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Regeneration of Cardiovascular Tissues using Tissue Engineering and Mesenchymal Stem Cells

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Abstract

Tissue engineering and stem cells show potentials to restore lost or malfunctioning human tissues or organs. Another cell source for tissue engineering of cardiovascular tissues is stem cell. This study reports the development of cardiovascular tissues using tissue engineering and mesenchymal stem cells. The blood vessels and heart valves were fabricated by culturing mesenchymal stem cells on biodegradable synthetic or natural matrices. Bone marrow was isolated from dogs or rats and mesenchymal stem cells were cultured. The cells were seeded onto biodegradable synthetic or natural matrices and implanted in dogs. Histological and immunohistochemical analyses were performed to examine the regenerated cardiovascular tissues. Histological and immunohistochemical analyses showed the complete regeneration of blood vessels and heart valves. Fluorescent labeling of cells prior to implantation and fluorescence examination of the regenerated tissues revealed that the implanted cells reconstituted the cardiovascular tissues. This study demonstrates the potential of tissue engineering and mesenchymal stem cells for the regeneration of functional cardiovascular tissues or organs.

1. Introduction

Atherosclerotic diseases such as coronary artery diseases and peripheral vascular

diseases are the leading cause of death and have continuously increased in the modern societies. Coronary or peripheral artery bypass grafting has been performed over approximately 550,000 every year. Thus, the demand of vascular grafts which are indispensable for coronary arteries bypass or replacement of diseased vessel segments has enormously increased in recent years. Autologous arteries or veins in patients are the best substitutes for diseased vessels. However, the number of available vessels for bypass or replacement is limited.

Artificial vascular grafts made of synthetic polymers have been developed and clinically utilized. The synthetic polymeric materials include Dacron (polyethelyne terephthalate) and ePTFE (expanded polytetrafluoroethylene). Although these polymeric grafts have been successfully utilized to replace blood vessels with internal diameter(ID) larger than 6 mm, these materials cannot be used as grafts for treatment of small-diameter (ID<6mm) vascular diseases due to thrombus formation [1]. Practically, when replaced into aorta, the grafts had the patency rate of 85 to 95% 5 years post-implantation, but in case of infrapopliteal position, the patency rate fell to 30% [2]. Coating of intimal sides with anti-thrombogenic materials such as heparin, polyethylene oxide, or endothelial cells, has been tried to solve this problem, but these approaches were unsuccessful [3-6].

Recently, several studies have been performed to develop small-caliber vascular grafts using tissue-engineering technique. The culture of smooth muscle cells, fibroblasts, and endothelial cells without exogenous materials and the culture of smooth muscle cells and endothelial cells on synthetic biodegradable polymer scaffolds resulted in the formation of blood vessels, but these grafts developed thrombosis shortly after implantation *in vivo* [7,8]. Another drawback of this approach is the graft fabrication procedure involving a second surgery to obtain blood vessels for vascular cell isolation. Collagen grafts constructed from small intestine submucosa (SIS) were remodeled into physiologically functional blood

vessels by ingrowth of the host endothelial cells in animal models [8], but this approach may not be appropriate for small-caliber vascular grafts for clinical settings because of limited ingrowth of human endothelial cells at anastomotic sites.

Another cell source for tissue engineering of vascular grafts is stem cell. Recently, vascular grafts fabricated by seeding endothelial progenitor cells derived from peripheral blood on collagen matrices were shown to maintaine patent for 130 days in ovine models [10]. However, seeding only with endothelial cells would not induce smooth muscle regeneration in the vascular grafts. Bone marrow stromal cells(BMSCs) demonstrate the ability to differentiate into multiple mesenchymal cell lineages [11] and may offer an alternative cell source for vascular tissue engineering. In this study, we demonstrate the feasibility of using BMSCs as an alternative cell source for tissue engineering of small-diameter (inner diameter= 3mm) blood vessels. The fabricated blood vessels were implanted in canine models and a vascular function and regenerated vascular structures were investigated.

2. Materials and methods

For BMSC isolation, four healthy mongrel dogs were anesthetized and 10-mL bone marrow was aspirated from each dogs iliac bone and immediately mixed with heparin (10 IU/mL) to prevent blood coagulation. Bone marrow was mixed with the equal volume of PBS and mononuclear cells were isolated with the Ficoll-Paque density gradient method. The cells were cultured in Medium 199 containing 20% fetal bovine serum, 1% penicillin and streptomycin supplemented with vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) at 37℃ under 5% CO2.

Autologous BMSCs were uniformly seeded onto small- diameter (3mm in inner diameter, 4cm in length) collagen matrices and maintained *in vitro* for 1 week at

37℃ under 5% prior to implantation.

To examine cell seeding onto the vascular scaffolds, the seeded-vascular grafts were examined with scanning electron microscopy(SEM). The samples were fixed in 1% glutaraldehyde and 1% formalin for 1 hour and 1 day, respectively, dehydrated with a series of ethanol, dried, coated with platinum by a Sputter Coater (Cressington 108), and examined with SEM (JSM-6330F, JEOL).

Four grafts were anastomosed to carotid arteries in dogs. Four healthy mongrel dogs were anesthetized and ventilated with mixture of O2, N2 and isoflurane during the operation. Through a longitudinal midneck incision, common carotid arteries were exposed for easy replacement by the tissue-engineered vascular grafts. Prior to the arterial clamping, heparin (100 IU/kg) was systemically administered by intravenous injection. 2 cm segments in the common carotid arteries was removed and replaced by the vascular grafts by an end-to-end anastomosis using 6-0 prolene suture material (Ethicon). The implanted graft patency was monitored by arterial digital subtraction angiography. All care and handling of animals were performed according to the Guide for the Care and Use of Laboratory Animals. Four collagen grafts without cell seeding served as controls.

For histological analysis, representative segments of the grafts were fixed with 10% buffered formalin solution, dehydrated with a series of ethanol, embedded in paraffin, sectioned, and stained with hematoxyline and eosin (H&E). Elastin layers and collagen in the regenerated vascular tissue sections were stained by van Gieson method and Massons trichrome method, respectively. The tissue sections were also stained immunohistochemically for vWF, smooth muscle -actin, and CD34 using avidin-biotin complex immunoperoxidase kit (Vectastain Elite ABC kit, Vector).

The suture retention strength of the vascular grafts was measured using an

Instron mechanical tester. The one end of specimens was fixed by stage clamp of the Instron tester and the other end was connected to the other clamp by suture material(4-0 prolene). A tensile force was applied until the grafts completely were torn and the vessel rupture stress was recorded.

3. Results

Bone marrow mononuclear cells were isolated by Ficoll-Paque gradient protocol, and BMSC fraction was cultured in medium adjusted to a condition appropriate for culture of both vascular endothelial cells(ECs) and smooth muscle cells(SMCs). The cultured BMSCs stained positively with anti-von Willebrand Factor (vWF) and anti-smooth muscle -actin. This demonstrates that ECs and SMCs are co-existed in the culture condition.

Ex vivo expanded BMSCs were seeded onto intimal sides and outsides of the scaffolds with a length of 40 mm and an internal diameter of 3 mm. After the cell seeded-vascular grafts were maintained in vitro for 1 week to insure cell adhesion onto the scaffolds, luminal surfaces of the grafts were examined by SEM, and cross-sections of the grafts were analyzed by H&E staining. Through these analyses, it was confirmed that seeded cells well adhered onto the luminal surfaces of the vascular matrices and the seeded cells uniformly distributed throughout the vascular grafts.

Suture retention strength was measured to determine whether the grafts endure forces exerted in anastomosis. The average value of suture retention strength of the vascular grafts was 606 98 g, which was slightly lower but insignificantly ($p>0.05$) than that of native canine carotid arteries (753 ± 112 g). Practically, the vascular grafts were anastomosed to carotid arteries in dogs without graft rupture.

After implantation, the animals were periodically investigated by arterial digital

subtraction angiography to determine the graft patency. Angiogram showed the vascular grafts seeded with autologous BMSCs maintained patent for up to 8 weeks. In contrast, all control groups, which were not seeded with cells, occluded within 2 weeks.

Histological analyses of the sections of retrieved vascular grafts revealed the regeneration of the three elements of blood vessel, endothelium, media, and adventitia. Elastin staining by van Giesons method displayed well-preserved internal elastic lamina and external elastic lamellae layers in the vascular grafts. Massons trichrome collagen staining showed that the media of the grafts were filled with collagen. Many cells in the vascular graft tissues including endothelium, media, and adventitia stained positively for CD34. Immunostaining analysis using antibodies for vWF and SM -actin revealed the regeneration of endothelium in intima and smooth muscle in media, respectively.

4. Discussion

It is urgently required to develop artificial small-diameter vascular grafts (ID≤6 mm) due to dramatic increase in vascular disease incidence. Although there have been enormous efforts to develop small-diameter vascular grafts, trials using synthetic materials such as Dacon and ePTFE, which have high success rates in many clinical treatments for large-diameter (ID>6 mm) vascular diseases, have failed due to thrombosis. Although patients own blood vessels are most appropriate for vascular grafts, many patients do not have suitable vessels. The present study reports a new approach for the development of small-diameter vascular grafts, which combines tissue engineering and adult stem cells.

The plasticity of bone marrow cells that can differentiate into various tissue cells was successfully applied to this study. We identified culture conditions that allow for the proliferation and/or differentiation of multiple types of cells

necessary for vascular tissue regeneration. Immunostaining for vWF and smooth muscle -actin demonstrated that cultured BMSCs were the mixed population of ECs and SMCs.

BMSCs would be an ideal cell source for development of autologous vascular grafts. Using patients own bone marrow cells, autologous grafts could be easily constructed. This advantage provides a non-immunogenic vascular graft that is most appropriate for individual patients without additional surgeries to obtain autologous vascular cells.

The suture retention strength tests verified that the vascular grafts developed in this study have appropriate mechanical strengths that can endure forces exerted by sutures during surgery and can be used clinically without graft rupture. The average suture retention strength of the vascular grafts was approximately 600 g, which is considered to be sufficient value for clinical use. This value is much higher than that of previously described vascular grafts constructed from synthetic polymeric materials (PGA) [8].

The animal studies showed the feasibility of the small-diameter vascular grafts developed in this study. Compared to the 2 week patency of control grafts which had no cell seeding prior implantation, the 8 week patency of the BMSC-seeded grafts showed that BMSCs can be a cell source for tissue engineering of vascular tissues.

Histological analyses of the retrieved grafts show the regeneration of vascular structure similar to that of native artery. The retrieved grafts retained complete internal elastic lamina and external elastic lamellae structures. The grafts also had collagen proteins, most of which were regenerated between medial elastic layers. CD34-positively stained sections indicate that most cells in endothelium, media and adventitia in the grafts may be derived from transplanted BMSCs, rather than host vascular cells. The cells lining the lumen of the grafts stained positively for

vWF, an endothelial cell specific marker. The cells in media in the grafts expressed smooth muscle α-actin, a smooth muscle cell specific marker. These immunohistochemical analyses show the successful regeneration of endothelium in intima and smooth muscle in media in the grafts.

The patency of the grafts developed in this study needs to be improved for clinical applications. The graft occlusion may be due to incomplete EC seeding on the lumen side of the grafts. It is well known that vascular ECs play an important role in prevention of thrombus formation by various mechanisms.23 Moreover, ECs secret factors which inhibit the in-growth of vascular smooth muscle cells. Scanning electron microscopic observation indicates that complete endothelium formation was not achieved in the lumen of the grafts. Therefore, the problem of the graft occlusion at 8 weeks after implantation by thrombus formation and intimal hyperplasia could be solved by an improved EC seeding technique. Cell seeding and *in vitro* graft maintenance in static condition may not be efficient methods for complete endothelium formation. Dynamic cell seeding on scaffolds [12] and preconditioning of seeded scaffolds [13] may improve complete endothelium formation and, in turn, graft patency.

This study demonstrates the feasibility of tissue-engineered blood vessels for small-diameter vascular grafts. The grafts were fabricated using an approach combining tissue engineering and stem cell. BMSCs were successfully expanded *ex vivo* and exhibited the phenotypes of ECs and SMCs. The vascular graft had appropriate mechanical strengths, which can endure forces exerted by sutures during surgery. The grafts contained vascular structures similar to those of native artery, containing the three elements of artery, endothelium, media, and adventitia. These vascular grafts showed the possibility as a small-diameter vascular graft in animal studies.. Followed by improvement on EC expansion and seeding methods to improve graft patency, this approach can lead to artificial small-

diameter vascular grafts appropriate for clinical implantation.

4. References

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