PVA/DNA complexes encapsulating inorganic particles prepared by UHP method for DNA delivery.

identified immunohistologically as smooth muscle cells and fibroblasts. And no calcific deposition was seen in the explanted graft after 3 months of the implantation. Currently long-term implantation experiments, 6 and 12 M, are in progress.

The processed graft may have better ability to promote cell infiltration and tissue remodeling compared with the acellular tissue without elastin digestion. We conclude that the collagenic vascular graft developed in this study may be adapted to the vascular tissue regeneration.

(115) Development of the Regenerative Vascular Graft Having an *In Vivo* Repopulationality

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Although an artificial blood vessel is in general use, the development of regenerative vascular grafts is strongly desired especially for the pediatric patients. In this study, regenerative collagenic vascular grafts were developed from porcine aorta by removing cells and structural proteins except collagen from the tissue.

Porcine aorta was isolated from the Clawn miniature pig (Japan Farm, Co. Ltd.). The tissue was placed in a vacuum oven at 120°C to cross-link collagen fibers. Elastin fibers were then taken away form the tissue by enzymatic digestion using elastase of 0.56 u/ml in tris buffer solution at 37°C with gentle stir. The tissues were incubated in 80% ethanol solution for 3 days at 37°C to remove phospholipids from the inside. The obtained tissues were subjected to histological and biomechanical studies. The vascular grafts made of miniature pig descending aorta were transplanted allogenically.

There was no thrombus on the intimal surface and aneurysm formation even after 3 months of the implantation. A large amount of the cell migration into the graft was observed. These cells were

surization, the collagen fibrils maintained their normal banding pattern, based on TEM observation. Also, the mechanical property of the decellularized aortic tissue was similar to that of native one. From these results, the decellularization method using UHP technology could be useful for preparing tissue-engineered scaffold.

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(136) Effect of the Pressurizing Process on the Decellularized Aortic Tissue Using Ultra High Hydrostatic Pressurization

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The decellularized tissues have been researched as a regenerative bio-scaffold. Generally, they have been prepared through chemical and biological processes using detergents and enzymes. However, some problems, such as ECM denaturation and the toxicity of residual reagents, have reported. So, we have developed a novel physical decellularization method using ultra high pressure (UHP) technology, in which the cells were disrupted by ultra high pressurization and the cellular debris were removed by washing process. The present study aim was to investigate the influence of the pressuring and washing processes on the decellularization and fabric structure of aortic tissue. Porcine aortic tissues were pressurized at different pressurization rate and temperature using a cold isostatic pressurizing machine, and then washed with cell culture medium for several periods. The characteristics of the decellularized aortic tissues were examined by H-E staining, DNA quantification, TEM observation, and mechanical test. The complete decellularization was achieved by the long term of the washing process. Rapid pressurization up to 980 MPa induced the structural disordering of collagen fibrils, in which the wide space of them was observed for H-E staining, whereas in the case of gradual pres-

(226) Implantation of Porcine Cornea Decellularized by Ultra High Hydrostatical Pressurization to Rabbit Cornea

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We have developed the porcine cornea decellularized by ultra high hydrostatical pressurization (UHP method), in which the cells were disrupted by ultra high pressurization and removed by washing process, to be utilized as novel artificial bio-cornea. We previously reported that the decellularization of porcine cornea was achieved by UHP method. In the present study, we investigated the implantation of porcine cornea decellularized by UHP method to rabbit cornea. Porcine corneal specimen with the thickness of 200 micro-meters was prepared using microkeratome. The specimen was pressed at 10,000 atm and 10 degree for 10 min, and then washed with cell culture medium containing 3.5% dextran for 72 hours. The semi-transparent cornea was obtained. By H-E staining, the complete removal of cells and the maintenance of the superstructure of collagen fibrils were confirmed. The porcine corneal discs having the diameter of 2 mm with/without the decellularization by the UHP method were implanted in a rabbit corneal pouch. It was found that the severe inflammation and vascularization occurred in the case of the native porcine cornea, which became opaque, in initial stage, whereas they were not observed for the implantation of the porcine acellular cornea, which became transparent. From these results, it suggests that the porcine cornea decellularized by the UHP method could be useful as an artificial corneal stroma for tissue regeneration.

Acknowledgements: We are grateful for funding from the Ministry of Health, Labor and Welfare, Japan.

For purpose to repair soft tissues, many research groups have been using porous scaffolds incorporated with cells *in vitro* or *in vivo*. General way for seeding cells in porous scaffold is dropping cell suspension on it, and then the cells may impenetrate into the scaffold spontaneously. However, it is not easy to seed cells completely inside of the scaffold having small pores. In this study, non-needle injector was applied to cell seeding into the tissue-derived acellular scaffolds.

The acellular cardiac muscle scaffolds were prepared by cold isostatic ultra-high pressure treatment (980 MPa for 10 min. at 4 degree Celsius) following washing steps. Cultured L929 cells were harvested from the culture dish and suspended in the PBS(-) at the density of 1.0×10^6 cells/mL. They were then injected into the acellular scaffold with the non-needle injector for percutaneous insulin administration (SHIMAJet®; Shimadzu Corporation, Japan) or the conventional syringe.

From the histological study, all nuclei were washed out from the scaffold and small pores in the range of 20–50 μm were observed among the cell skeletons. Most of the cells seeded into the scaffold with the injector were stained by calcein-AM as viable cells 24 hrs after the injection. They were scattered over a wide area in the scaffold, whereas the cells were located in cellular aggregation when injected by the conventional syringe. In conclusion, the non-needle injector may be suitable for the cell seeding into the small pore scaffolds.

(303) Novel Cell Seeding Method for the Tissue-derived Acellular Scaffolds

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(336) Preliminary Study of *In Vitro* Niche Effect on Differentiation of Rat Bone Marrow Stem Cells to Cardiomyocytes-Like Cells

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The adult heart does not regenerate after injury because the cardiac myocytes are terminally differentiated and lost their growth activity. Injection of the bone marrow stem cells into infarcted region is one of the most promising solutions proposed, and recent evidence has suggested that stem cell can differentiate into cardiomyocyte either *in vivo* or *in vitro*. However the efficiency of the differentiation process is still very low. In this study, we investigated the effect of various culture conditions including differentiation medium, cell culture matrices, and culture system (suspension or monolayer culture and static culture or dynamic culture) on the differentiation of rat mesenchymal stem cells (rMSCs) to cardiomyocytes-like cells under *in vitro* condition. First, the effect of 5-azacytidine, vitamin-C and human basic fibroblast growth factor (b-FGF) added to Dulbecco's modified Eagle's medium-low glucose (DMEM-LG) in static culture was determined. The rMSCs were obtained from femurs and tibias of male Sprague-Dawley (SD) rats (2-week-old) using our own method. rMSCs (6.0×10^5) at the third passage as seeded in the culture dish were then exposed to differentiation medium for 24 hours on day-3 of culture. The RT-PCR result shows that the expression of α-actin gene has increased to a peak value after 2 weeks induced by 5-azacytidine, vitamin-C, and b-FGF but the expression of MEF2C gene was low. Then, we treated the rMSCs suspended in DMEM containing 5-azacytidine, vitamin-C, and b-FGF. As a result, the expression of α-actin gene was forty times higher than control, whilst the MEF2C was forty times higher than the control. The effect of the other "niches" will be also discussed.

(337) Preparation and Characterization of Cornea Decellularized by Ultra High Pressurization

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Although corneal transplantation is an effective treatment for serious corneal diseases, the lack of supply is one of important problems. Also, inflammatory rejection has often occurred. In the present study, we developed the decellularized cornea by ultra high pressurization as novel artificial cornea. Porcine corneas were pressurized at 4,000 to 10,000 atm and 10 to 30 degrees C for 10 min, and then immersed in culture medium containing DNase I, antibiotics for 72 hours to remove the cellular components (UHP method). The decellularized corneas were subjected to histological study (H-E staining). The transparency, thickness and mechanical strength of them were investigated. When a porcine cornea was hydrostatically pressed at 4,000 or 10,000 atm at 10°C for 10 min, semi-transparent cornea was obtained by pressurization at 4,000 atm and 10°C. The transparency of cornea was decreased under the higher pressure and temperature condition. The swelling of them was observed for the washing process. By H-E staining, the complete removal of epithelial and stromal cells was confirmed in all of the pressurized corneas. The superstructure of their collagen fibrils was relatively maintained. Furthermore, the transmittance of the decellularized corneas by UHP method was recovered by the immersion of them in glycerol and the mechanical property similar to native cornea was shown. These results indicate that the decellularized cornea by UHP method would be useful as corneal scaffold for regeneration.

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P-1

超高圧処理技術を応用した人工角膜の作製と評価

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【はじめに】

人工角膜として、これまでに多くの材料研究が行われているが、移植後の感染や脱落により、長期間有用である人工角膜開発には至っていない。一方、異種組織から細胞を除去し、残存する基材を移植組織として用いる方法として、組織の脱細胞化が検討されている。これまで我々は、脱細胞化法として、超高圧印加により組織内の細胞を破壊し、洗浄により細胞残渣を除去する超高圧脱細胞化法を考案した。本手法で、細胞の除去による免疫反応の抑制と生体の微小構造の保持による適合性の向上が期待できる。本研究では、超高圧脱細胞化法による人工角膜の作製とその物性解析を行ない、角膜移植片としての可能性を検討した。また、他の脱細胞法である界面活性剤を用いた脱細胞化角膜の調製についても比較検討した。

【実験方法】

成体ブタの眼球((株)東京芝浦臓器)を購入し、角膜を採取した。超高圧印加装置を用い、10℃と30℃にて4,000～10,000気圧の超高圧印加を10分間行った。続いて3日間の洗浄を行い、細胞残渣を除去した。得られた組織を組織学的に観察することで脱細胞化を評価した。また、透過率、力学特性の測定およびウサギを用いた*in vivo*試験により基礎評価を行った。

【結果と考察】

超高圧処理による脱細胞化をHE染色で評価した。完全な細胞除去が達成され、またコラーゲン線維の配向も維持されていた。しかし、圧力の上昇に伴う透明性の低下と力学特性に変化が見られた。また、洗浄中での浸透圧変化による組織の膨潤が認められた。そこで、脱細胞角膜を高張液であるグリセロールにて浸漬した結果、組織の透明性は回復した。*in vivo*実験においては、移植直後の脱細胞化移植片は白濁しているが、4週間経過後で移植組織片は透明になり、8週間後でも透明性を保っていた。これらのことより、人工角膜としての可能性が示唆された。

図3に、ネイティブブタ心臓弁の円周方向と半径方向における代表的な応力歪み曲線を示した。図のように、円周方向と半径方向とで大きな異方性が存在していることがわかる。図4には、円周方向断面と半径方向断面のエラスチカ・ワン・ギーソン（EVG）染色写真を示した。図のように、円周方向には配向したコラーゲン線維が多く見られるのに対して、半径方向には連続した線維が見られず、このことが応力歪み曲線に顕著な異方性として現われたものと考えられる。この異方性は、大動脈弁の3尖弁（右冠尖、左冠尖、無冠尖）のいずれにも同様に認められ、弁尖の力学特性を評価する上で試験方向の重要性を示唆する結果である。

Fig. 1 Appearance of native porcine aortic valve. (a), right coronary cusp (RCC); (b), non coronary cusp (NCC); (c), left coronary cusp (LCC).

Fig. 2 Vaivular strips for tensile test in each direction. (a), circumference; (b), radial direction. Arrow in the figure points a tensile specimen.

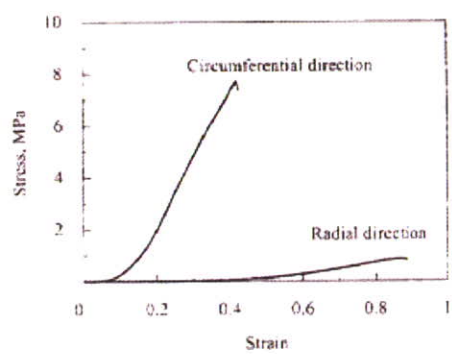

Fig. 3 Stress-strain curves of valvular strip in circumferential and radial direction.

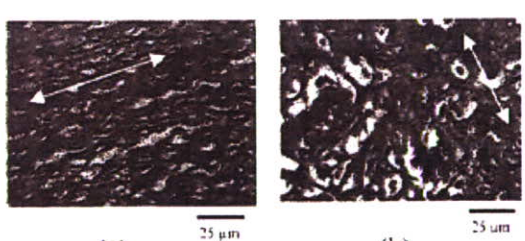

Fig.4 Elastica van Gieson staining of a cross-section on (a)circumferential and (b)radial directions of a native porcine valvular leaflet. Arrow in the figure shows each direction.

図5に、ネイティブヒト心臓弁とネイティブブタ心臓弁の、円周方向における応力歪み曲線を示した。図のように、ヒト弁尖の破断応力はブタ弁尖のそれよりも若干低い。生体組織の力学特性を評価する際、個体差の影響を無視することは出来ず、また、同一個体であっても年齢によって組織の物性は変化するため、さらに試料数を増やして検討する必要がある。

図6に、再生型心臓弁とネイティブブタ心臓弁の円周方向における応力ひずみ曲線を示した。図のように、再生型心臓弁の破断応力は、ネイティブの結果と比較して大幅に上昇していることがわかる。しかしながら、破断荷重で比較した場合、ネイティブ心臓弁と再生型心臓弁はほぼ同等の値を示していたことから、破断応力の差は脱細胞化処理による弁尖の厚さの減少を意味している。超高静水圧印加処理とその後の洗浄処理過程において、グリコサミノグリカンなど保水性成分が喪失したものと推察される。しかしながら、先述のように破断荷重に顕著な差は認められず、弁尖の強度に寄与するコラーゲン線維は脱細胞化処理後も保存されていると考えられる。

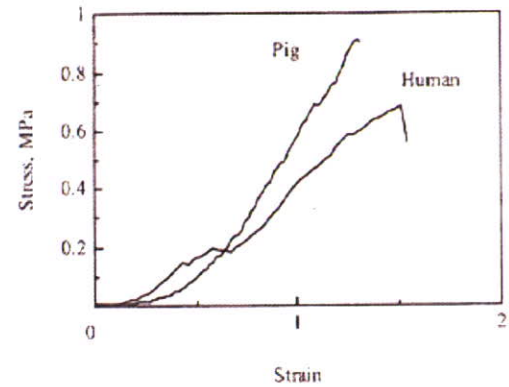

Fig. 5 Stress-strain curve of a human and a porcine valve leaflet. Tensile direction is parallel to circumferential direction of a valve leaflet.

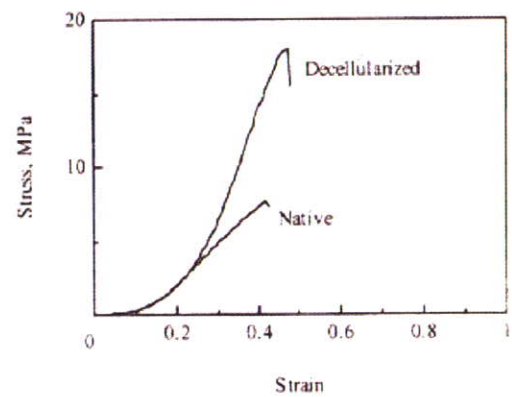

Fig. 6 Stress-strain curves of a decellularized and a native porcine valve leaflet. Tensile direction is parallel to radial direction of a valve leaflet.

今回の引張り試験の結果からは、我々の開発した再生型心臓弁は移植に耐えうる強度を有していると判断出来る。再生型心臓弁を臨床試験へ進めるため、さらに疲労耐久試験など他の試験も検討中である。

学会22

1112 再生型心臓弁の特性評価

Characterization of Regenerative Heart Valve.

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The purpose in this study is to characterize a regenerative heart valve mechanically which was developed from a porcine aortic valve with our ultra-high pressure method. The result of tensile test on a porcine native valve showed anisotropy in a leaflet. A maximum stress on a circumferential direction was much greater than that on a radial direction. This difference of maximum stress in each direction may depend on orientation of collagen fibers. The tensile property of a human valvular leaflet was similar to the property of a porcine valve. A maximum stress of the regenerative valvelar leaflet was considerably stronger than that of the porcine and the human native leaflet. The thickness of the regenerative valvular leaflet decreased because glycosaminoglycan and other contents were removed from the inside through the decellularization process. Consequently the maximum stress increased although maximum load of the regenerative leaflet was not so different from the native one. This result means that the property of collagen was not affected by our decellularization treatment. The results of this study showed that the regenerative valve could be implanted to a human from the point of tensile property.

Key words; heart valve, tensile test, regenerative medicine.

[緒　言]

我が国では、現在年間1万件（機械弁, 70%; 異種生体弁, 30%; 同種生体弁, <1%）を越える心臓弁置換術が行われているが、いずれの置換弁も、不全弁の機能を代行することのみを目的としているため、患者の体内では永久的に異物として存在し続ける。また、置換弁には患者の成長に合わせた成長能が備わっていないため、小児患者への適応には問題が残る。

近年、これらの問題を解決するために、組織工学的手法を用いた再生型移植用生体弁の研究が盛んに行われている。患者自身の自己弁組織を再構築し、その機能を回復させることを目的としており、再生弁組織には成長性の獲得も期待されている。一般的には、脱細胞化したブタ弁組織、あるいはポリマー製人工弁組織を用いた様々な研究が行われている。しかしながら、再生型移植弁に関する材料規格や試験方法は各研究室間で統一されておらず、臨床応用へ向けた規格の策定や、標準化された材料試験方法の確立が必要となってくる。そこで本研究では、再生型移植弁の開発を目的として作製された脱細胞化ブタ弁組織の力学評価を行い、材料試験法の標準化のための基礎的な知見を報告する。

[実　験]

試　料

ブタ心臓弁　屠殺直後（㈱ジャパンファーム）に採取した食用ブタの心臓を、保冷下で輸送し、解体して大動脈弁組織を分離した。生理食塩水で洗浄した後、試験実施までの間、4 ℃のPBS（5%ペニシリンストレプトマイシン含有）中で保存した。

再生型心臓弁　ブタ心臓弁に対して、10 ℃のPBS中で980 MPa（10,000 atm）の圧力を10分間印加した後、PBSをベースとした洗浄液中で14日間、振盪洗浄した。その後、80%エタノール/PBS中で3日間振盪し、さらにPBSで置換した後、試験に供した。

ヒト心臓弁　国立循環器病センター組織保存バンクより、凍結保存された移植用ヒト心臓弁組織の提供を受けた。このヒト組織は、細菌感染検査などの各種検査結果から、ヒトへの移植には不適とされたもので、研究への提供に同意されたものである。組織保存バンクより凍結状態で輸送された組織は、臨床で使用される場合と同じ手順で解凍され、試験に供した。

試料準備

試験実施直前に弁尖を基底部から切除し、引張り試験片形状に切り出して試験に供した。間隔3 mmとなるように並行に固定した2枚の剃刀刃を用いて、弁尖から各試験方向の引張り試験片を切り出した。デジタルマイクロメータを用いて試験片の長、短軸方向の長さを測定した後、試料表面の水分を濾紙で拭い、重量と比重を測定して断面積を算出した。

引張試験

各弁尖の円周方向および半径方向に対して引張試験（オリエンテック万能試験機）を行った（図1, 2）。試験片幅は3 mm、クロスヘッドスピードは20 mm/min、チャック圧は150 g/mm2とし、試験片・チャック間には、滑り防止のために耐水ペーパーを使用した。

[結果と考察]

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学会23

筋芽細胞の分化と細胞膜電位の変化

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現在，注目を集めている培養筋肉の医工学応用において，筋芽細胞の分化を制御することや多核筋管細胞の収縮動態を調べることが重要である．筋肉を構成する筋細胞は，筋芽細胞が分化し成熟したものであり，筋芽細胞の分化を制御するうえで分化の判定技術が大切である．現在実用的な判定方法として染色技術やDNAを調べる方法が挙げられる．しかしこれらの方法は，時間やコストの面から考えると今後改善する余地がある．そこで本研究では，時間とコストの改善と分化後の多核筋管細胞の収縮動態を調べる方法として細胞膜電位計測技術の応用を提案した．

1．はじめに

現在，次世代アクチュエータや医学における培養筋，工学に於ける人工筋のような培養筋肉の医工学応用が注目を集めている．本研究は，培養筋肉の状態を電気的に評価する方法を目的としている．培養筋肉を構成する筋細胞は，筋芽細胞が分化，成熟したものであり筋細胞の医工学応用の研究においては，細胞の分化を制御することが重要となる．分化を制御するうえで分化の判定技術が大切である．しかし実用的である染色やDNAを調べる方法は，コストや時間の面で今後改善する余地がある．そこで本研究では，分化を判定することと分化後の細胞を電気的に評価することを考慮にいれ細胞膜電位計測技術を応用した新しい判定技術を開発する．細胞膜電位とは，細胞膜の内側空間と外側空間の電位差であり，細胞膜上に多数分布しているタンパク質であるイオンチャネルによって発生する．[1]

2．実験方法

培養筋芽細胞は，マウス由来筋芽細胞株C2C12細胞を用いた．10%FBS含有DMEMで増殖培養し，分化誘導は，60mmディッシュにサブコンフルトとなった細胞を2%HS含有DMEMで行った．

外径 1[mm]の中空ガラス管を熱で加工し，先端径が1[μm]以下の針状に作製した．このガラス管内に高濃度電解質の3[M]KCl水溶液を満たしてPt線を浸し電極とした．計測には，電極抵抗値が10[MΩ]前後のものを用いた．倒立顕微鏡下でガラス電極を細胞内に刺入したときの細胞の内側空間と外側空間の電位差を計測し，高入力インピーダンス増幅器へ導いた．そしてこのときの電位差をオシロスコープにて波形を観測した．

3．結果

C2C12細胞の細胞膜電位を計測した．計測結果の平均値と標準偏差を図1にまとめた．

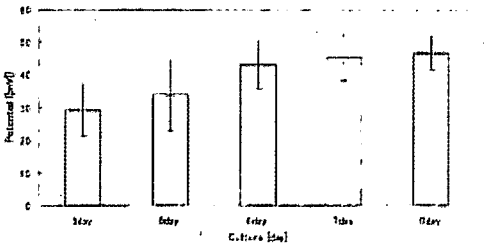

図1　細胞膜電位の平均値と標準偏差

図1より分化と共に細胞膜電位が大きくなっていることが確認できた．詳細は，当日報告する．

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