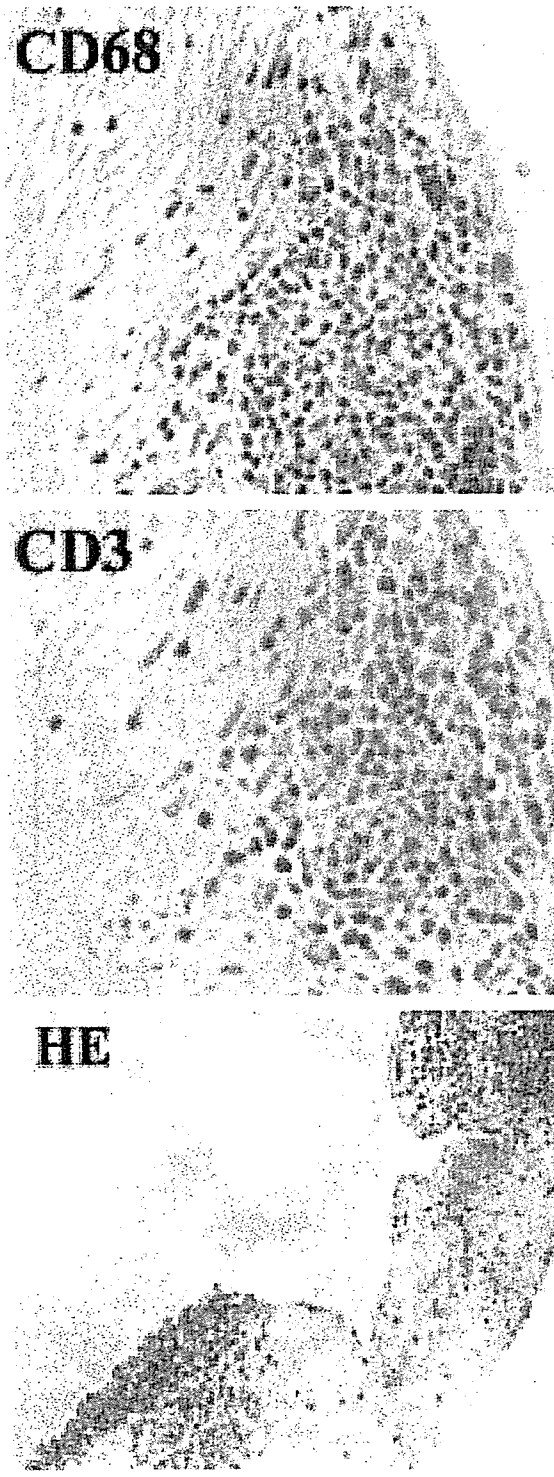

Figure 2: Histological and immunohistochemical findings of pig #6 (cryopreserved graft; not decellularized, not cell-seeded). There were two to three regions of focal spindle or round cell proliferation in the cusp. CD68-positive cells were observed focally at the interstitium of the cusps, together with CD3-positive cells. HE: Hematoxylin and eosin staining.

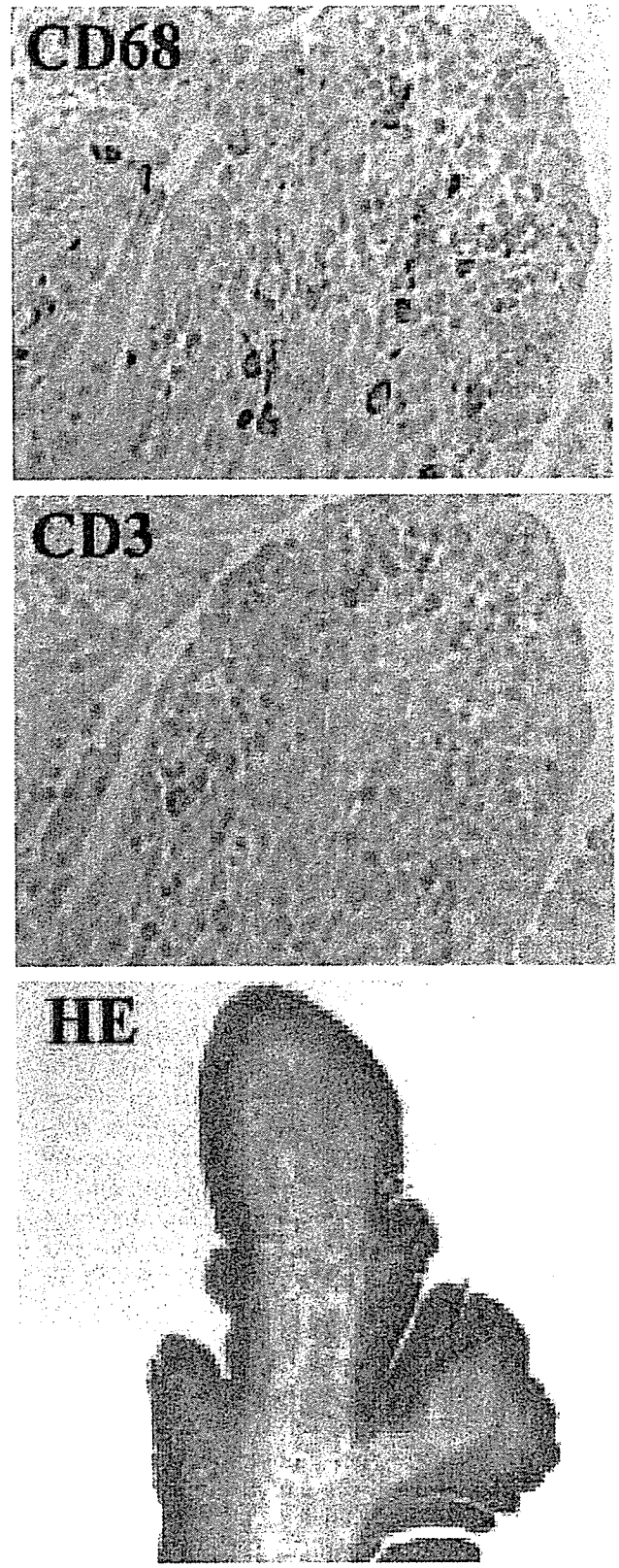

Figure 3: Histological and immunohistochemical findings of pig #5 (cryopreserved graft but not decellularized or cell-seeded). There were multi-layer cell linings predominantly localized at the cusp surface. CD68-positive cells were observed at the multi-layer cells, and CD3-positive cells were also present. HE: Hematoxylin and

cells were also present around these cells (Table II; Fig. 3). No polymorphonuclear neutrophil leukocytes were observed in the valve tissue of either of these pigs.

Discussion

The preservation, clinical application, and long-term outcome of cryopreserved homografts have been evaluated previously (9-11). It has also been shown that fibroblast viability persists in cryopreserved allografts after implantation, and that these cells express the genes for fibroblast growth factor and procollagen (12), which can maintain the structure of the tissue. It has also been demonstrated that collagen synthesis of the cryopreserved allograft was relatively well maintained (13). However, fibroblasts in the donor allograft were unable to survive for long, because of apoptosis (14). The durability of the homograft valve is related to the viability of the fibroblasts that maintain the valve matrix (15), and therefore the loss of fibroblasts may result in a loss in homograft durability.

Endothelial cells exhibit strong antigenicity, but cannot survive under ischemic conditions. Moreover, during the cryopreservation process endothelial cells lose their ability to proliferate (16,17). This raises the possibility that homograft durability is related to the extent of endothelial cell denudation. Thus, the homograft will lose its endothelial cells and fibroblasts, and eventually become a non-viable tissue.

The fate of implanted cryopreserved homografts has been demonstrated in previous clinical and pathological studies (18,19). After implantation, the homograft loses its endothelium, cellularity, and the layered architecture, after which calcified deposits and hematoma/mural thrombi are seen to form. The homograft cusps become flattened and thinned, with obliteration of the usual corrugated pattern. These investigators concluded that the cryopreserved allograft is morphologically non-viable, and also suggested that the early influx of macrophages and T lymphocytes was limited after implantation. Over the longer term, inflammatory cells were not found, and thus the degeneration could not be attributed to immunological responses.

The results of previous studies have indicated that, after allograft implantation, donor cells disappear rapidly and are then replaced by recipient endothelial and interstitial cells (20-22). It would be expected that influx of the recipient cells would progress into the allograft and maintain valvular function. Thus, the present authors speculated that if valvular tissue were to be decellularized prior to implantation, then the recipient cells would readily repopulate and proliferate upon this substrate.

In the present study in a mini-pig model, right ventricular outflow tract replacement was performed with a cryopreserved and decellularized allograft and an 'only-cryopreserved' allograft, and the short-term results evaluated. In the decellularized group, H&E staining revealed that the surfaces of the graft were repopulated by endothelial cells for up to one month. By contrast, in the only-cryopreserved group, endothelial cells were almost completely lost, the trilaminar tissue architecture had disappeared, and macrophages and T-lymphocytes were found in the valve. Endothelialization of the decellularized cusps occurred more densely than in the only-cryopreserved cusps. Furthermore, in the decellularized group, inflammatory cells infiltrated less than in the only-cryopreserved cusps. Decellularization may precede repopulation of the recipient cells.

In the present study, no significant differences were observed between the cell-seeded and non-cell-seeded groups. The endothelial cells play an important role in preservation of the subendothelial cellular and matrix components (23), and this influence may become apparent after implantation. With the static seeding approach used in the present investigation, the endothelial cells were seeded heterogeneously.

Steinhoff et al. (7) reported that with only static reseeding, complete recellularization with endothelial cells and myofibroblasts occurred, at four and 12 weeks after implantation, although the unseeded group recellularized only with endothelial cells. The duration of reseeding used by these authors was longer (8 days) than was used in the present study (2 days). Furthermore, these other investigators performed the reseeding with myofibroblasts for six days and with endothelial cells for two days, whereas only endothelial cells were used for seeding in the present study. For further evaluation of these procedures, the duration of static reseeding and the type of reseeding cells used are important. It is possible that confluent endothelial cell coverage before implantation might be preferable, and if the grafts were to be seeded using a bioreactor (24,25), then confluent cell coverage might be established.

Several points regarding this graft procedure remain unclear. The first is thrombogenicity - the evaluation of which is difficult because the operative procedure itself is associated with thrombus formation. In addition, in the present study neither anticoagulation nor anti-platelet therapy were initiated, and a limited period of anticoagulation therapy may indeed be necessary with this graft procedure. Furthermore, at the microscopic level the surface of the graft was found to be rough, and this may provide a suitable substrate for the thrombus formation observed in the present study. If confluent coverage of the graft surface with endothelial cells were to be established before implantation, then thrombus formation may be reduced.

In conclusion, a decellularized allograft was recellu-

larized with endothelial cells for up to four weeks. In comparison with the cryopreserved allograft, the inflammatory response was reduced by decellularization.

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EXPERIMENTAL INVESTIGATION Circ J 2005; 69: 850–857

Bone Marrow Cell-Seeded Biodegradable Polymeric Scaffold Enhances Angiogenesis and Improves Function of the Infarcted Heart

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Background The present study examined whether a bioengineered polyglycolic acid cloth (PGAC) impregnated with bone marrow cells (BMC) improved the function and angiogenesis of the infarcted heart.
Methods and Results The coronary artery was ligated in Lewis rats and the infarcted area was covered with a PGAC in group 1 (n=8), with a PGAC containing basic-fibroblast growth factor (b-FGF) in group 2 (n=11) and a PGAC containing b-FGF and freshly isolated BMC in group 3 (n=10). In addition, BMC derived from transgenic mice expressing green fluorescent protein (GFP)-BMC were seeded into a PGAC, which was sutured over the infarcted area of C57BL/6 mice (n=5). In the rat study, developed and systolic pressures, dp/dt max and dp/dt min) were the highest in group 3, as were the capillary density in the PGAC and infarcted area. In the mouse study, there were few GFP-BMC in the PGAC, but none in the infarcted area.
Conclusions A PGAC with BMC improved cardiac function by inducing angiogenesis without migration of BMC. Freshly isolated BMC work as angiogenic inducers and a PGAC is useful as a "drug delivery system". (*Circ J* 2005; **69:** 850–857)

Key Words: Angiogenesis; Biomaterial; Bone marrow cells; Ischemic heart disease

When the myocardium is damaged by acute myocardial infarction, the heart compensates with hypertrophy of the remaining cardiomyocytes[1] However, with global ischemia the heart dilates, cardiac performance deteriorates and finally there is chronic heart failure.

Angiogenesis attempts to restore cardiac function after myocardial infarction. Growth factors such as vascular endothelial growth factor (VEGF) or basic-fibroblast growth factor (b-FGF) induce angiogenesis at the border area of the infarcted myocardium, and cardiac function is improved[2] Recently, bone marrow cell (BMC) transplantation has been investigated[3–5] because BMC have many growth factors[6,7] and are currently recognized as clinical "drugs" for hematopoietic diseases[8,9]

In most previous studies cell transplantation was by direct injection using BMC, skeletal myoblasts, and fetal cardiomyocytes as cell sources. However, it may be difficult to treat the globally damaged heart by the direct injection technique and cell-seeded biomaterials have been used to replace the damaged area of the heart in animal studies![10]

We hypothesized that a BMC-seeded biomaterial would induce angiogenesis and improve the function of the infarcted heart. In addition, we wanted to investigate whether the seeded BMC would migrate and create new capillary vessels in the scar tissue.

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Methods

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). All procedures were approved by the Animal Care Committee of National Cardiovascular Center, Osaka, Japan. Lewis rats (250–300 g) and C57BL/6 mice at the age of 8 weeks old were purchased from a licensed vendor. Transgenic mice expressing green fluorescent protein (GFP) (C57BL/6Tg14 (act-EGFP) OsbY01: GFP mouse) were from Dr Okabe![11] Animals were housed in an air-conditioned room with free access to food and water at all times.

Rat Study

Model of Myocardial Ischemia Briefly, 29 Lewis rats were anesthetized with an intramuscular injection of ketamine (22 mg/kg body weight) and an intraperitoneal injection of phenobarbital (30 mg/kg body weight)![12] Under mechanical ventilation, the chest was opened under sterile conditions and the left anterior descending coronary artery was ligated with 7-0 prolene. The chest was closed in 3 layers.

Fig 1. (a) Polyglycolic acid cloth (PGAC) with type I collagen hydrogel. (b) Suturing of the PGAC over the infarct in the left ventricular free wall.

Isolation of BMC Under general anesthesia, the femora and tibiae were collected from other Lewis rats[3] After removing the connective tissue around the bone, both ends were cut and the bone marrow plugs were flushed out with phosphate-buffered saline (PBS, pH 7.4; Nissui Pharmaceutical Co Ltd, Tokyo, Japan). The cells were centrifuged at 1,500 rpm for 5 min and then resuspended with PBS. The cells were preserved on ice until seeding. We did not select or pre-treat the BMC.

Creation of Cell-Seeded-Biodegradable Scaffold The scaffold was a cloth made of polyglycolic acid solution (PGAC, Gunze Co Ltd, Kyoto, Japan)[13,14] The average fiber diameter, pore volume fraction, and density of the PGAC were 12 μm, 85%, and 0.23 g/cm^3, respectively (Fig 1a). It was cut into a 1-cm diameter circle with a thickness of 0.5 mm. The PGAC was immersed in 70% ethanol overnight, dried and then irradiated with UV light in the culture hood for 30 min. For group 1 (n=8), the sterilized PGAC was washed with PBS and then impregnated with type I collagen hydrogel prepared from 0.3% acidic collagen solution (Cell Matrix® Type I-A, Nitta Gelatin Co Ltd, Osaka, Japan), minimal essential medium, and sodium bicarbonate at 4°C, and gelated in a 37°C incubator. For group 2 (n=11), the PGAC was impregnated with type I collagen hydrogel containing 0.2 μg of b-FGF. For group 3 (n=10), the PGAC was impregnated with type I collagen hydrogel containing 0.2 μg of b-FGF and freshly isolated BMC (1×10^7 cells) as described earlier. We seeded the PGAC with BMC immediately before rapid transplantation of the bio-cloth.

Implantation of the PGAC At 1 month after artery ligation 29 rats were randomly divided into 3 groups and the conditioned PGACs implanted as follows[15] Briefly, under general anesthesia, the heart was approached through a median sternotomy and the PGAC was sutured over the infarcted left ventricular free wall with 7-0 prolene (Fig 1b). The chest was closed in 3 layers.

Assessment of Cardiac Function At 4 weeks after transplantation, the rats were anesthetized with ketamine and pentobarbital as previously described[3] The heart function of each group was measured using a Langendorff apparatus. Briefly, after midline sternotomy, the heart was removed and suspended on a Langendorff apparatus while in Krebs-Henseleit buffer (in mmol/L: NaCl, 118; KCl, 4.7; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 25; and glucose, 11; pH 7.4) equilibrated with 5% CO_2 and 95% O_2 at 100 mmHg. A latex balloon was passed into the left ventricle through the mitral valve and connected to a pressure transducer (model P231D, Gould Instrument System Inc, Statham, USA), a transducer amplifier (model AP-641G, Nihon Koden, Tokyo, Japan), and a differentiator amplifier (model EQ-601G, Nihon Koden). After a 30-min stabilization, systolic and diastolic pressures were recorded at 0, 10, and 20 mmHg of end-diastolic pressure and developed pressures, dp/dt max, and dp/dt min, were calculated.

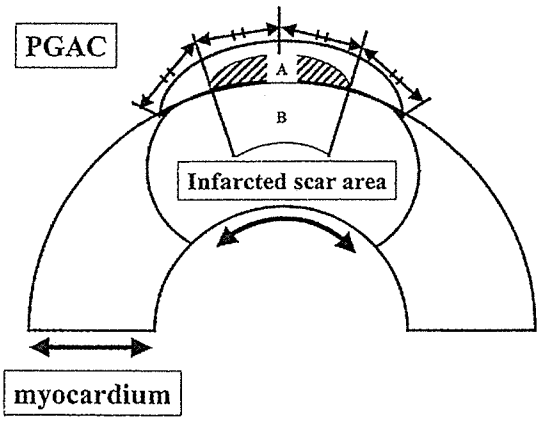

Fig 2. Schema of the infarct scar and the polyglycolic acid cloth (PGAC). The area of the scar and the PGAC were divided into four equal parts and the number of capillaries was counted at the center of the cloth over the scar area (A) and at the center of the infarcted area (B) at ×400 magnification.

Histological Study After measuring heart function, the heart was fixed with 10% neutralized formalin and cut into 3-mm slices, which were embedded in paraffin and cut into 6-μm slices[3] Sections were then stained with hematoxylin and eosin (HE), and Masson's trichrome for identifying the degree of fibrosis. They were also stained with antibody against von Willebrand factor (Dako, CA, USA) to quantitate capillary density in both the PGAC and the scar. To avoid compromised results from the surface of the PGAC, the number of capillaries was counted at the center of the cloth and in the area of the infarct scar by an observer unaware of the treatment group (Fig 2). Capillary density was calculated as the number of capillaries per square

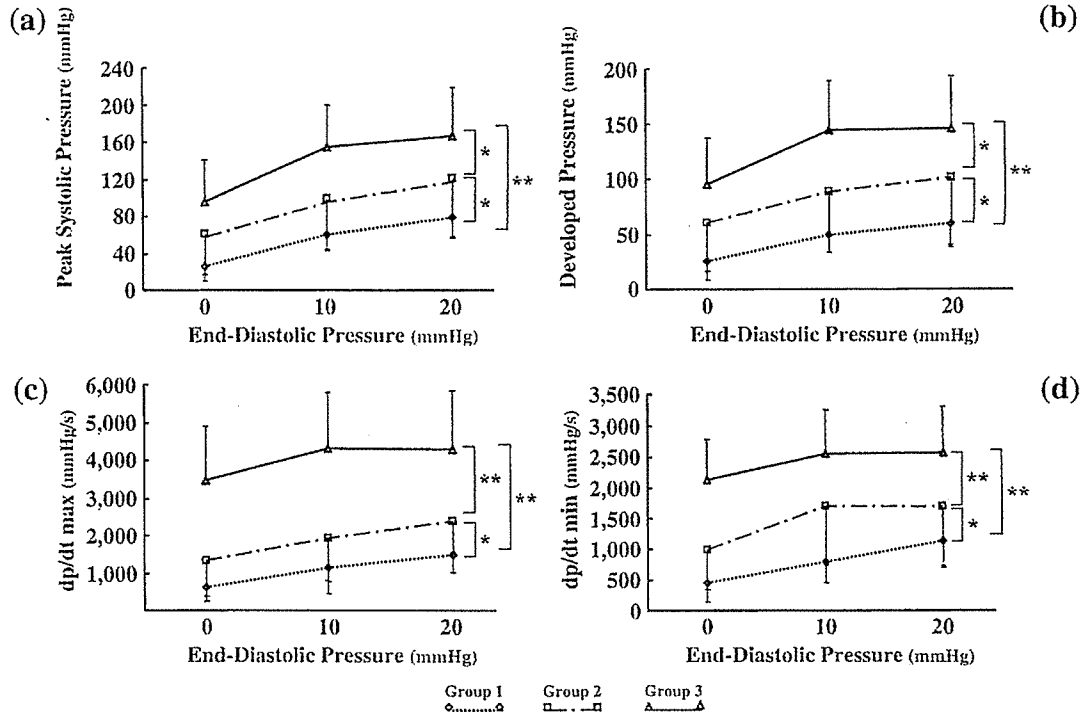

Fig 3. Cardiac function using the Langendorff apparatus. (a) Peak systolic pressure, (b) developed pressure, (c) dp/dt max, and (d) dp/dt min. All cardiac parameters were the highest in group 3. All cardiac parameters in group 2 were higher than those of group 1 (*p<0.001, **p<0.0001) (◇) Group 1, (□) Group 2, (△) Group 3.

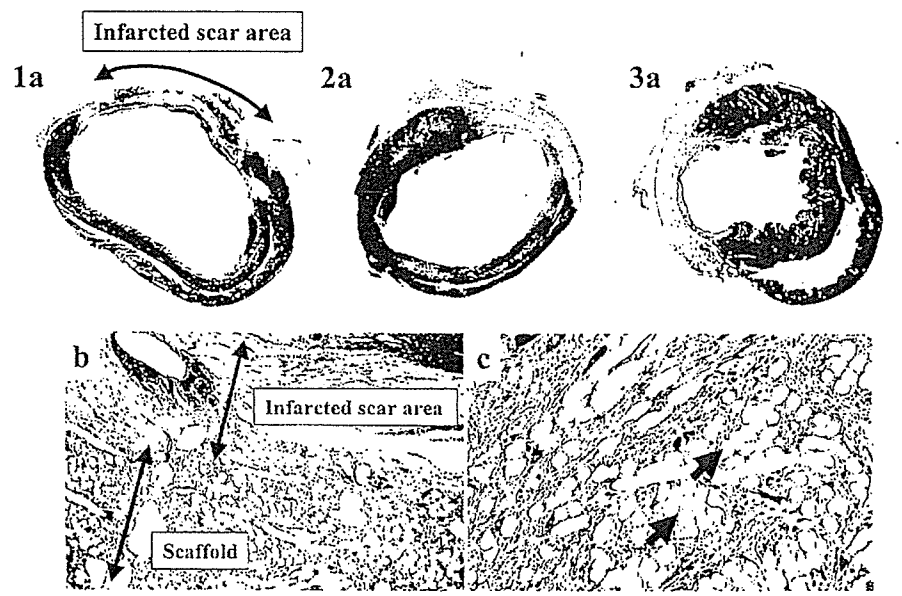

Fig 4. Histology of the polyglycolic acid cloth (PGAC) and the heart at 4 weeks after implantation. (a) No. 1 is Masson's trichrome staining for group 1, No. 2 group 2, and No. 3 group 3 (×0.5). Arrow in 4.1a indicates the PGAC over the infarcted scar area. (b) PGAC and infarcted scar area in group 3 (HE, ×100). (c) PGAC partially absorbed, and infiltrating inflammatory cells and fibrous strands of collagen in group 3 (arrows) (Masson's trichrome staining, ×200).

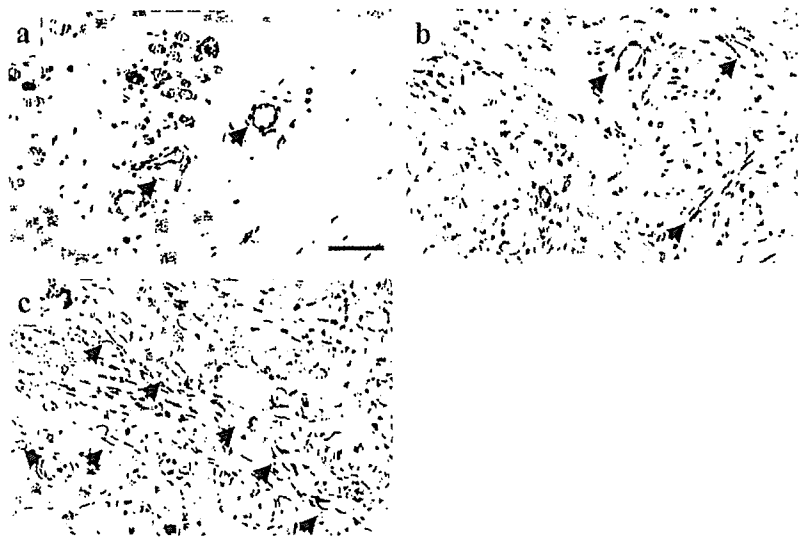

Fig 5. Von Willebrand factor staining at the center of the polyglycolic acid cloth (PGAC) (a: group 1, b: group 2, c: group 3). Arrows indicate capillaries. Greater capillary density was observed in groups 3, 2, and 1 in that order (Bar = 50 μm).

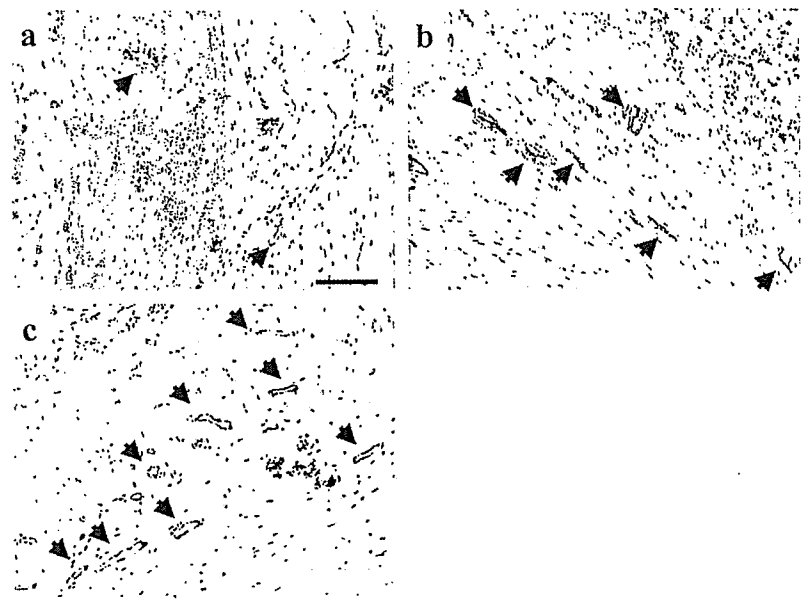

Fig 6. Von Willebrand factor staining at the center of the infarcted area (a: group 1, b: group 2, c: group 3). Arrows indicate capillaries. Greater capillary density was observed in groups 3, 2, and 1 in that order (Bar = 50 μm).

millimeter and the diameter of a capillary was defined as 5–10 μm (>10 μm was defined as a "small vessel").

Stem cells were recognized by mouse monoclonal antibody against nestin (BD Bioscience, USA)[16] diluted 1:100. The cell proliferation cycle was evaluated using a rabbit polyclonal antibody against Ki-67 (DAKO),[17] diluted 1:100. We counted the number of positive cells at the center of both the infarcted area and the PGAC under a light microscope.

Statistical Analysis

Statistical analysis was performed using StatView® 5.0 (SAS Institute Inc, Cary, NC, USA). All values are expressed as mean ± SD. Heart function and capillary density were assessed by one-way ANOVA among groups. If there was a significant difference, the Bonferroni method was used to evaluate differences between 2 groups. Correlation between the numbers of nestin- and Ki67-positive GFP-BMC was assessed by Pearson's coefficient, and regression analysis was performed. A p-value <0.05 was considered statistically significant.

Mouse Study

In order to evaluate the fate of the seeded BMC we used 8-week-old C57BL/6 mice in which the coronary artery was ligated with 8-0 prolene under general anesthesia, as described earlier, and GFP-mouse-derived BMC (1×10^7 cells)[3] were seeded into a PGAC (5×5 mm) with collagen

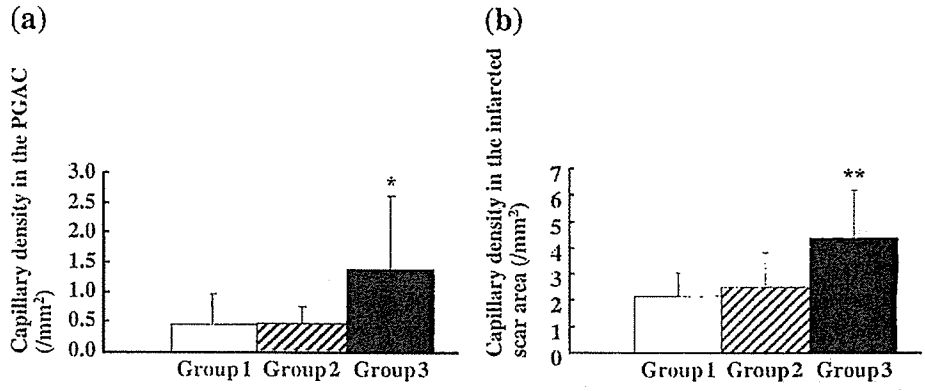

Fig 7. Capillary density (a) at the center of the polyglycolic acid cloth (PGAC) and (b) at the center of the infarcted scar area. Capillary density in group 3 was the greatest among the groups for the PGAC (*p<0.05) and in the infarcted area (**p<0.01).

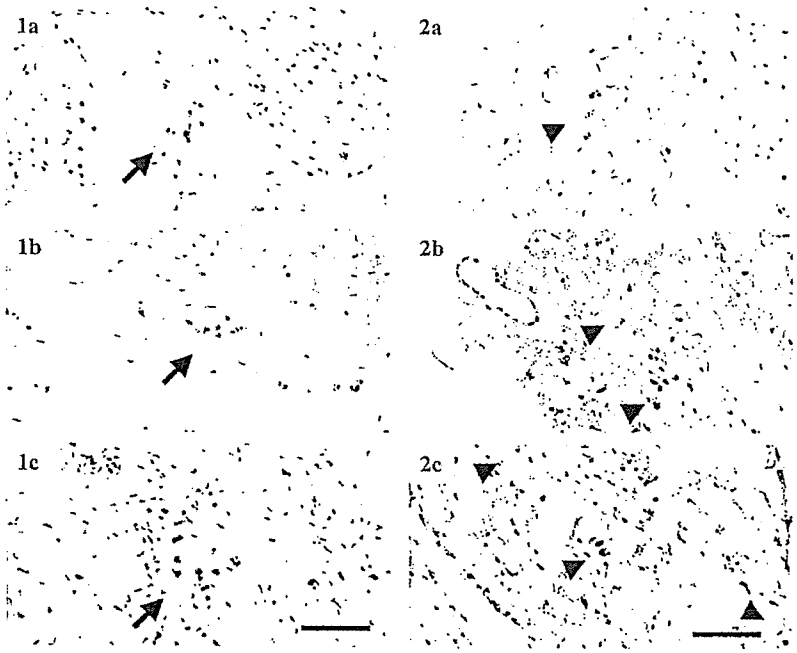

Fig 8. Nestin-positive and Ki-67 positive staining of the infarcted area (a: group 1, b: group 2, c: group 3). No. 1 is immunostaining for nestin, and No. 2 for Ki-67. Nestin-positive cells (arrows) and Ki-67-positive cells (arrowhead) at the margins of the scar (Bar=50μm).

gel and b-FGF (0.2μg). The conditioned PGAC was sutured over the infarcted area at 4 weeks after infarction. The heart was harvested, frozen, and sectioned into 6μm slices 4 weeks later. The sections were observed under a fluorescent microscope. In addition, the sections were stained with antibody against von Willebrand factor (DAKO) to evaluate capillaries originating from the GFP-BMC.

Results

Rat Study

Cardiac Function All cardiac parameters (systolic pressure, developed pressure, dp/dt max and dp/dt min) at 0, 10, 20 mmHg of end-diastolic pressure in were highest in group 3 ($p<0.001$). All cardiac parameters in group 2 were higher than those of group 1 ($p<0.001$) (Fig 3a–d).

Histological Study The PGAC were fit on the scar, and the surface of the PGAC was covered with connective tissue. There were no cases in which the PGAC detached from the surface of the heart. The infarcted scar area was thin (Fig 4) and the PGAC were partially absorbed, and foreign giant cells were gathered around the residual scaffold (Fig 4b). Masson's trichrome staining revealed inflammatory infiltrates and fibrous strands of collagen in the PGAC over the infarcted scar (Fig 4c).

Capillary Density There were many capillaries on the inner side of the PGAC and on the outer side of the infarcted scar area. Vessel size ranged from 10 to 200μm in diameter. Capillaries were observed near the edge of the PGAC. The capillary density of group 3 at the center of both

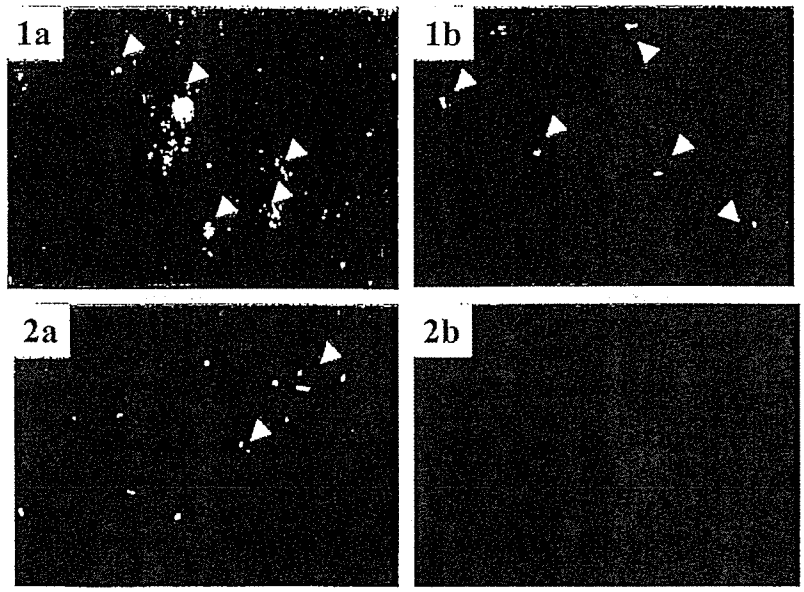

Fig 9. Capillary formation in the polyglycolic acid cloth (PGAC) and infarcted area in the mouse study. Sections were stained for von Willebrand factor, and positive cells were visualized as red (1) Section of the PGAC by fluorescent microscopy (a: red excitation at 524–640 nm, b: green at 515–540 nm). (2) Section of the infarcted area by fluorescent microscopy conditions as in (9.1a, b). There were capillaries in both the PGAC (9.1a) and scar (9.2a), but they were not derived from green fluorescent protein bone marrow cells (GFP-BMC) (×200).

the PGAC (1.39±1.24/mm^2) ($p<0.05$) (Figs 5c,7a) and the infarcted scar area (4.36±1.82/mm^2) ($p<0.01$) (Figs 6c,7b) was the greatest among all groups.

Nestin- and Ki-67-Positive Cells in the PGAC and Infarcted Area Nestin-positive cells existed at the margin, but not in the normal myocardium. There were no significant differences in the number of nestin-positive cells in the PGAC and infarcted areas among the 3 groups. In contrast, Ki-67-positive cells existed at margin, but not at the center of the scar and PGAC (Fig 8). The number of Ki-67-positive cells of group 3 (2.67±3.33/mm^2) ($p<0.05$) and group 2 (2.69±4.33/mm^2) ($p=0.11$) in the infarcted scar area were higher than that of group 1 (0.05±0.13/mm^2).

Mouse Study

Identification of GFP-BMC in the PGAC and Heart In the mouse study, morphological changes in the PGAC and the infarcted scar area were similar to those in the rat study with HE staining. There were 0–6 GFP-BMC in the PGAC at ×200 magnification, but none in the infarcted area. In the infarcted scar area, we observed capillaries that had not originated from GFP-BMC (Fig 8).

Discussion

Cell therapy for the globally ischemic heart has been investigated during the past decade, but the usual technique of direct injection can affect only a limited area of myocardium. A large amount of cell suspension cannot be implanted[18] and it is difficult to transplant cells to many sites. Moreover, the direct injection technique may lacerate the myocardium and cause embolization[19] or arrhythmia.[20] Under hypoxic conditions, directly-injected cells might die after transplantation and it is difficult to inject cells into the thin myocardium of the endstage heart.

To overcome these issues we propose BMC-seeded bioengineered scaffolds, which we think has several advantages. First, the procedure does not injure the infarcted area. Second, we can apply the PGAC can be used not only for a regional infarct but also for a global infarcted area. In addition, the cell density and concentration of b-FGF in the sheet can be modified. We previously reported that a collagen sponge scaffold with chondrocytes and b-FGF induced angiogenesis and enhanced cartilage regeneration because the newly formed blood vessels helped the implanted cells to survive.[13] The PGAC impregnated with collagen hydrogel including b-FGF (0.2μg) induced significant angiogenesis and maintained the function of transplanted Langerhans islet cells.[14] The PGAC enables cells to attach to its surface and moreover, the collagen hydrogel can trap cells efficiently and carry b-FGF for slow release. The PGAC normally resolves at 15 weeks after implantation in vivo, but more detailed histological and long-term implantation are needed.

Based on our previous study, we modified the PGAC for an ischemic heart model. We used 10^7 BMC, which is 10-fold the number of the common dosage in the direct-injection technique for the rat heart model. We used them as the candidate cell for transplantation and as a source of growth factors such as VEGF and b-FGF.[6,7] BMC can be taken easily and autologously. Hamano et al reported that the growth factor from BMC was enhanced under hypoxia, and induced angiogenesis.[21] Although we still do not know which fraction is the source regarding angiogenesis, bone marrow mononuclear cells supply angiogenic ligands (b-FGF, VEGF, angiotensin-1) and cytokines (interleukin-1β and tumor necrosis factor-α).[22] Orlic et al reported that the lineage-negative, c-kit-positive cells differentiated into cells of cardiogenic lineage, including coronary arterioles and capillaries.[23] Recently, Sata reported that bone marrow-derived precursors can give rise to vascular cells that contribute to the repair, remodeling, and lesion formation of the arterial wall.[24]

Bioengineered scaffolds can also be used as a controlled drug-delivery system that induces the transplanted cells to migrate into the infarcted area and release growth factors augmenting angiogenesis and aiding new tissue generation.[25] However, it is difficult to maintain appropriate

concentrations of growth factors because their biological activities last only a few minutes in the circulation?[6] We used the same dosage of b-FGF reported previously,[4] which was not a large quantity. A scaffold that includes a large amount of growth factors may be costly.

In the present study, the b-FGF and BMC-seeded-PGAC group (group 3) showed the best cardiac function in the Langendorff apparatus. There are several possible reasons for this result. We used BMC as the source of growth factors[6,7] that could induce angiogenesis. The capillary density of group 3 was the greatest in the comparison of all the groups, which suggests that the growth factor in the BMC is involved in the formation of capillary at the border of the damaged myocardium. The growth factor might be enhanced under hypoxia?[1] Nishida et al reported the importance role of cardioprotection against myocardial death of b-FGF in AMI?[7] Although we do not know that the growth factor directly contributed to the improvement in the contraction of residual myocardium, we speculate that when angiogenesis increases, residual cardiomyocytes might be rescued and extracellular matrix and cell density might be maintained. In particular, the border of the infarct is the main area from which the beneficial effect extends and thus the infarcted scar area might be prevented from dilating. Prevention of dilatation of the left ventricle might be linked to maintenance of heart function. These interactions might contribute to the improvement in cardiac function. Surprisingly, there were no GFP-BMC inthe infarcted scar area. We did not detect 4',6-diamidino-2-phenylindole-labeled BMC in the scar area of the rat model either (unpublished data). Taking all our findings together, seeded BMC did not affect angiogenesis by migration or vasculogenesis, but may have exerted their influence via the paracrine pathway. Many more angiogenic factors might be released when seeded BMC were exposed to ischemic conditions?[1] Although the transplanted cells did not survive under ischemic conditions, they played a role in inducing angiogenesis. In the rat model, there were several implanted cells, small vessels, and proliferating (Ki-67-positive) EPC-like cells in the infarcted or border areas, but not in the GFP-mouse models. Therefore, we speculate that angiogenesis or vasculogenesis is induced by cooperation between the implanted b-FGF, other angiogenesis factors (eg, VEGF, hepatocyte growth factor, angiotensin-1 etc), and the seeded BMCs. This is a new concept of using cells and biomaterials under ischemic conditions and may also be used in other ischemic organs.

Nestin and Ki-67 were identified in the infarcted area and the PGAC. Nestin, an intermediate-filament protein, is basically expressed in the embryonic heart;[16] and implies the presence of stem cells?[8] We previously reported that bone marrow was one of the origins of regenerated myocardium in a self-renewal system?[9] The present findings raise a new hypothesis that exogenous BMC implantation would trigger endogenous stem cells to regenerate myocardium. When we detected the expression of the nestin-positive cells in the infarcted area, not in normal myocardium, we assumed there was a correlation between the nestin-positive cells and ischemia, although the reason for the nestin positive cells in the PGAC remains unknown. Ki-67 is a nuclear antigen expressed in all phases of the cell cycle except G_0;[17] and it is associated with cell division. There were more Ki-67-positive cells in group 3, which suggested that BMC-derived growth factors, such as VEGF and b-FGF, diffused into the infarcted scar area and enhanced proliferation of residual cells?[1] As well as nestin, the expression of the Ki-67-positive cells might correlate with angiogenesis. Moreover, the formation of connective tissue was related to Ki-67-positive cells. These cells were spindle- and thin-shaped or corresponded to vessels. Therefore we speculated that these cells might be endothelial progenitor cells and fibroblasts.

Study Limitations

We did not establish a control group with an infarcted heart without intervention. Also, there were several unsolved issues. First, we did not examine the interaction of capillary density and the quantities of b-FGF and BMC. Second, we did not measure growth factor in the BMC, tissue, and blood. Third, long-term results remain unknown. Lastly, we quantified the capillary density without measuring the blood flow.

Conclusion

A bioengineered PGAC with b-FGF and fresh BMC improved cardiac function by inducing angiogenesis without migration of BMC. Fresh BMC worked as angiogenic inducers, and the biomaterial as a "drug delivery system" for treating the globally ischemic heart.

Acknowledgments

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Regenerative Medicine 5

再生医療用材料

Biomaterials for tissue engineering

Keywords
biomaterials
polymer
collagen
biological tissue
decellularization

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Summary

Biomaterials used in tissue engineering field are reviewed in this article. The first generation of the biomaterials for tissue engineering scaffold is simple biodegradable polymers such as poly (lactic acid) and collagen. Second generation is the combination of materials with biological substances, such as growth factors and genetic vector, which promotes the regeneration of tissues. Nowadays, several researches for the third generation biomaterials are preceded. One of the most clinically applicable one is the acellularized tissue, named "bioscaffold". Tissue engineered grafts based on acellular xenogenic matrices have been widely studied to have more durability with growth potential and less immunogenicity than the current bioprostheses. However, they have still several problems to be solved such as degradation control of biodegradable polymeric scaffolds and transfer of unknown animal related infectious diseases. Our novel tissue processing of decellularization by ultrahigh pressure treatment for the safe valve transplantation was introducted briefly.

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はじめに

Langer と Vacanti らが "Tissue Engineering" のコンセプトを示して以来[1]，細胞，増殖因子，足場材料は組織工学，再生医療の 3 つの柱として開発研究が進められている。足場材料は当初，ポリ乳酸を主体とする生分解性材料を指したが，再生医療の広がりとともに，使用される材料の種類も広がっている。

本稿では，材料工学の観点から再生医療およびそれに関連した研究分野に用いられている材料について概観し，さらに我々が注目している生物由来組織を用いた機能性再生医療用材料について紹介する。

組織再生の方法には大きく分けて，*in vitro* での人為的な再構築と *in vivo* での生体による再生の 2 つがある。いずれの場合にもそれぞれについて特徴的な材料を用いる。多くの場合，生体

内で分解することが主要な機能であるが，用いられるそれぞれの場面で種々の機能が必要とされる。これらの概要を以下に説明する。

再生医療用Scaffold材料

人工細胞外マトリックスには，組織再生後の慢性期異物反応を回避するために生体吸収性の材料が用いられる。また，細胞を組み込むためにスポンジ状あるいは繊維集合体に加工されて用いられる。広く用いられている人工細胞外マトリックスの代表的な素材は，コラーゲンのような天然高分子，グリコール酸・乳酸系高分子のような合成高分子，ハイドロキシアパタイトのような無機物質である。

1．合成高分子材料

主な生分解性材料を表と図1にまとめた。最も研究されている生体吸収性の合成高分子はポリ乳酸(PLA)，ポリグリコール酸(PGA)，および乳酸-グリコール酸共重合体である。PLAにはモノマーが不斉炭素を有し光学活性なためD体(PDLA)とL体(PLLA)，およびラセミ体(PDLLA)が存在する。PDLLAは非結晶性のため生体内では速やかに分解する。PGA繊維は外科用縫合糸として，また，高強度のPLLAは骨プレートや骨固定スクリューとして応用されている。これらの高分子の分解生成物の乳酸とグリコール酸は生体内の代謝物であるため，安全であり，分子量と組成を制御

分　類	化学構造	例
天然高分子		
1．植物由来	多糖	セルロース，デンプン，アルギン酸
2．動物由来	多糖	キチン，キトサン，ヒアルロン酸，
	蛋白質	コラーゲン(ゼラチン)，アルブミン，フィブリン
3．微生物由来	ポリエステル	ポリ(3-ハイドロキシアルカノエート)，デキストラン，ポリ(γ-グルタミン酸)
	多糖	ヒアルロン酸
合成高分子		
1．脂肪族ポリエステル	重縮合系	ポリブチレンサクシネート
	ポリラクチド類	ポリグリコール酸(PGA)，ポリ乳酸(PLA)
	ポリラクトン類	ポリ(ε-カプロラクトン)(PCL)
	その他	ポリブチレンテレフタレート・アジペート
2．ポリオール		ポリビニルアルコール
3．ポリカーボネート		ポリエステルカーボネート
4．その他		ポリ酸無水物，ポリシアノアクリレート，ポリオルソエステル，ポリフォスファゼン
無機材料		リン酸三カルシウム(TCP)，炭酸カルシウム

表　主な生分解性材料

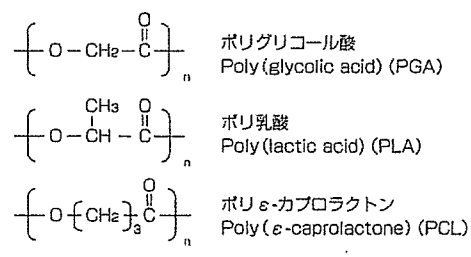


図1　脂肪族ポリエステル系生分解性材料の構造式

することで強度や分解速度の異なる種々の材料を自由に合成できる。たとえば、柔軟性が必要な場合には乳酸とε-カプロラクトンとの共重合体が用いられる。LangerとVacantiらは、生体から単離した肝細胞や軟骨細胞をPGAメッシュ内に播種し、生体外で培養した後に生体に移植する手法が、組織の再建に有効であることを示した[1]。その後、軟骨や半月板[2]、網膜色素細胞[3]、筋肉組織[4]、神経組織[5]など幅広い応用が試みられている。

2．無機材料

ハイドロキシアパタイト、リン酸カルシウム、あるいは炭酸カルシウムなどの無機材料は生体親和性に優れ、主として骨再生用Scaffoldとして検討が進んでいる。アパタイトは、人工骨・人工歯根・骨充填材のほかに、ハイブリッド型人工骨として骨髄造血巣の誘導に関する研究、あるいは経皮デバイスとしての可能性が研究されている。Yamashitaらはハイドロキシアパタイトなどのある種のセラミックスは、熱電気的な操作によりセラミックス内部にイオン分極を発生させることができ、この結果大きな電荷を長期間にわたって表面に誘起できることを示した[6]。この表面誘起電荷の効果はmmオーダーの限定された領域において有効であり、これをエレクトロベクトル効果と名付けた。このエレクトロベクトル効果をもつエレクトロベクトルセラミックスは骨誘導能を有することが報告されており、骨再生誘導材料として期待されている。

3．生体由来材料

生体の細胞外マトリックスの主成分であるコラーゲンやプロテオグリカンなどが単離され、再生医療用材料として検討されている。これらはいずれも水溶性で分解速度が速いために、そのままではScaffoldとして用いることができない。そこで、グルタルアルデヒドやエポキシなどで架橋することで不溶化して用いられる[7]。Shimizuらはコラーゲンスポンジを用いて人工気管、人工食道の再建について優れた成果をあげている[8]。これらの生体由来材料は優れた生体活性を有するものの、その反面、化学架橋剤の影響、抗原性、およびウイルスやプリオンに代表されるような感染の問題がある。

組織再生因子の徐放のためのキャリア材料

組織再生に細胞増殖因子の利用は非常に効果的である。しかし、いくつかの細胞増殖因子は非常に不安定で投与後に速やかに消失してしまったり、またそれらの水溶液を目的の部位に注入しても直ちにその部位以外に拡散してしまったりし、組織の再生の効果が得られない場合が多い。そこで、キャリアを用いたDDS(ドラッグデリバリーシステム)技術によって細胞成長因子を徐放化させたり[9]、目的の細胞成長因子の遺伝子を細胞に送り込んで発現させ、組織中に分泌させたりする試みが行われている。

新しい機能性再生医療用材料開発の試み

再生医療の研究は、その範囲が日々拡大しており、応用される目的に応じた特徴ある材料の要求が高まっている。ここではいくつかの新しい機能を有する再生医療用材料について紹介する。

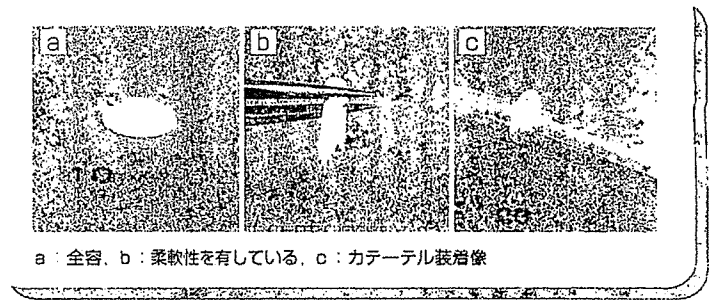
1．感温性培養基材

細胞を分散した状態でScaffoldに複合化するのではなく、*in vitro*で培養し細胞が密集して細胞間の連絡ができるようになると、機能が高まる例がある。しかし従来の培養法では、たとえ機能が亢進した状態になっても、生体に用いる場合には、培養基材から分離するために再び分散する必要があった。Okanoらは、感温性高分子であるポリ*N*-イソプロピルアクリルアミド(PNIPAAm)をプラスチック製培養皿にグラフトし、温度変化によって密集状態のまま細胞を回収する方法を考案した[10][11]。細胞塊は機能を保持したままシート状で回収でき、階層構造の形成も可能である。彼らはこれらの技術を用いて「細胞シート工学」を展開している。

2．有機・無機ハイブリッド材料

アパタイトは骨再生材料だけでなく、軟組織への高い接着性を有し、幅広い応用が期待できる。しかし、物性が軟組織と著しく異なるためバルク状態で用いるには困難である。近年、ア

パタイトなどの無機材料を有機高分子材料と分子レベルで複合化する有機・無機ハイブリッド材料の研究が精力的に行われており，再生医療への応用も試みられている。Furuzonoらはアパタイトの微粒子を化学結合でシリコーン表面に固定化し，アパタイトの有する高い軟組織接着性とシリコーンの柔軟性を合わせ持つ新しい材料を考案した(図2)[14]。



a：全容，b：柔軟性を有している，c：カテーテル装着像

図2 アパタイト-シリコーン複合体からなる経皮デバイス

3．生体Scaffold

生体組織そのものをScaffoldとして用いようとする試みも行われている。これは，同種もしくは異種の動物の血管，心臓弁などから細胞組織を除去し，組織のみを用いて移植を行うものである[15]。わが国でも筆者らを含め，大阪大学，防衛医科大学のグループが同様の研究を進めている。Knebelらは独自の脱細胞技術で作製した心臓弁の臨床応用について報告した[16]。次項で，この脱細胞化心臓弁について紹介する。

脱細胞化生体Scaffoldによる再生医療

1．背　景

わが国では年間約9千件の心臓弁置換術が施行されており，代用弁としてパイロライトカーボン製の機械弁が80％，ブタやウシ組織をグルタルアルデヒドで固定化した異種生体弁が20％使用されている[16]。機械弁は1960年代初頭の実用化以来，形状や材質の改良が重ねられ，現在使用されているものは一生の使用に耐えられる物理的強度を有している。しかし，機械弁では依然として抗血栓性の問題が解決されていない。抗凝固のための生涯にわたる厳重なワーファリン服用のコントロール，血栓付着による弁機能不全，脳塞栓症および催奇形性などの問題がある。

異種生体弁も1960年代後半の登場以来，抗凝固剤の服用が不必要であるというQOL上の利点から，特に最近は使用例が増えている。異種生体弁は石灰化などによる構造的劣化の問題を抱え，高齢者では15～20年程度の耐久性を有するが，若年者では5～10年程度の耐久性しか有せず，米国心臓病学会および心臓病協会のガイドラインでは65歳以上の高齢者に使用が奨励されている。

欧米では1980年代半ばから，わが国でも近年，凍結保存による組織バンクが整備されたことで，死体から提供された凍結保存同種弁が臨床で使用されつつある。しかし機械弁や異種生体弁と同様に，凍結保存同種弁でも成長性を有しないため，小児患者の場合では再移植となる場合が多い。

以上のようなことから，自己弁と同等の抗凝固性，耐久性，成長性などを兼ね備えた次世代型の代用弁の開発のために再生医療の方法論が試みられている。

2．テーラーメード心臓弁

現在，心臓弁の再生医療に関して研究されているScaffold材料としては，吸収性縫合糸などで利用されているポリグリコール酸やポリ乳酸などの生体内分解吸収性人工材料と，同種あるいは異種心臓弁組織からドナー由来細胞を除去した脱細胞化マトリックスとがある。

人工材料製Scaffoldを用いた代表例としてShinokaらの報告がある。ヒツジを用いた実験で，ポリグリコール酸製のシート状メッシュScaffoldに末梢血管壁の細切によって得た血管内皮細胞，平滑筋細胞，および線維芽細胞を

播種することで，再生型心臓弁葉を作成した。ヒツジの肺動脈弁の一葉を再生医療心臓弁葉と置換したところ，6週後には正常組織と同様の組織が再生し，9週以降は力学特性も正常組織と同等であったと報告している[17]。

脱細胞化マトリックスを用いた先駆例としては，米国CryoLife社によるSynerGraft心臓弁がある。1999年から脱細胞化ブタ大動脈弁の臨床使用を開始し，2001年には世界初の再生型心臓弁と称して欧州で市販を開始した。移植後数ヵ月間で自己細胞が組織内に浸潤し，自己組織化すると報告している[18]が，移植後に破断の生じた例も報告されている。ドイツ・ハノーバー医科大学のHaverichらは，1998年より異種生体弁から動物由来細胞を除去し，代わりにレシピエントの自己血管内皮細胞を播種する動物実験を行い，界面活性剤であるTriron X-100や蛋白分解酵素であるトリプシン溶液を細胞除去に用いている[19]。英国リーズ大学のInghamらは種々の薬液で細胞除去効果を検討し，Sodium Dodecyl Sulfate (SDS)が最も細胞除去に適していると報告している[20]。また，ドイツ・フンボルト大学のKonertzらはヒツジを用いた6ヵ月間の実験で，脱細胞化ブタ肺動脈弁に自己内皮細胞を播種することで弁の変形や石灰化もみられなかったと報告しており，臨床使用も開始している[21]。

我々は2000年から，脱細胞化した生体弁に患者自身の細胞を組み込むテーラーメード心臓弁の開発を開始した。免疫反応の主因を成すドナー由来細胞を除去し，コラーゲン線維や弾性線維などの構造マトリックスからなる脱細胞化Scaffoldに患者の自己細胞を組み込むことで，生体適合性を高めるとともに，自己修復性や成長性を有する代用弁が創製できると期待できる(図3)。このためには，完全な脱細胞化処理法の開発による安全な脱細胞化Scaffoldの開発が重要である。

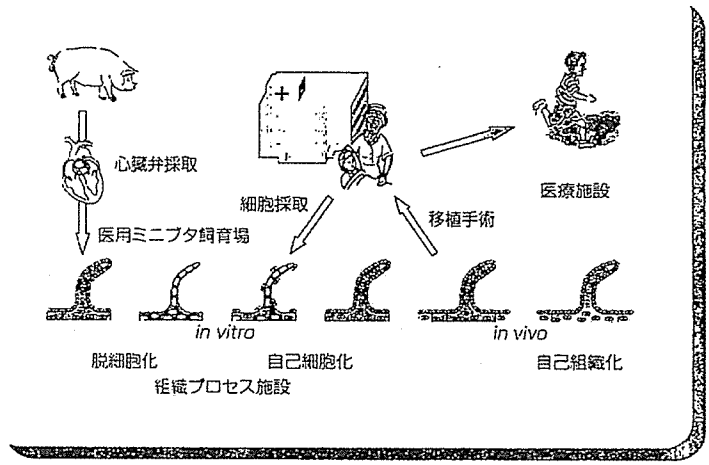


図3　脱細胞化組織を用いた再生医療のスキーム図

3．新規脱細胞化処理

前述のように，ほとんどのグループは界面活性剤や蛋白分解酵素などの薬液処理によって細胞を除去している。我々も当初，Triton X-100溶液による界面活性剤浸漬処理を検討した。その結果，厚さ数百μmの弁葉内においては処理6時間後には細胞核は染色されなくなったが，弁葉基部の組織内細胞の核は処理24時間後でも表面から1mm以遠の組織深部では染色されており，界面活性剤溶液の組織内浸透性が悪いためであると考えられた(図4中)。また，細胞毒性を示すTriton X-100を洗浄除去するために数週間以上の時間を要し，その間における生体力学特性の変化や汚染の危険性についても注意が必要であった。そこで我々は，より完全な細胞除去法について検討し，液体を圧力媒体として等方圧力を加える冷間等方圧加圧法による超高圧印加処理法を開発した(図5)。多くの蛋白質や酵素は300MPa程度の高圧処理によって変性するとともに，酵母や芽胞をもたない細菌は500MPaの処理で細胞膜が破壊され，殺菌される。また，HIVなどのエンベロープをもつウイルスは600MPaの処理でほぼ完全に不活化される[22]。清潔下にて摘出し

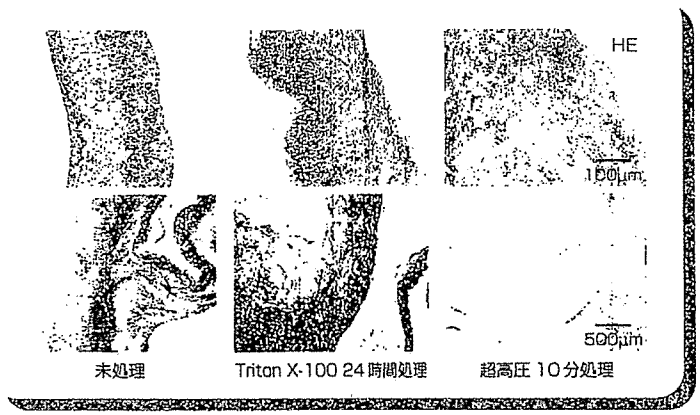


図4　脱細胞化処理された心臓弁の組織断面（上：弁葉，下：弁基部）
（→巻頭Color Gravure参照）

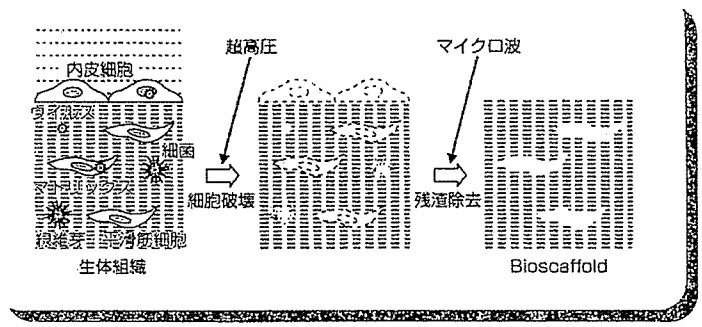


図5　新規な脱細胞処理法

たミニブタ心臓より肺動脈弁を採取し，低温下にて980MPa（10,000気圧）の超高圧印加処理（4℃，10分間）を行い，続いて洗浄処理したところ，組織深部まで完全に細胞を除去することができた（図4右）。力学特性を検討したところ，界面活性剤浸漬処理では，処理時間に伴って強度，弾性率とも増加する傾向を示したが，超高圧処理では力学特性への影響がみられなかった（図6）。現在，超高圧処理および複数種の自己細胞を組み込んだテーラーメード心臓弁の動物実験に取り組んでいる。

再生医療用材料の今後

今後の再生医療に望まれるテクノロジーとしては，①材料・細胞の空間的配置，②時間的制御のための物理刺激法（温度以外の），③成形法および④再生組織の評価があげられる。

①は材料および細胞の空間的配置を制御する技術で，たとえば肝臓や腎臓などでは，働きの異なる細胞が三次元的に階層構造をとっている。このような複雑な構造を工学的な技術で再構築するには，三次元的に細胞を配置するための精緻な加工法とそれに対応した材料開発が必要である。②については，組織再生のカスケードについては，発生学の進展により次第に明らかになってきているが，これによると，種々の蛋白質や遺伝子が時系列順に発現することが明らかになっている。これらの生理的な状況を，再生医療の場で実現するためには，それぞれの因子の作用を時系列的に制御する技術が必要である。一つの手法は，DDSであり，材料の特性を用いたものが考えられる。もう一つの手法は，外部から物理的な刺激によって制御するものである。すでに温度，磁場，超音波などによる薬剤などの放出のOn-Off制御の検討が行われている。今後は全体でなく，空間的な制御法を組み込んだ精緻な位置特異的な放出制御技術の開発が期待される。③については，現在の多孔性もしくは繊維による構築技術だけでなく，細胞の空間配置制御を可能にする

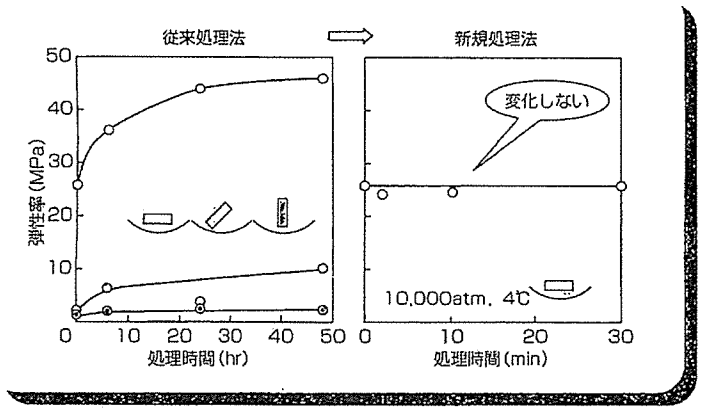


図6　界面活性剤および新規法による脱細胞化弁葉の力学特性評価

ような新しい材料加工法が必要となると思われる。

このように、再生医療における材料の重要性は今後ますます増大していくと思われる。

おわりに

現状の再生医学のための工学技術は、足場材料を含んだバイオマテリアルの開発や細胞を増やすためのバイオリアクターの開発が主体である。発生工学的研究などにより組織再生のメカニズムの全容が明らかになるまでは、材料工学だけでなくすべての工学技術を結集した生体工学技術の発展が、再生医療を支えていくものと思われる。材料研究者と医学研究者の共通する基盤として、再生医療の今後のより一層の発展を祈念する。

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特集「異種移植」

ブタ組織の脱細胞化

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はじめに

一般に組織の置換や欠損部の修復を同種あるいは異種組織移植により行おうとする場合，そのままでは激しい拒絶反応が引き起こされ，組織は壊死・脱落する。この拒絶反応の原因となる同種・異種の主要抗原は主に組織中の細胞膜に存在するため，組織から細胞成分を完全に除去できれば抗原性はほとんど消失すると考えられる。そこで脱細胞化した組織を移植し，これをscaffold（足場）として宿主細胞により組織が再構築されれば，拒絶反応に対する免疫抑制治療の必要なく，場合によっては成長性を期待することもできる新たな再生医療となりえる。

これまで生体組織の脱細胞化法として，急速な凍結・溶解による細胞破壊，界面活性剤や蛋白分解酵素による細胞除去など試みられてきたが，細胞成分の残存，ウィルスを含む病原微生物の存在，組織力学特性の変化，使用薬剤の毒性，組織保存・輸送にかかる費用などの点でそれぞれ一長一短であった。

われわれは，上記諸問題をクリアする脱細胞化方法として新たに超高圧処理法を開発し，各種組織においてその移植可能性を検討しているので，ここにその一部を紹介する。

超高圧処理による脱細胞化

生体組織に液体を圧力媒体として温度上昇を抑えながら等方圧力を加えていくと，多くの機能性蛋白は300 MPa程度の加圧により失活・変性し，酵母や芽胞のない細菌も500 MPaで死滅する。さらに600 MPaではHIVなどのエンベロープを有するウィルスまで不活化させることができる[1]。

そこでわれわれは，低温を保ちながら等方圧力を加えることのできる冷間等方圧印加処理装置（図1）を用い，ブタから採取した各種組織を4℃にて980 MPa（10,000気圧）で10分間加圧し，続いてPBSを主体と