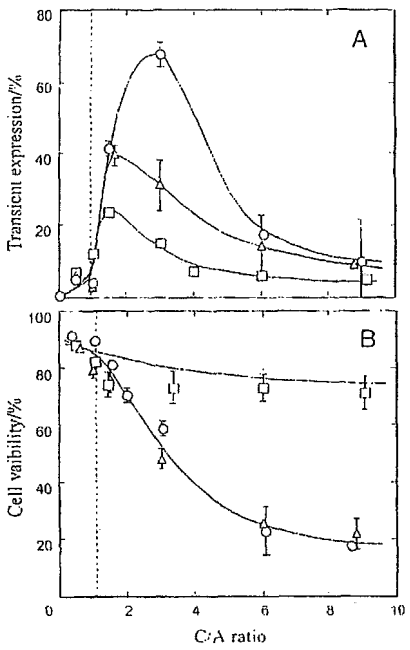

FIG. 2. A Transient expressio of *lacZ* introduced into COS-1 cells; B cytotoxicity after osmotic shock using DEAE-dextran with the substitution ratio for DEAE groups of: ○ 55, △ 31, and □ 22 %

Cherng et al. 1996) have also been proposed but there is little information on the correlation between their chemical structure and transfection efficacy. In 1995, Boussif et al. reported that polyethyleneimine (PEI) can be effectively used as a non-viral gene vector for the purpose of gene therapy (Boussif et al. 1995). Godbey et al. (1999a) reported the effect of molecular weight of PEI. Low-molecular-weight (1,800) PEI resulted in no gene expression but transfection efficacy increased with increasing molecular weight, with the most effective results obtained at 70,000.

2.2 Molecular Shape

When linear PEI and branched PEI were compared with respect to their transfection efficacy, the results were dependent on the adopted transfection procedure (Fig. 1) (Plank et al. 1999). They showed the efficiency of the branched structure, while Ohashi et al. reported more efficient gene transfer using linear PEI than branched PEI (Ohashi et al. 2001). Thus, further experiments are necessary to investigate the relationship between the molecular shape of polymers and gene expression.

In 1993, Haensler and Szoka reported on the effectiveness of hyper-branched polyamidoamine (PAMAM) dendrimers, a well-defined class of cascade polymers from methyl acrylate and ethylenediamine (Fig. 1) . They achieved excellent gene expression using heat-treated dendrimers (fractured dendrimers), which are a degraded form of the intact dendrimers, at the amide linkage (Tang et al. 1996) (see Chaps. 1–5).

Various amphiphilic block polymers, such as PEG ylated PLL, which self-organize into micelles in aqueous solution, have been used as carriers. DNA can form PICs with hydrophilic chains of block polymers. Micelles are formed from the core of these PICs surrounded by hydrophobic chains of block polymers (Erbacher et al. 1999).

3 Transfection Protocol Using Polyplexes

3.1 Various Inhibitors in In Vitro Gene Delivery

The roles of endosomes and lysosomes in gene transefer have been discussed in the literature (Wattiaux et al. 2000). Polyplexes are taken up via endocytosis and then travel through various intracellular pathway via endosomes and lysosomes. In order to avoid degradation of internalized polyplexes in these structures, the activity of the lysosomal enzymes must be suppressed by adding various endosomal or lysosomal inhibitors as shown in Table 1.

Weak bases, such as chloroquine and ammonium chloride, inhibit the acidication of endosomal or lysosomal environments as well as the degradation of polyplexes in endosomes and lysosomes (Maxfield 1982; Cotten et al. 1990; Choi et al. 1998). In a study comparing chloroquine and several other weak bases, it was found that only chloroquine enhances transgene expression, which may well be related to dissociation of the complexes (Erbacher et al. 1996). Bafilomycin A_1 and concanamycin A act as inhibitors of vacuolar ATPases known to block the endosomal proton pump. Photosensitizing compounds, such as AlPcS2a and TPPS2a, destabilize endosomes following photochemical reactions of these agents with visible light. Endosomal and lysosomal inhibitors thus improve the release of polyplexes from these organelles.

3.2 Mechanical Methods

Mechanical transfection, such as electroporation (Magin-Lachmann et al. 2004), microinjection (Zauner et al. 1999), and osmotic shock (Takai and Ohmori 1990; Okada and Rechsteiner 1982), are useful methods especially for studying transgene expression following endosomal escape. We use osmotic shock because, compared to microinjection, a larger number of cells can be easily treated and the polyplexes are directly delivered into the cytosolic compartment (Kimura et al. 2002). The method is applicable even for lymphoid cells. Briefly, cell suspensions are incubated with polyplexes for 30 min–1 h at 37°C, and a highly osmotic solution containing 1 M sucrose,

TABLE 1. Endosomal inhibitors

Inhibitors	Action	References
Chloroquine	Raises endosomal pH	(Erbacher et al. 1996; Cotten et al. 1990; Murphy et al. 1984)
Ammonium chloride		(Erbacher et al. 1996; Fredericksen et al. 2002; Maxfield 1982)
Monensin, FCCP		
Bafilomycin	ATPase inhibition	(Fredericksen et al. 2002; Drose and Altendorf 1997)
Fusogenic peptides	Membrane fusion	(Collins and Fabre 2004)

20% PEG 4000, 210 mM NaCl, and 70 mM Tris-HCl buffer (pH 7.3) is then added. After incubating the cells for a given period of time, they are rinsed twice with serum-free culture medium and exposed to hypotonic culture medium. After a 48-h incubation, transgene expression can be evaluated.

4 Biological Barriers

The mechanism of polyplex-mediated gene transfer is thought to follow the general endocytotic process. In order to lead to successfully high transgene expression, various biological barriers must be crossed: (1) interaction with the plasma membrane, (2) internalization, (3) escape from the endosome, (4) trafficking into the nucleus, and (5) dissociation of the complexes and/or DNA recognition by transcription factors (Fig. 3).

4.1 Internalization

Since positively charged complexes interact with the cell surface by an electrostatic interaction with anionic substances on the cell surface, such as sialic acid and proteoglycan, the zeta potential of polyplexes is important. Polyplexes composed of polycations with molecular weights of several thousands exhibit significantly higher zeta potentials (Ruponen et al. 1999; Jeong et al. 2001; Ahn et al. 2002; Wolfert et al. 1996; Toncheva et al. 1998; Cherng et al. 1996; Wolfert et al. 1999; Putnam et al. 2001; Howard et al. 2000), irrespective of the polycations used .

Internalization events also seem to be affected by polyplex size and zeta potential. The size of the polyplexes has been assessed by transmission electron microscopy

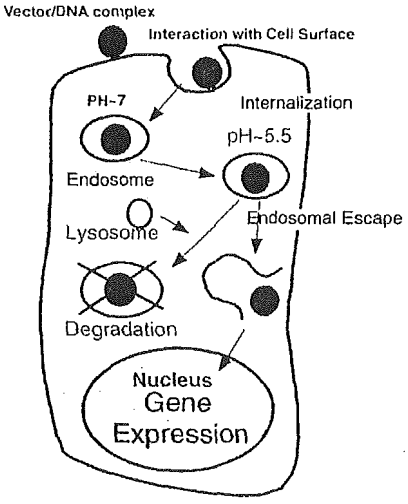

FIG. 3. Intracellular trafficking of polyplexes

(TEM) (Mannisto et al. 2002), atomic force microscopy (AFM) (Wolfert et al. 1996; Toncheva et al. 1998; Choi et al. 1999a), and dynamic light scattering (DLS) (Jeong et al. 2001; Cherng et al. 1996; Ogris et al. 1999). Generally, aggregation of the complexes makes their internalization difficult because of their large size. Tang and Szoka (1997) studied the aggregation properties of linear PLL, intact PAMAM dendrimers, fractured dendrimers, and branched PEI in forming complexes with DNA. These polycations formed similar complexes in terms of size and zeta potential but high-level gene expression was induced only by fractured dendrimers and branched PEI, due to the stability of the complexes and absence of aggregation (Tang and Szoka 1997). Despite these investigation, the correlation between transfection efficiency and size of the polyplexes remains unclear. Aggregation of the polyplexes in serum-containing medium or in blood is also a problem. In order to decrease both aggregation and the toxicity of polyplexes, conjugation with PEG has been proposed (Mannisto et al. 2002; Choi et al. 1999b). For example, the solubility of PEG ylated PEI and DNA polyplexes at higher therapeutic concentration was not only improved without aggregation but the in vivo toxicity was also reduced (Ogris et al. 1999; Kursa et al. 2003).

4.2 Receptor-Mediated Gene Delivery

In 1987, Wu et al. developed a system for targeting foreign genes to hepatocytes, which possess a unique receptor that binds and internalizes galactose-terminal asialoglycoproteins, through receptor-mediated endocytosis. It was shown that asialoorosomucoid-PLL carriers delivered pSV2-CAT plasmid DNA specifically to HepG2 hepatoma cells but not to other receptor (−) cell lines (Wu and Wu 1987, 1988a, b). Consequently, site-specific gene delivery has received much attention, especially in vivo direct gene transfer using various biologically active moieties, such as sugar (Midoux et al. 1993; Erbacher et al. 1996, 1997, 1995; Wu and Wu 1988a, b), transferrin (Wagner et al. 1991a,b), and LDL (Table 2).

Some types of cell, such as nonadherent primary hematopoietic cells, are well known to be difficult or almost impossible to transfect with foreign genes linked to conventional carriers because the endocytotic activity of these cells is quite low. Birnstiel and coworkers developed a system in which transferrin served as ligand and named the system "transferrinfection" (Wagner et al. 1991). The authors synthesized tranferrin-PLL conjugates using various molecular weights of PLL and different modification ratios of transferrin. A strong correlation was found between DNA condensation, evaluated using electron microscopy, and cellular DNA uptake.

Other candidates for receptor-mediated gene delivery are the receptors for integrin (Erbacher et al. 1999), insulin (Rosenkranz et al. 1992), and some growth factors (Fisher et al. 2000). Interestingly, polycations bound to VEGF (vascular endothelial growth factor) could not deliver DNA into nucleus but bFGF (basic fibroblast growth factor) could. The PEI derivatives conjugated to the integrin-binding peptide CYG-GRGDTP via a disulfide bridge led to transgene expression in integrin-expressing epithelial cells (Hela) and fibroblasts (MRC5) at an expression level 10- to 100-fold higher than obtained with PEI. The advantage of receptor-mediated endocytosis is not just the cell-type specificity of the gene transfer but also the controlled intracellular trafficking of the complexes (Erbacher et al. 1999).

TABLE 2. Biological signal-mediated gene therapy

Barriers	Ligand	Base polymer	References
Plasma membrane	Galactose	PLL	(Wu and Wu 1987, 1988a, b; Nishikawa et al. 1998; Zanta et al. 1997)
		Vinyl polymer	(Lim et al. 2000)
	Lactose	PLL / PLL-PEG	(Erbacher et al. 1997; Midoux et al. 1993; Klink et al. 2001) / (Choi et al. 1999b)
	Folate	PLL / PLL-PEG	(Mislick et al. 1995; Leamon et al. 1999)
		PEI	(Guo and Lee 1999)
	Transferin	PLL / PLL-PEG / PEI-PEG	(Wagner et al. 1991a,b 1990; Cotten et al. 1990;) / (Ogris et al. 1999; Vinogradov et al. 1999; Kursa et al. 2003)
	RGD	PLL	(Harbottle et al. 1998)
		PEI	(Erbacher et al. 1999)
	LDL	PLL	(Kim et al. 1998)
		PEI	(Furgeson et al. 2003)
Nuclear membrane	NLS	PLL	(Chan and Jans 1999; Chan et al. 1999)
		None	(Fritz et al. 1996; Balicki et al. 2002)

RGD; Arg-Gly-Asp tripeptide
LDL; low density lipoprotein
NLS; nuclear localization signal
PLL; poly(L-lysine)
PEG; poly(ethylene glycol)
PEI; polyethyleneimine

4.3 Endosomal Escape

The internal pH of endosomes containing polyplexes gradually decreases to about 5.5. Then, the endosomes fuse with lysosomes, resulting in the formation of secondary lysosomes, in which the incorporated DNA is normally hydrolyzed by lysosomal enzymes. DNA digestion at this step is one of the biggest barriers to effective gene transfer. When a foreign gene is transferred by microinjection or osmotic shock (Takai and Ohmori 1990), by which the transgene is compulsorily delivered into the cytosol, gene expression is generally much higher than obtained using the coculture method because there is no lysosomal digestion. If a non-viral vector has the ability to disrupt or fuse with the endosomal membranes, transferred foreign gene can escape from the endosome into the cytosol, and effective transgene expression should occur.

One promising strategy to release internalized complexes from the endosome is osmotic endosomal disruption. In 1995, Boussif et al. pointed out a novel mechanism, the "proton sponge hypothesis", which resulted in high-level expression of a transgene introduced into the cell using PEI as vector (Boussif et al. 1995). At pH 5.5.–7, PEI has a greater buffering capacity than PLL and other polycations. When PEI is used as carrier and is internalized into the endosome with DNA, a larger amount of H^+ should influx into the endosome, thus reducing the pH and raising the internal osmotic pressure, resulting in osmotic rupture of the endosome. In fact, fluorescence imaging showed that there was no overlap of PEI and labeled lysosomes while PLL was found together with lysosome when labeled PEI/DNA or PLL/DNA polyplexes

were transfected into cells (Godbey et al. 2000; Remy-Kristensen et al. 2001). Recently, it was reported that DNA with PEI (linear or branched) showed rapid endosomal escape (Itaka 2003). Hennink studied the effect of pKa of the cationic groups on their transfection efficacy using various cationic vinyl polymers (Zuidam et al. 2000; van de Wetering et al. 1999). The pKa ranges from 7.5 (for poly(2-(dimethylamino)ethyl methacrylate)) to 8.8 [for poly(3-(dimethylamino)propyl methacrylamide)], and the lower the pKa the higher the amount of gene expression. The pKa of the cationic groups is also influenced by their arrangement based on the polymer effect of the adjacent charged groups (van de Wetering et al. 1999).

4.4 Nuclear Transport

Transgenes must be transported to nucleus by some means. When lipoplexes are injected into the nucleus, gene expression is strongly suppressed by the cationic lipids, while polyplexes lead to strong gene expression after nuclear microinjection (Pollard et al. 1998). These results indicate that the intracellular trafficking and gene expression mechanisms for polyplexes and lipoplexes differ from each other.

One possible mechanism is the transportation through the nuclear membrane pore but this seems unlikely because the sizes of the complexes are too large, being usually around 100 nm. Another possibility is the accumulation of the complexes during the mitotic event accompanying nuclear membrane disappearance. Zauner et al. compared the role of mitosis in the transfection of confluent, contact-inhibited primary human cells using polyplexes and lipoplexes. It was shown that lipoplexes cannot lead to high-level gene expression at the confluent stage but that polyplexes can (Zauner et al. 1999). Godbey et al. (1999b) reported another mechanism of nuclear transport for PEI/DNA complexes. They suggested a mechanism in which the polyplexes come into contact with phospholipids of the endosome; the membrane then becomes permeabilized and bursts due to osmotic swelling, resulting in the polyplexes becoming coated with the phospholipids. The coated complexes could then enter the nucleus via fusion with the nuclear envelope.

In order to enhance trafficking through the nuclear pore, several nuclear localization signals (NLS) were utilized (Garcia-Bustos et al. 1991; Yoneda 1997). NLSs are oligopeptides mainly composed of cationic residues; they are 5–20 amino acids long and different sequences have been found in many species. NLS bound to PLL has been evaluated by many researchers as a gene carrier and was shown to be effective (Table 2) (Chan and Jans 1999).

4.5 Transcription of the Transgene

The delivered polyplexes may require disassembly in order to be transcribed but electrostatic polyplexes dissociate with difficulty under normal physiological conditions. In contrast, it is also possible that the DNA in the polyplexes is recognized without prior disassembly. In any case, the polyplex should possess adequate characteristics allowing these events. The tendency towards polyplex dissociation can be estimated by adding another polyanion, such as heparin sulfate, polyvinyl sulfate, and heparin, into the polyplex suspension (Erbacher et al. 1999; Wolfert et al. 1996; Ruponen et al. 1999). Kabanov et al. reported that under these conditions a polyion interexchange

reaction occurs, resulting in free DNA release when an adequate amount of polyanion is added to polyplex suspensions (Vinogradov et al. 1998). The interexchange reaction of the complexes depends on the kind of polyanions added, such as poly(vinyl sulfonate) (Katayose and Kataoka 1998), poly(aspartic acid), or glycosaminoglycans (GAGs) (Mannisto et al. 2002). DNA was more easily released from pDMAEMA than from poly[(trimethylamino)ethyl methacrylate chloride)] (pTMAEMA) and the former showed high transfection efficiency than the latter (Arigita et al. 1999).

We reported that only cationic polymers containing nonionic hydrophilic groups lead to higher amounts of gene expression (Yamaoka et al. 1994). These groups seem to impart a hydrophilic nature to the complexes since they are not involved in complex formation. Gene expression in these complexes was higher even in an in vitro transcription/translation system using rabbit reticulocyte lysate. Based on this observation, we have attempted to improve the carrier ability of PLL (Yamaoka et al. 2000; Kimura et al. 2002). As shown in Fig. 4, PtmLS, which is a PLL derivative containing 25 mol % serine residues and quaternary ammonium groups, greatly enhanced transgene expression. PtmLS complexes were easily disassembled and were also transcribed in an in vitro translation system. Recently, low-molecular-weight PEI (LMW-PEI) was reported to be a promising carrier for gene transfer under in vitro and in vivo conditions compared with high-molecular-weight PEI (HMW-PEI). Poly-

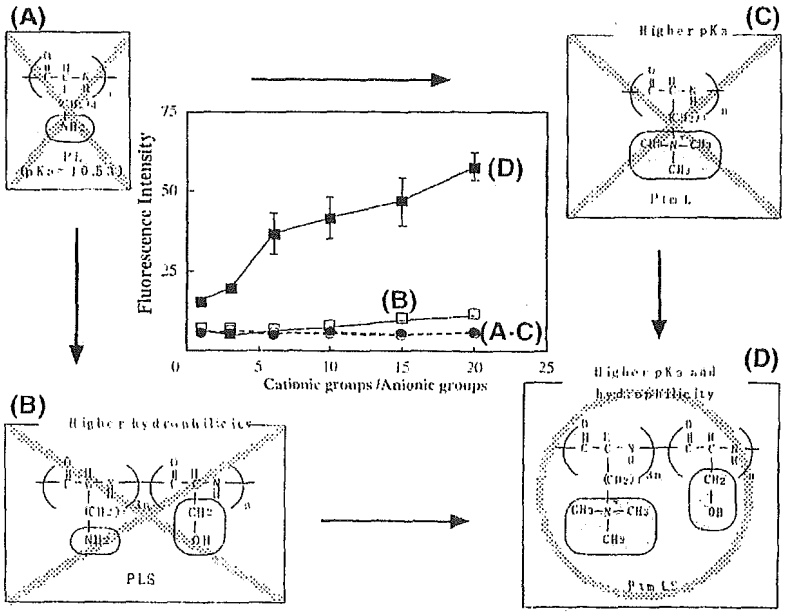

FIG. 4. Fluorescence intensity of EGFP expressed by COS-1 cells 40 h after osmotic shock of pEGFP using ○ PL, • PLS, □ PtmL, and ■ PtmLS

plexes formed from LMW-PEI also showed significantly reduced condensation and were reported to induce an higher transfection efficiency (Kunath et al. 2003).

5 Conclusions

The physicochemical features of the polyplexes are extremely important for designing effective non-viral carriers. Recently, several new systems have been described but are not well tested with respect to intracellular trafficking and transcription event. For example, non-condensing polyplexes are advantageous for transcription but, at the same time, are disadvantageous for nuclease digestion of DNA. In order to design and develop, new effective carriers, each step of intracellular trafficking route should be analyzed quantitatively.

References

Ahn CH, Chae SY, Bae YH, Kim SW (2002) Biodegradable poly(ethylenimine) for plasmid DNA delivery. J Control Release 80:273–282

Arigita C, Zuidam NJ, Crommelin DJ, Hennink WE (1999) Association and dissociation characteristics of polymer/DNA complexes used for gene delivery. Pharm Res 16: 1534–1541

Aubin RA, Weinfeld M, Mirzayans R, Paterson MC (1994) Polybrene/DMSO-assisted gene transfer. Generating stable transfectants with nanogram amounts of DNA. Mol Biotechnol 1:29–48

Balicki D, Putnam CD, Scaria PV, Beutler E (2002) Structure and function correlation in histone H2A peptide-mediated gene transfer. Proc Natl Acad Sci USA 99:7467–7471

Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. Proc Natl Acad Sci USA 92:7297–7301

Chan CK, Jans DA (1999) Enhancement of polylysine-mediated transferrinfection by nuclear localization sequences: polylysine does not function as a nuclear localization sequence. Hum Gene Ther 10:1695–702

Cherng JY, van de Wetering P, Talsma H, Crommelin DJ, Hennink WE (1996) Effect of size and serum proteins on transfection efficiency of poly ((2-dimethylamino)ethyl methacrylate)-plasmid nanoparticles. Pharm Res 13:1038–1042

Choi JS, Lee EJ, Choi YH, Jeong YJ, Park JS (1999a) Poly(ethylene glycol)-block-poly(L-lysine) dendrimer: novel linear polymer/dendrimer block copolymer forming a spherical water-soluble polyionic complex with DNA. Bioconjug Chem 10:62–65

Choi YH, Liu F, Choi JS, Kim SW, Park JS (1999b) Characterization of a targeted gene carrier, lactose-polyethylene glycol-grafted poly-L-lysine and its complex with plasmid DNA. Hum Gene Ther 10:2657–2665

Choi YH, Liu F, Kim JS, Choi YK, Park JS, Kim SW (1998) Polyethylene glycol-grafted poly-L-lysine as polymeric gene carrier. J Control Release 54:39–48

Collins L, Fabre JW (2004) A synthetic peptide vector system for optimal gene delivery to corneal endothelium. J Gene Med 6:185–194

Cotten M, Langle-Rouault F, Kirlappos H, Wagner E, Mechtler K, Zenke M, Beug H, Birnstiel ML (1990) Transferrin-polycation-mediated introduction of DNA into human leukemic cells: stimulation by agents that affect the survival of transfected DNA or modulate transferrin receptor levels. Proc Natl Acad Sci USA 87:4033–4037

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.

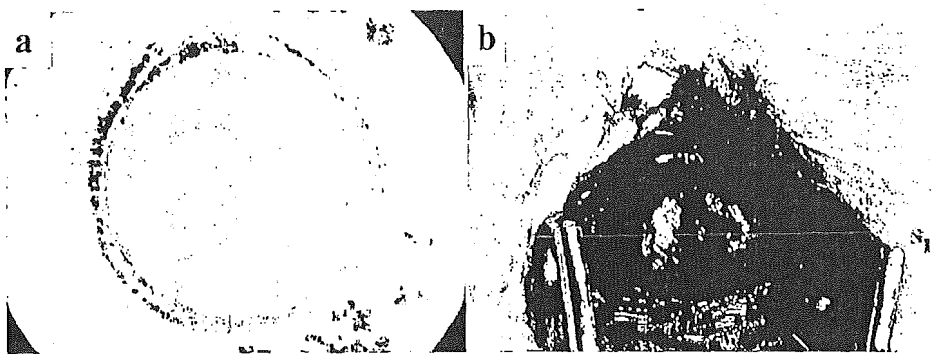

Fig 1. (a) Polyglycolic acid cloth (PGAC) with type I collagen hydrogel. (b) Suturing of the PGAC over the infarct in the left ventricular free wall.

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.

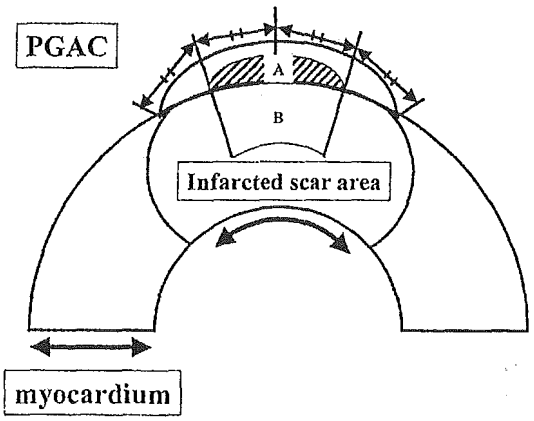

Fig 2. Schema of the infarct scar and the polyglycolic acid cloth (PGAC). The area of the scar and the PGAC were divided into four equal parts and the number of capillaries was counted at the center of the cloth over the scar area (A) and at the center of the infarcted area (B) at ×400 magnification.

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

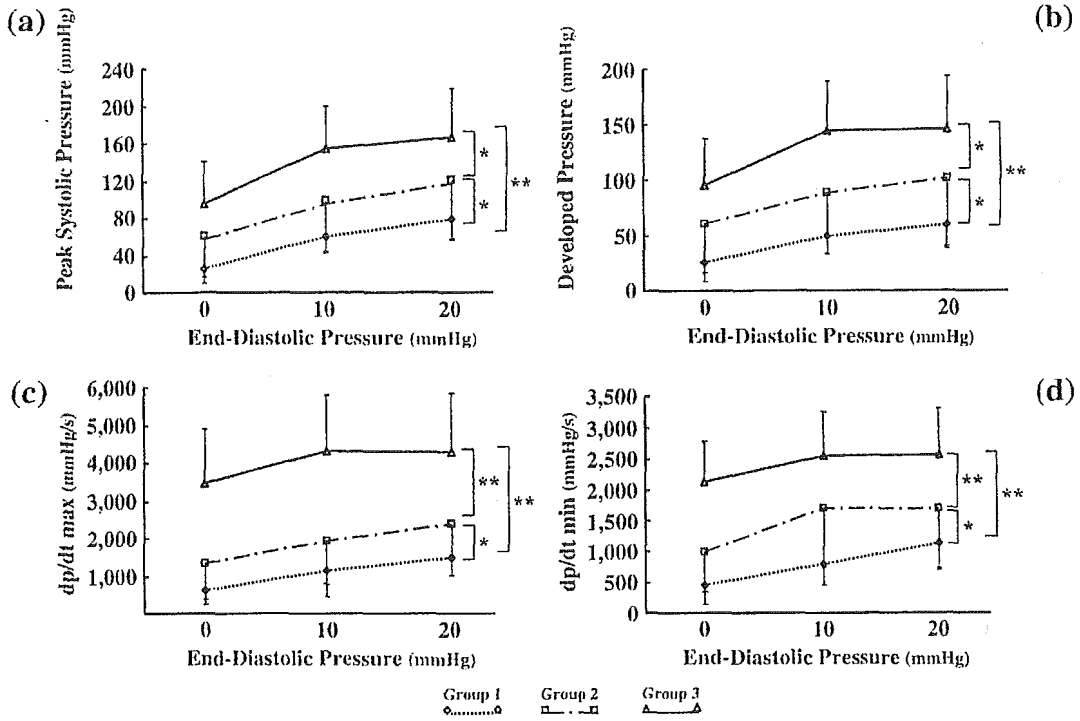

Fig 3. Cardiac function using the Langendorff apparatus. (a) Peak systolic pressure, (b) developed pressure, (c) dp/dt max, and (d) dp/dt min. All cardiac parameters were the highest in group 3. All cardiac parameters in group 2 were higher than those of group 1 (*p<0.001, **p<0.0001) (◇) Group 1, (□)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

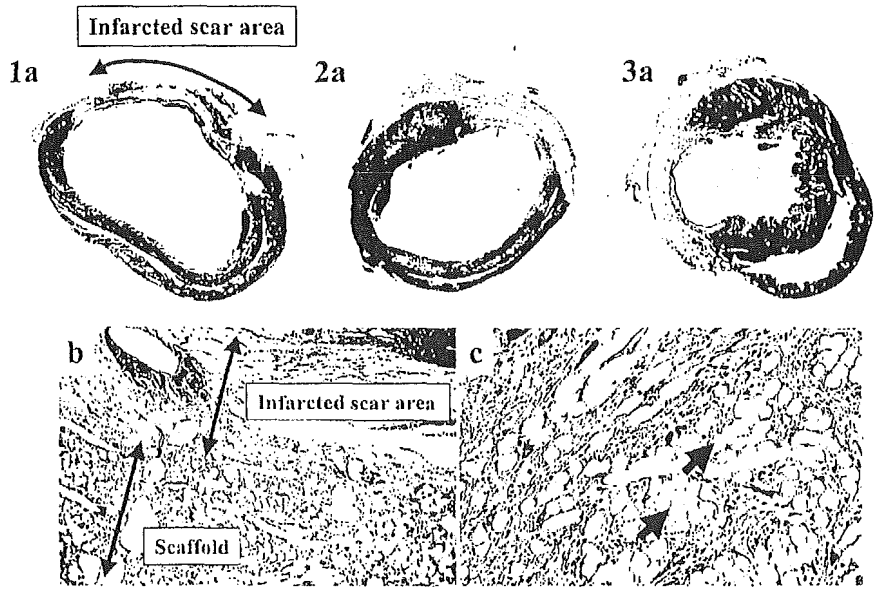

Fig 4. Histology of the polyglycolic acid cloth (PGAC) and the heart at 4 weeks after implantation. (a) No. 1 is Masson's trichrome staining for group 1, No. 2 group 2, and No. 3 group 3 (×0.5). Arrow in 4.1a indicates the PGAC over the infarcted scar area. (b) PGAC and infarcted scar area in group 3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

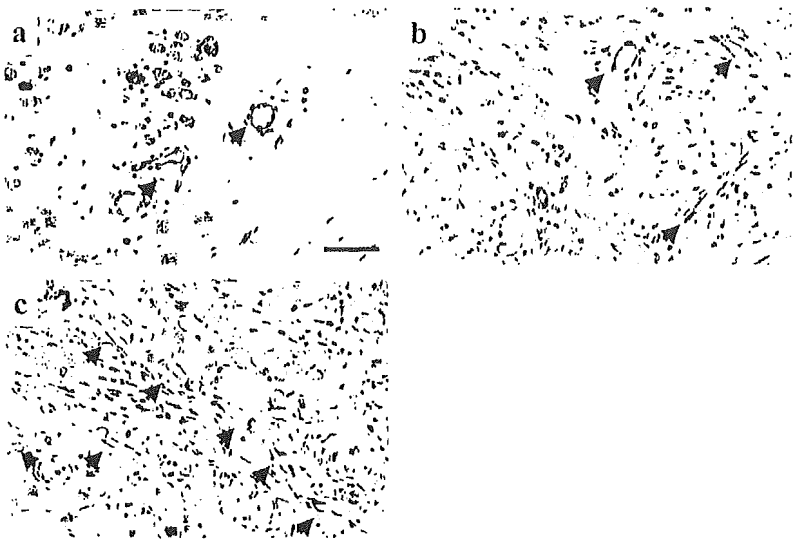

Fig 5. Von Willebrand factor staining at the center of the polyglycolic acid cloth (PGAC) (a: group 1, b: group 2, c: group 3). Arrows indicate capillaries. Greater capillary density was observed in groups 3, 2, and 1 in that order (Bar=50 μm).

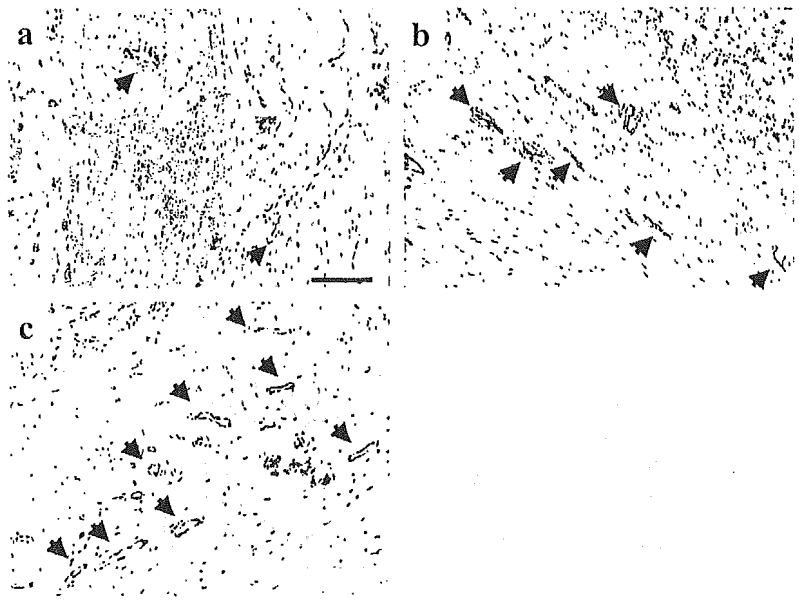

Fig 6. Von Willebrand factor staining at the center of the infarcted area (a: group 1, b: group 2, c: group 3). Arrows indicate capillaries. Greater capillary density was observed in groups 3, 2, and 1 in that order (Bar= 50 μm).

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

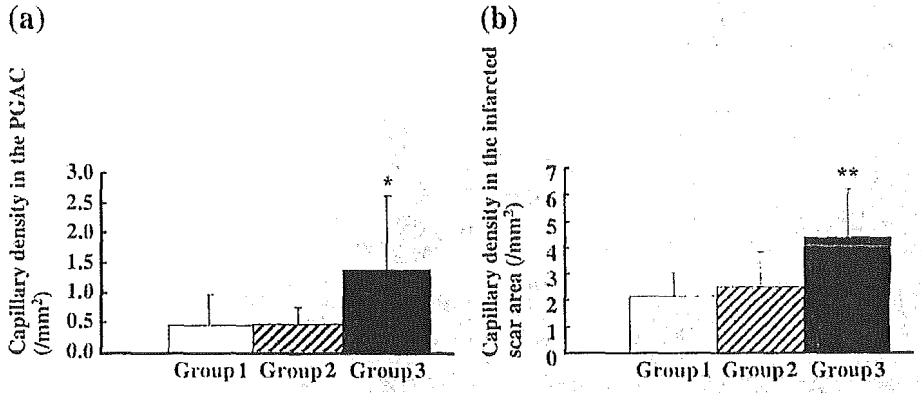

Fig 7. Capillary density (a) at the center of the polyglycolic acid cloth (PGAC) and (b) at the center of the infarcted scar area. Capillary density in group 3 was the greatest among the groups for the PGAC (*p<0.05) and in the infarcted area (*p<0.01).

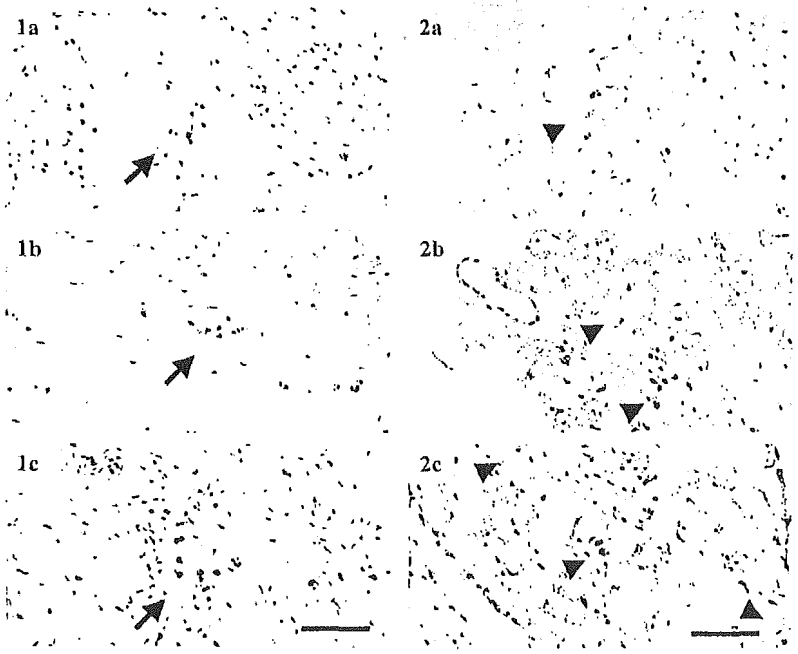

Fig 8. Nestin-positive and Ki-67 positive staining of the infarcted area (a: group 1, b: group 2, c: group 3). No. 1 is immunostaining for nestin, and No. 2 for Ki-67. Nestin-positive cells (arrows) and Ki-67-positive cells (arrowhead) at the margins of the scar (Bar = 50 μm).

gel and b-FGF (0.2 μg). The conditioned PGAC was sutured over the infarcted area at 4 weeks after infarction. The heart was harvested, frozen, and sectioned into 6 μm 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 200 μm in diameter. Capillaries were observed near the edge of the PGAC. The capillary density of group 3 at the center of both

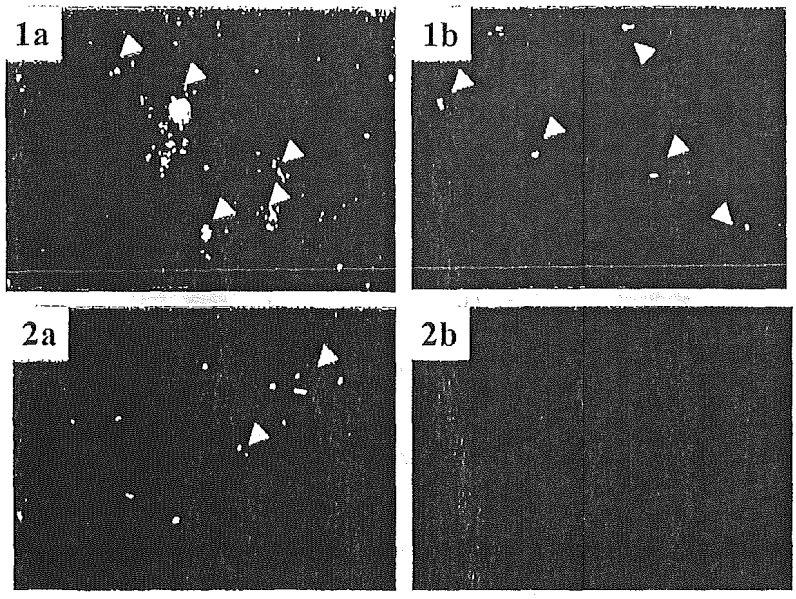

Fig 9. Capillary formation in the polyglycolic acid cloth (PGAC) and infarcted area in the mouse study. Sections were stained for von Willebrand factor, and positive cells were visualized as red (1) Section of the PGAC by fluorescent microscopy (a: red excitation at 524–640 nm, b: green at 515–540 nm). (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/mm²) ($p<0.05$) (Figs 5c, 7a) and the infarcted scar area (4.36±1.82/mm²) ($p<0.01$) (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/mm²) ($p<0.05$) and group 2 (2.69±4.33/mm²) ($p=0.11$) in the infarcted scar area were higher than that of group 1 (0.05±0.13/mm²).

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 Go,[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

We thank Ms K. Hattori for her help in breeding the GFP mice, and Mr Masuda for his help in the histological study. This study was partially supported by the Ministry of Health, Labor, and Welfare, Health Sciences Research Grants, Research for Cardiovascular Diseases (13C-1), Research on the Human Genome, Tissue Engineering Food Biotechnology (12-007), Japan Society for the Promotion of Science, Grant-in-Aid for Scientific Research (B) and Grand-in-Aid for Exporatory Research.

References

1. Mann DL. Mechanisms and models in heart failure: A combinatorial approach. *Circulation* 1999; **100:** 999–1008.
2. Takeshita S, Zheng LP, Brogi E, Kearney M, Pu LQ, Bunting S, et al. Therapeutic angiogenesis: A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. *J Clin Invest* 1994; **93:** 662–670.
3. Tomita S, Li RK, Weisel RD, Mickle DAG, Kim EJ, Sakai T, et al. Autologous transplantation of bone marrow cells improves damaged heart function. *Circulation* 1999; **100:** II-247–II-256.
4. Tomita S, Mickle DA, Weisel RD, Jia ZQ, Tumiati LC, Allidina Y, et al. Improved heart function with myogenesis and angiogenesis after autologous porcine bone marrow stromal cell transplantation. *J Thorac Cardiovasc Surg* 2002; **123:** 1132–1140.
5. Kamihata H, Matsubara H, Nishiue T, Fujiyama S, Tsutsumi Y, Ozono R, et al. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. *Circulation* 2001; **104:** 1046–1052.
6. Noishiki Y, Tomizawa Y, Yamane Y, Matsumoto A. Autocrine angiogenic vascular prosthesis with bone marrow transplantation. *Nat Med* 1996; **2:** 90–93.
7. Kobayashi T, Hamano K, Li TS, Katoh T, Kobayashi S, Matsuzaki M, et al. Enhancement of angiogenesis by the implantation of self bone marrow cells in a rat ischemic heart model. *J Surg Res* 2000; **89:** 189–195.
8. Martin PJ, Hansen JA, Storb R, Thomas ED. Human marrow transplantation: An immunological perspective. *Adv Immunol* 1987; **40:** 379–438.
9. Beatty PG, Anasetti C, Hansen JA, Longton GM, Sanders JE, Martin PJ, et al. Marrow transplantation from unrelated donors for treatment of hematologic malignancies: Effect of mismatching for one HLA locus. *Blood* 1993; **81:** 249–253.
10. Li RK, Yau TM, Weisel RD, Mickle DA, Sakai T, Choi A, et al. Construction of a bioengineered cardiac graft. *J Thorac Cardiovasc Surg* 2000; **119:** 368–375.
11. Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. 'Green mice' as a source of ubiquitous green cells. *FEBS Lett* 1997;

407: 313–319.
12. Pouzet B, Vilquin JT, Hagège AA, Scorsin M, Messas E, Fiszman M, et al. Intramyocardial transplantation of autologous myoblasts: Can tissue processing be optimized? *Circulation* 2000; **102**: III-210–III-215.
13. Fujisato T, Sajiki T, Liu Q, Ikada Y. Effect of basic fibroblast growth factor on cartilage regeneration in chondrocyte-seeded collagen sponge scaffold. *Biomaterials* 1996; **17**: 155–162.
14. Fujisato T, Sada M, Tsuji T, Rosenberg L. Development of artificially vascularized tissue scaffolds for islet cell transplantation. *Sixth World Biomaterials Congress Transactions* 2000; 1496.
15. Sakai T, Li RK, Weisel RD, Mickle DAG, Kim ETG, Jia ZQ, et al. The fate of a tissue-engineered cardiac graft in the right ventricular outflow tract of the rat. *J Thorac Cardiovasc Surg* 2001; **121**: 932–942.
16. Kachinsky AM, Dominov JA, Miller JB. Intermediate filaments in cardiac myogenesis: Nestin in the developing mouse heart. *J Histochem Cytochem* 1995; **43**: 843–847.
17. Scholzen T, Gerdes J. The Ki-67 protein: From the known and the unknown. *J Cell Physiol* 2000; **182**: 311–322.
18. Langer R, Vacanti JP. Tissue engineering. *Science* 1993; **260**: 920–926.
19. Ishida M, Tomita S, Nakatani T, Kagawa T, Yamaguchi T, Suga Y, et al. Acute effects of direct cell implantation into the heart: Analysis of cardiac function by pressure-volume study. *J Heart Lung Transplant* 2005 (in press).
20. Menasche P, Hagege AA, Scorsin M, Pouzet B, Desnos M, Duboc D, et al. Myoblast transplantation for heart failure. *Lancet* 2001; **357**: 279–280.
21. Hamano K, Li TS, Kobayashi T, Kobayashi S, Matsuzaki M, Esato K. Angiogenesis induced by the implantation of self-bone marrow cells: A new material for therapeutic angiogenesis. *Cell Transplant* 2000; **9**: 439–443.
22. Kamihata H, Matsubara H, Nishiue T, Fujiyama S, Tsutsumi Y, Ozono R, et al. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. *Circulation* 2001; **104**: 1046–1052.
23. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Pickel J, Mckay R, et al. Bone marrow cells regenerate infarcted myocardium. *Nature* 2001; **410**: 701–705.
24. Sata M. Molecular strategies to treat vascular diseases: Circulating vascular progenitor cell as a potential target for prophylactic treatment of atherosclerosis. *Circ J* 2003; **67**: 983–991.
25. Iwakura A, Tabata Y, Tamura N, Doi K, Nishimura K, Nakamura T, et al. Gelatin sheet incorporating basic fibroblast growth factor enhances healing of devascularized sternum in diabetic rats. *Circulation* 2001; **104**: I-325–I-329.
26. Whalen GF, Shing Y, Folkman J. The fate of intravenously administered bFGF and the effect of heparin. *Growth Factors* 1989; **1**: 157–164.
27. Nishida S, Nagamine H, Tanaka Y, Watanabe G. Protective effect of basic fibroblast growth factor against myocyte death and arrhythmias in acute myocardial infarction in rats. *Circ J 2003; 67: 334–339.*
28. Orlic D, Kajstura J, Chimenti S, Limana F, Jakoniuk I, Quaini F, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. *Proc Natl Acad Sci USA* 2001; **98**: 10344–10349.
29. Fukuhara S, Tomita S, Ohtsu Y, Ishida M, Yutani C, Kitamura S, et al. G-CSF promotes bone marrow cells to migrate into infarcted mice heart, and differentiates into cardiomyocytes. *Cell Transplant* 2005; **13**: 741–748.

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[1]，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

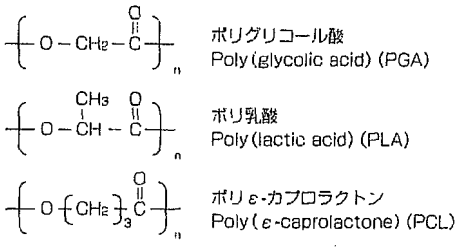


図1　脂肪族ポリエステル系生分解性材料の構造式

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Ⅲ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。

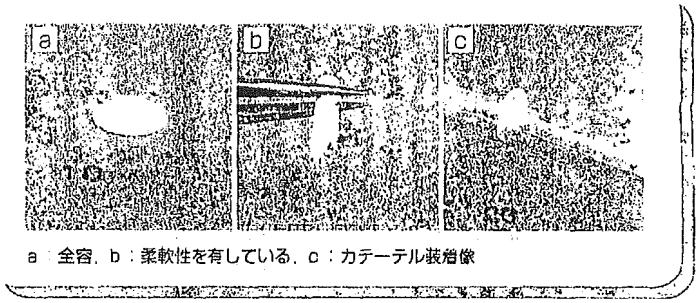
1．感温性培養基材

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]。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）[10]。



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[11]。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[12]。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[13]。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