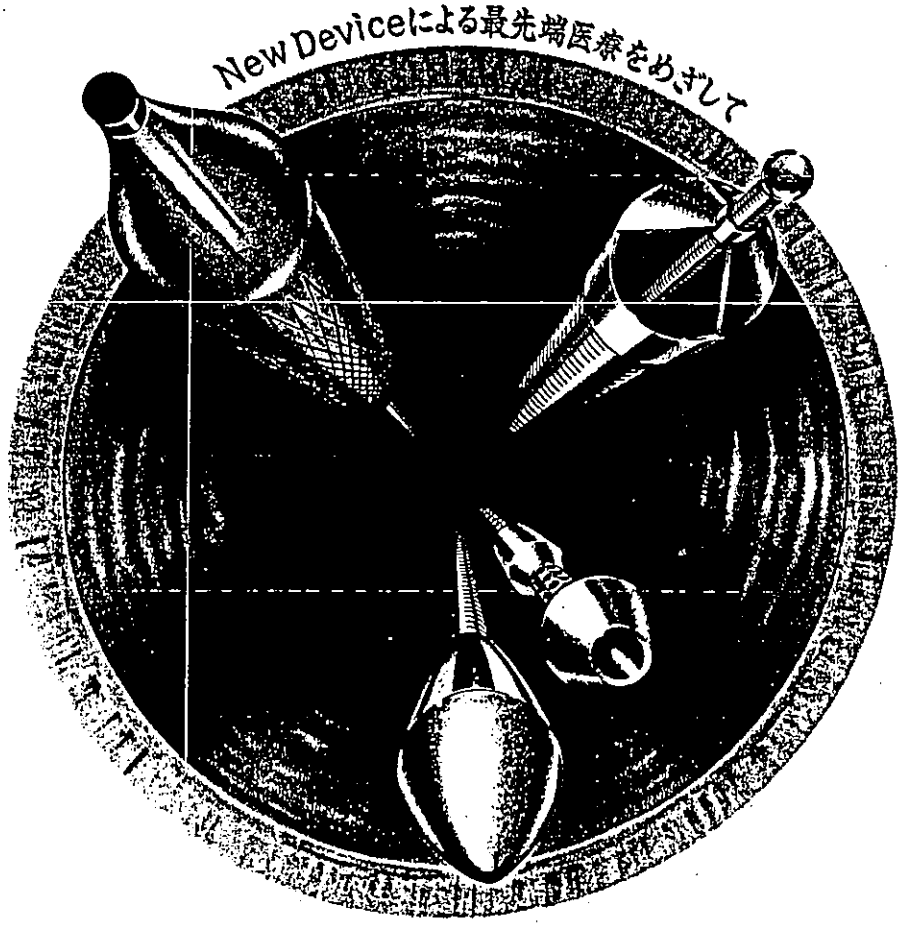
Volume 4, Number 1, 2003 ISSN 1345-2673

日本血管内治療学会誌

Japanese Journal of Endovascular Intervention

Official Journal of the Japanese Society for Endovascular Intervention

第4号



日血内治療会誌
Jpn. J. Endovasc. Intervent.

日本血管内治療学会

高機能ステントグラフトによる実験的動脈瘤の閉塞
ーその有用性と展望ー

Development of a High-Performance Stent Graft for Extracranial Aneurysms
– its Usefulness –

高槻赤十字病院　脳神経外科[1)]　西　正吾[1)]　Shogo Nishi[1)], M.D.
国立循環器病センター研究所　生体工学[2)]　中山　泰秀[2)]　Yasuhide Nakayama[2)], M.D.
国立循環器病センター病院　病理部[3)]　植田　初江[3)]　Hatsue Ueda[3)], M.D.
九州大学大学院医学系研究科　医用工学分野[4)]　松田　武久[4)]　Takehisa Matsuda[4)], M.D.

Department of Neurosurgery, Takatsuki Red Cross Hospital[1)]
Department of Bioengineering, National Cardiovascular Center Research Institute[2)]
Department of Pathology, National Cardiovascular Center Hospital[3)]
Department of Biomedical Engineering, Graduate School of Medicine, Kyushu University[4)]

Our newly developed stent grafts with micropores and heparin coating were used for embolization of experimental carotid aneurysms in dogs. Aneurysms were formed on both common carotid arteries by using an external jugular vein patch. At 1 month after aneurysm formation, the aneurysms were occluded with stent grafts. Carotid arteries were removed with the aneurysms, immediately (2 aneurysms), 1 week (4), 1 month (3), and 3 months (4) after embolization, and studied angiographically and histologically to determine patency and endothelialization over the intraluminal surface of the thin film. Even at 1 week after embolization, endothelialization on the surface of the stent graft on the lumen side was confirmed. At 1 month and 3 months, all treated aneurysms were filled with organized thrombi and completely occluded. The parent carotid arteries were enough patent. The stent graft that we have developed appears to be promising for the treatment of extracranial aneurysms, especially with respect to immediate termination of blood inflow and early endothelialization in the neck of the aneurysm.

Key words : stent graft, covered stent, heparin coating, micropores, extracranial aneurysms

【はじめに】

頭頚部動脈瘤の治療に対しては外科的治療、血管内治療が行われている[1,2)]。外科的手法と血管内手法の組み合わせにて互いの利点、欠点を克服しながら治療にあたっているのが現状と思われる。

最近の血管内治療の発達はめざましいものの、同領域の動脈瘤に対しては、未だにその手法は確立されていない。血管内手術で動脈瘤のみを閉塞し親血管を温存する際、その動脈瘤の解剖学的位置、大きさ、neckの形状により、手技の難易度は異なる。現在、プラチナコイル，ステントの組み合わせ[3,4,5)]、更には液体塞栓材料の組み合わせ[6)]にて、そのtherapeutic windowは広がりつつある。

ステントグラフトによる動脈瘤閉塞が可能になれば、症例は限定されるものの、プラチナコイルに比べ、そのcost-performanceは飛躍的に増し、更にtime-savingにもなるものと思われる。

今回、我々はそのカバー材に2つの工夫を凝らしたステントグラフトを作製し[7)]、犬の実験的頚動脈瘤に用いて良好な閉塞効果と親動脈の開存性を得たので紹介するとともに今後の展望を述べたい。

【対象と方法】

断片化ポリウレタン薄膜に微細孔を設けさらに表面にヘパリンを被覆して従来のステント骨格に縫合固定しステントグラフトを作成した[7)]。予め作成しておいた犬頚部動脈瘤に対して作製したステントグラフトを用いて閉塞を行い、長期閉塞効果と親動脈の開存性を調べた。

【ステントグラフトの作製】

厚さ30ミクロンの断片化ポリウレタン薄膜（Sheedom Co., Tokyo, Japan）にエキシマレーザーにてCAD systemを用い直径100ミクロンの微細孔を孔間隔250ミクロンで設けた[8,9)]。

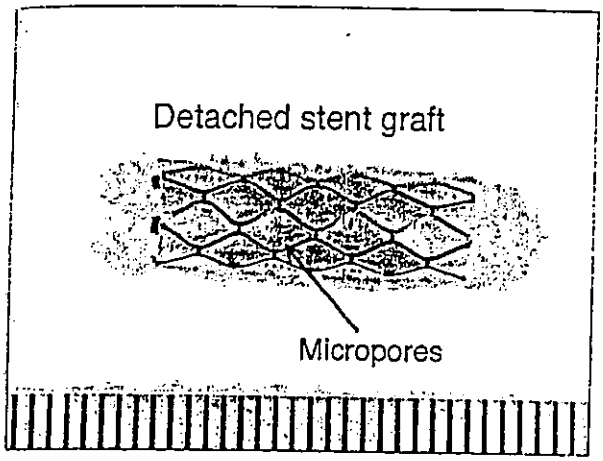


Fig.1　Macroscopic stent graft
An inflated and detached stent graft shows micropores on a surface of a thin SPU film mounted on a Palmaz stent.

その後、光反応性ゼラチンにヘパリンを包埋固定し薄膜カバーの作製を終えた。手術用顕微鏡を用いてPalmaz stent（Cordis, J & J, Japan）に縫合固定し最終断端をホルムアマイドにて溶剤固定した（Fig.1）。ステントグラフトを使用する際に0.5%マスキンアルコールにて浸潤滅菌後、生食にて洗浄した。

【動脈瘤の作製】

雑犬6匹を用いて両側頚部頚動脈動脈瘤を作製した[10~12]。気管内挿管の下、全身麻酔下にて頚部正中切開を行い、両側胸鎖乳突筋の内側縁に進入し総頚動脈を露出した。右の外頚静脈を露出しvein patch用に切断、採取した。取り出した頚静脈片を両側頚動脈動脈瘤作製の為に、2つに切断した。各頚静脈片の一方の断端を2-0絹糸、6-0ナイロンにて巾着縫合した。他方の断端と頚動脈の側面を端側吻合を行い動脈瘤を作製した（Fig.2）。

broad neckになるように縦方向の頚動脈の切開を長めにした。顕微鏡下にて7-0ナイロンにて断続的に縫合し

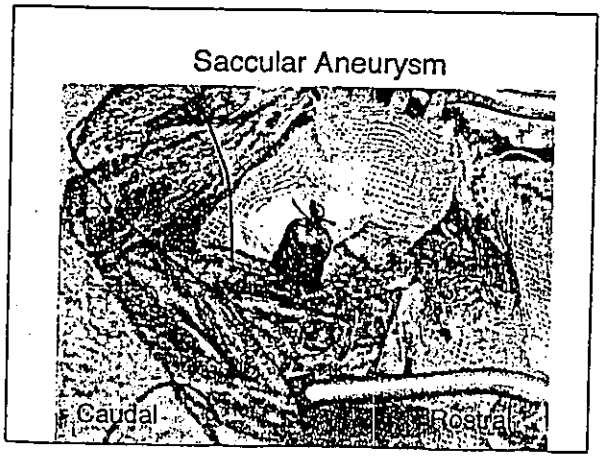


Fig.2　A prepared saccular aneurysm
Right carotid artery is anastomosed to a vein patch, end to side. A prepared aneurysm is shown .

た。瘤への良好な血流の存在と吻合部からの出血の無いのを確認後、閉創した。

【ステントグラフトの留置】

ステントグラフトの留置1週間前からチクロピヂン100mgとアスピリン81mgを経口投与した。硫酸アトロピン0.5mgとケタラール10ccにて麻酔導入を行った。ペントバルビタール50mg/1ccの静脈注射にて麻酔を維持した。鼡径部を切開し大腿動脈を露出した。末梢側を結紮し、中枢側をブルドッグ鉗子にてクランプし動脈穿刺にて8Fシースを挿入した。作製したステントグラフトを5F Power Flex （Cordis, J & J, Japan）にmountしシース径と同じストレーナーを用いてシース内に容易に挿入した。透視下にて、既に動脈瘤を作製してあった頚動脈に進めた。透視や血管撮影にて動脈瘤頚部の位置、形状を確認後、ステントグラフトを進め、適正な位置で拡張し切り離した。血管撮影後、対側の動脈瘤の閉塞を同様に行い処置を終了した。術後、3週間は上記の経口薬剤を同様に投与した。

【術後観察】

ステントグラフト留置直後、1週間後、1ヶ月後、3ヶ月後に鼡径部からSeldinger法にて血管撮影を行った。ステントグラフト留置部の血管-動脈瘤の標本を採取した後で、安楽死の処置を施した。HE染色、Masson's Trichrome染色を行い　動脈瘤の閉塞状況の評価、親血管である頚動脈の開存の程度を評価した。

【結果】

血管撮影による評価

ステントグラフトによる動脈瘤閉塞は直後1匹（2動脈瘤）、1週間後2匹（4動脈瘤）、1ヶ月後2匹（3動脈瘤）、1ヶ月後2匹（4動脈瘤）であった。ほとんどはステントグラフト留置直後に瘤の閉塞をみた。1週間後、1ヶ月後、3ヶ月後の血管撮影でも瘤の完全閉塞はみられ、また親動脈の頚動脈も十分な開存性を保っていた。

【動脈瘤の閉塞状況】

留置直後のHE染色では瘤と親血管の間は完全にステントグラフトで遮断されており、瘤内は新鮮血栓にて閉塞され、更に親動脈内腔はステントグラフトにて平滑に被覆されていた（Fig.3）。留置1週間後では瘤と親動脈の間は直後と同様にステントグラフトにて完全に遮断されていた。瘤のneckを遮断しているステントグラフト部は血管内膜から微細孔を通して進入してきた組織により瘤側、血流側ともに被覆されていた。瘤内は古い血栓にて閉塞されていた（Fig.4）。留置1ヶ月では瘤と親血管の間は完全に遮断され、瘤内は器質化された血栓により完全に閉塞されていた。親血管は内膜の肥厚も軽度で十

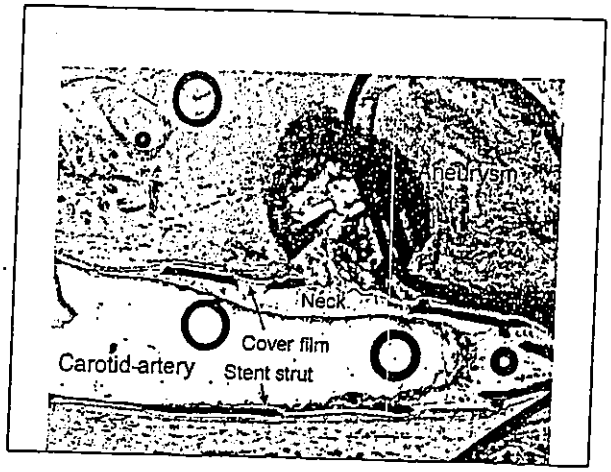


Fig.3　Histological sagittal view (immediately)
··· low power field
This microscopic sagittal view shows an aneurysm and parent artery immediately after embolization. The neck of the aneurysm is bordered by the stent graft, and the aneurysm is also completely packed with fresh thrombi.

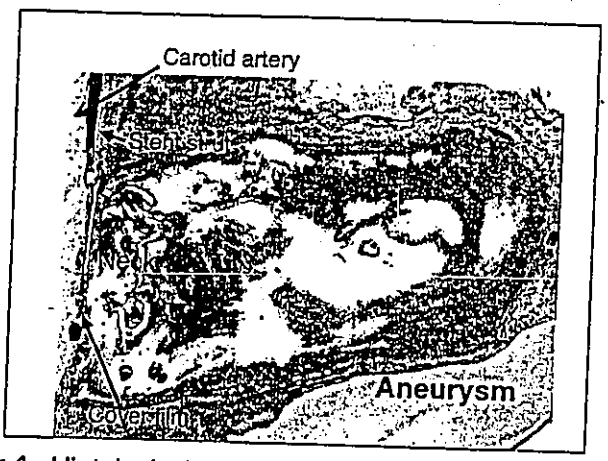


Fig.4　Histological sagittal view (1 week)
··· low power field
The neck of the aneurysm is completely packed with old thrombi. There is intimal ingrowth through the micropores on inner and outer surfaces of a stent graft in the neck of the aneurysm. A parent carotid artery is enough patent.

分な開存性が得られた (Fig.5)。留置3ヶ月は1ヶ月後と同様の所見で動脈瘤の完全な閉塞と親動脈の十分な開存性が得られた。走査電子顕微鏡所見では留置1週間後で既に血流側ステントグラフト内面は内皮細胞によって十分被覆されていた。

【考察】

我々は、独自にステントグラフトを作製し[7]犬での実験的頚動脈瘤に応用しその閉塞効果と親動脈の十分な開存性を示し、更にその臨床応用への可能性を示唆した。臨床的に実際、使用されているバルーン拡張型ステントを骨格として使い断片化ポリウレタン薄膜をカバー材に用い、そのカバーに2つの工夫を施した。1つにはエキ

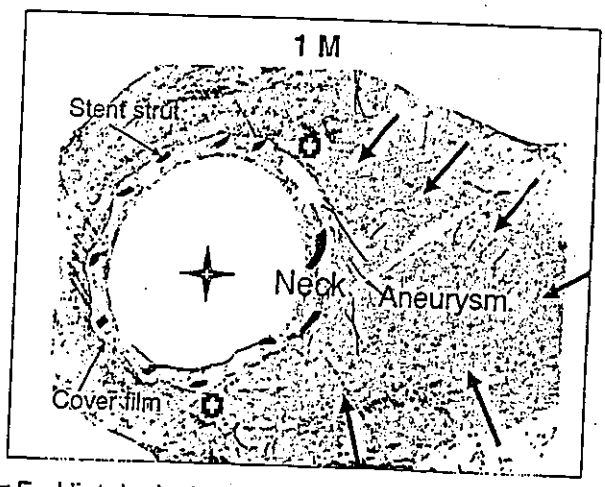


Fig.5　Histological axial view (1month)
The neck (cross) of the aneurysm (long arrows) is completely packed with old organized thrombi. A parent carotid artery is enough patent (asterisk).

シマレーザーにて微細孔を設けることにより早期の内皮を含めた周辺組織のステントグラフト内への早期誘導を行い血栓の予防を図り[13,14]、また、中長期においてはその微細孔により周辺組織の進入を抑制し内膜肥厚を調節することで再狭窄を予防をめざした。2つにはカバーの血流側に光反応性ゼラチンを用いてヘパリンを包埋固定することにより留置早期における血栓形成を阻止し閉塞を予防することである[7,15]。

頭蓋外（サイフォン部以下の内頚動脈と頚部頚動脈）動脈瘤に対する治療は外科的治療と血管内治療[1,2]とに分かれる。こと頭蓋底手術を駆使しての手術はその大きさ、解剖学的位置、neckの形状により難しい場合も容易に想定される。また、血管内治療にしても動脈瘤頚部の形状・大きさにより難しい場合がある。現在、プラチナコイルを使った手技が主流となり、動脈瘤のneckがはっきりしていて更にsmall neckであればプラチナコイルによる閉塞が適している[1,16,19]。しかし、術中の出血、原理的には残存腔ができる可能性がありそこからの出血、coil compactionによる再開通などが問題である。broad neckやfusiform-typeの動脈瘤に対してステントを骨格にして頚部を形成後プラチナコイルを用いて閉塞したり[17,18,20]、また、欧米で最近行われているステントを骨格にして同様に頚部を形成し液体塞栓材料を使用して閉塞するという特殊な手技[6]も開発されている。いずれにしても症例により難しさがあり、未だ解決しなければならない点が残されている。ステントグラフトを用いることにより瘤内へのアプローチをすることなく閉塞可能でプラチナコイルによる難点を解決する事ができる。自家静脈、GoaTex、Dacronを使用したステントグラフトの動脈瘤への臨床的使用が散見される[21~23]。しかし、未だ緊急避難的な使用にとどまったり、また、留置後の閉塞や再狭窄に課題が残されている。犬を用いた実験的頚動脈瘤閉塞モデルでは、ステントグラフトの留置後1, 3ヶ月で血管

撮影上はもちろんのこと、組織学的にも器質化した血栓により瘤内は充満され親血管腔は十分保たれていた。今回示したステントグラフトは実験的頚動脈瘤に対する効果を検討したものであるが、近い将来、ステントグラフトを進めることが可能な頭頚部の動脈瘤閉塞に使われるようになれば、これまでの塞栓材料に比べてcost-performanceが高く、更にtime-savingになるものと考えられる。

[文献]

1) Byrne JV, Sohn M, Molyneux AJ: Five-year experience in using coil embolization for ruptured intracranial aneurysms: outcomes and incidence of late bleeding. J Neurosurg, 1999, 90: 656-663.
2) Moret J, Cognard C, Weil A, et al: Reconstruction technique in the treatment of wide-neck intracranial aneurysms: Long-term angiographic and clinical results-Apropos of 56 cases. J Neuroradiol, 1997, 24: 30-44.
3) Massoud TF, Turjman F, Ji C, Vinuela F, Guglielmie G, Gobin YP, Duckwiler GR: Endovascular aneurysms with stents and coils: technical feasibility in a swine model AJNR, 1995, 16: 1953-1963.
4) Szikora I, Geterman LR, Wells KM, Hopkins LN: Combined use of stent and coils to treat experimental wide-necked carotid aneurysms; preliminary results. AJNR, 1994, 15: 1091-1102.
5) Turjman F, Massoud TF, Ji C, Guglielmi G, Vinuela F, Robert J: Combined stent implantation and endosaccular coil placement for treatment of experimental wide-necked aneurysms: a feasibility study in swine. AJNR, 1994, 15: 1087-1090.
6) Szikora I, Geterman, LR, Standard SC, Wakhloo AK, Hopkins LN: Endovascular treatment of experimental aneurysms with liquid polymers: The protective potential of stents. Neurosurgery, 1996, 38: 339-347.
7) Nishi S, Nakayama Y, Ueda H, Ishikawa M, Matsuda T: Newly developed stent graft with micropored and heparin impregnated SPU film, long-term follow-up study in vivo, Interventional Neuroradiology, 2001, 7 (Suppl 1) : 161-166.
8) Doi K, Nakayama Y, Matsuda T/: Novel compliant and tissue-permeable microporous polyurethane vascular prosthesis fabricated using an excimer laser ablation technique. J Biomed Mater Res, 1996, 31:27-33.
9) Matsuda T, Nakayama Y: Surface microarchitectual design in biomedical application: In vitro transmural endothelialization on microporous segmented polyurethane films fabricated using an excimer laser. J Biomed Mater Res, 1996, 31: 235-242.
10) German WJ, Black SPW: Experimental production of carotid aneurysms. New England J. Med, 1954, 250: 104-106.
11) Graves VB, Strother CM, Rappe AH: Treatment of experimental canine carotid aneurysms with platinum coils, AJNR, 1993, 14: 787-793.
12) Kerber AV, Buschman RW: Experimental carotid aneurysms: 1. simple surgical production and radiographic evaluation. Invest Radiol, 1976, 12: 154-157.
13) Bjornsson TD, Dryjski M, Tluczek J, Mennie R, Ronan J, Mellin TN, Thomas KA: Acidic fibroblast growth factor promotes vascular repair. Proc. Natl. Acad. Sci USA, 1991, 88: 8651-8655.
14) Schwartz SM, Stemerman MB, Benditt EP: The Aortic Intima, II. Repair of the aortic lining after mechanical denudation. Am J Pathol, 1975, 81: 15-42.
15) Palmaz JC: Intravascular stents: Tissue-stent interaction and design consideration. AJR, 1993, 160: 613-18.
16) Brilstra EH, Rinkel GJE, Graaf Y, Rooij WJJ, Algra A: Treatment of intracranial aneurysms by embolization with coils. A systematic review. Stroke, 1999, 30: 470-6.
17) Kuether TA, Nesbit GM et al: Clinical and angiographical outcomes, with treatment data, for patients with cerebral aneurysms treated with Guglielmi detachable coils: a single center experience. Neurosurgery, 1998, 43: 1016-1023.
18) Guglielmi G, Vinuela F, Dion J, Duckwiler G: Electrothrombosis of saccular aneurysms via endovascular approach: Part 2-Preliminary clinical experience. J Neurosurg, 1991, 75: 8-14.
19) Vinuela F, Duckwiler G, Mawad M: Guglielmi detachable coil embolization of acute intracranial aneurysms: perioperative anatomical and clinical outcome in 403 patients. J Neurosurg, 1997, 86: 475-82.
20) Raymond J, Roy D: Safety and efficacy of endovascular treatment of acutely ruptured aneurysms. Neurosurg, 1997, 41: 1235-1245.
21) Criado E, Marston W, Ligush J, Mauro M, Keagy B: Endovascular repair of peripheral aneurysms, pseudoaneurysms and arteriovenous fistulas. Ann Vasc Surg, 1997, 11: 256-263.
22) Nicholson A, Cook A, Dyet J, Galloway J: Case report: Treatment of a carotid artery pseudoaneurysm with a polyester covered nitinol stent. Clin Radiol, 1995, 50: 877-875.
23) Tepe Gunnar, Duda H Stephan,Hnake Hartmut, Schulze Stephan, Hagmeier Sonja, Briuck Birgit, Schott Ulrich, Betz Eberhard, Schmahl Friedrich-Wilhelm, Claussen D Claus: Preliminary report, Covered stents for restenosis, Experimental and Clinical results with different stent designs. Investigate Radiology, 1996, 31: 223-229.

Cell Transplantation, Vol. 13, pp. 439–449, 2004
Printed in the USA.

0963-6897/04 $20.00 + .00

In Vivo Tissue-Engineered Small-Caliber Arterial Graft Prosthesis Consisting of Autologous Tissue (Biotube)

Yasuhide Nakayama,* Hatsue Ishibashi-Ueda,† and Keiichi Takamizawa*

*Department of Bioengineering, National Cardiovascular Center Research Institute, Fujishiro-dai 5-7-1, Suita, Osaka 565-8565, Japan
†Department of Pathology, National Cardiovascular Center Hospital, Fujishiro-dai 5-7-1, Suita, Osaka 565-8565, Japan

In this study, vascular-like tubular tissues called biotubes, consisting of autologous tissues, were prepared using in vivo tissue engineering. Their mechanical properties were evaluated for application as a small-caliber artificial vascular prosthesis. The biotubes were prepared by embedding six kinds of polymeric rods [poly(ethylene) (PE), poly(fluoroacetate) (PFA), poly(methyl methacrylate) (PMMA), segmented poly(urethane) (PU), poly(vinyl chloride) (PVC), and silicone (Si)] as a mold in six subcutaneous pouches in the dorsal skin of New Zealand White rabbits. For rods apart from PFA, biotubes were constructed after 1 month of implantation by encapsulation around the polymeric implants. The wall thickness of the biotubes ranged from about 50 to 200 μm depending on the implant material and were in the order PFA < PVC < PMMA < PU < PE. As for PE, PMMA, and PVC, the thickness increased after 3 months of implantation and ranged from 1.5-to 2-fold. None of the biotubes were ruptured when a hydrostatic pressure was gradually applied to their lumen up to 200 mmHg. The relationship between the intraluminal pressure and the external diameter, which was highly reproducible, showed a "J"-shaped curve similar to the native artery. The tissue mostly consisted of collagen-rich extracellular matrices and fibroblasts. Generally, the tissue was relatively firm and inelastic for Si and soft for PMMA. For PMMA, PE, and PVC the stiffness parameter (β value; one of the indexes for compliance) of the biotubes obtained was similar to those of the human coronary, femoral, and carotid arteries, respectively. Biotubes, which possess the ability for wide adjustments in their matrices, mechanics, shape, and luminal surface design, can be applied for use as small-caliber blood vessels and are an ideal implant because they avoid immunological rejection.

Key words: Biotube; Arterial graft prosthesis; Tissue engineering; Autologous transplant; Small caliber

INTRODUCTION

It is ideal to design transplantation tissues consisting solely of the patient's own cells and matrix components, including appropriate mechanical properties and shapes. In this study, we have attempted to develop a novel in vivo tissue-engineering technique for the design and preparation of vascular tissues with high patency at a chronic phase for autotransplantation using the patient's own cells and extracellular matrix components. It is known that when artificial materials are embedded in the body, fibroblasts appear around the implanted materials due to the action of the body's own biological defense system. This results in the formation of capsular tissues consisting of a collagen-rich extracellular matrix produced by the fibroblasts. The principle of our study was to utilize capsular tissues.

The encapsulation phenomenon has been well documented since the 1930s. Peirce et al. attempted to utilize capsular tissues as artificial vascular vessels (19,27). In the latter half of the 1960s, Sparks et al. investigated the clinical application of grafts consisting of a combination of capsular tissues and artificial Dacron blood vessels (8,34). In their study, a Dacron fabric graft with a silicone tube inside the graft was embedded into the subcutaneous tissue of a patient. When the patient's tissue gradually covered the Dacron graft, the silicone tube in the graft was removed to give an autotissue tube around the Dacron graft as a scaffold. The tube was used as an arterial bypass by anastomosis of both ends of the tube with an occluded native artery. However, because the luminal surface of the grafts was exposed with collagenous fibers, which promote thrombus formation, occlusion occurred within the early stage of implantation in almost all of the cases.

Recently, Campbell et al. investigated utilizing capsular tubular tissues alone for artificial blood vessels (5). After intraperitoneal indwelling of silicone rods for sev-

Address correspondence to Yasuhide Nakayama, Department of Bioengineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan. Tel: +81-6-6833-5012; Fax: +81-6-6872-8090; E-mail: nakayama@ri.ncvc.go.jp

eral weeks in rats, mice, rabbits, and dogs, tubular tissues that possessed a medial wall with several layers of myofibroblasts and a collagen-rich extracellular matrix covered with a single layer of mesothelial cells were obtained. By inverting the tubular tissues, an inner lining of the mesothelial cells was acquired as a nonthrombotic luminal surface. Autotransplantation of these tissues (3–5 mm) as grafts resulted in high patency for several months, suggesting the possible application of capsular tissues alone for arterial blood vessels at an early stage of implantation.

The patency rate after implantation of small-caliber artificial grafts is much lower than medium-to large-diameter artificial grafts due to thrombosis in early stage and neointimal hyperplasia in chronic stage. Among the many factors determining the patency of small-caliber artificial grafts, the compliance mismatch between the native artery and the artificial grafts has been discussed as a major detrimental factor of graft failure (1,17,18, 28,36). Indeed, it has been demonstrated that compliance matching of elastomeric segmented poly(urethane) (PU) tubes by microporing markedly inhibited intimal hyperplasia in an animal model (6).

In this study, the mechanical properties of the tissues were investigated as the first stage of utilizing tubular tissues formed by encapsulation for small-caliber arterial blood vessels. At first, various rod-form polymeric implants were embedded in subcutaneous pouches in the dorsal skin of rabbits to obtain tubular tissues, called biotubes, that were formed by encapsulation. The mechanical properties, including pressure resistance, pulse follow-ability, and compliance of the biotubes, were measured after histological analysis of their components. The designs of the matrix, including luminal surface, mechanics, and shape of the biotubes for specific transplantation sites, were also discussed.

MATERIALS AND METHODS

Preparation of Biotube

The experimental animals were nine New Zealand White rabbits, weighing 2.0–2.5 kg. The investigations were performed according to the Principles of Laboratory Animal Care (formulated by the National Society for Medical Research) and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication, No. 56-23, revised 1985). Anesthesia was induced by intramuscular injection of a mixture of ketamine (62.5 mg/kg) and xylazine (8.3 mg/kg). The dorsum of the rabbits was shaved and sterile prepped with iodine (Meiji Seika, Ltd., Tokyo, Japan). Six small incisions (approximately 1 cm long) were made laterally on the dorsal skin using a surgical blade. Subcutaneous pouches were made away from each incision using blunt scissors. Six kinds of polymers in the shape of rods (length: 20 mm; diameter: 3 mm) were placed inside each pouch. The polymer materials used were: low-density poly(ethylene) (PE, Yamaichi Co. Ltd., Osaka, Japan; specific gravity: 0.92 g/cm^3, tensile strength: 110 kg/cm^2, elongation: 650%), soft poly(vinyl chloride) (PVC, Yamaichi Co. Ltd.; tensile strength: 1.6 kgf/mm^2, elongation: 281%), poly(fluoroacetate) (PFA, Toho Kasei Co. Ltd.; specific gravity: 2.12 g/cm^3, tensile strength: 20.5 MPa, elongation: 244%), and silicone (Si, Tigers Polymer Co. Ltd., Osaka, Japan; specific gravity: 1.16, tensile strength: 10.8 MPa, elongation: 510%). All rods apart from poly(methyl methacrylate) (PMMA) and PU rods were fabricated by thermally end-capping of the tubes prepared by an extrusion molding method. PMMA rod was fabricated by Daisan Kako Co. Ltd. (Osaka, Japan) by an extrusion molding method of PMMA pellet (Delpet 60N, Asahi Chemical Co. Ltd., Tokyo, Japan; specific gravity: 1.19 g/cm^3, tensile strength: 72 MPa, elongation: 5%). All polymeric tubes and PMMA rod were obtained from Sanplatec Co. Ltd. (Osaka, Japan). Segmented PU rod was prepared by thinly dip-coating around the PMMA rod from a tetrahydrofuran solution of PU purchased from Nihon Miractrane Co. Ltd. (E980MNAT, Kanagawa, Japan). The coating thickness was about 50 μm. The incisions were closed with a 3.0 ethilon suture (Ethicon, Somerville, NJ).

At specific time points (1, 2, and 3 months) after insertion of the polymer rods, the rabbits were anesthetized with a mixture of ketamine (62.5 mg/kg) and xylazine (8.3 mg/kg). The dorsum of the rabbits was shaved and sterile prepped with iodine. Six small incisions (approximately 2 cm long) were made in the neighborhood of the implanted part on the dorsal skin. The encapsulated six implants, which were weakly covered with a membrane tissue in the subcutaneous layer, were harvested with surrounding tissues from the dorsum of each of rabbits using a surgical blade. The surrounding fragile membrane tissues covered on the obtained firm encapsular tissues were carefully removed by scissors. The biotubes that formed around the polymeric rods as a capsule were removed from the rod by trimming one end.

Histological Examination

The created biotubes were explanted and fixed by 10% buffered formalin solution and embedded in paraffin. Tissue sections were cut into 3–5-μm-thick pieces for routine hematoxylin and eosin for histological evaluation. Immunohistochemistry was also performed for identification of the muscular component of the biotubes. Antibodies to α-smooth muscle actin (α-SMA, DAKO, 1:100 dilution), vimentin (DAKO, 1:100 dilu-

tion), desmin (DAKO, 1:100 dilution), and RAM11 for rabbit macrophages (DAKO, 1:50 dilution) were applied to the serial sections of the tubular tissues using a labeled avidin-biotin (LAB) system (DAKO, Japan). Diaminobenzidine (DAB) was used as a reacting color. The thickness of the biotube walls was measured using a microscopic monometer.

Mechanical Properties

The luminal pressure–diameter relationship was determined using an apparatus designed by Takamizawa and Hayashi (37). One end of the biotube was cannulated to a fixed stainless steel connector for pressure loading and the other to a sliding connector, and the biotube was restored to in situ length in a bath filled with Krebs-Ringer solution held at 37°C. The biotube was gradually inflated with a pressurized Krebs-Ringer solution using a pump, recording the intraluminal pressure from 0 to around 200 mmHg at a rate of 5 mmHg/s. The luminal pressure, P, measured with a pressure transducer (N5901; Nihon Denki Sanei, Inc., Tokyo, Japan), and the external diameter at the center of the tube, D, were determined using an apparatus consisting of a video camera (C2400; Hamamatsu Photonics, Inc., Shizuoka, Japan), TV monitor, and width analyzer (C3160, Hamamatsu Photonics, Inc) and all were simultaneously recorded.

The compliance of the tube was determined by the stiffness parameter (β) as defined by Hayashi et al. (9,10), which is described according to the equation: $\ln(P/P_s) = \beta(D/D_s - 1)$, where P, P_s, D, and D_s denote luminal pressure, standard pressure (100 mmHg in this study), external diameter, and diameter at the pressure P_s, respectively. The logarithm of the normalized pressure $[\ln(P/P_s)]$ was plotted against the normalized external diameter (D/D_s). The β value was determined as the approximate slope of the plot in the physiological blood pressure range from 60 to 140 mmHg.

RESULTS

Preparation of Biotubes

The six types of polymeric round rods, consisting of either PMMA, Si, PU, PE, PVC, or PFA, were used as a mold. In all nine rabbits, the polymeric rods were inserted separately into each subcutaneous pouch via a small incision on the rabbit's dorsal skin. After 1 month of embedding all rods were found to be encapsulated by a membrane tissue in the subcutaneous layer (Fig. 1A). The capsule that formed around the implants adhered weakly to the subcutaneous membrane tissue, making it easy to excise the implants with the surrounding capsulated tissue from the subcutaneous tissue (Fig. 1B). When one end of the tissue was opened the inner implants and the tubular tissues were not adhered and the inner implants could be easily removed without injuring the tissues, thereby obtaining tubular tissues (biotubes) whose walls were thin and relatively firm. The outer surface of the biotubes had a slightly rough appearance due to adhesion with the subcutaneous membrane tissue, but the luminal surfaces were extremely smooth. At 3 months of embedding, all implants were still covered with capsular tissues and were impregnated in the subcutaneous membrane tissue. Generally, the biotubes obtained after 3 months of embedding showed a firmer wall form than those obtained after 1 month of embedding.

Components of the Biotubes

Cross sections from the obtained biotubes were histologically investigated. For all biotubes the size of the inner diameter that formed around the polymeric round rods after 1 month of embedding was similar to the outer diameter of the mold, about 3 mm (Fig. 2A), and the wall thickness was almost uniform. The thickness of the biotube walls was around 70 μm after 1 month of embedding when Si, PVC, or PFA implants were used and around 100–150 μm when PU or PMMA implants were used (Figs. 3 and 4). The wall was thickest (around 200 μm) when the PE implant was used. For all implants the inner diameter did not change after 3 months of embedding compared with 1 month of embedding. However, the wall thickness increased (1.5-to 2-fold) for PMMA, PVC, and PE (Fig. 3). The structure of the biotube walls formed after 3 months of embedding is shown in Figure 4. The biotube walls that formed around the PFA rod had sparse collagen fibers and contained relatively abundant component spindle cells consistent with fibroblasts. Regarding the Si rod, the biotube wall was thin but collagen fibers with a close mesh structure were layered and almost no component cells were observed. Regarding the PMMA, PU, and PVC rods, the walls formed a moderate thickness and relatively large collagen fibers formed around the mesh structures. Fibroblasts as component cells were abundant and the biotubes that formed around the PU and PVC bases contained a number of inflammatory cells. Regarding the PE rod, the wall was very thick but almost no regular mesh structure of collagen fibers formed.

From immunohistochemical studies vimentin, a mesenchymal tissue marker, was positive for all tubular tissues around the various rods after 1 and 3 months of embedding (Fig. 5). α-SMA was intensely positive for all tubular tissues after 3 months. Desmin as a cytoskelton of matured muscle was negative in all tubular tissues after 1 and 3 months. Inflammatory cells such as lymphocytes and foreign body giant cells were observed in

Figure 1. (A) Macroscopic observation of the biotube, impregnated in the rabbit dorsal subcutaneous tissue, which was formed by implantation of the PMMA round rod (diameter: 3 mm, length: 2 cm) for 1 month into a subcutaneous pouch prepared on rabbit dorsal skin. (B) The external view of the biotube with the PMMA rod inside extracted from the rabbit dorsal subcutaneous tissue.

the tubular tissues of PE and PU as described above. RAM11, a marker of rabbit macrophages, was observed for a small number of macrophages among the tubular tissues of PU, PFA, and PMMA after 3 months of implantation.

Mechanical Properties of the Biotubes

Changes in the outer diameter of the biotubes were measured when both ends of the biotubes were closed and a water pressure was continuously added to the lumen. None of the biotubes ruptured even after 200 mmHg inner pressure, showing pressure resistance after only 1 month of implantation (Fig. 6). The external diameter of the biotubes around the Si rod became slightly dilated when exposed to water at low pressure but did not change significantly with increased pressure (about 20 mmHg or higher) (Fig. 6). In contrast, the external diameter of the biotube that formed around the PMMA rod became dilated at low-pressure ranges and gradually increased with pressure up to a high range, indicating "J"-shaped curves (Fig. 6). The dilatation rate of the outer diameter at a water pressure of 200 mmHg was about 5% for Si and about 25% for PMMA.

Water pressure was repeatedly loaded and removed in the lumen of the biotube that formed around the PMMA rods within a range of 0 to 200 mmHg, and changes in the external diameter were investigated (Fig. 7). The external diameter of the biotube was about 2.7 mm before loading and dilated to about 3 mm after loading at several 10-mmHg water pressure and thereafter continuously dilated slowly with an increase in inner pressure load, reaching about 3.2 mm at a luminal pressure of 200 mmHg. When the water pressure was removed the outer diameter gradually decreased and

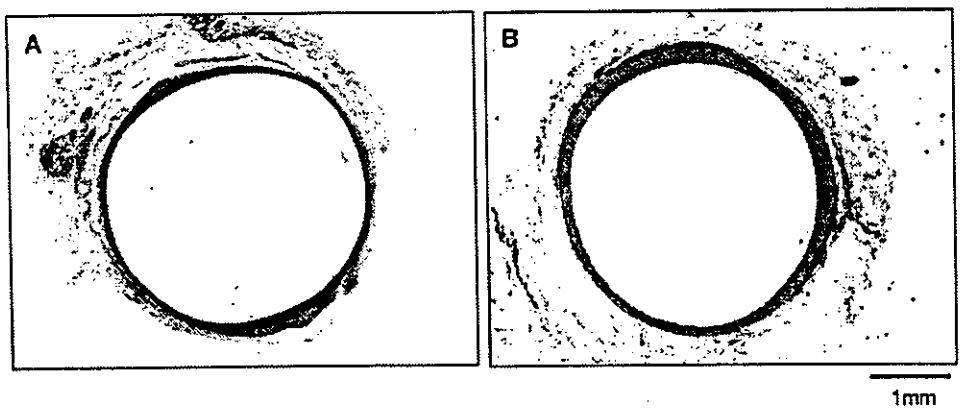


Figure 2. Circumferential sections of the biotubes formed by implantation of the PMMA round rod in the rabbit dorsal subcutaneous pouch for (A) 1 month or (B) 3 months (hematoxylin and eosin stain).

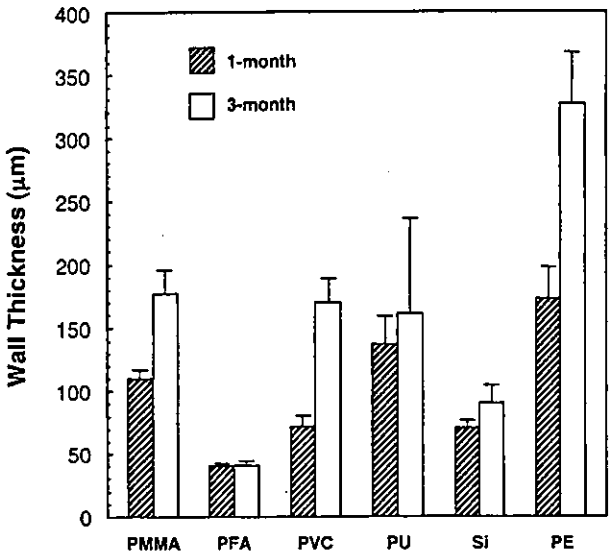


Figure 3. Implantation period-dependent changes of wall thickness of the biotubes formed around the six kinds of polymers.

reached about 3 mm at several 10 mmHg then rapidly decreased and returned to its original diameter before loading water pressure began, about 2.7 mm at 0 mmHg. Changes in the outer diameter with luminal pressure were basically the same with repeated pressure loadings in the lumen. In the case of biotubes prepared using other implants, repeat experiments produced almost identical changes.

The relationship between logarithmic value of the relative pressure and relative outer diameter was obtained from the relationship between outer diameter and luminal pressure. Because a linear relationship was obtained within a physiological range of pressure, β values were calculated from the slope of the line at a point near the standard inner pressure (100 mmHg). After 1 month of implantation, the highest β value was obtained from the biotube formed around the Si base and the β value decreased in the order of PMMA, PE, and PVC (Fig. 8). The biotube that formed around the PMMA rod exhibited a β value close to that of the human coronary artery whereas the β values of the biotubes formed around the PE and PVC bases were close to those of the human femoral and common carotid arteries, respectively. The β value of the biotube formed around the PMMA rod was about 30 after 1 month of embedding and increased linearly to about 80 after 3 months of embedding (Fig. 9).

DISCUSSION

Considering problems such as immunological rejection, it is important that tissues and organs for transplantation should consist solely of autotissues. However, it is sometimes difficult to obtain appropriate biocompatible tissues for transplantation, even for blood vessels. For example, the patient's own vessels (autografts) are used

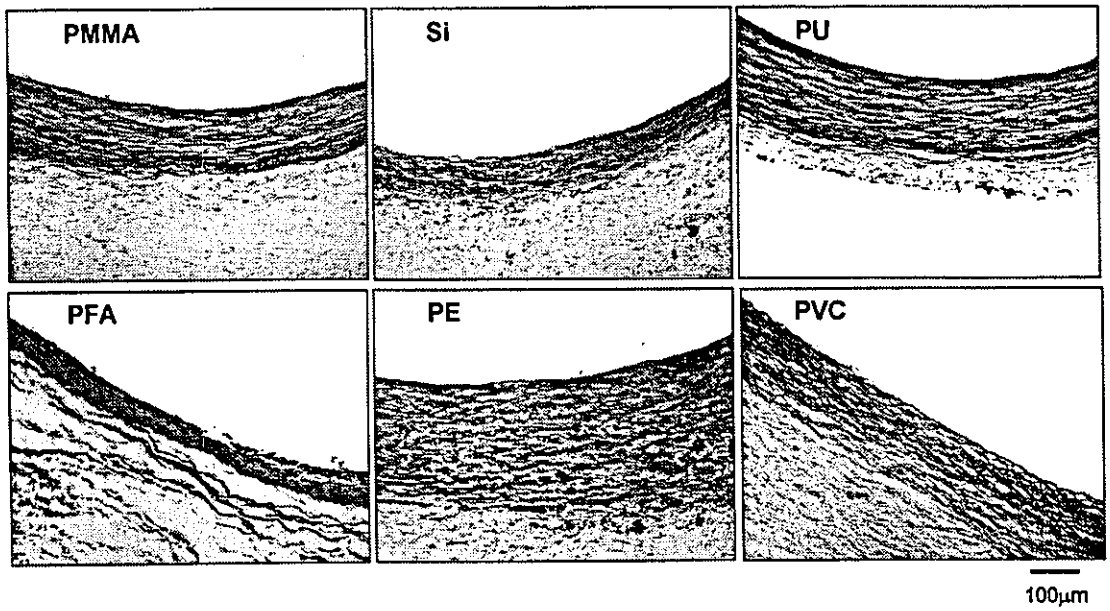


Figure 4. Circumferential sections of the biotubes formed by 3 months of implantation of six kinds of polymer round rods in the rabbit dorsal subcutaneous pouch (hematoxylin and eosin stain). Upper side of the wall of the biotube in each photo indicates luminal surface.

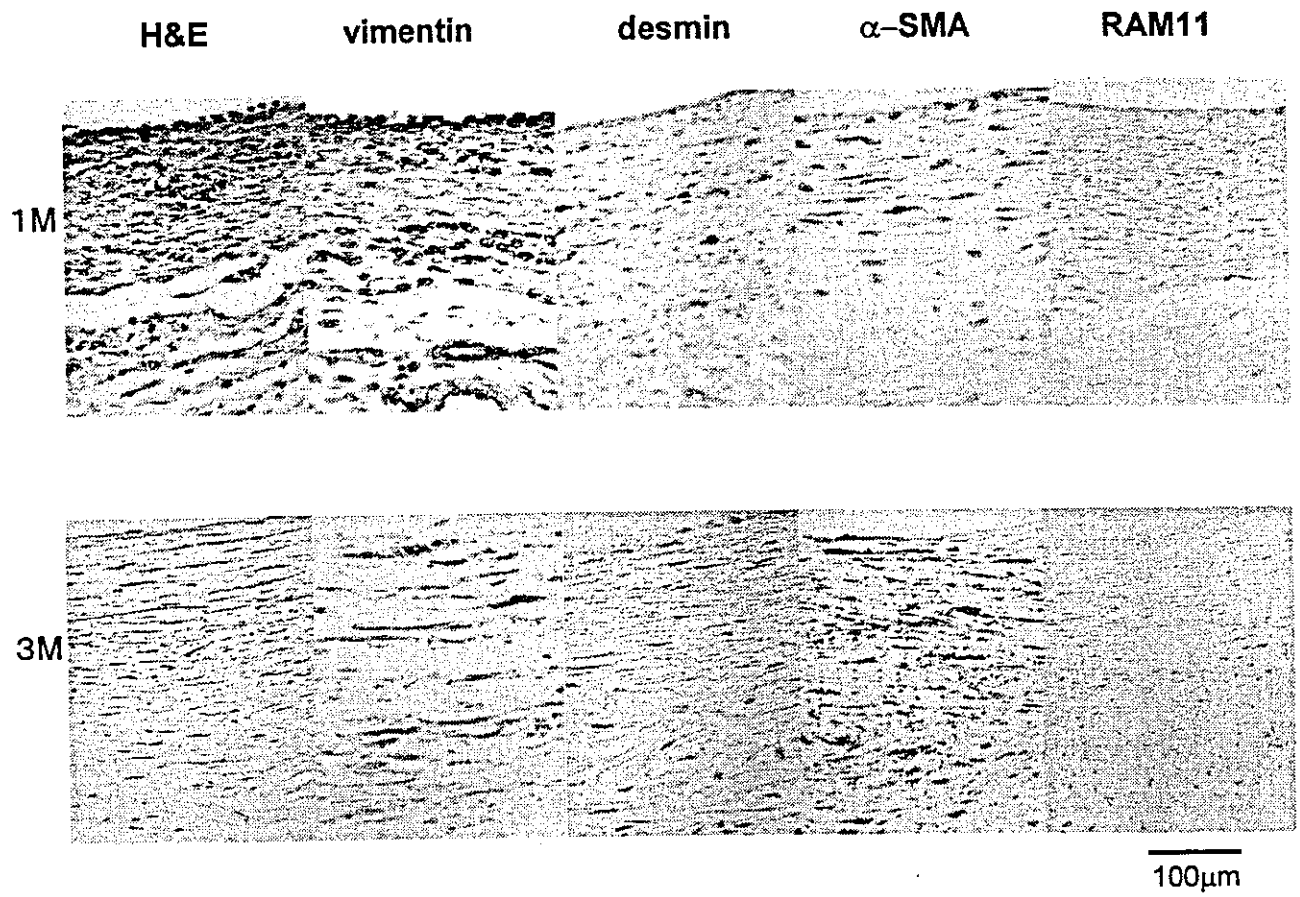


Figure 5. Immunohistochemistry of the circumferential sections of the biotubes formed around the PMMA rods after 1 month (upper columns) and 3 months (lower columns) of implantation. In two implantation periods, the fibroblasts and collagen fibers, the component of the biotube walls, are positive for vimentin and α-SMA but not for desmin. α-SMA is intense at 3 months. Some macrophages (RAM11) are recognized perifibrous tissue at 3 months.

for bypass operations for angina pectoris and myocardial infarction as a graft. The patient's great saphenous vein in the lower leg and internal thoracic artery are generally harvested for grafts. Gastroepiploic arteries and radial arteries are also recruited when other vessels are inappropriate. In addition, elderly patients may have second or third bypass operations. However, it is sometimes difficult to obtain a sufficient amount of grafts that are of the correct size or length due to the patient's limited supply of vessels. Therefore, autologous grafts are not always available.

In the field of large-caliber artificial blood vessels, with inner diameters more than 5 mm, grafts are used that are composed solely of artificial materials such as Dacron fabric grafts and expanded poly(tetrafluoroethylene) (ePTFE). However, when these materials are experimentally used for small-caliber blood vessels, occlusions occur within a short period after implantation due to their thrombogenicity. Thus, the development of hybrid-type artificial blood vessels combining artificial and biological materials is in progress. A technique to construct a hierarchical layer with three cell types resembling biological vascular walls in vitro has been developed as a hybrid artificial blood vessel (13,20,21). The technique involves sequentially embedding component cells of an excised autovascular wall into collagen gel and culturing. These layered hybrid blood vessels with a diameter of 4 mm were found to retain very high patency after 1 year of autotransplantation in a canine carotid artery. Other hybrid artificial blood vessels have been prepared by seeding and culturing mixed suspensions of vascular wall component cells of the auto-great saphenous vein in a poly-L-lactic acid (PLLA) tubular sponge, and have successfully been used in reconstruction of the pulmonary artery in humans (11,30,31). Such progress greatly increases the possibility of reconstruction utilizing in vitro tissue-engineering techniques. Recently, it has been proposed that the preparation of vari-

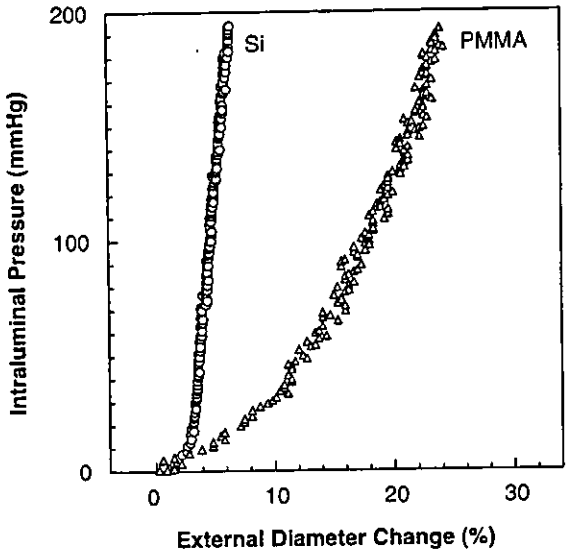


Figure 6. Pressure–diameter relationship of the biotubes formed around the PMMA rod and the Si rod after 1 month of implantation. Water pressure was loaded in the lumen of the biotube up to 200 mmHg.

ous hybrid artificial blood vessels could be achieved by in vitro tissue-engineering techniques using stem cells, including progenitor vascular endothelial cells (2,15) and embryonic stem (ES) cells (25,39), as a cell source (16,32).

When a foreign body such as an artificial material is embedded under the skin, two major reactions based on the biological defense system occur in an effort to reject it. One representative reaction is biodegradation and the other is capsulation. When a foreign body releases inflammatory substances, inflammatory cells such as leukocytes and macrophages accumulate around the foreign body. The foreign body is then phagocytosed and degraded by these cells. When a foreign body is less inflammatory and rather large, fibroblasts accumulate around the foreign body and produce collagen, resulting in the formation of a capsule consisting of collagen-rich extracellular matrix that wraps the foreign body. Although this capsulation phenomenon has been long known, details of the relationship of artificial materials with the amount and physical properties of capsules formed have not been investigated. Accordingly, no systemic explanation has been put forward regarding what type of capsulation occurs for different artificial materials.

Si is widely used as a biomaterial and has high biocompatibility (7,38). It is well known that embedding of Si bases causes capsulation, and tubular tissues form around the embedded tubular Si base. Use of this tubular tissue as a vascular substitute (artificial blood vessel) has been investigated for a long time, and clinical applications of the tissue strengthened with artificial Dacron grafts for arterial bypass in the lower limbs have been attempted (8,34). The tubular tissue was found to acquire vascular function at an early stage of the transplantation (8,34). However, the tube was occluded within a short period in most cases because the luminal surface was exposed with collagen fibers, which promote thrombus formation. Because of this, the technique has been dormant for nearly 30 years. However, it has recently been reported that mesothelial cells, which have an antithrombotic function, were arranged on the luminal surface of tubular tissue and were obtained using Si bases (5). The patency rate was relatively high, about 70%, after 2 months of transplantation of the tubular tissues in animal experiments. This result showed that the antithrombotic

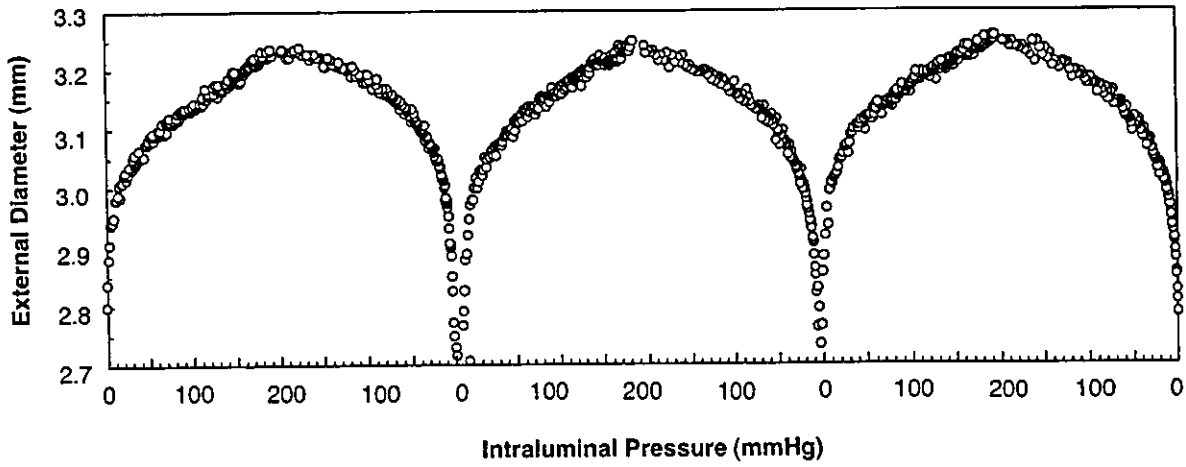


Figure 7. External diameter change by repeatable process in loading and removing of water pressure within a range 0–200 mmHg to the lumen of the biotube formed around the PMMA rod after 3 months of implantation.

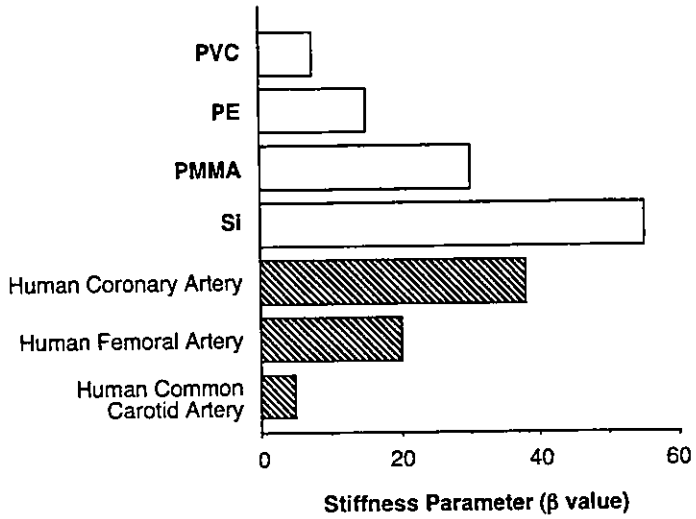


Figure 8. The stiffness parameters (β values) of the biotubes by 1 month of implantation. The biotubes formed around the PMMA, PE, and PVC rods exhibited β values close to those of the human coronary artery, the human femoral artery, and common carotid artery, respectively.

treatment allowed tubular tissues alone to acquire vascular function even a short time after implantation.

We have attempted to develop small-caliber artificial blood vessels that have high patency by combining the tubular tissue (biotube) preparation technique, which utilizes capsulation and involves in vivo tissue engineering, with recent cytokine engineering and nanotechnology to develop a clinical application. Utilizing capsulation, it may be possible to prepare biotubes that are appropriately mechanically designed and shaped according to the vascular transplantation site in the patient's body. Because biotubes consist of the patient's own cells and extracellular matrix components, transplantation of the tubular tissue is equivalent to autotransplantation. Therefore, immunorejection of the tubular tissue is avoided. Moreover, the tissue may become self and grow after transplantation in the body. In addition, grafts can be abundantly prepared, resolving the lack of donor blood vessels that is the greatest problem of current bypass procedures using auto-blood vessels.

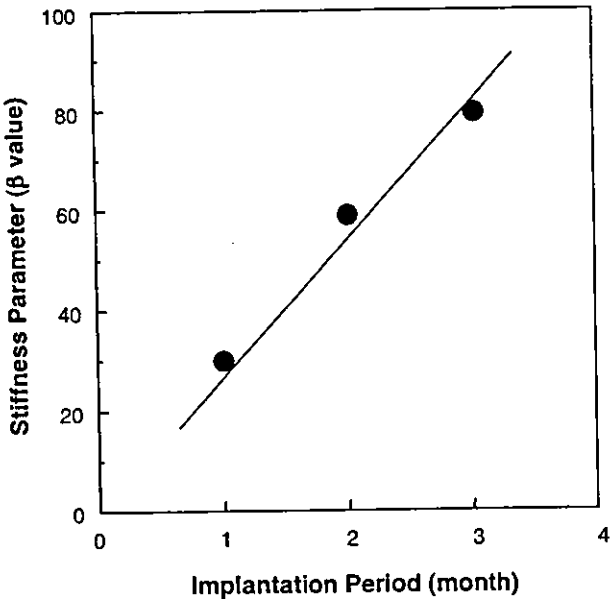


Figure 9. Implantation period-dependent stiffness parameter (β value) change of the biotubes formed around the PMMA rods. The compliance increased linearly up to 3 months of implantation.

For patency of artificial blood vessels, particularly those with a small caliber (5 mm or less inner diameter), the following are required. 1) Resistance to blood pressure, 2) antithrombotic properties to avoid thrombotic occlusion in the early stage of transplantation, and 3) mechanical compatibility including compliance matching and pulse follow-ability to avoid occlusion due to intimal hyperplasia in the chronic stage. In this study various general polymeric rods as a mold were embedded in the body. Tissue formation, which was dependent on the type of polymeric rod material used, was investigated for its encapsulation ability with regard to the above requirements for small-caliber artificial blood vessels 1) and 3). The materials used for the polymeric rods were PMMA, PU, PVC, PE, and Si, all of which are hydrophilic, and PFA, which is water repellent, and all are used as biomaterials. When these rods were embedded under the dorsal skin of rabbits, biotubes were formed around the implants even after 1 month. The luminal

surfaces of the biotubes did not adhere to the implants, but the outer surfaces were covered weakly with the subcutaneous membrane tissue. The biotubes were impregnated in the surface of the subcutaneous tissue, but were easily exfoliated and excised with the implants inside without injury (Fig. 1). Excluding the tube that formed around the PFA rod, none of the biotubes ruptured with 200-mmHg inner pressures. The walls of these biotubes were mainly composed of a collagen-rich extracellular matrix containing fibroblasts (Fig. 4). However, in the tube that formed around PFA the collagen fiber density was extremely low and the majority of the cells were inflammatory cells, forming almost no capsule (Fig. 4). It is known that protein adsorption and cell adhesion are inhibited on PFA surfaces. Therefore, capsulation may have been prevented due to an inhibition of fibroblast adhesion. The collagen mesh structure accumulated in an irregular manner in the biotube that formed around PE (Fig. 4), but mesh structures that were relatively rough formed around PMMA, PU, and PVC (Fig. 4). In the tube that formed around PU, inflammatory cell infiltration was noted (Fig. 4). Although PU is used as a blood-compatible material for artificial hearts (40), when transplanted as an artificial blood vessel granulation may often be recognized, caused by inflammation on its outer surface (29,33). PU is considered to have strong tissue reactivity. A dense collagen mesh structure was formed around Si (Fig. 4). The wall thickness of the biotubes after 1 month of implantation decreased in the order of PE > PU > PMMA > PVC > Si > PFA and increased with transplantation period apart from PFA, PU, and Si.

The stiffness parameter (β) is one of the indexes for compliance of blood vessels and indicates the mechanical property under physiological blood pressure (9,10). Lower values in the β value indicate the material is soft and flexible. Within the polymers used in this study, the β value decreased in the order of Si > PMMA > PE > PVC. Therefore, it can be said that the synergistic action of the wall thickness and inner structure of the walls determines the mechanical property of the biotubes. The biotube that formed around Si was relatively firm and inflexible, while the biotube that formed around PMMA was elastic within a low-pressure range and less extensible at a high-pressure range, showing a mechanical property similar to that of biological arteries. The relationship between intraluminal pressure and external diameter showed a "J"-shaped curve, similar to the native artery. The pulse follow-ability was good for both these biotubes. The biotube obtained after 1 month using PMMA, PE, and PVC exhibited compliance similar to that of the human coronary artery, human femoral artery, and human carotid artery, respectively. Selection of specific rod materials and embedding period allow the design of artificial blood vessels with matching of mechanical properties with biological blood vessels. This matching is expected to prevent intimal hyperplasia-causing occulusion in the chronic stage. However, differences in mechanical properties among biotubes formed around various implants have not been clarified. The following causes can be considered: 1) chemical composition of the implant surface, 2) physical microstructure of the implant surface, 3) mechanical strength of implant, and 4) biochemical activity of implants. We have started to investigate the effect of surface chemistry on biotube formation. The chemical composition of the outermost surface (about a few hundred nanometers) of PMMA, which was standardized as the implant material, was changed by surface grafting using surface nanotechnology (23).

On the other hand, embedding for at least 1 month is necessary for biotube formation. Accordingly, this method cannot deal with urgent cases such as myocardial infarction. To promote tissue formation, bioactive implants are being prepared by immobilizing several cytokines such as bFGF and HGF (22,24,26). Furthermore, it is also required to provide antithrombotic properties to the luminal surface for inhibition of occlusion in the early stage of transplantation. The most effective method is early induction of vascular endothelial cells on the luminal surface of the biotube after transplantation to obtain complete endothelization of the luminal surface. For this purpose, immobilization of VEGF on the rod surface is being investigated. It has been reported that a slow release of VEGF promotes angiogenesis (35) and is effective for neovascularization therapy for peripheral and cardiac vascular ischemia (3,14). In our preliminary study, slow release of VEGF from the rod surface promoted induction of much neovascularization in the biotube tissue (data not shown). When these biotubes containing abundant neovasculars are transplanted, endothelial cells in the biotubes may migrate to the luminal surface of the biotubes and early endothelization after transplantation may occur.

Another advantage of the biotube preparation method is that the shape can be freely designed. Manipulation of the shape of the rods that are to be embedded may induce capsulation along their particular shape and a biotube with a complex shape may be obtained. In a preliminary study, when a branched rod as a mold was embedded under a rabbit's skin, a biotube with a branched shape was obtained (data not shown). This method may allow easy preparation of arterial grafts with shapes specific to the transplantation site.

Biotubes as artificial blood vessels can be prepared in the patient's body using the patient's cells and extracellular matrix components. Furthermore, it is possible to design specific mechanics and it is easy to match the

host's vascular tissue. There is no concern for limited supply and there are no problems of immunorejection because it is autotransplantation. Biotubes are expected to grow with the host's blood vessels and are an ideal vascular graft. We are planning to establish a biotube preparation method that combines surface design as described above and demonstrate the usefulness of biotubes as small-caliber artificial blood vessels by animal transplantation experiments. These results will be reported in the near future.

ACKNOWLEDGMENTS: The authors thank Ms. Michiko Hayashi for her technical assistance. This study was supported by Research Grants for "Advanced Medical Technology," "Human Genome, Tissue Engineering and Food Biotechnology," and "Aging and Health," and Cardiovascular Grant (13C-1) from the Ministry of Health, Labour and Welfare of Japan, and by Grant-in-Aid for Scientific Research (B2-13450302) from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

1. Abbott, W. M.; Megerman, J.; Hasson, J. E.; L'Italien, G.; Warnock, D. F. Effect of compliance mismatch on vascular graft patency. J. Vasc. Surg. 5:376–382; 1987.
2. Asahara, T.; Isner, J. M. Endothelial progenitor cells for vascular regeneration. J. Hematother. Stem Cell Res. 11: 171–178; 2002.
3. Baumgartner, I.; Isner, J. M. Stimulation of peripheral angiogenesis by vascular endothelial growth factor (VEGF). Vasa 27:201–206; 1998.
4. Brodbeck, W. G.; Patel, J.; Voskerician, G.; Christenson, E.; Shive, M. S.; Nakayama, Y.; Matsuda, T.; Ziats, N. P.; Anderson, J. M. Biomaterial adherent macrophage apoptosis is increased by hydrophilic and anionic substrates in vivo. Proc. Natl. Acad. Sci. USA 99:10287–10292; 2002.
5. Campbell, J. H.; Efendy, J. E.; Campbell, G. R. Novel vascular graft grown within recipient's own peritoneal cavity. Circ. Res. 85:1173–1178; 1999.
6. Doi, K.; Matsuda, T. Significance of porosity and compliance of microporous, polyurethane-based microarterial vessel on neoarterial wall regeneration. J. Biomed. Mater. Res. 37:573–584; 1997.
7. Habal, M. B. The biologic basis for the clinical application of the silicones. A correlate to their biocompatibility. Arch. Surg. 119:843–848; 1984.
8. Hallin, R. W.; Sweetman, W. R. The sparks' mandril graft. A seven year follow-up of mandril grafts placed by Charles H. Sparks and his associates. Am. J. Surg. 132: 221–223; 1976.
9. Hayashi, K.; Handa, H.; Nagasawa, S.; Okumura, A.; Moritake, K. Stiffness and elastic behavior of human intercranial and extracranial arteries. J. Biomech. 13:175–184; 1980.
10. Hayashi, K.; Nakamura, T. Material test system for the evaluation of mechanical properties of biomaterials. J. Biomed. Mater. Res. 19:133–144; 1985.
11. Hibino, N.; Shin'oka, T.; Kurosawa, H. Long-term histologic findings in pulmonary arteries reconstruction with autologous pericardium. N. Engl. J. Med. 348:865–867; 2003.
12. Higashi, J.; Nakayama, Y.; Marchant, R. E.; Matsuda, T. High-spatio resolved microarchitectural surface prepared by photograft copolymerization using dithiocarbamate: Surface preparation and cellular responses. Langmuir 15: 2080–2088; 1999.
13. Ishibashi, K.; Matsuda, T. Reconstruction of a hybrid vascular grafs hierarchically layered with three cell types. ASAIO J. 40:M284–M290; 1994.
14. Isner, J. M.; Asahara, T. Angiogenesis and vasculogenesis as therapeutic strategies for postnatal neovascularization. J. Clin. Invest. 103:1231–1236; 1999.
15. Isner, J. M.; Kaka, C.; Kawamoto, A.; Asahara, T. Bone marrow as a source of endothelial cells for natural and iatrogenic vascular repair. Ann. NY Acad. Sci. 953:75–84; 2001.
16. Kaushal, S.; Amiel, G. E.; Guleserian, K. J.; Shapira, O. M.; Perry, T.; Sutherland, F. W.; Rabkin, E.; Moran, A. M.; Schoen, F. J.; Atala, A.; Soker, S.; Brischoff, J.; Mayer, J. E., Jr. Fabrication of small-diameter neovessels created using endothelial progenitor cells expanded ex vivo. Nat. Med. 7:1035–1040; 2001.
17. Kidson, I. G.; Abbott, W. M. Low compliance and arterial graft occulusion. Circulation 58(Suppl.):I1–I4; 1978.
18. Kinley, C. E.; Marble, A. E. Compliance: A continuing problem with vascular grafts. J. Cardiovasc. Surg. 21: 163–170; 1980.
19. Kott, I.; Peirce, E. C., II.; Mitty, H. A.; Geller, S. A.; Jacobson, J. H., II. The tissue tube as a vascular prosthesis. Arch. Surg. 106:206–207; 1973.
20. Matsuda, T.; Miwa, H. A hybrid vascular model biomimicking the hierarchic structure of arterial wall: Neointimal stability and neoarterial regeneration process under arterial circulation. J. Thorac. Cardiovasc. Surg. 110:988–997; 1995.
21. Miwa, H.; Matsuda, T. An integrated approach to the design and engineering of hybrid arterial prostheses. J. Vasc. Surg. 19:658–667; 1994.
22. Nakayama, Y.; Takatsuka, M.; Matsuda, T. Surface hydrogelation using photolysis of dithiocarbamate or xanthate: Hydrogelation, surface fixation, and bioactive substance immobilization. Langmuir 15:1667–1672; 1999.
23. Nakayama, Y.; Matsuda, T. In situ observation of dithiocarbamate-based surface photograft copolymerization using quartz crystal microbalance. Macromolecules 32:5405–5410; 1999.
24. Nishi, S.; Nakayama, Y.; Hashimoto, N.; Matsuda, T. Basic fibroblast growth factor impregnated hydrogel microspheres for embolization of cerebral arteriovenous malformations. ASAIO J. 44:M405–M410; 1998.
25. Nishikawa, S. Embryonic stem cells as a source of hematopic and vascular endothelial cells in vitro. J. Allergy Clin. Immunol. 100:S102–S104; 1997.
26. Okino, H.; Nakayama, Y.; Tanaka, M.; Matsuda, T. In situ hydrogelation of photocurable gelatin and drug release. J. Biomed. Mater. Res. 59:233–245; 2002.
27. Peirce, E. C., II. Autologous tissue tubes for aortic grafts in dogs. Surgery 33:648–657; 1953.
28. Pevec, W. C.; Darling, R. C.; L'Italien, G. J.; Abbott, W. M. Femoropopliteal reconstruction with knitted, non velelour Dacron versus expanded polytetrafluoroethylene. J. Vasc. Surg. 16:60–65; 1992.
29. Seifalian, A. M.; Salacinski, H. J.; Tiwari, A.; Edwards, A.; Bowald, S.; Hamilton, G. In vivo biostability of a poly(carbonate-urea)urethane graft. Biomaterials 24:2549–2557; 2003.
30. Shin/oka, T.; Imai, Y.; Ikada, Y. Transplantation of a tis-

sue engineered pulmonary artery. N. Engl. J. Med. 344: 532–533; 2001.
31. Shin'oka, T.; Shum-Tim, D.; Ma, P. X.; Tanel, R. E.; Isogai, N.; Langer, R.; Vacanti, J. P.; Mayer, J. E. Creation of viable pulmonary artery autografts through tissue engineering. J. Thorac. Cardiovasc. Surg. 115:536–546; 1998.
32. Shirota, T.; He, H.; Yasui, H.; Matsuda, T. Human endothelial progenitor cell-seeded hybrid graft: Proliferative and antithrombogenic potentials in vitro and fabricated processing. Tissue Eng. 9:127–136; 2003.
33. Sonoda, H.; Takamizawa, K.; Nakayama, Y.; Yasui, H.; Matsuda, T. Coaxial double-tubular compliant arterial graft prosthesis: Time-dependent morphogenesis and compliance changes after implantation. J. Biomed. Mater. Res. 65A:170–181; 2003.
34. Sparks, C. H. Silicone mandril method of femoropopliteal artery bypass. Clinical experience and surgical techniques. Am. J. Sur. 124:244–249; 1972.
35. Street, J.; Bao, M.; deGuzman, L.; Bunting, S.; Peale, F. V., Jr.; Ferrara, N.; Steinmetz, H.; Hoeffel, J.; Cleland, J. L.; Daugherty, A.; van Bruggen, N.; Redmond, H. P.; Carano, R. A.; Filvaroff, E. H. Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. Proc. Natl. Acad. Sci. USA 99: 9656–9661; 2002.
36. Stewart, S. F.; Lyman, D. J. Effects of a vascular graft/natural artery compliance mismatch on pulsatile flow. J. Biomech. 25:297–310; 1992.
37. Takamizawa, K.; Hayashi, K. Stain energy density function and uniform strain hypothsis for arterial mechanics. J. Biomech. 20:7–17; 1987.
38. Tang, L.; Eaton, J. W. Inflammatory responses to biomaterials. Am. J. Clin. Pathol. 103:466–471; 1995.
39. Yamashita, J.; Itoh, H.; Hirashima, M.; Ogawa, M.; Nishikawa, S.; Yurugi, T.: Naito, M.; Nakao, K.; Nishikawa, S. Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. Nature 408:92–96; 2000.
40. Zdrahala, R. J.; Zdrahala, I. J. Biomedical applications of polyurethanes: A review of past promises, present realities, and a vibrant future. J. Biomater. Appl. 14:67–90; 1999.

Three-Dimensional Cardiac Tissue Engineering Using a Thermoresponsive Artificial Extracellular Matrix

HIROSHI NAITO,* YOSHIAKI TAKEWA,* TOSHIHIDE MIZUNO,* SHOJI OHYA,† YASUHIDE NAKAYAMA,† EISUKE TATSUMI,* SOICHIRO KITAMURA,* HISATERU TAKANO,* SHIGEKI TANIGUCHI,‡ AND YOSHIYUKI TAENAKA*

The purpose of this study was to try to reconstitute three-dimensional cardiac tissue using a thermoresponsive artificial extracellular matrix, poly (N-isopropylacrylamide)-grafted gelatin (PNIPAM-gelatin), as the scaffold. PNIPAM-gelatin solution gels almost immediately when heated above 34°C. We thought this property could become advantageous as scaffolding for reconstituting three-dimensional tissue. Because PNIPAM-gelatin solution gels so quickly, all seeded cells in PNIPAM-gelatin solution would become entrapped and uniformly distributed toward three dimensions. Thus it would be possible to reconstitute three-dimensional tissue by a very simple method of mixing cells and PNIPAM-gelatin solution. Fetal rat cardiac cells were mixed with PNIPAM-gelatin solution, incubated at 37°C to allow the mixture to gel, and cultured *in vitro*. To define suitable culture conditions the following parameters were tested: (1) PNIPAM-gelatin concentration, 0.04~0.125 mg/ml; (2) cell seeding density, 1~50 × 10^6 cells/ml; and (3) addition or not of hyaluronic acid. With a PNIPAM-gelatin concentration of 0.05 mg/ml, a cell seeding density of 50 × 10^6 cells/ml, and the addition of hyaluronic acid, tissue was reconstituted and it contracted synchronously. After hematoxylin and eosin staining, the cells reconstituted three-dimensional tissue, and the tissue cross-section was approximately 60 μm thick. *ASAIO Journal* 2004; 50:344–348.

Because of the shortage of donor organs for heart transplantation, cell transplantation for the end stage of chronic heart failure is expected to become reality. Recently, there have been many studies demonstrating that cells transplanted into the infarcted myocardium improved heart function.[1-3] Clinically, a myoblast transplantation has been performed,[4] but it is controversial whether these effects observed were caused by the ability of transplanted cells to create sufficient amounts of new myocardium-like tissue within the infarcted area and to participate in synchronized heart contraction.[5] In these studies, donor cells were delivered by means of direct injection into the infarcted myocardium. We believe, however, that if we expect to observe contractile activity in cells transplanted into the infarcted myocardium, we will have to transplant sufficient numbers of donor cells into the scarred and thin infarcted myocardium. We believe that it is difficult to create a functional tissue equivalent in such a myocardium without the use of scaffolding. We must, therefore, seek effective transplantation methods. A promising approach to repairing large areas of scar may be the use of tissue engineered cardiac grafts. In tissue engineering, both the cells and the scaffolding material are important for reconstituting three-dimensional tissues. In this study, we used a thermoresponsive artificial extracellular matrix, poly(N-isopropylacrylamide) (PNIPAM)-gelatin,[6-9] for scaffolding to reconstitute three-dimensional cardiac tissue. PNIPAM-gelatin solution gels quickly with simple heating above 34°C. Our hypothesis was that the cells mixed in the PNIPAM-gelatin solution would be entrapped uniformly in PNIPAM-gelatin gel by heating and that the mixture would allow reconstitution of three-dimensional cardiac tissue.

The purpose of this study was to demonstrate that three-dimensional cardiac tissue may be reconstituted by mixing fetal rat cardiac cells with PNIPAM-gelatin and to establish the optimal conditions for doing so.

Materials and Methods

Care of Animals

All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985).

Cardiac Cells

Fetal rat cardiac cells were isolated from 18 day gestational Wistar rats. The ventricles of the fetuses were digested in three rounds of 10 minute incubations in a solution consisting of 0.05% trypsin in phosphate buffered saline. To reduce non-myocyte contamination cells, isolated cardiac cells were plated on a glass dish and cultured for 2 hours in the culture medium. The supernatant containing suspended cells was collected and centrifuged at 1,000 rpm for 5 minutes. The viability of the cells was calculated by trypan blue staining, and these cells were used for the following studies.

Scaffold

Thermoresponsive artificial extracellular matrix, poly (N-isopropylacrylamide) (PNIPAM) grafted gelatin,[6-9] here called PNIPAM-gelatin (a gift from Shoji Ohya), was used for the

From the Departments of *Artificial Organs and †Bioengineering, National Cardiovascular Center Research Institute, Osaka, Japan; and the ‡Department of Surgery III, Nara Medical University, Nara, Japan.

Correspondence: Hiroshi Naito, Department of Artificial Organs, National Cardiovascular Center Research Institute, 5-7-1 Fujishiro-dai Suita-city, Osaka, 565-8565, Japan.

DOI: 10.1097/01.MAT.0000132656.42232.E3

scaffold material. PNIPAM-gelatin was prepared by graft polymerization of PNIPAM on gelatin. PNIPAM-gelatin is water soluble at room temperature and immediately precipitates at temperature above approximately 34°C, so PNIPAM-gelatin solution gels as soon as it is heated above 34°C. A mixture was prepared at room temperature of various amounts of PNIPAM-gelatin and culture medium. The PNIPAM-gelatin concentrations were 0.04, 0.05, 0.075, 0.1, and 0.125 mg/ml. We studied the effects of the different PNIPAM-gelatin concentrations while using a cell seeding density of 10×10^6 cells/ml.

Three-Dimensional Cardiac Tissue

The reconstitution mixture was prepared by pouring PNIPAM-gelatin solution mixed with cardiac cells on a culture dish at room temperature. The reconstitution mixture was incubated at 37° to allow it to gel, and thereafter culture medium was added to each dish. These dishes were then placed in a humidified incubator at 37°C with 5% carbon dioxide and 95% air. The proportions of cardiac cells in the reconstituted mixture, the cell seeding densities, were studied at five different densities (1, 2, 5, 10, and 50×10^6 cells/ml). We studied the effects of various cell seeding densities using a PNIPAM-gelatin concentration of 0.05 mg/ml.

Supply of Hyaluronic Acid

Hyaluronic acid (HA) (0.003 mg/ml) was supplied to the culture medium to make HA supplied PNIPAM-gelatin solution. HA supplied reconstituted mixtures were made using the same methods as mentioned previously. We studied the effect of supplying HA for making cardiac tissue using a PNIPAM-gelatin concentration of 0.05 mg/ml and a cell seeding density of 50×10^6 cells/ml.

Macroscopic and Microscopic Observation

Macroscopic observation of the contractile activities of the reconstituted mixtures was performed daily with the unaided eye. Microscopic observation of cells' survival and the contractile activities of the reconstituted mixtures was performed daily by phase contrast microscopy. These views were recorded with a digital video camera.

Histologic Analysis

After 2 weeks of culturing, the reconstituted mixtures were fixed in 10% neutral buffered formalin, dehydrated in a graded series of ethanol, embedded in paraffin, and sectioned into 10 μm slices (perpendicular to the plane of the tissue). For basic morphology, hematoxylin and eosin (HE) staining was performed by conventional methods.

Results

PNIPAM-Gelatin Concentration

When the PNIPAM-gelatin concentration was 0.04 mg/ml, the PNIPAM-gelatin solution did not gel, even with heating above 34°C, so it could not be used as scaffold. At a PNIPAM-gelatin concentration of 0.05 mg/ml, the PNIPAM-gelatin solution did gel, and scattered asynchronously contracting isolated cells and clusters were observed in the reconstituted mixture after 2 weeks of culturing (**Figure 1**). When the PNIPAM-gelatin concentration was higher than 0.075 mg/ml, the PNIPAM-gelatin solution did gel, but the seeded cells could not survive in the reconstituted mixture (**Figure 2**).

Cell Seeding Density

When the cell seeding density was lower than 10×10^6 cells/ml, scattered asynchronously contracting isolated cells and clusters were observed in the reconstituted mixture after 2 weeks of culturing (**Figure 1**). These clusters were neither gathering nor becoming larger. When the cell seeding density was 50×10^6 cells/ml, more cells were connected, and tissue like constructs were observed in some parts of the reconstituted mixture, but they were too small for us to observe their contractions macroscopically.

Supply of Hyaluronic Acid

When HA was supplied to the reconstituted mixtures, spontaneously and synchronously contracting tissue was observed macroscopically from 1 week after culture, and the frequency of contraction was approximately between 30 and 250 beats/min as estimated by microscopic inspection. The macroscopic views were recorded by a digital video camera after 2 weeks of culturing (**Figure 3**). We also observed the same tissue using phase contrast microscopy. The tissue was made of many cells, and synchronous contractions of these cells were observed (**Figure 4**). With HE staining, cells reconstituted three-dimensional tissue with a cross-section as thick as 60 μm, and all cells in the tissue were viable and uniformly spread not only at the periphery but also in the center lesion (**Figure 5**).

Discussion

In vitro engineering of three-dimensional cardiac tissue has emerged as a technology with potential for tissue replacement therapy.[10-21] Successful engineering of cardiac tissue has been previously demonstrated by many methods of cultivation on

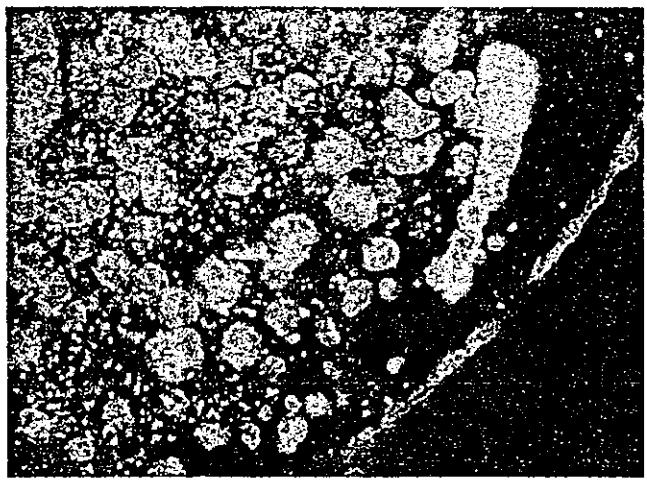


Figure 1. A phase contrast microscopic view of cardiac cells in PNIPAM-gelatin gel (PNIPAM-gelatin concentration, 0.05 mg/ml; initial cell seeding density, 10×10^6 cells/ml) after 2 weeks of culturing. Original magnification × 4. PNIPAM-gelatin, poly (N-isopropylacrylamide)-grafted gelatin.

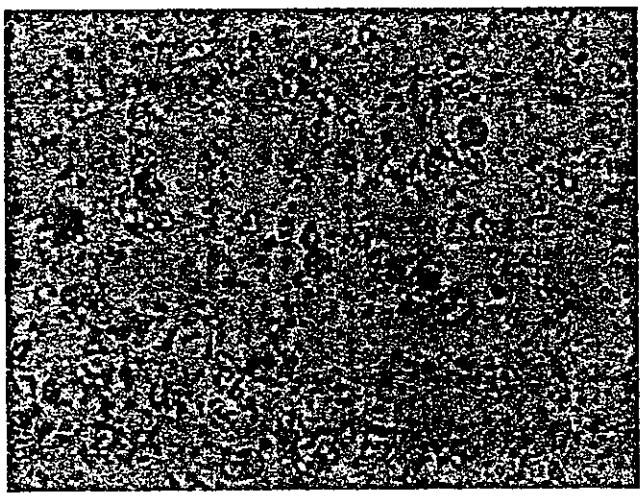


Figure 2. A phase contrast microscopic view of cardiac cells in PNIPAM-gelatin gel (PNIPAM-gelatin concentration, 0.075 mg/ml; initial cell seeding density, 10×10^6 cells/ml) after 2 weeks of culturing. Original magnification × 10. PNIPAM-gelatin, poly (N-isopropylacrylamide)-grafted gelatin.

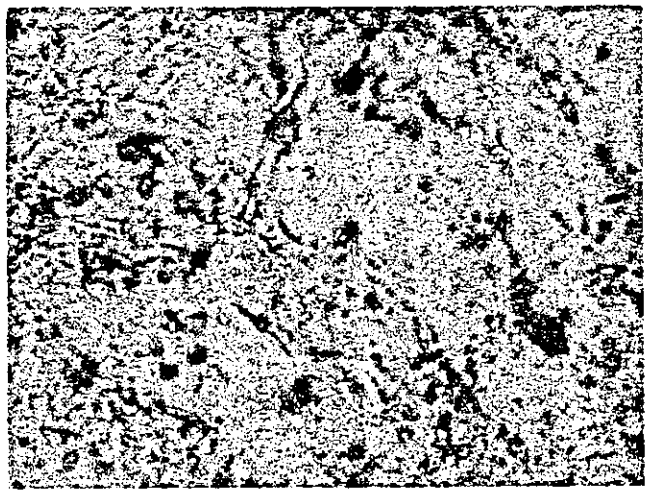


Figure 4. A phase contrast microscopic view of the tissue (PNIPAM-gelatin concentration, 0.05 mg/ml; initial cell seeding density, 50×10^6 cells/ml; hyaluronic acid, addition) after 2 weeks of culturing. Original magnification × 20. PNIPAM-gelatin, poly (N-isopropylacrylamide)-grafted gelatin.

biodegradable preformed three-dimensional mesh,[10-16] on polystyrene microcarrier beads in bioreactors,[17] in collagen gels,[18,19] on micropatterned laminin surfaces,[20] and in layered cardiomyocyte sheets.[21] To make widespread use of cardiac tissue reconstitution for tissue replacement therapy, cardiac tissues need to be made by a simple method. Cultivation on biodegradable, preformed, three-dimensional scaffolding is simple and the most popular method, but when the cells are seeded onto the scaffold, some will be unattached and lost for further tissue development.[22] Also, the problem of a spatially nonuniform distribution of cells, resulting in tissue formed only at the surface layer while the construct interior remained largely acellular,[14] remains. No new simple method of cultivation in collagen gels had been reported after Souren's report in 1992,[23] which stated that rat cardiac cells did not reconstitute cardiac tissue in collagen, but there have been recent reports of the reconstitution of cardiac tissue by the culture of cardiac cells in suspension with type I collagen[18,19] and the culture of smooth muscle cells in PNIPAM-gelatin gel.[9] PNIPAM-gelatin solution gels almost immediately by simply heating it above 34°C,[6-9] so we thought that PNIPAM-gelatin would be advantageous as a scaffold material for reconstituting three-dimensional tissue. Because PNIPAM-gelatin solution gels so quickly and easily, all of the seeded cells in PNIPAM-gelatin solution should be entrapped and uniformly distributed toward three dimensions in PNIPAM-gelatin gel. It should be possible to reconstitute three-dimensional tissue by a very simple method of mixing cells and PNIPAM-gelatin solution. We were not sure, however, whether cardiac cells would reconstitute cardiac tissue in PNIPAM-gelatin gel.

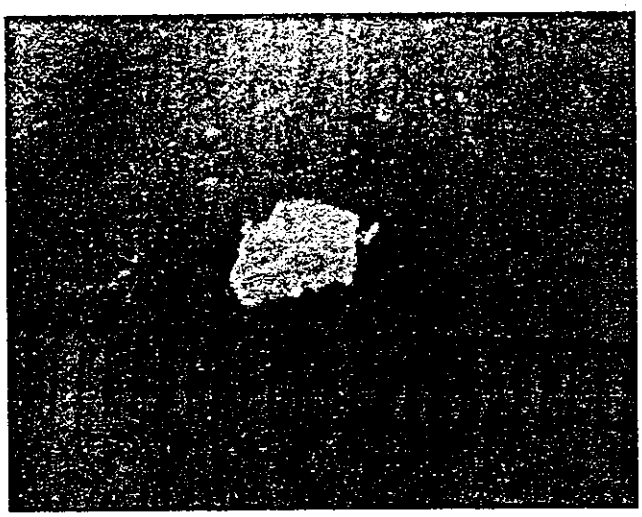


Figure 3. A macroscopic view of the tissue (PNIPAM-gelatin concentration, 0.05 mg/ml; initial cell seeding density, 50×10^6 cells/ml; hyaluronic acid, addition) after 2 weeks of culturing. PNIPAM-gelatin, poly (N-isopropylacrylamide)-grafted gelatin.

Several studies have made clear that variations in cell seeding density and culture conditions affected whether cardiac cells reconstituted cardiac tissue[23] and also affected the structure of engineered cardiac tissue.[15,16,19] In the present study, at low cell seeding density (lower than 10×10^6 cells/ml), we observed scattered asynchronously contracting isolated cells and clusters (**Figure 1**), and at high cell seeding density (50×10^6 cells/ml), synchronously contracting small tissue like constructs were observed. These results indicate that the distances between seeded cells might be too great to contact other cells at low cell seeding densities, as was noted in Souren's report.[23] High cellularity is known to enhance cell to cell contact and communication, which are factors for cardiac tissue formation *in vivo*, and this high cell seeding density might result in good distances between cells for connecting with neighbor cells. The high cell seeding density we used was closer to that in adult rat myocardium than that reported by many others, so we expected the twitch tension of this tissue to be near that of adult rat myocardium.

When the PNIPAM-gelatin concentration was higher than 0.075 mg/ml, the seeded cells could not survive in PNIPAM-gelatin gel (**Figure 2**). The cells' survival depends upon the

Figure 5. Cross-sectional view of the tissue (PNIPAM-gelatin concentration, 0.05 mg/ml; initial cell seeding density, 50×10^6 cells/ml; hyaluronic acid, addition) with hematoxylin and eosin staining after 2 weeks of culturing. PNIPAM-gelatin, poly (N-isopropylacrylamide)-grafted gelatin.

diffusion of oxygen and nutrition and adequate space in the three-dimensional culture. Smooth muscle cells could survive in PNIPAM-gelatin gel even at 0.2 mg/ml PNIPAM-gelatin concentration,[9] so the space in PNIPAM-gelatin gel should be sufficient for cardiac cells. We thought that cardiac cells might be more weakened by the poor diffusion of oxygen and nutrition than smooth muscle cells. At a PNIPAM-gelatin concentration of 0.05 mg/ml, PNIPAM-gelatin solution gels, and the seeded cells could survive in PNIPAM-gelatin gel (**Figure 1**). However, even with the same concentration and a cell seeding density of 50×10^6/ml, the seeded cells make small tissue like constructs in some parts of the reconstituted mixture but not in other parts of the mixture. It seemed that that was because the microenvironment of the seeded cells must vary in different parts of the reconstituted mixture and that the variation affected the cells' growth and ability to connect with neighbor cells. Our aim was to improve the microenvironment of the seeded cells so that it would be uniform throughout the reconstituted mixture for reconstituting large cardiac tissue.

Living cells require a continuous supply of nutrients and removal of metabolites. The scaffold must have pores that are larger than 10–30 μm, preferably 200–400 μm.[24] In the present study, we did not confirm the pore size made by PNIPAM-gelatin because of technical problems, but we thought it would improve microenvironment for cardiac cells in PNIPAM-gelatin gel, and we supplied HA. HA makes the pore size larger by holding water, which has been shown to improve the control of oxygen, pH, nutrients, and metabolites. The resulting less tight network made by HA provides more free room for the formation of a new tissue and extracellular matrix synthesis.[25] By supplying HA, we were able to reconstitute large cardiac tissue, and we could observe the contractions of tissue macroscopically (**Figure 3**). HE staining revealed that cells reconstituted three-dimensional tissue with a cross-section as thick as 60 μm, and all of the cells were viable and uniformly spread at not only the periphery but also in the center lesion of the tissue (**Figure 5**). Therefore, it seemed that by supplying HA, the microenvironment of the all seeded cells was improved in all parts of the reconstituted mixture. The seeded cells survived and grew at the same place where they were distributed uniformly.

Conclusions

Spontaneously contracting three-dimensional cardiac tissue was reconstituted using a simple method of mixing cardiac cells and PNIPAM-gelatin *in vitro*. A limitation of the study was that we were not able to evaluate cell viability in the tissue by the specific property of PNIPAM-gelatin. When the tissue was incubated in trypsin solution, PNIPAM-gelatin gelled, and we could not isolate cells from the tissue to evaluate cell viability. Also in this study, we made three-dimensional tissue that was less than 100 μm thick. Some reports have noted that engineered tissues greater than 100 μm thick that are highly active metabolically and are supplied with nutrients solely by diffusion may have insufficient transport to and from the cells.[14] Further studies of cell viability in the tissue and the limitations of tissue thickness are needed.

References

1. Li RK, Jia ZQ, Weisel RD *et al*: Cardiomyocyte transplantation improves heart function. *Ann Thorac Surg* 62: 654–661, 1996.
2. Scorsin M, Hagege AA, Marotte F *et al*: Dose transplantation of cardiomyocytes improve function of infracted myocardium? *Circulation* 96 (Suppl III): 188–193, 1997.
3. Taylor DA, Atkins BZ, Hungspreugs P *et al*: Regenerating functional myocardium: improved performance after myoblast transplantation. *Nat Med* 4: 929–933, 1998.
4. Menasche P, Hagege AA, Scorsin M *et al*: Myoblast transplantation for heart failure. *Lancet* 357: 279–280, 2001.
5. Reinlib L, Field L: Cell transplantation as future therapy for cardiovascular disease? A workshop of the National Heart, Lung, and Blood Institute. *Circulation* 101: 182–187, 2000.
6. Ohya S, Nakayama Y, Matsuda T: Thermoresponsive artificial extracellular matrix for tissue engineering: Hyaluronic acid bioconjugated with poly(N-isopropylacrylamide) grafts. *Biomacromolecules* 2: 856–863, 2001.
7. Matsuda T: Molecular design of functional artificial extracellular matrix: Thermoresponsive gelatin. *Jpn J Artif Organs* 28: 242–245, 1999.
8. Ohya S, Nakayama Y, Matsuda T: Artificial extracellular matrix design in tissue engineering: Synthesis of thermoresponsive hyaluronic acid and its supramolecular organization. *Jpn J Artif Organs* 29: 446–451, 2000.
9. Ohya S, Nakayama Y, Matsuda T: Matrix design in tissue engineering: in vitro and in vivo evaluation of thermoresponsive gelatin for cell scaffold. *Int J Artif Organs* 25: 685, 2002.
10. Bursac N, Papadaki M, Cohen RJ *et al*: Cardiac muscle tissue engineering: toward an in vitro model for electrophysiological studies. *Am J Physiol* 277: H433–444, 1999.
11. Li RK, Jia ZQ, Weisel RD, Mickle DAG, Choi A, Tau TM: Survival and function of bioengineered cardiac grafts. *Circulation* 100 (Suppl II): 63–69, 1999.
12. Leor J, Aboulafia-Etzion S, Dar A *et al*: Bioengineered cardiac grafts A new approach to repair the infarcted myocardium? *Circulation* 102 (Suppl III): 56–61, 2000.
13. Kofidis T, Akhyari P, Boublik J *et al*: In vitro engineering of heart muscle: artificial myocardial tissue. *J Thorac Cardiovasc Surg* 124: 63–69, 2002.
14. Carrier RL, Rupnick M, Langer R, Schoen FJ, Freed LE, Vunjak-Novakovic G: Perfusion improves tissue architecture of engineered cardiac muscle. *Tissue Eng* 8: 175–188, 2002.
15. Papadaki M, Bursac N, Langer R, Merok J, Vunjak-Novakovic G, Freed LE: Tissue engineering of functional cardiac muscle: molecular, structural, and electrophysiological studies. *Am J Physiol Heart Circ Physiol* 280: H168–178, 2001.
16. Dar A, Shacher M, Leor J, Cohen S: Optimization of cardiac cell