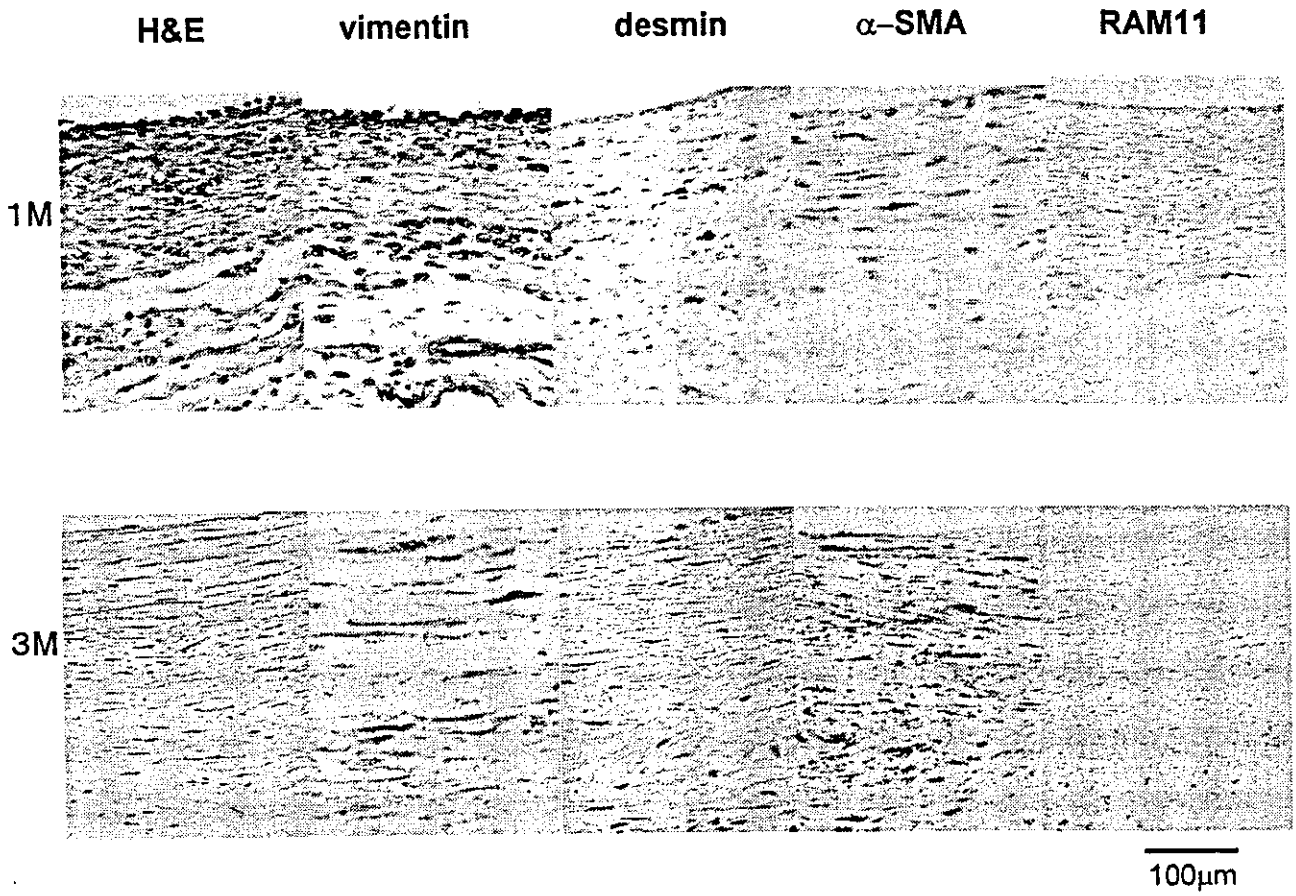

Figure 5. Immunohistochemistry of the circumferential sections of the biotubes formed around the PMMA rods after 1 month (upper columns) and 3 months (lower columns) of implantation. In two implantation periods, the fibroblasts and collagen fibers, the component of the biotube walls, are positive for 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-

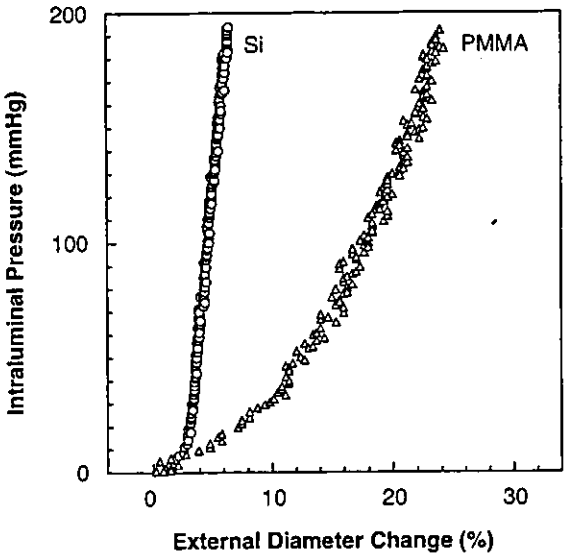

Figure 6. Pressure–diameter relationship of the biotubes formed around the PMMA rod and the Si rod after 1 month of implantation. Water pressure was loaded in the lumen of the biotube up to 200 mmHg.

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

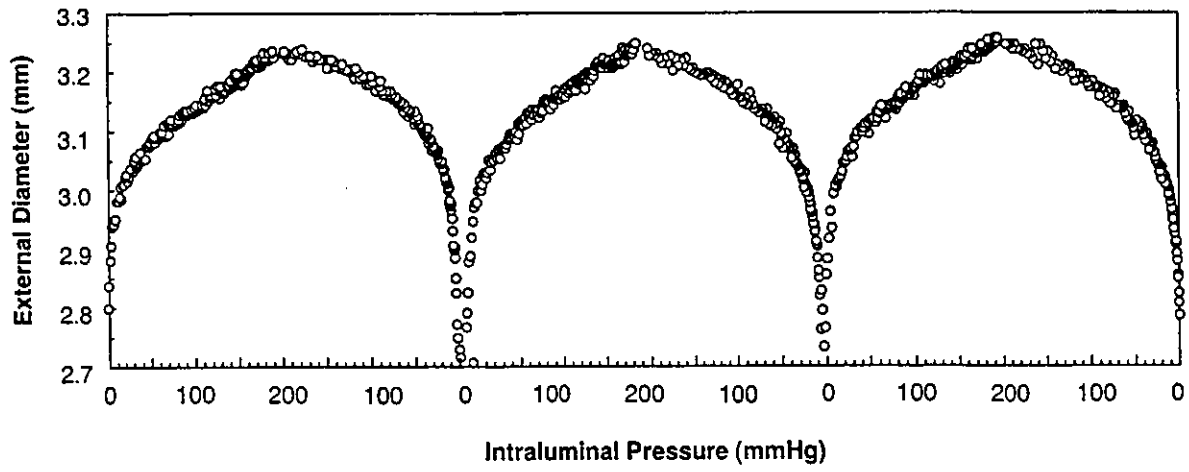

Figure 7. External diameter change by repeatable process in loading and removing of water pressure within a range 0–200 mmHg to the lumen of the biotube formed around the PMMA rod after 3 months of implantation.

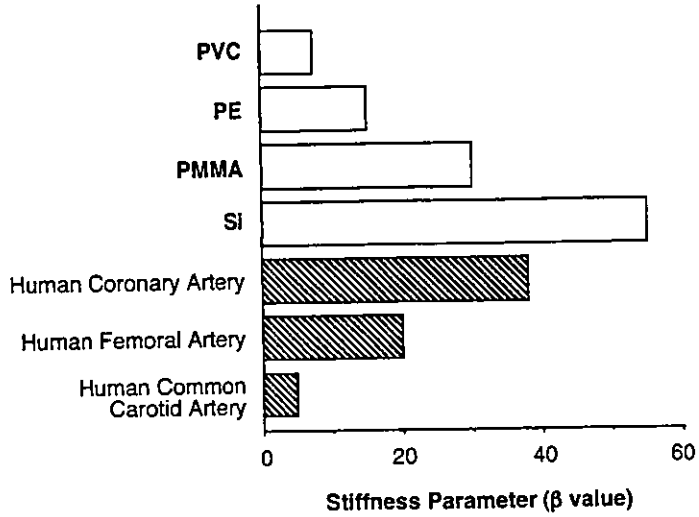

Figure 8. The stiffness parameters (β values) of the biotubes by 1 month of implantation. The biotubes formed around the PMMA, PE, and PVC rods exhibited β values close to those of the human coronary artery, the human femoral artery, and common carotid artery, respectively.

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.

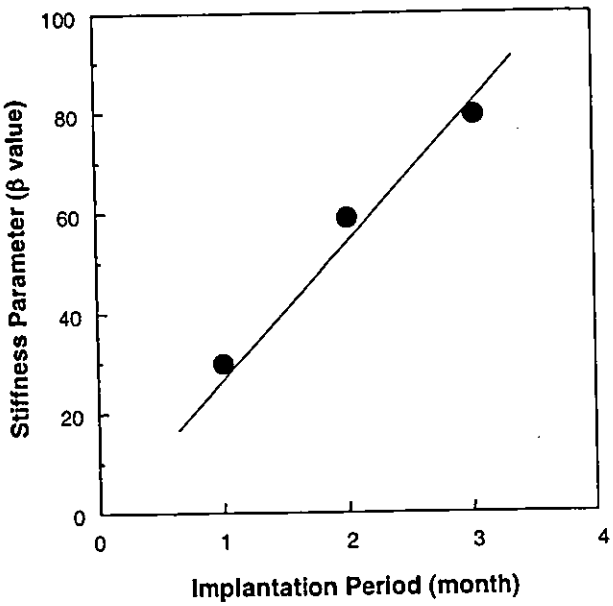

Figure 9. Implantation period-dependent stiffness parameter (β value) change of the biotubes formed around the PMMA rods. The compliance increased linearly up to 3 months of implantation.

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

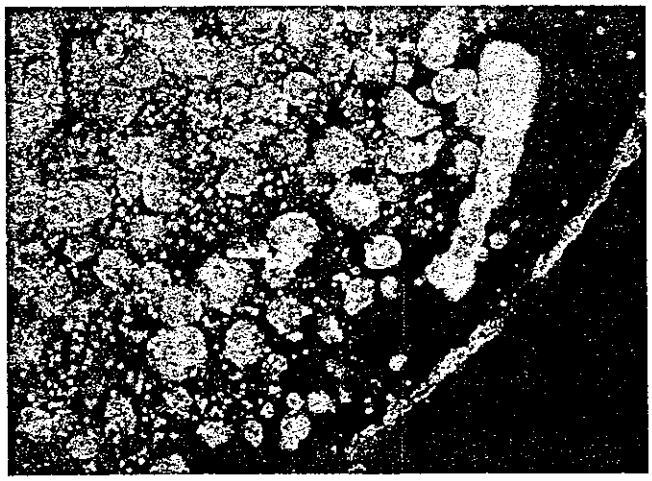

Figure 1. A phase contrast microscopic view of cardiac cells in PNIPAM-gelatin gel (PNIPAM-gelatin concentration, 0.05 mg/ml; initial cell seeding density, 10×10^6 cells/ml) after 2 weeks of culturing. Original magnification × 4. PNIPAM-gelatin, poly (N-isopropylacrylamide)-grafted gelatin.

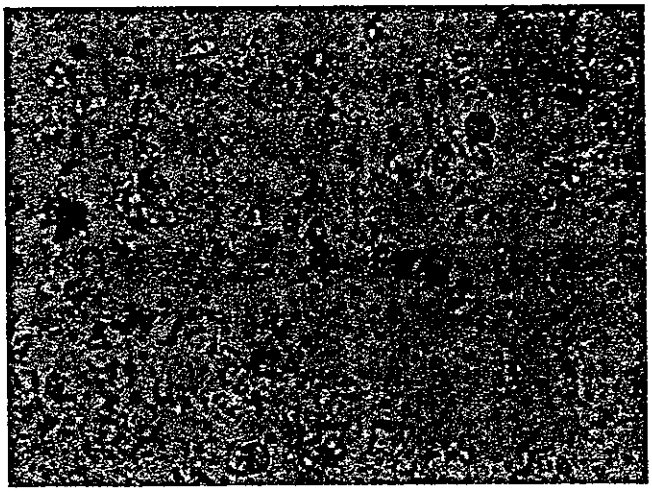

Figure 2. A phase contrast microscopic view of cardiac cells in PNIPAM-gelatin gel (PNIPAM-gelatin concentration, 0.075 mg/ml; initial cell seeding density, 10×10^6 cells/ml) after 2 weeks of culturing. Original magnification × 10. PNIPAM-gelatin, poly (N-isopropylacrylamide)-grafted gelatin.

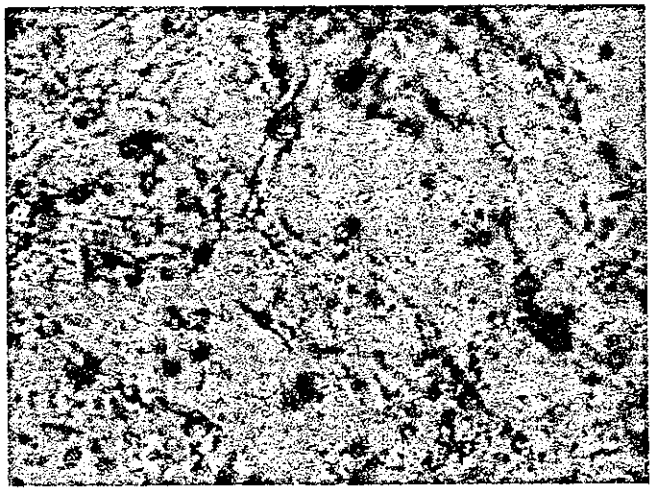

Figure 4. A phase contrast microscopic view of the tissue (PNIPAM-gelatin concentration, 0.05 mg/ml; initial cell seeding density, 50×10^6 cells/ml; hyaluronic acid, addition) after 2 weeks of culturing. Original magnification × 20. PNIPAM-gelatin, poly (N-isopropylacrylamide)-grafted gelatin.

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.

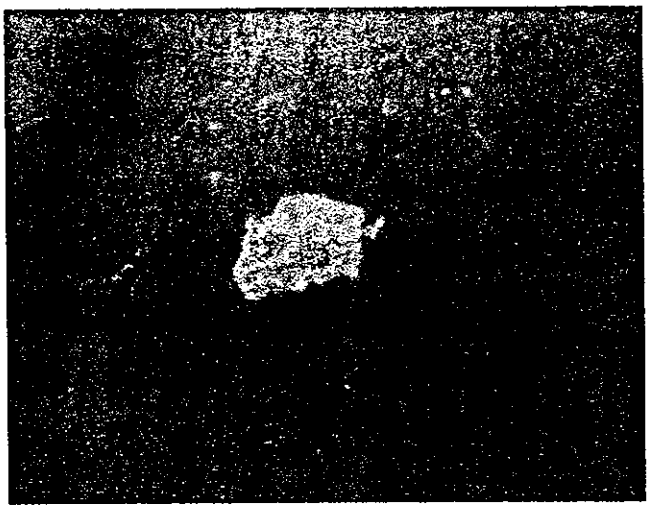

Figure 3. A macroscopic view of the tissue (PNIPAM-gelatin concentration, 0.05 mg/ml; initial cell seeding density, 50×10^6 cells/ml; hyaluronic acid, addition) after 2 weeks of culturing. PNIPAM-gelatin, poly (N-isopropylacrylamide)-grafted gelatin.

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

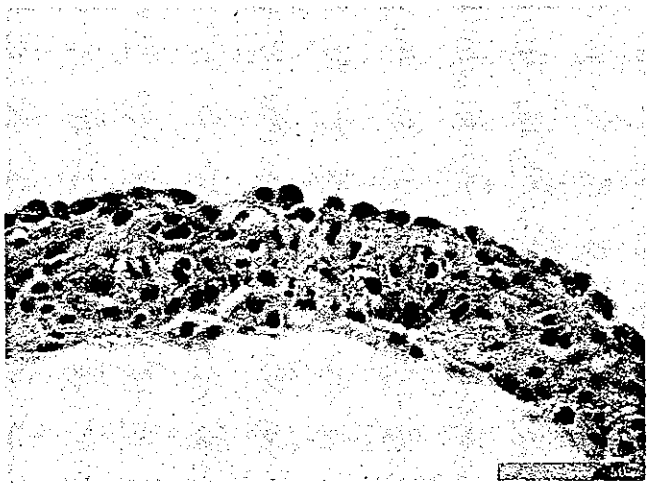

Figure 5. Cross-sectional view of the tissue (PNIPAM-gelatin concentration, 0.05 mg/ml; initial cell seeding density, 50×10^6 cells/ml; hyaluronic acid, addition) with hematoxylin and eosin staining after 2 weeks of culturing. PNIPAM-gelatin, poly (N-isopropylacrylamide)-grafted gelatin.

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 II): 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

seeding and distribution in 3D porous alginate scaffolds. *Biotechnol Bioeng* 80: 305–312, 2002.
17. Akins RE, Schroedl NA, Gonda SR *et al*: Neonatal rat heart cells cultured in simulated microgravity. *In Vitro Cell Dev Biol-Animal* 33: 337–343, 1997.
18. Zimmermann WH, Schneiderbanger K, Schubert P *et al*: Tissue engineering of a differentiated cardiac muscle construct. *Circ Res* 90: 223–230, 2002.
19. Zimmermann WH, Fick C, Kralisch D, Remmers U, Weil J, Eschenhagen T: Three-dimensional engineering heart tissue from neonatal rat cardiac myocytes. *Biotechnol Bioeng* 68: 106–114, 2000.
20. McDevitt TC, Angello JC, Whitney ML *et al*: In vitro generation of differentiated cardiac myofibers on micropatterned laminin surfaces. *J Biomed Mater Res* 60: 472–479, 2002.
21. Shimizu T, Yamato M, Isoi Y *et al*: Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. *Circ Res* 90: 40–48, 2002.
22. Ye Q, Zund G, Benedikt P *et al*: Fibrin gel as a three dimensional matrix in cardiovascular tissue engineering. *Eur J Cardio-thorac Surg* 17: 587–591, 2000.
23. Souren JEM, Schneijdenberg C, Verkleij AJ, Wijk RV: Factors controlling the rhythmic contraction of collagen gels by neonatal heart cells. *In Vitro Cell Dev Biol* 28A: 199–204, 1992.
24. Landers R, Hubner U, Schmelzeisen R, Mulhaupt R: Rapid prototyping of scaffolds derived from thermoresponsive hydrogels and tailored for applications in tissue engineering. *Biomaterials* 23: 4437–4447, 2002.
25. Miralles G, Baudoin R, Dumas D *et al*: Sodium alginate sponges with or without sodium hyaluronate: In vitro engineering of cartilage. *J Biomed Mater Res* 57: 268–278, 2001.

J Artif Organs (2004) 7:181–186
DOI 10.1007/s10047-004-0265-9

ORIGINAL ARTICLE

Shoji Ohya, MS · Yasuhide Nakayama, PhD
Takehisa Matsuda, PhD

In vivo evaluation of poly(*N*-isopropylacrylamide) (PNIPAM)-grafted gelatin as an in situ-formable scaffold

Abstract We examined whether poly(*N*-isopropylacrylamide)-grafted gelatin (PNIPAM-gelatin) with a lower critical solution temperature of approximately 34°C, which was prepared by quasi-living radical graft polymerization, can serve as an in situ-formable three-dimensional extracellular matrix or cell scaffold. A mixture of fibroblasts stained with fluorescent dye and PNIPAM-gelatin in Dulbecco's modified Eagle's medium solution was injected into the subcutaneous tissue of Wistar rats, and immediately formed a white, opaque cell-incorporated gel. Fibroblasts immediately after injection were spherical in shape and were homogeneously distributed in the gel. Fibroblasts in the gel 2 weeks after injection had spread and proliferated. One day after injection, many macrophages and neutrophiles were observed around the gel. As the implantation period proceeded, the inflammation reaction subsided. One week after injection, fibroblasts in the native tissue and macrophages migrated into the gel. From 6 to 12 weeks after injection, some degree of calcification in the solid tissue was intermittently observed. The weight of the gel 6 weeks after implantation was reduced to almost one-half of the weight of the originally injected sample. The potential usefulness of PNIPAM-gelatin as an injectable scaffold is discussed.

Key words Poly(*N*-isopropylacrylamide)-grafted gelatin · Thermoresponsiveness · In situ-formable scaffold · Cell viability

Received: May 7, 2004 / Accepted: August 18, 2004

S. Ohya (✉) · Y. Nakayama
Department of Bioengineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan
Tel. +81-6-6833-5012; Fax +81-6-6872-8090
e-mail: ohya@ri.ncvc.go.jp

T. Matsuda
Division of Biomedical Engineering, Graduate School of Medicine, Kyushu University, Fukuoka, Japan

Introduction

Engineered tissues composed of cells and extracellular matrices (ECMs) have been fabricated ex vivo and in vivo to heal or replace diseased tissues or to replace lost tissues.[1–5] Biomacromolecules and synthetic polymers have been utilized for designing the extracellular space and as structural platforms or scaffolds. However, the difficulty is to precisely fabricate custom-made or complex tissue shapes. In situ-formed gel prepared from its aqueous solution can be used to fabricate hybrid tissues with desired shapes.[6,7] The materials, which are induced to form a gel by a physiologically permitted stimulus such as temperature[8–11] or pH change[12–14] and light irradiation,[15–17] have been utilized for artificial three-dimensional (3D) ECM or cell scaffolds and drug delivery vehicles.

Gelatin, denatured collagen, exhibits cell adhesiveness and biodegradability, but is soluble in water at physiological temperatures. Insolubilization of gelatin at physiological temperatures is an essential requirement for tissue-engineered platforms, cell scaffolds, and artificial ECM. However, chemically or photochemically driven cross-linking reactions leading to gelation are more or less harmful or toxic. Instead, we utilized thermoresponsive sol-to-gel characteristics with an inverse phase transition temperature. Poly(*N*-isopropylacrylamide) (PNIPAM) precipitates in water above 32°C but is water-soluble at room temperature.[18] Because of this unique thermoresponsive feature, PNIPAM has been utilized for thermoresponsive tissue culture dishes,[19–21] drug delivery vehicles,[8] hemostasis,[10] and 3D ECM materials.[9,11]

In our previous study, we prepared PNIPAM-grafted gelatin (PNIPAM-gelatin), which was obtained by quasi-living radical polymerization initiated from the dithiocarbamyl group derivatized on a lysine residue of gelatin, as a thermoresponsive 3D ECM material.[21,22] PNIPAM-gelatin gel, prepared from its buffer suspended with bovine smooth muscle cells, produced a viable cell-entrapped hybrid tissue. The cell viability and cell proliferation potential were found to be markedly influenced by the PNIPAM graft density,

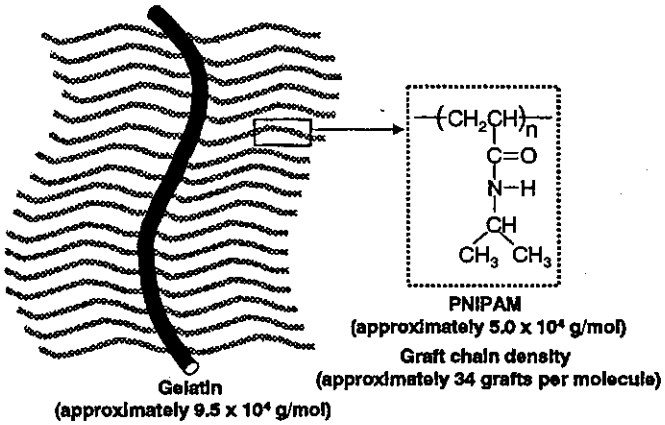

Fig. 1. Structure of poly(*N*-isopropylacrylamide)-grafted gelatin (*PNIPAM*-gelatin). The approximate number of graft chains per gelatin molecule was 34 and the average molecular weight of each PNIPAM graft was approximately 5.0×10^4 g/mol

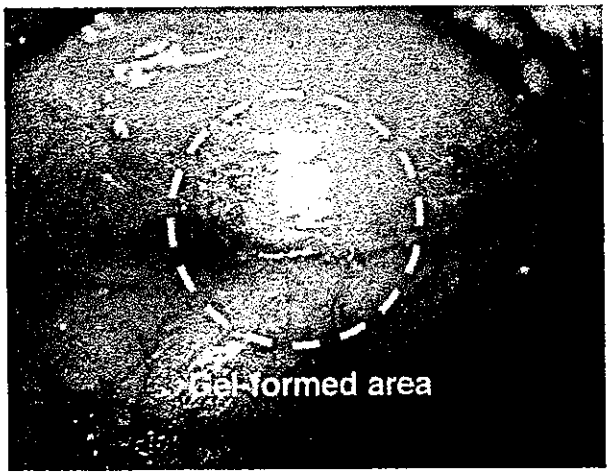

Fig. 2. PNIPAM-gelatin gel formation in rat subcutaneous tissue. A buffer solution of PNIPAM-gelatin suspended with rat fibroblasts was injected and spontaneously formed a white, opaque gel

the molecular weight of the graft chain, and the concentration of PNIPAM-gelatin. Our previous study showed that higher graft density and higher molecular weight of the graft chain in PNIPAM-gelatin enhanced cell viability and cell proliferation potential in 3D culture.[23] Among the designed PNIPAM-gelatins, the highest potential was found for PNIPAM-gelatin with each graft chain having a length of 5.0×10^4 g/mol and a graft density of approximately 34 grafts per molecule (Fig. 1).

In this study, using this PNIPAM-gelatin, the potential usefulness of PNIPAM-gelatin as an in situ-formable scaffold was examined in vivo. In situ characteristics of hybrid tissue formed in rat subcutaneous tissue, including cell viability, cell proliferation potential, natural ECM productivity, and degradability of the gel, as functions of implantation period, were studied.

Materials and methods

Materials

Gelatin (molecular weight: approximately 9.5×10^4 g/mol, from bovine bone) and sodium *N,N*-diethyldithiocarbamate trihydrate were obtained from Wako (Osaka, Japan). Solvents and other reagents (special reagent grade) were also purchased from Wako and used after conventional purification.

Cell morphology and matrix formation in PNIPAM-gelatin gel

We conducted the experiments in accordance with Industrial Guidance for the Care and Use of Laboratory Animals. The Wistar rats used were 10 weeks old. Rat fibroblasts harvested from rat subcutaneous tissue by the collagenase digestion method were stained with fluorescent dye of (benzoxazolium, 3-octadecyl-2-[3-(3-octadecyl-2(3H)-benzoxazolylidene)-1-propenyl], perchlorate, DiO, Molecular Probes, Eugene, OR, USA) before use. A phosphorous buffered saline (PBS) solution of PNIPAM-gelatin (concentration: 5 w/v%), prepared as previously described[21–23] and suspended with fluorescently stained fibroblasts (cell density: 1.2×10^6 cells/ml), was injected into a rat's subcutaneous tissue under anesthesia using ketamine (1 ml/Kg). Three rats were used in this study. Tissue containing gels were extirpated and fixed with 10% formalin neutral solution (pH = 7.4) at 37°C for 7 days and then rinsed with buffer. Specimens were dehydrated with a graded series of ethanol, embedded in paraffin, and cut into sections. Cell morphology in gels was observed by confocal laser scanning microscopy (Radiance 2100, Biorad, Hercules, CA, USA). The specimens for light microscopy were stained with hematoxylin and eosin (H&E) and Masson's trichrome (MT). Cell morphology and secreted collagen were observed by light microscopy (Vanox-S, Olympus, Tokyo, Japan).

Histological analysis of PNIPAM-gelatin gel

A PBS solution of PNIPAM-gelatin without cells was injected into a rat's subcutaneous tissue. Tissues containing gel were dissected 1 week after injection. Specimens were stained with H&E, MT, periodic acid schiff stain (PAS), and von Kossa's method. The interfaces between tissue and gel were observed using light microscopy.

Bioresorbability of PNIPAM-gelatin gel

The bioresorbability of PNIPAM-gelatin gels (20 w/v%) was examined by weight loss after incubation. Gels formed ex vivo were implanted into a rat's subcutaneous tissue. After a predetermined period, the weight loss of gels was measured after freeze-drying. Two rats, in which two gels were placed, were used for measuring the weight loss.

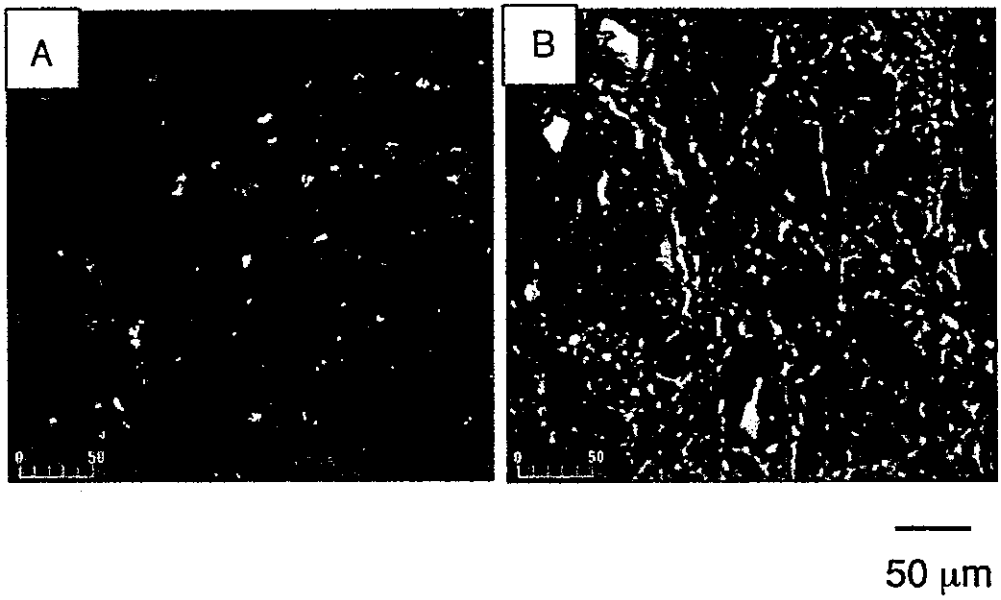

Fig. 3. Confocal laser microscopic observation of rat fibroblasts stained with fluorescent dye (DiO) in PNIPAM-gelatin gel immediately (**A**) or 2 weeks (**B**) after injection. Initially injected cell density: 1.2×10^6 cells/ml

Results

A buffer solution of PNIPAM-gelatin suspended with fluorescently stained fibroblasts was injected into a rat's subcutaneous tissue using a syringe. A white, opaque elastic gel that entrapped fibroblasts was immediately formed (Fig. 2). Confocal laser scanning microscopic observation of tissue samples showed that fluorescently labeled fibroblasts in the gel were homogeneously dispersed and remained spherical in shape when injected (Fig. 3A). Cell aggregation and elongated cell shapes were observed 2 weeks after injection (Fig. 3B). Higher cell proliferation was noticed compared with cells initially injected. Histochemical observation showed an accumulated ECM, which must have been produced by inoculated and proliferated cells and probably by cells migrating from surrounding tissues (Fig. 4).

The weight of PNIPAM-gelatinous tissues, which were easily harvested from the injected sites of native tissue and were subsequently freeze-dried, gradually decreased with incubation time, indicating that PNIPAM-gelatin was biodegraded and sorbed over the implantation period (Fig. 5). The harvested tissue weighed around only one-half of the weight of the initially injected sample on a solid basis 6 weeks after injection.

Cell-free PNIPAM-gelatin gel, prepared in rat subcutaneous tissue by injection, was histologically examined for up to 12 weeks after injection (Fig. 6). At the very early implantation period (1 day after injection), many cells responsible for inflammation such as neutrophils and macrophages were observed around the gel. One week after injection, fibroblasts and macrophages, both stained with PAS, began to penetrate into the gel. Although PAS-stained cells appeared to be rare at 1 week after injection, the cells resided on and in the biodegrading gels at 6 and 12 weeks. Masson's trichrome-stained tissue showed that collagen fibers locally accumulated around the gel and a fibrous granulation tissue was observed at 6 and 12 weeks after injection (Fig. 6). Microparticles stained by H&E and von Kossa's method, identified as calcified tissue, intermittently aggregated or scattered in the tissue, were observed 6 and 12 weeks after injection.

Discussion

In situ-formed gel, induced by a physiologically permitted stimulus, can function as cell scaffold and may be very useful in fabricating a custom-made or complex shaped hybrid tissue in tissue engineering applications. The required properties for in situ-formed 3D ECM materials are (1) rapid gelation from their aqueous solutions in living tissues, (2) high cell viability and cell proliferation potential, (3) low inflammatory reaction, and (4) suitable biodegradability, an additional property to materials required for in vitro use. Our injectable 3D scaffold is gelatin grafted with PNIPAM, thereby incorporating bioactive function and thermoresponsive gelation with the designed scaffold. In vitro study showed that cell viability and cell proliferation in the 3D gel were enhanced by a suitable graft chain density and molecular weight of graft chain.

In this study, we evaluated the potential as an in situ-formed ECM material in vivo of the PNIPAM-gelatin that exhibited the highest cell proliferation potential among PNIPAM-gelatins designed with different molecular components. A cell suspension of PNIPAM-gelatin in a medium, when injected into rat subcutaneous tissue, was immediately converted into a cell-incorporated white, opaque gel (Fig. 2). Fibroblasts in the gel extended and proliferated and secreted collagen 2 weeks after injection (Figs. 3 and 4), indicating that the PNIPAM-gelatin gel functions as a cell scaffold in vivo as expected from our previous experiments in vitro.

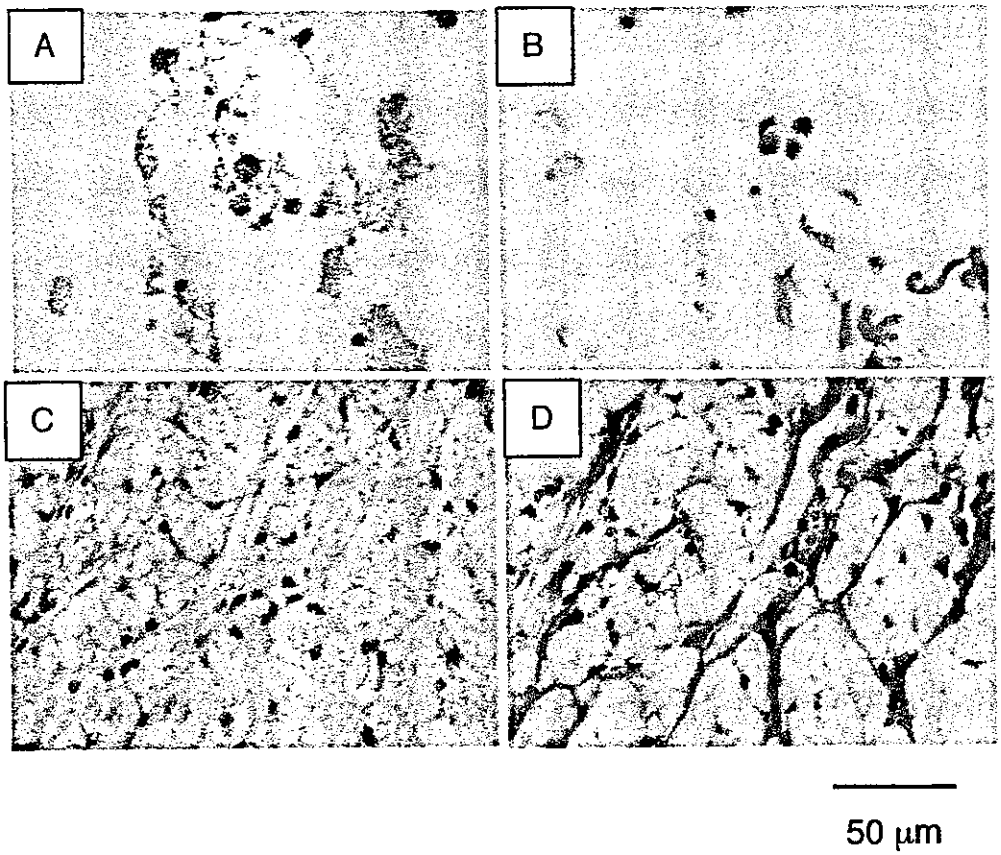

Fig. 4. Light microscopic observation of fibroblasts in PNIPAM-gelatin gel immediately (**A**, **B**) or 2 weeks (**C**, **D**) after injection. The specimens were stained with hematoxylin and eosin (**A**, **C**) and Masson's trichrome (**B**, **D**)

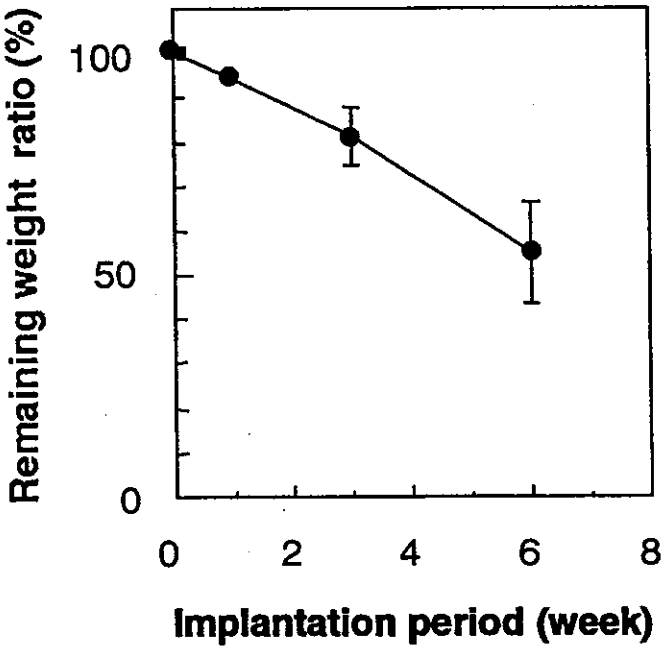

Fig. 5. Examination of dry weight loss of PNIPAM-gelatin gel in rat subcutaneous tissue. The weight gradually decreased with the implantation period

Histological evaluation of PNIPAM-gelatin gel showed that the wound healing process is accompanied by the presence of neutrophils, macrophages, and fibroblasts, and gel degradation and new tissue formation occurred throughout the implantation time. As shown in Fig. 5, the weight of the gel decreased with implantation time, resulting in approximately 50% weight reduction 6 weeks after implantation, probably the result of the balance between the weight gain of the ingrown and regenerated tissue and the weight loss caused by the degradation of the gel. Appropriate tissue formation and simultaneous gel degradation for tissue regeneration depend on the cell type and injection site. The gel degradation period may be controlled by the PNIPAM to gelatin ratio. Calcification was found 6 and 12 weeks after injection. In general, the occurrence of calcification is detrimental in a soft tissue regeneration technology. Only limited studies on gel-induced calcification have been reported. Pioneering studies by Imai[24–26] and Kopecek[27,28] et al. revealed that hydrogels such as poly(2-hydroxyethyl methacrylate) or poly(acrylamide) and its copolymer with a hydrophobic monomer (butyl methacrylate) induced hydroxyapatite formation on and in the gel upon implantation into subcutaneous tissues of young rats. In young rats, calcification was induced at very high probability regardless of the type of implanted material. However, such calcification did not occur in adult rats or other large animals such as dogs or goats, indicating that the occurrence of calcification largely depends on the age of the rat and the animal species. The rats implanted in this study were young, i.e., 10 weeks old. A separate experiment showed that no calcification occurred upon extensive implantation of injectable cartilage tissues containing chondrocytes in rabbits using PNIPAM-gelatin as an injectable scaffold over a period of 1 year.[29] To translate the evidence of the occurrence of

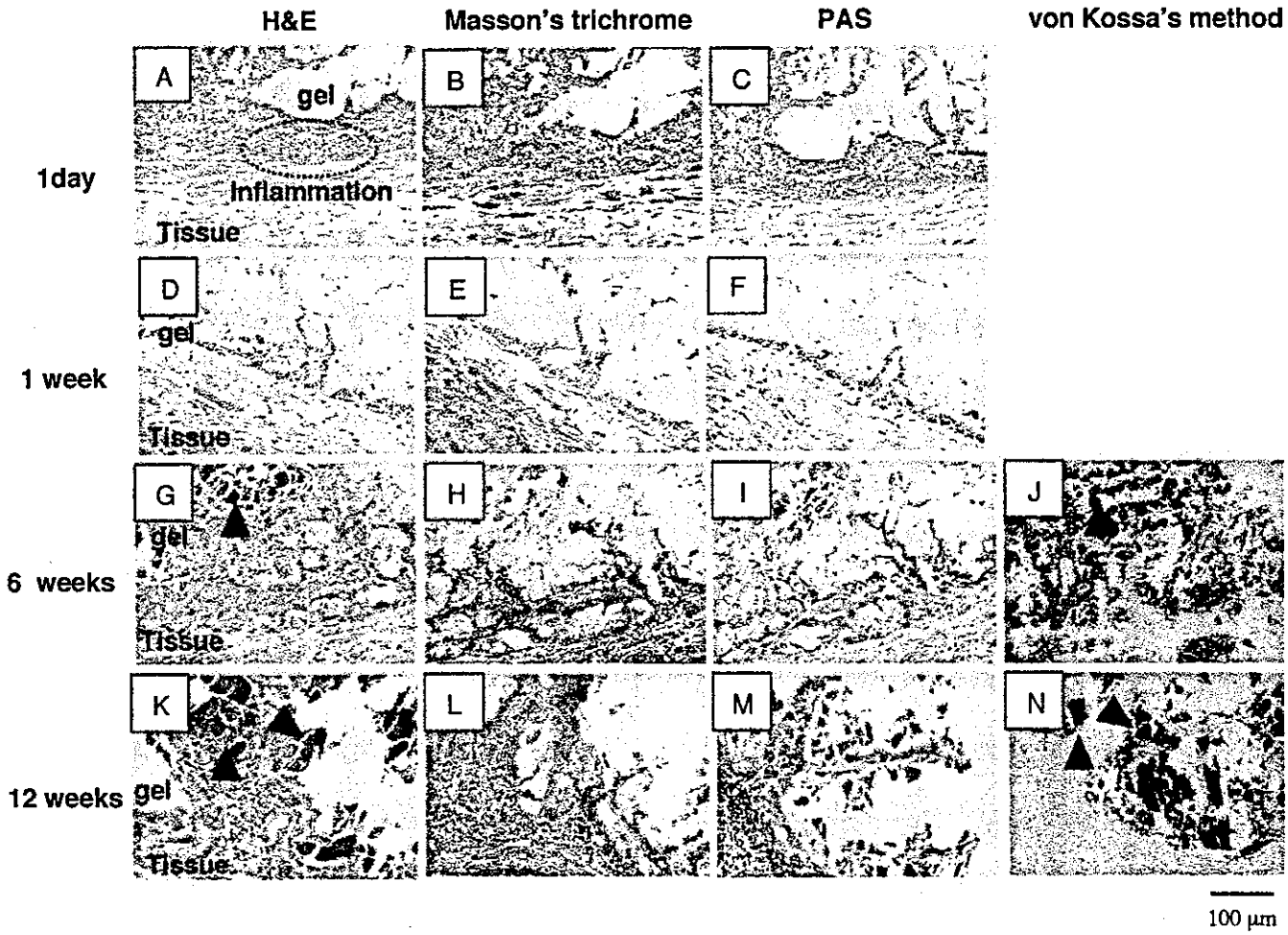

Fig. 6. Histological analysis of PNIPAM-gelatin gel in rat subcutaneous tissue 1 day (**A–C**), 1 week (**D–F**), 6 weeks (**G–J**), and 12 weeks (**K–N**) after injection. Staining was with hematoxylin and eosin (**A, D, G, K**), Masson's trichrome (**B, E, H, L**), periodic acid schiff (**C, F, I, M**), and von Kossa's method (**J, N**). *Black triangles* show the calcification points

calcification in rats in the present study to humans may not be appropriate or not valid at this time, but further study is required for elucidation. Our ongoing study using PNIPAM-gelatin is focusing on soft tissue augmentation technology, and will be reported in the near future.

Conclusions

PNIPAM-gelatin served as an injectable, in situ-formed scaffold on and in which fibroblasts spread and proliferated. PNIPAM-gelatin was biodegraded over time without excessive inflammatory reactions. It is suggested that the calcification occurring in the later period of implantation was the result of using young rats. Further study will elucidate the suitability of PNIPAM-gelatin as a soft tissue augumentation technology.

Acknowledgments This study was financially supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan under Grants No. 14780665 and No. 15200038.

References

1. Bell E. Strategy for the selection of scaffolds for tissue engineering. Tissue Eng 1995;1:163–179
2. Hutmacher DW, Goh JC, Teoh SH. An introduction to biodegradable materials for tissue engineering applications. Ann Acad Med Singapore 2001;30(2):183–191
3. Lee CH, Singla A, Lee Y. Biomedical applications of collagen. Int J Pharm 2001;221(1–2):1–22
4. Rao KP. Recent developments of collagen-based materials for medical applications and drug delivery systems. J Biomater Sci Polym Edn 1995;7(7):623–645
5. Uludag H, Vos PD, Tresco PA. Technology of mammalian cell encapsulation. Adv Drug Deliv Rev 2000;42(1–2):29–64
6. Gutowska A, Jeong B, Jasionowski M. Injectable gels for tissue engineering. Anat Rec 2001;263:342–349
7. Jeong B, Bae YH, Kim SW. Biodegradable block copolymers as injectable drug-delivery systems. Nature 1997;388:860–862
8. Hoffman AS, Afrassiabi A, Dong L. Thermally reversible hydrogels: II. Delivery and selective removal of substances from aqueous solutions. J Controlled Release 1986;4:213–222
9. Iwata H, Amemiya H, Akutsu T. *N*-isopropylacrylamide and methacrylate copolymer for encapsulation of islets. Artif Organs 1990;14 (suppl. 3):7–10

10. Matsuda T, Moghaddam MJ. Development of thermosensitive peptidyl cell substrate synthesis and artificial fibrin glue. ASAIO Trans 1991;37(3):M196–M197
11. Vernon B, Kim SW, Bae YH. Thermoreversible copolymer gels for extracellular matrix. J Biomed Mater Res 2000;51:69–79
12. Dong L, Hoffman AS. A novel approach for preparation of pH-sensitive hydrogels for enteric drug delivery. J Controlled Release 1991;15:141–152
13. Serres A, Baudys M, Kim SW. Temperature and pH-sensitive polymers for human calcitonin delivery. Pharm Res 1996;13(2):196–201
14. Bromberg L. Intelligent polyelectrolytes and gels in oral drug delivery. Curr Pharm Biotechnol 2003;4(5):339–349
15. Okino H, Nakayama Y, Tanaka M, Matsuda T. In situ hydrogelation of photocurable gelatin and drug release. J Biomed Mater Res 2002;59(2):233–245
16. Nakayama Y, Matsuda T. Newly designed hemostatic technology based on photocurable gelatin. ASAIO J 1995;41(3):M374–M378
17. Nakayama Y, Matsuda T. Photocurable surgical tissue adhesive glues composed of photoreactive gelatin and poly (ethylene glycol) diacrylate. J Biomed Mater Res (Appl Biomater) 1999;48:511–521
18. Heskins M, Guillet JE. Solution properties of poly(*N*-isopropylacrylamide). J Macromol Sci Chem 1968;A2:1441–1455
19. Yamada N, Okano T, Sakai H, Karikusa F, Sawasaki Y, Sakurai Y. Thermo-responsive polymeric surfaces; control of attachment and detachment of cultured cells. Macromol Chem, Rapid Commun 1990;11:571–576
20. Takezawa T, Mori Y, Yoshizato K. Cell culture on a thermo-responsive polymer surface. Bio/Technology 1990;8:854–856
21. Morikawa N, Matsuda T. Thermoresponsive artificial extracellular matrix: *N*-isopropylacrylamide-graft-copolymerized gelatin. J Biomater Sci Polym Ed 2002;13(2):167–183
22. Ohya S, Nakayama Y, Matsuda T. Material design for artificial extracellular matrix: cell entrapment in poly(*N*-isopropylacrylamide) (PNIPAM)-grafted gelatin hydrogel. J Artif Organs 2001;4:308–314
23. Ohya S, Matsuda T. Poly(*N*-isopropylacrylamide) (PNIPAM)-grafted gelatin as a thermoresponsive three-dimensional artificial extracellular matrix: Molecular and formulation parameters versus cell proliferation potential. J Biomater Sci Polym Edn 2005; in press
24. Imai Y. Effect of age on calcification of poly(hydroxyethyl methacrylate) in animals. Artif Organs 1985;5:255–258
25. Imai Y, Watanabe A. Effect of hydrophilicity and chemical structure of hydrogels on calcification. Artif Organs 1985;5:994–997
26. Imai Y, Masuhara E. Long-term in vivo studies of poly(2-hydroxyethyl methacrylate). J Biomed Mater Res 1982;16:609–617
27. Sprincl L, Vacik J, Kopecek J, Lim D. Biological tolerance of poly(*N*-substituted methacrylamides). J Biomed Mater Res 1971;5(3):197–205
28. Sprincl L, Vacik J, Kopecek J. Biological tolerance of ionogenic hydrophilic gels. J Biomed Mater Res 1973;7(1):123–136
29. Ibusuki S, Iwamoto Y, Matsuda T. System-engineered cartilage using poly(*N*-isopropylacrylamide)-grafted gelatin as in situ-formable scaffold: in vivo performance. Tissue Eng 2003;9(6): 1133–1142

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biomaterials 26 (2005) 655–659
Biomaterials
www.elsevier.com/locate/biomaterials

Technical note

The potential of poly(*N*-isopropylacrylamide) (PNIPAM)-grafted hyaluronan and PNIPAM-grafted gelatin in the control of post-surgical tissue adhesions

Shoji Ohya[a,*], Hiromichi Sonoda[a,b], Yasuhide Nakayama[a], Takehisa Matsuda[b]

[a] *Department of Bioengineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan*
[b] *Division of Biomedical Engineering, Graduate School of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*

Received 12 February 2004; accepted 13 March 2004

Abstract

Poly(*N*-isopropylacrylamide)-grafted hyaluronan (PNIPAM–HA) and PNIPAM-grafted gelatin (PNIPAM–gelatin), which exhibit sol-to-gel transformation at physiological temperature, were applied as control of tissue adhesions: tissue adhesion prevention material and hemostatic aid, respectively. The rat cecum, which was abraded using surgical gauze, was coated with PNIPAM–HA-containing PBS (concentration: 0.5 w/v%). The coated solution was immediately converted to an opaque precipitate at body temperature, which weakly adhered to and covered the injured rat cecum. One week after coating, tissue adhesion between the PNIPAM–HA-treated cecum and adjacent tissues was significantly reduced as compared with that between non-treated tissue and adjacent tissues. On the other hand, the coating of bleeding spots of a canine liver with PNIPAM–gelatin-containing PBS (concentration: 20 w/v%) resulted in spontaneous gel formation on the tissues and subsequently suppressed bleeding. Although these thermoresponsive tissue adhesion prevention and hemostatic materials are still prototypes at this time, both thermoresponsive biomacromolecules bioconjugated with PNIPAM, PNIPAM–HA and PNIPAM–gelatin, may serve as a tissue adhesion prevention material and hemostatic aid, respectively.

Keywords: Poly(*N*-isopropylacrylamide)-grafted biomacromolecules; Gel; Thermoresponsiveness; Tissue adhesion prevention; Hemostasis

1. Introduction

The prompt management of normal wound healing during and after surgical treatment may predict the post-surgical healing of tissues. Hemostatic control during surgical operation and tissue adhesion prevention after surgery are two critical issues in wound healing. To this end, various approaches and materials have been developed and tested over the years. However, "ideal" wound-healing materials have not been realized as yet.

Post-surgical tissue adhesion, which results from malignant healing response of a damaged tissue to a non-injured tissue, often causes life-threatening complications or necessitates re-operation. To reduce tissue adhesion, the use of physical barrier membranes to separate adjacent tissues during the healing process has been proposed and examined [1]. Carboxymethylcellulose [1,2], dextran [3] and oxidized regenerated cellulose [1–4] films have been clinically used as such membranes with some therapeutic effects. Hyaluronan (HA), which is an extracellular matrix component, is known to temporarily prevent tissue adhesion [5] when such a solution is coated on damaged tissue. However, HA is rapidly biodegraded by hyaluronidase and removed away from the injury sites [6].

On the other hand, tissue adhesive glue or hemostatic aids have been used when bleeding cannot be controlled during surgery. Fibrin glue has been clinically used in these cases. However, its major drawbacks are its low mechanical strength and potential infection risk inherent to blood origin. Semisynthetic and synthetic materials such as cyanoacrylate derivatives [7,8], gelatin–resorcinol–formaldehyde [8], and fluorinated hexamethylene diisocyanate-based urethane prepolymers [9] have been applied as surgical adhesives. Although they have

*Corresponding author. Tel.: +81-6-6833-5012; fax: +81-6-6872-8090.
E-mail address: ohya@ri.ncvc.go.jp (S. Ohya).

0142-9612/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2004.03.002

appropriate tissue adhesiveness, cytotoxicity and severe inflammatory reactions with the use of the former two glues and very slow degradation with the last glue are the major drawbacks, respectively.

Regardless of tissue adhesion prevention or hemostatic aids, rapid sol-to-gel formation is necessary to cover the injured or bleeding sites of a tissue. Such a phase transition is desired to occur within a few minutes after application at physiological temperature. We previously prepared poly(*N*-isopropylacrylamide) (PNIPAM)-grafted hyaluronan (PNIPAM–HA) [10,11] and PNIPAM-grafted gelatin (PNIPAM–gelatin) [12–14]. These were soluble in water at room temperature but precipitated or gelled at physiological temperature due to thermoresponsive phase transition characteristics of PNIPAM. In this study, we explored the potential applicability of thermoresponsive PNIPAM–HA and PNIPAM–gelatin as a tissue adhesion prevention material or hemostatic aid, respectively.

2. Materials and methods

2.1. Material

Sodium hyaluronate (HANa, molecular weight: ca. 5.0×10^5 g/mol) was supplied by Seikagaku Kogyo Co. Ltd., Gelatin (molecular weight: ca. 9.5×10^4 g/mol, from bovine bone) and the solvents, which were of special reagent grade, were purchased from Wako Pure Chemical Industry Ltd., (Osaka, Japan) and used after conventional purification.

2.2. Cell adhesion on PNIPAM–HA film

An aqueous solution of PNIPAM–HA (concentration: 0.5 w/v%) was coated onto a circular cover glass (diameter: 14.5 mm, Matsunami Glass Co. Ltd., Osaka, Japan) and dried at room temperature. Rat fibroblasts at a density of 2.0×10^4 cells/ml were seeded on PNIPAM–HA films. After 3 h of incubation, cell morphology was observed by phase-contrast microscopy (Diaphoto, Nikon, Tokyo, Japan). All the procedures including cell culture were carried out at 37°C.

2.3. Tissue adhesion prevention efficacy of PNIPAM–HA

Tissue adhesion prevention efficacy was assessed using a rat cecum abrasion model [1,4]. Anesthesized Wistar rats were subjected to laparotomy. Each rat cecum was abraded with a surgical gauze. A PBS (0.5 ml) of PNIPAM–HA (concentration: 0.5 w/v%) was coated onto the cecum. One week after application, the incidence and severity of adhesions of the cecum to adjacent tissues were evaluated according to the following system: after harvesting the cecum and fixing it in formalin neutral buffer solution (pH 7.4, Wako Pure Chemical Industry Ltd., Osaka, Japan) at 37°C, the specimens stained with hematoxylin–eosin (H&E) and Masson's trichrome were observed by light microscopy (VANOX-S, Olympus, Tokyo, Japan).

0: No cecum adhesions
1: Firm adhesion with easily dissectable plane
2: Adhesion with dissectable plane causing mild tissue trauma
3: Fibrous adhesion with difficult tissue dissection
4: Fibrous adhesion with non-dissectable tissue planes.

2.4. Histological analysis of tissue adhesion prevention efficacy for PNIPAM–HA precipitate

For histological analysis, PNIPAM–HA-treated rat ceca, after seven days, were fixed with 10% formalin neutral buffer solution (pH 7.4) for more than seven days, dehydrated in a graded ethanol series, embedded in paraffin, and sectioned at 5 μm thickness. After staining with H&E or Masson's trichrome, the specimens were evaluated by light microscopy.

2.5. Hemostatic characteristics of PNIPAM–gelatin

The hemostatic characteristics of PNIPAM–gelatin were evaluated using a canine liver model (weight: 25 kg) and a Wistar rat aorta model (average weight: 250 g). The canine liver was abraded with trephine in laparotomy and the rat aorta was clamped and punctured using a 23-gage needle. A PBS of PNIPAM–gelatin (concentration: 20 w/v%) was coated on the bleeding spot. The efficacy of hemostasis was determined by gross observation.

3. Results

3.1. PNIPAM–HA

When rat fibroblasts were seeded and cultured on PNIPAM–HA film, cast from their aqueous solution, a markedly reduced adhesion and suppressed spreading (mostly round shape) were observed (Fig. 1), indicating that PNIPAM–HA is a non-cell-adhesive matrix.

The efficacy of the PNIPAM–HA film for tissue adhesion prevention was evaluated using a rat cecum abrasion model [1,4]. When a PBS solution of PNIPAM–HA was coated on a rat cecum, an opaque PNIPAM–HA precipitate was immediately formed around the cecum at body temperature. One week after coating, ceca without PNIPAM–HA coating strongly adhered to adjacent tissues (Figs. 2 and 3). When the adhesion incidence of each rat was scored according to the scoring

rate described in Section 2, the average overall score (4: severe adhesion, 0: non-adhesion) was 2.2 ± 0.7 ($n=9$) and the experimental sample number over score 2, which shows tissue adhesion, was counted as eight out of nine rats examined (Table 1). On the other hand, the reduced adhesion of the cecum to adjacent tissues was observed, although the PNIPAM–HA-treated ceca weakly adhered to adjacent tissues (Figs. 2 and 3). The average overall score was 1.3 ± 0.5 ($n=8$). The experimental sample number over score 2 was two out of eight rats examined (Fig. 2 and Table 1). These results indicate that the in situ formed PNIPAM–HA precipitate significantly reduced the degree of adhesion and occurrence of tissue adhesion of the rat cecum to adjacent tissues.

3.2. PNIPAM–gelatin

A PBS solution of PNIPAM–gelatin (20 w/v%) was coated on the bleeding spots generated by pricking a canine liver and a rat aorta with a needle. The solution was immediately converted to an elastic hydrogel on the bleeding spots (Fig. 4). The hydrogel weakly adhered to and covered the injured sites, resulting in hemostasis, which was completed within a minute after coating (Fig. 4). Pulsation was maintained, and no bleeding was observed within the experimental time observed (1–2 h after application).

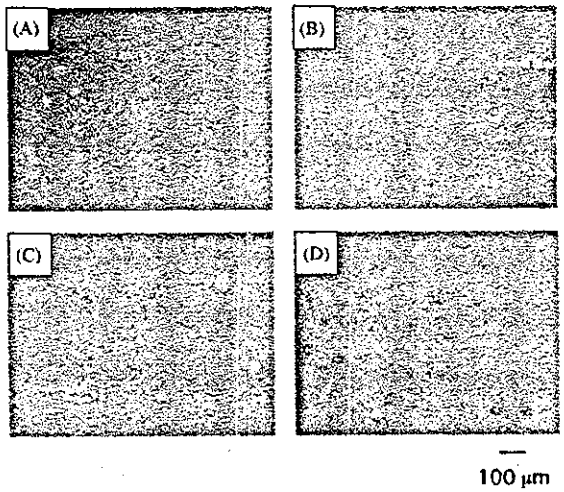

Fig. 1. Phase-contrast micrographs of rat fibroblasts (seeding density: 2.0×10^4 cells/ml) on glass (A)(C), PNIPAM–HA (B)(D) surfaces at 37°C immediately (A)(B) or after 3-h incubation (C)(D).

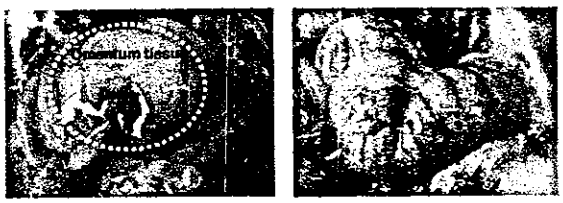

Fig. 2. Gross observation of PNIPAM–HA-treated cecum adhering to adjacent tissues one week after coating. Left: Non-PNIPAM–HA-treated cecum where omentum tissue adhered to and covered the injured cecum. Right: PNIPAM–HA-treated cecum without tissue adhesion.

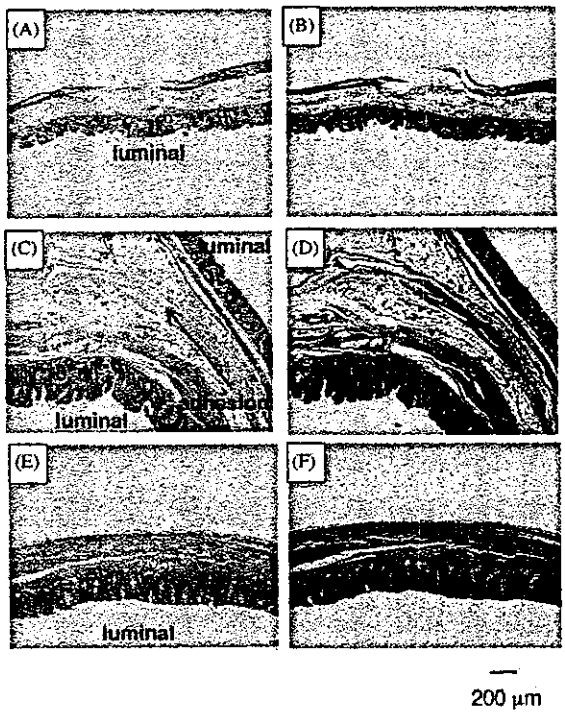

Fig. 3. Histological analysis of PNIPAM–HA-treated cecum adhering to adjacent tissues. Specimens were stained with H&E (A)(C)(E) and Masson's trichrome (B)(D)(F). PNIPAM–HA-treated cecum: (A) and (B), immediately after covering (E) and (F), 7 days after coating. Non-PNIPAM–HA-treated cecum: (C) and (D), 7 days after coating.

Table 1
Adhesion score of rat cecum

Sample	Adhesion score[a]	n	Ratio ≥score 2
Non-treated	2.2 ± 0.7	9	8/9
PNIPAM-HA-treated	1.3 ± 0.5	8	2/8

[a] Adhesion scores are as follows: 0: No cecum adhesions, 1: Firmly adhesion with easily dissectable plane, 2: Adhesion with dissectable plane causing mild tissue trauma, 3: Fibrous adhesion with difficult tissue dissection, 4: Fibrous adhesion with nondissectable tissue planes.

4. Discussion

Fundamental requirements for "ideal" wound-healing materials are as follows: (1) viscous liquid form to