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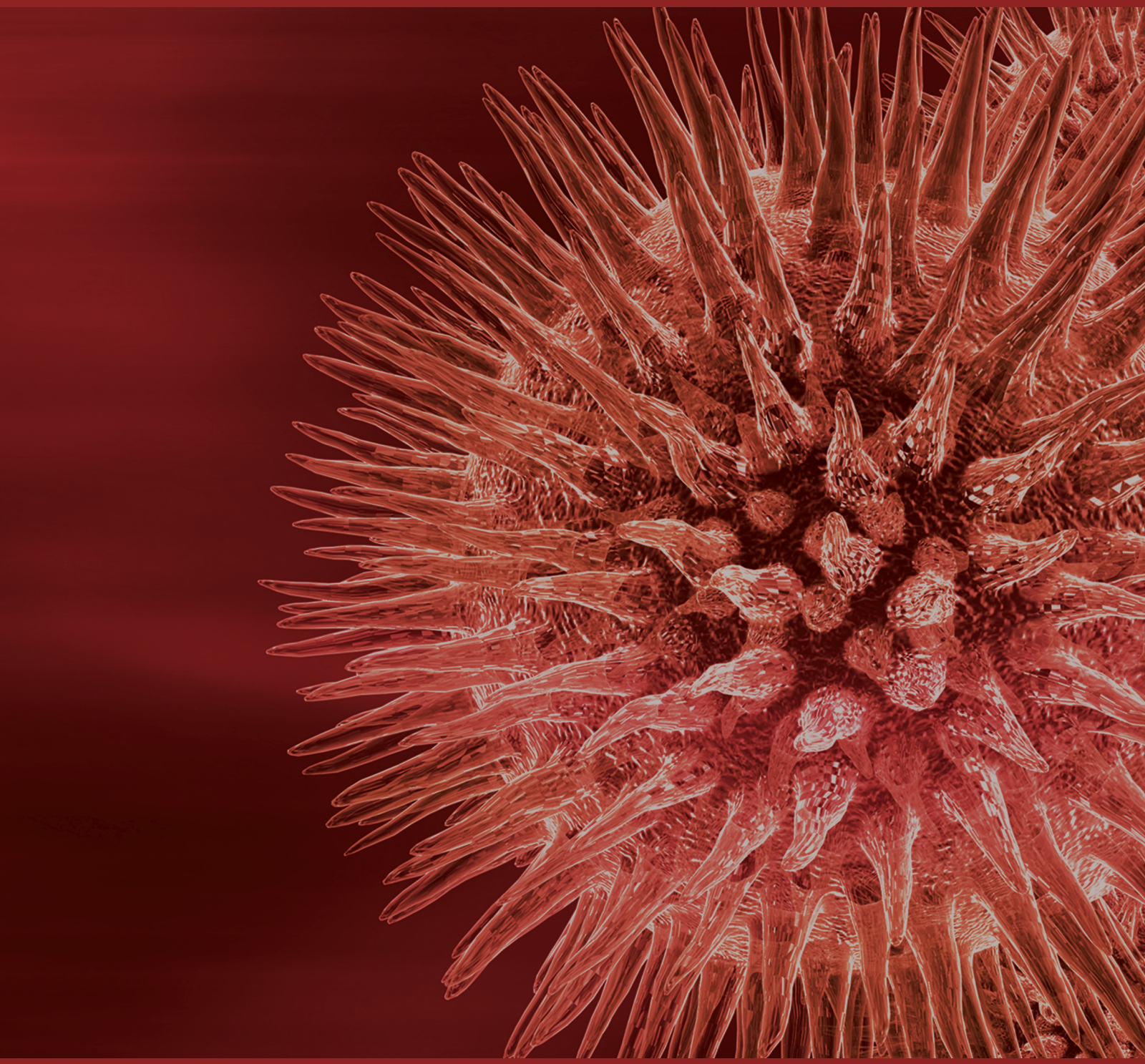
Regenerative Medicine

Guest Editors: Ryuichi Morishita, Yasufumi Kaneda, Yoshiki Sawa, Ken Suzuki, Masanori Aikawa, and Koji Nishida



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Editorial

Regenerative Medicine

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Regenerative medicine would be the most exciting area in the recent scientific topics. In the history of human gene therapy, the severe combined immunodeficiency caused by the absence of adenosine deaminase (SCID-ADA) was the first monogenic disorder for which gene therapy was developed in 1990. Over 30 patients have been treated worldwide using the current protocols, and recent trials provided the demonstration of long-term clinical efficacy of HSC gene therapy for SCID-ADA in combination with gene and cell therapy. We have now several options, such as gene therapy and/or cell therapy with advanced technology (i.e., cell sheet or drug delivery system), to fight against the severe diseases.

The main focus of this special issue will be on the new and exciting therapy based on stem cell biology, tissue engineering, and gene therapy technology toward regenerative medicine. This special issue contains seven papers, and three papers focus on vascular medicine. J.-I. Kawabe and N. Hasebe described the role of vasa vasorum and vascular stem cells as resident stem cells in atherosclerosis, which might be a novel therapeutic approach for atherosclerosis. M. Shimamura et al. summarized the results of therapeutic angiogenesis in comparison with gene therapy and cell therapy including endothelial progenitor cells. We will reach the final conclusion of therapeutic angiogenesis concept for critical limb ischemia in near future. Y. Saito et al. described the novel findings of therapeutic lymphoangiogenesis which may reduce the edema in patients. Two papers focus on heart regeneration. C. Ikebe and K. Suzuki mainly focused on optimization of cell preparation protocols, especially mesenchymal stem cells, which might be an important process for clinical application. Y. Sawa and S. Miyagawa introduced the experience of their cell sheet-based heart regeneration because their group is the top leader of heart regenerative therapy based on sheet technology. Y. Oie and K. Nishida also show the regenerative medicine for the cornea based on cell sheet technology. They are also top leaders of cornea regeneration therapy based on sheet technology. K. Saga and Y. Kaneda proposed the novel concept of virosome which presents multimodel immunotherapy without viral replication in cancer.

As a result, this special issue includes the topics of atherosclerosis, angiogenesis, and lymphoangiogenesis, heart regeneration, cornea regeneration, and immunotherapy for cancer. We hope that the mixed discussion of these different fields may produce the innovation leading to regenerative medicine in future.

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We would like to express appreciation to the authors and editors for their excellent contribution and patience in assisting us.

Ryuichi Morishita

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Review Article

Role of the Vasa Vasorum and Vascular Resident Stem Cells in Atherosclerosis

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Atherosclerosis is considered an "inside-out" response, that begins with the dysfunction of intimal endothelial cells and leads to neointimal plaque formation. The adventitia of large blood vessels has been recognized as an active part of the vessel wall that is involved in the process of atherosclerosis. There are characteristic changes in the adventitial vasa vasorum that are associated with the development of atheromatous plaques. However, whether vasa vasorum plays a causative or merely reactive role in the atherosclerotic process is not completely clear. Recent studies report that the vascular wall contains a number of stem/progenitor cells that may contribute to vascular remodeling. Microvessels serve as the vascular niche that maintains the resident stem/progenitor cells of the tissue. Therefore, the vasa vasorum may contribute to vascular remodeling through not only its conventional function as a blood conducting tube, but also its new conceptual function as a stem cell reservoir. This brief review highlights the recent advances contributing to our understanding of the role of the adventitial vasa vasorum in the atherosclerosis and discusses new concept that involves vascular-resident factors, the vasa vasorum and its associated vascular-resident stem cells, in the atherosclerotic process.

1. Introduction

Atherosclerosis, a chronic progressive inflammatory disease of the arterial wall, has traditionally been considered an "inside-out" response in which injury to intimal endothelial cells initiates the adhesion/invasion of inflammatory cells in the subendothelial space. Subsequently atherosclerotic plaques grow by the accumulation of inflammatory cells and lipid substances and the proliferation of vascular smooth muscle cells [1, 2]. Clinical evidence indicates that instability rather than plaque size affects the prognosis of cardiovascular diseases [3, 4]. However, the underlying mechanism driving the conversion of an asymptomatic stable atheroma to a lesion vulnerable plaque is not fully explained by the "inside-out" theory.

The adventitia is no longer viewed as a passive support structure in large vessels. The vascular adventitia, which harbors a wide variety of components such as fibroblasts, inflammatory cells, stem/progenitor cells, and vasa vasorum, can act as a biological central processing unit in vessel wall function. Recent emerging evidence proposes a new paradigm regarding the sites/direction of the atherosclerotic process, the "outside-in" hypothesis. Under this paradigm, vascular inflammation is initiated in the adventitia and progresses toward the intima [5, 6]. The vasa vasorum is an adventitial microvascular network that supplies oxygen and nutrients to the blood vessel walls. Numerous studies report that changes in vasa vasorum characteristics are closely associated with the development of atheromatous plaques. Consequently, it is proposed that the vasa vasorum appears to play key role in the "outside-in" model of atherosclerosis [5–7]. However, whether the vasa vasorum plays a causative or reactive role in the atherosclerotic process is not clearly understood.

A number of studies have demonstrated that the vascular walls act as a perivascular niche for stem/progenitor cells that contribute to vascular repair, fibrosis, and atherosclerosis (Table 1) [8, 9]. In particular, stem cells that associated with microvessels have been identified. Pericytes, also known as vascular mural cells, surround endothelial cells in capillaries

Table 1: Resident progenitor cells within the vasculature.

Location	Name	Selection method	Markers	Comments	Reference
Adventitia	Vascular progenitor cells	**Sca1** positive cells from cells outgrown from mouse thoracic aorta adventitia	Sca1+	Differentiate into **SMCs**, contributing to the formation of hyperplasia of ApoE-deficient atherosclerotic lesions	Hu et al., 2004 [19]
	Vascular progenitor cells (Saphenous vein-derived progenitor cells; **SVPs**)	**CD34+ CD31**− cells from human adult saphenous vein	CD34+ After culture in the presence of serum, CD34 were subsided, and the following markers were increased:	**MSC**- and **NSC**-like differentiation potential, but no endodermic differentiation was detected *at the clonal level*	Campagnolo et al., 2010 [20]
		(CD34+ cKit+ cells were located at perivascular sites of the **vasa vasorum**)	CD29+, CD44+, CD105+, SOX2+, Nestin+, NG2+ (CD146−)	Act as **PCs**, formed capillary structures (attached to EC-tubes), and improve ischemic hind limb through forming capillaries	
	Adventitial stem cells	**CD34+ CD146− cells** from human stromal adipose tissue around vascular adventitia	CD34+ CD31−, CD146−, and CD45−	CD34+ CD146− cells display **MSC**-like features *at the clonal level* These cells acquired a **PCs**-like phenotype (NG2+, CD146+, etc.) in the presence of angiopoietin 2	Corselli et al., 2012 [21]
	Adventitial pericyte progenitor cells	**NG2+ CD146+cells** from vascular adventitia	NG2+, CD146+, PDGFR+ Some of these cells also express CD29+, CD90+	**MSC-like PCs** contribute to restenosis following arterial injury NG2/CD146+ cells were increased in the adventitia of the injured vasculature	Tigges et al., 2013 [22]
Vasculogenic zone	Vascular wall resident **EPCs**	**CD34+ CD105**− cells from human internal thoracic artery (CD34+ cells located at the "**vasculogenic zone**")	CD34+, KDR (VEGFR2)+, Tie2+ CD105−, CD144−	Differentiate into mature **ECs** forming new vessels and **hematopoietic cells** including macrophages	Zengin et al., 2006 [23]
	Angiogenic MSCs	Adherent culture condition (cells were isolated from human thoracic artery, and select the adherent cells forming colony) (CD34+ cKit+ cells were enriched in the **vasculogenic zone**)	MSC markers (CD44+, CD90+, CD105+, etc.) CD45−, CD146−, vWF−	Differentiate into **ECs** in the presence of VEGF, and form capillary-like structure (These cells were heterogenous) It is uncertain whether the isolated cells are equivalent of CD34− stained cells within thoracic aorta	Pasquinelli et al., 2007 [24]

Table 1: Continued.

Location	Name	Selection method	Markers	Comments	Reference
Media	Side population-progenitor cells	Side population of cells from the tunica media of mouse aorta	Sca1+, cKit(dim), CD34−, lineage negative	Differentiate into **ECs** and **SMCs** to form vascular-like branching structures on Matrigel (These cells were heterogenous)	Sainz et al., 2006 [25]
	Multipotent vascular stem cells (**MVSCs**)	**SM-MHC (smooth muscle myosin heavy chain)—negative** cells from media of carotid artery of rats.	SM-MHC(−), Sox1+, Nestin+ CD146−, CD34−, CD31−	**MSC**- and **NSC**-like multipotency at the clonal level Differentiate into **SMCs** and **chondrogenic cells**, contributing to vascular remodeling and neointimal hyperplasia	Tang et al., 2012 [26]

EPCs: endothelial progenitor cells; MSCs: mesenchymal stem cells; NSCs: neuronal stem cells; ECs: endothelial cells; SMCs: smooth muscle cells; PCs: pericytes.
Vasculogenic zone: the border between the media and adventitial layer.
Stem cell/hematopoietic markers: CD34, Sca1, and cKit.
MSC markers: CD29, CD44, CD90, and CD105.
EC markers: CD31, vWF, and VEGFR.

and microvessels [10, 11]. Several recent studies demonstrated that a subpopulation of pericytes is multipotent. Multipotent pericytes can differentiate into mesodermal and ectodermal cell lineage, including smooth muscle cells, osteoblasts, adipocytes, and skeletal muscle cells [12–16]. Endothelial progenitor cells (EPCs) also exist within populations of tissue-resident endothelial cells [17]. Recently, an endothelial cell-like stem cell population has been identified as a side population of CD31+ cells that are located at the inner surface of preexisting microvessels and macrovessels [18]. Therefore, the vasa vasorum can contribute to vascular remodeling through not only its conventional function as a blood conduit tube, but also its newly proposed function as a stem cell reservoir. This brief review highlights recent advances in our understanding of the role of the adventitial vasa vasorum and its associated vascular stem cells in atherosclerosis and discusses progress toward an integrated view of adventitial function in atherosclerotic plaques.

2. Vasa Vasorum and Atherosclerotic Plaques

The vasa vasorum is a microvascular network that supplies oxygen and nutrients to the walls of large vessel. These conduits consist of a lumen lined by endothelial cells that are surrounded by pericytes or smooth muscle cells. Recent technological advances in image analysis have revealed that the enhanced vascularization in plaques is closely associated with the prognosis of acute arterial occlusion [6, 27]. Because of the histological characteristics of the vasa vasorum in plaque, it has been proposed that neovascularization of the vasa vasorum plays a role in the progression and associated complications of atherosclerotic plaques. The vasa vasorum is primarily located in the adventitial layer of large vessel and extends into atherosclerotic plaques.

In atherosclerotic plaques, the vasa vasorum is considered immature, a characteristic that leads to the microvascular leakage that is responsible for plaque hemorrhage. Because of its high permeability, the vasa vasorum also serves as a conduit for the delivery of inflammatory cells into the plaques. Plaque hemorrhage and inflammatory cell delivery are the key mechanisms underlying the persistence of chronic vascular inflammation and the rapid expansion or rupture of atherosclerotic plaques [28, 29].

2.1. The Association between the Vasa Vasorum and Neointimal Thickening. Experimental studies using atherosclerotic models, such as apolipoprotein E- (ApoE-) deficient mice, clearly demonstrate a correlation between vasa vasorum neovascularization and plaque progression [30–32]. A study aimed at visualizing and quantifying the three-dimensional spatial patterns of the vasa vasorum in normal and balloon-injured porcine coronary arteries demonstrated that the amount of adventitial neovascularization is proportional to the degree of injured arterial stenosis [33]. Tanaka et al. demonstrated that angiogenesis in the adventitia, induced by the local administration of the angiogenic factor bFGF, promoted the growth of atherosclerotic plaques in ApoE-deficient mice, supporting the notion that vasa vasorum formation plays a crucial role in the pathogenesis of atherosclerosis [32]. A major factor that induces pathological angiogenesis is the accumulation of inflammatory cells within plaques, which causes oxidative stress. The overexpression of a critical component of NAD(P)H oxidase, p22-phox, in the smooth muscle cells of transgenic mice causes oxidative stress in carotid lesions and triggers an *in vivo* angiogenic switch associated with experimental plaque progression and angiogenesis [30].

2.2. Does Expansion of the Vasa Vasorum Cause or React to Neointimal Thickening? The findings highlighted above are consistent with an emerging concept suggesting that the expansion of the vasa vasorum causes the progression of atherosclerotic plaques; however, it is still controversial whether the vasa vasorum plays a causative or reactive role in the atherosclerotic process. In some cases, even a low density of vasa vasorum induces neointimal thickening. Khurana et al. reported that the application of the angiogenesis stimulator VEGF to injured rat arterial walls results in, but does not initiate, a marked increase in neointimal thickening [34]. In crossbred swine fed a high cholesterol diet, low-density vasa vasorum territories within the coronary vessel wall became susceptible to hypoxia, oxidative stress, and microinflammation, potential triggers of early atherogenesis [35].

In atherosclerotic plaques, neovascularization is the primary compensatory response to hypoxia and inflammatory conditions. Neointimal thickening causes ischemia, which strongly induces angiogenesis. Although the expansion of the vasa vasorum in response to neointimal thickening should improve intraplaque ischemia, it does not. Recently, Rademakers et al. investigated the vasa vasorum in plaques of atherosclerotic carotid arteries from aged ApoE-deficient mice by performing *in vivo* functional imaging using multiphoton laser-scanning microscopy. Interestingly, the enhanced plaque-associated immature vasa vasorum not only showed increased permeability, leukocyte adhesion, and intraplaque hemorrhage, but also showed reduced blood flow within the plaques [36]. In accordance with this finding, hyperglycemia altered the structure, but not the density of the vasa vasorum, and accelerated atherosclerosis [37]. In normoglycemic ApoE-deficient mice, atherogenesis is associated with vasa vasorum expansion, likely corresponding to the increasing blood supply demands of the thickening artery wall. By contrast, in the hyperglycemic group, there was no significant neovascularization of the vasa vasorum despite the fact that the lesions were significantly larger [37]. Therefore, it should be noted that not only the density, but also the structural features of the vasa vasorum affect atherogenesis, and the role of the adventitial vasa vasorum may vary depending on time elapsed after vascular injury.

3. Vasa Vasorum and Vascular-Resident Stem Cells

3.1. Vascular-Resident Stem Cells and Atherosclerosis. The contribution of vascular-resident stem/progenitor cells to atherosclerosis progression has been confirmed in recent studies utilizing animal models of atherosclerosis with vascular injury. The vascular-resident stem cells are capable of differentiating into myofibroblasts that subsequently migrate to the intima and contribute to the development of neointimal hyperplasia [8, 9]. Adventitial Sca1+ cells carrying a *lacZ* reporter gene were transferred to the adventitial side of vein grafts in ApoE-deficient mice. β-gal-positive transplanted cells were found in atherosclerotic lesions in the intima, and these cells enhanced the development of these lesions. Consequently, the authors proposed that a large population of vascular progenitor cells residing in the adventitia can differentiate into the vascular smooth muscle cells that contribute to atherosclerosis [19]. Human autopsies have demonstrated the presence of CD34+Sca1+CD133− cells within neointimal lesions and the adventitia of atherosclerotic plaques, which may be a source of endothelial and vascular smooth muscle cells that form atherosclerotic lesions [38].

3.2. The Contribution of the Vasa Vasorum as a Stem Cell Reservoir in Plaque Formation. In the adventitia, multipotent pericytes and endothelial progenitor cells exist as structural cells of the vasa vasorum. Capillary microvessels also provide a vascular niche to house perivascular stem cells [39]. Therefore, the adventitial vasa vasorum might serve as a major reservoir for vascular-resident stem cells. An expanded vasa vasorum may contribute to vascular remodeling by serving as a reservoir for vascular stem cells and a conduit for not only the delivery of inflammatory cells, but also the circulation of stem cells and resident stem cells in plaques [40–42].

Previously, Diaz-Flores et al. reported that in rat femoral arteries that had the adventitial layers removed, the pericytes and endothelial cells of adventitial growing microvessels served as a source of myointimal cells at the intimal thickening and endothelium at the luminal surface, respectively [43]. Recent evidence suggests that the vasa vasorum-associated stem cells affect the prognosis of atherosclerosis. Using a mouse vein graft model, Chen et al. examined the effect of vasa vasorum-associated progenitor cells on atherosclerosis [44]. In ApoE-deficient mice, transplantation of $Sca1^+$ cells that were in close proximity to the vasa vasorum to the outer layer of vein grafts enhanced atherosclerosis, contributing approximately 30% of the neointimal smooth muscle cells. Recently, Tigges et al. reported that adventitial multipotent pericytes participate in the restenotic response in mice with femoral arterial injuries [22]. These multipotent pericytes are increased in adventitia in response to vascular injury and contribute to restenosis in injured arteries. Adventitial pericytes have mesenchymal stem cell-like features and are potentially an important cellular source that contributes to intimal hyperplasia in rat aortic allograft models with transplantation-derived arteriosclerosis [45]. Collectively, these findings suggest that the expansion of the vasa vasorum contributes to a pool of vascular stem cells, including multipotent pericytes, and participates in the atherosclerotic process, in part, by supplying plaque-forming cells, includingsmooth muscle cells (Figure 1).

It is well documented that vascular stem cells migrate to the intimal sites and differentiate into myofibroblasts, contributing to neointimal thickening. When $Sca1^+$ cells are transplanted to the adventitial side of vein grafts in ApoE-deficient mice, the cells migrate into the intima and differentiate into smooth muscle cells [19]. Tigerstedt et al. examined vascular cell kinetics in response to intimal injury *ex vivo* [46]. There is an influx of adventitial precursor cells in the intimal layer that occurs after rat aortic denudation injury. Cell migration was found to contribute to neointimal hyperplasia more than cell proliferation [46]; however, it is

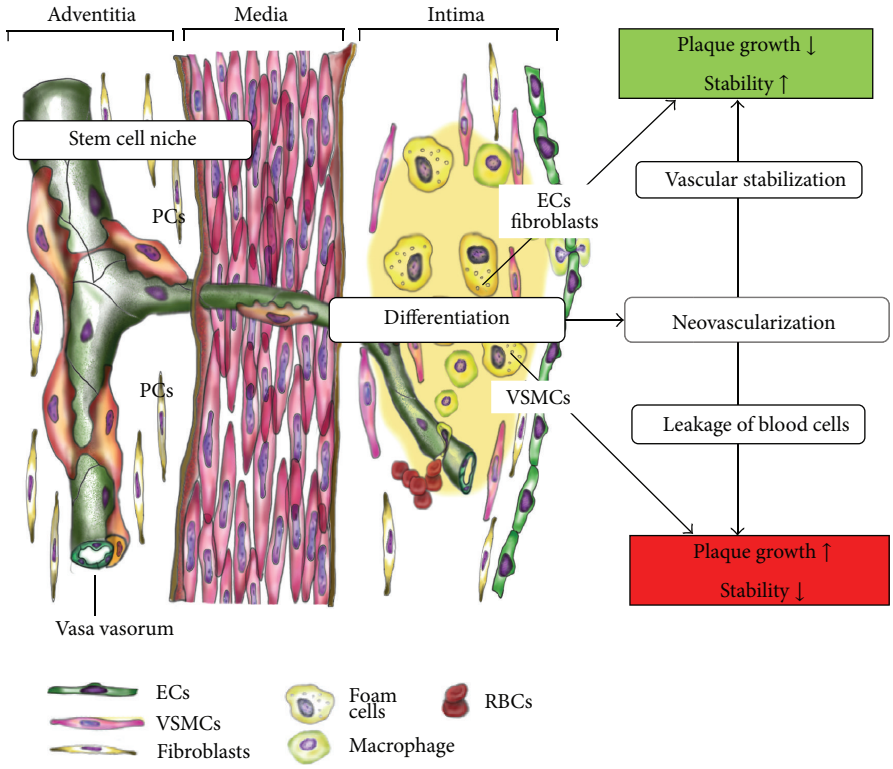

Figure 1: Role of the vasa vasorum in atherosclerosis. In atherosclerotic plaque, the vasa vasorum leads to the microvascular leakage that is responsible for hemorrhage and accumulation of inflammatory cells within plaque. Vasa vasorum also serves as the vascular niche for the vascular-resident stem cells (VSCs), including multipotent pericytes and endothelial progenitor cells. Vasa vasorum acts not only as the blood conduit tube but also as a stem cell reservoir to supply VSCs into the intima. VSCs can differentiate into several cells, such as vascular smooth muscle cells (VSMCs), endothelial cells (ECs), and fibroblasts, and can contribute to the atherosclerotic remodeling. Some of VSCs act as pericytes (PCs) to stabilize the vasculature, which attenuate the leakage of blood cells within plaques.

unclear how these stem cells migrate through the vascular walls. Díaz-Flores jr et al. investigated the vasa vasorum as a source of supplementary cells during intimal thickening by tracing labeled cells within vascular walls. This study provides evidence that adventitial microvascularization contributes to the delivery of supplementary population of fibroblast-like and myointimal cells into the neointima [47].

3.3. The Role of Vascular-Resident Stem Cells in Neovascularization. It is well documented that vascular stem cells have potent angiogenic effects through the paracrine effect and/or the differentiation into endothelial cells or pericytes (Table 1). Therefore, vascular stem cells may contribute to the growth of the vasa vasorum within atherosclerotic lesions. In normal human arteries, vascular progenitor-committed nestin+ cells are located in small-sized vasa vasorum. This could represent a valid evidence for the vasculogenic niche and potentially represents the main source for neovasculogenesis during atherosclerosis [48]. As discussed previously, plaque neovessels are characterized as thin-walled with less investment by pericytes and are often of larger caliber than normal capillaries. This fragile structure could be regarded as sufficient in itself to render these vessels prone to the delivery of inflammatory cells, hemorrhage, and reduced blood flow. Pericytes play an important role in the regulation of vascular contractility and support their maturation and stability that fragile blood vessels become firm to suppress the leakage of the blood cells or hemorrhage [10, 49]. In addition to their potent regenerative activity, multipotent pericytes also play a role in vascular stabilization by structurally and functionally interacting with endothelial cells (Figure 1). Coculture of CD34+ multipotent pericytes with endothelial cells on Matrigel leads to the cooperative assembly of an endothelial network with enhanced stability [15]. Vascular-resident CD44+ multipotent stem cells give rise to pericytes, smooth muscle cells, and contribute to the vessel maturation [50]. To date, it is unclear whether angiogenesis of vascular-resident stem cells acts beneficially or detrimentally in the atherosclerotic pathogenesis. This may be changed depending on the kinds of stem cells and differentiated cells, that is, endothelial cells or pericytes, and the environmental conditions.

4. Antiatherosclerotic Therapy Based on Vasa Vasorum Biology

4.1. Antiangiogenesis Therapy. Antiatherosclerotic therapeutic strategies have been proposed based on findings describing the biology of the vasa vasorum in atherosclerotic plaques [5, 51]. Because a major determinant of plaque vulnerability

and progression is the leakage of red blood cells from the vasa vasorum, the targeted inhibition of plaque angiogenesis may constitute a valuable therapeutic approach toward plaque stabilization and regression.

Moulton et al. reported that blocking vasa vasorum angiogenesis with angiostatin reduces the accumulation of macrophages in plaques and around the vasa vasorum and reduces the progression of atherosclerosis [52]. They propose that the inhibition of plaque angiogenesis and the secondary reduction of macrophages may have beneficial effects on plaque stability. Antiangiogenic PAI-1_{23}, a truncated isoform of plasminogen activator inhibitor-1 promotes vasa vasorum regression and reduces atherosclerotic plaques in hypercholesterolemic mice through a plasmin-dependent mechanism [53, 54].

It is widely recognized that cholesterol-lowering statin drugs have potent antiatherosclerotic activity [55]. In addition to their cholesterol lowering effect, statins have pleiotropic pro- and antiangiogenic properties [56]. There is abundant evidence from both animal and human studies examining the effects of statins on angiogenesis in ischemic heart disease and stroke, but statins also have a potent antiangiogenic effect on atherosclerotic neovasculature. Independent of its cholesterol-lowering effects, simvastatin attenuated vasa vasorum neovascularization in a pig model fed a high cholesterol diet [57]. Similarly, independent of lowering cholesterol levels, atorvastatin significantly inhibited the development of adventitial vasa vasorum and the progression of atherosclerosis in a rabbit model of atherosclerosis [58].

4.2. Vascular Normalization Therapy. In addition to the density/expansion of the vasa vasorum, its structural and functional impairment play crucial roles in atherosclerotic plaque development. Fragile neovessels are formed within plaques, reducing perfusion flow regardless of the expansion of intraplaque vasa vasorum that contributes to plaque growth [36]. Therefore, the mere reduction of vasa vasorum density may decrease oxygenation and increase oxidative stress, initiating cascades of inflammation and intimal proliferation. Alternatively, the deletion of microvessels results in the depletion of the tissue-specific stem cell niche that subsequently becomes prematurely exhausted and unable to maintain organ function [59, 60]. Therefore, normalization of the impaired vasa vasorum would be an attractive therapeutic strategy rather than mere antiangiogenesis therapy. Although analytical techniques to image the vasa vasorum have advanced, histological methods to specifically visualize the vasa vasorum are limited. Therefore, experiments aimed at investigating vascular maturation/stabilization in the vasa vasorum, particularly in pathological settings, are limited.

The intimate interaction between pericytes and endothelial cells tightly correlates with vascular growth, maturation/stabilization, and remodeling of vessels [61]. Several external factors also may affect this interaction to regulate vascular maturation [62]. Recently, we developed an *in vivo* angiogenesis assay using collagen-coated tubes (CCTs) to observe the vasa vasorum of the injured mouse femoral artery. Using this novel angiogenesis assay, we found that nerve growth factors (NGF) had potent angiogenic effects on the microvessels around the injured artery and, more specifically, induced the maturation/stabilization of microvessels and the regeneration of perivascular nerves [63]. Lastly, we propose new strategies for the normalization of vasa vasorum by peripheral nerve innervation.

5. Conclusion

In this review, we discussed the effect of the vasa vasorum on the progression of atherosclerotic plaques with respect to its function not only as a conduit structure that delivers blood components, but also as a stem cell reservoir. A clearer understanding of adventitial vasa vasorum biology would provide insight that would lead to a better understanding of atherosclerotic pathogenesis and improved therapeutic strategies to combat atherosclerotic diseases. Pharmacological inhibition of angiogenesis in atherosclerotic plaques reportedly inhibits lesion progression in animal models. However, it is important to consider that the vasa vasorum acts as either a causative or responsive factor in neointimal formation depending on the atherosclerotic stage. Prior to designing clinical studies aimed at regulating angiogenesis in atherosclerotic diseases, the stage-dependent role of the vasa vasorum in atherosclerotic plaque development should be fully elucidated.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Mesenchymal Stem Cells for Regenerative Therapy: Optimization of Cell Preparation Protocols

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Administration of bone marrow-derived mesenchymal stem cells (MSCs) is an innovative approach for the treatment of a range of diseases that are not curable by current therapies including heart failure. A number of clinical trials have been completed and many others are ongoing; more than 2,000 patients worldwide have been administered with culture-expanded allogeneic or autologous MSCs for the treatment of various diseases, showing feasibility and safety (and some efficacy) of this approach. However, protocols for isolation and expansion of donor MSCs vary widely between these trials, which could affect the efficacy of the therapy. It is therefore important to develop international standards of MSC production, which should be evidence-based, regulatory authority-compliant, of good medical practice grade, cost-effective, and clinically practical, so that this innovative approach becomes an established widely adopted treatment. This review article summarizes protocols to isolate and expand bone marrow-derived MSCs in 47 recent clinical trials of MSC-based therapy, which were published after 2007 onwards and provided sufficient methodological information. Identified issues and possible solutions associated with the MSC production methods, including materials and protocols for isolation and expansion, are discussed with reference to relevant experimental evidence with aim of future clinical success of MSC-based therapy.

1. Introduction

Recent research has extensively shown the potential of bone marrow- (BM-) derived mesenchymal stem cells (MSCs) for regenerative therapies in various organs including the heart [1]. The effects from this approach are dependent on their potency of secretion of beneficial cytokines and growth factors for tissue repair/regeneration and immunomodulation and/or their differentiation for regenerating damaged organs [2]. Since the first clinical trial of BMC injection in 1995 [3], more than 2,000 patients have been administered with allogeneic or autologous MSCs for the treatment of various diseases, including graft-versus-host disease, hematologic malignancies, cardiovascular diseases, neurologic diseases, autoimmune diseases, organ transplantation, refractory wounds, and bone/cartilage defects [4]. More than 200 clinical trials of MSC-based therapy, completed or ongoing, have been listed on the website of the United States National Institute of Health (http://www.ClinicalTrial.gov/) as of July 2013. The cells used are, strictly speaking, mesenchymal stromal cells, which include MSCs and other cells; but, in most cases they are simply referred to as MSCs. Previous preclinical studies and clinical trials have shown feasibility and safety of MSC-based therapy; however, the therapeutic effects observed in clinical trials to date appear to be inconsistent and remain inconclusive [5].

MSCs were first described in 1976 by Friedenstein and colleagues [6] and are more recently defined by The International Society of Cellular Therapy based on three cellular properties: (1) adherence to plastic, (2) positive expression of CD105, CD73, and CD90 and negative expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA class II, and (3) differentiation potential to mesenchymal lineages including osteocytes, adipocytes, and chondrocyte [7]. Unfortunately, the frequency of MSCs in BM is low; MSCs represent 0.001–0.01% of BM mononuclear cells or lower [8].

Although the optimal dosage of MSCs in therapeutic applications is still unclear and will be dependent upon the type of therapy, $1.0–2.0 \times 10^6$ MSCs per kg body weight is generally used [8]. Direct collection of such a large number of MSCs from BM is not practical. Therefore, it is necessary to expand isolated MSCs *in vitro* to obtain a sufficient number for therapeutic approaches.

MSCs have a rapid proliferation ability, achieving a thousandfold expansion of cell number in a two- to three- week period. However, inappropriate expansion may reduce the quality of MSCs. It is known that extensive *in vitro* culture induces cellular senescence that is associated with growth arrest and apoptosis [9]. In addition, particular therapeutic properties of MSCs may be lost during prolonged culture; for example, the cardioprotective effect of passage 5 MSCs is significantly reduced compared to passage 3 MSCs [10]. However, protocols of MSC preparation used in clinical studies remain inconsistent and suboptimal. There are surprisingly different protocols used in current clinical studies, in terms of culture materials (flasks, culture media, and supplements), seeding density, passaging, and storage. These factors can influence the important properties of MSCs, leading to reduced or unexpected therapeutic results [11]. In addition, such inconsistent protocols make comparison of the results between clinical studies difficult.

Establishment of optimal, standardized protocols for MSC isolation and expansion will therefore be a key for MSC-based therapies to become widespread, generic approaches. For this aim, understanding of currently used protocols with their scientific justification is essential. We hereby carefully searched the protocols used in recent clinical trials of MSC-based therapy by referring PubMed. As a result, a total of 47 reports, which sufficiently describe MSC-preparation methods, were found, published from January 2007 onwards (see Supplementary Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/951512). This review article summarizes the information obtained from these clinical trials with further referencing to relevant experimental studies, highlighting issues and solutions associated with current protocols of MSC isolation and expansion.

2. Background of Clinical Trials of MSC-Based Therapy Analyzed in This Review

By literature search using PubMed, we selected 47 reports of clinical trials of BM-derived MSC-based therapy published between January 2007 and June 2013, which sufficiently describe the methods of isolation and expansion of MSCs (Supplementary Table 1). Most reports provide parts (not all) of the methodological information of interest to us. The trials aimed to treat a range of diseases, including oncological diseases (38%), followed by neurological diseases (26%) and cardiovascular diseases (11%) (Figure 1(a)). 66% of the studies used autologous MSCs, while the remaining 34% used allogeneic MSCs (Figure 1(b)). The number of MSCs injected ranged from 0.34 to 2.3×10^6 cells/kg body weight; the majority of the reports administered $1\text{-}2 \times 10^6$/kg body weight MSCs (Figure 1(c) and Supplementary Table 1). All these trials successfully supported feasibility to obtain the aimed number of MSCs, but with using a variety of isolation and expansion protocols. Furthermore, regardless of the protocols to prepare MSCs and cell number injected, no major safety issues that were directly caused by MSC, were reported.

3. Isolation of MSC from BM

3.1. BM Preparation for MSC Isolation. Possible techniques to isolate MSCs from BM materials include cell adherence-based methods and cell-sorting methods, with the vast majority of previous clinical trials using the former method. The latter including fluorescence-activated cell sorting and immune-magnetic bead cell sorting [12] has the advantage of collecting a more purified MSC population. However, they are hardly used in clinical trials because of the lack of appropriately specific simple surface markers for MSCs, possible cellular damage, more expensive cost, and more demanding labor. For the adherence-based methods, either whole BM cells or BM mononuclear cells separated by density gradient centrifugation were used. The use of whole BM cells is clearly easier and yields higher numbers of adhered cells on plastic dishes with reduced loss of MSCs compared to density gradient separation methods. However, cells collected by an adherence method represent a heterogeneous mixture of cells, including not only MSCs but also hematopoietic cells at different differentiation/commitment stages, endothelial cells and endothelial progenitor cells. Although many of these contaminating cells may be removed during passaging, such contamination would affect the expansion of MSCs as well as the overall effect of the therapy. In order to isolate a more homogeneous initial MSC population, BM mononuclear cells can be separated from whole BM cells by density gradient centrifugation using either Ficoll (Paque, Hypaque, or Paque Premium) or Percoll (both available from GE Healthcare, Uppsala, Sweden). In the current studies we have analyzed, 62% used Ficoll-based density gradient separation, 16% used whole BM cells without separation, and another 9% employed Percoll-based density gradient separation (Figure 2(a)).

Percoll and Ficoll have usually been used at densities of 1.073 g/mL [13] and 1.077 g/mL [14], respectively, to isolate MSCs with high proliferative and differentiative potential. Percoll is a suspension of colloidal silica particles (diameter 15–30 nm), which has been widely used for separating cells, organelles, viruses, and other subcellular particles in basic science experiments, but it is not produced as a good manufacturing practice (GMP) grade reagent. Ficoll, a polymer of sucrose with a high synthetic molecular weight, is generated at GMP grade and has been frequently used for separating mononuclear cells and lymphocytes from peripheral blood in clinical practice for several decades, indicating clinical safety of the reagent. Recently Mareschi et al. compared MSCs collected via Percoll-separated mononuclear cells, Ficoll-separated mononuclear cells, and whole BM cells and found no significant differences in terms of gross morphology, dif-

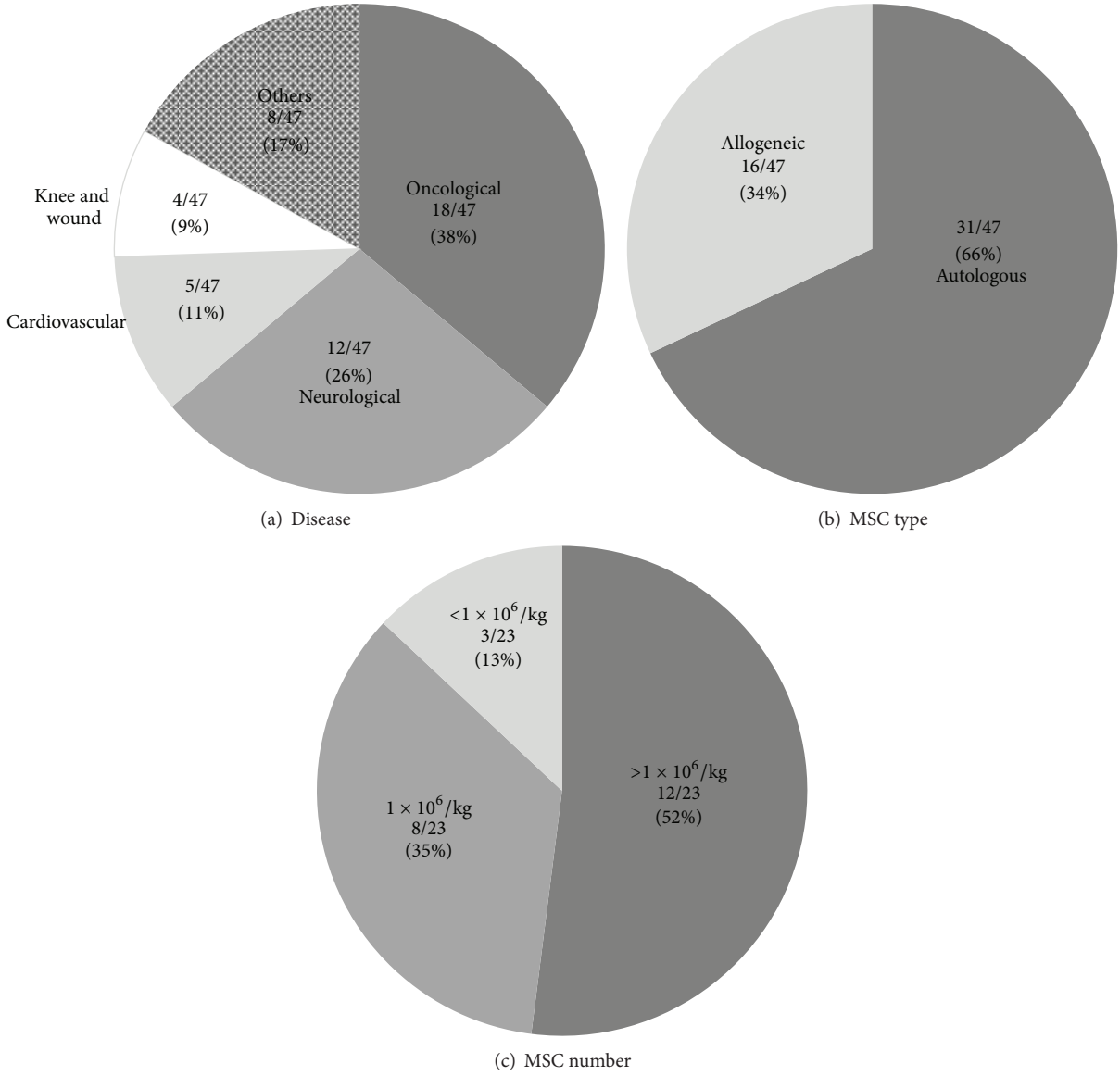

Figure 1: Background of the clinical trials of MSC-therapy reviewed in this article. A total of 47 clinical trials that have reported sufficient information on the MSC preparation were selected to review in this article. (a) A wide range of diseases were targeted by MSC therapy. (b) Both autologous and allogeneic MSCs were used for MSC therapy. (c) The number of MSCs administered was 1×10^6/kg body weight or more in the majority of clinical trials. Some trials repeated the injection. See Supplementary Table 1 as well.

ferentiation potential, or immunophenotype between the collected cells [15]. However, the whole BM cell method apparently resulted in a greater Colony-Forming Unit-Fibroblast (CFU-F) number and improved cellular growth compared to gradient-separated cell methods. Given the other advantages in being less demanding in cost and labor, it is proposed that the whole BM cell method would be the first-choice method for MSC isolation from BM samples.

3.2. Flask for MSCs Isolation. There are many manufacturers that produce plastic flasks suitable for MSC isolation by the adherence-based method including Corning, Falcon, Nunc, and Greiner. Sotiropoulou et al. compared the effect of these 4 culture flasks to adhere MSCs [16] and indicated that greater numbers of MSCs were acquired in Corning flasks followed by Falcon, Nunc, and Greiner at 7 days after plating (without passaging). All these types of flasks are produced from polystyrene permanently rendered hydrophilic with corona discharge, using high voltage to create a reactive gas plasma [17]. This process for Falcon flasks takes place in a closed chamber, thus creating a consistent treatment surface. On the other hand, during manufacturing of the flasks from other companies, the gas is exposed to ambient air and therefore

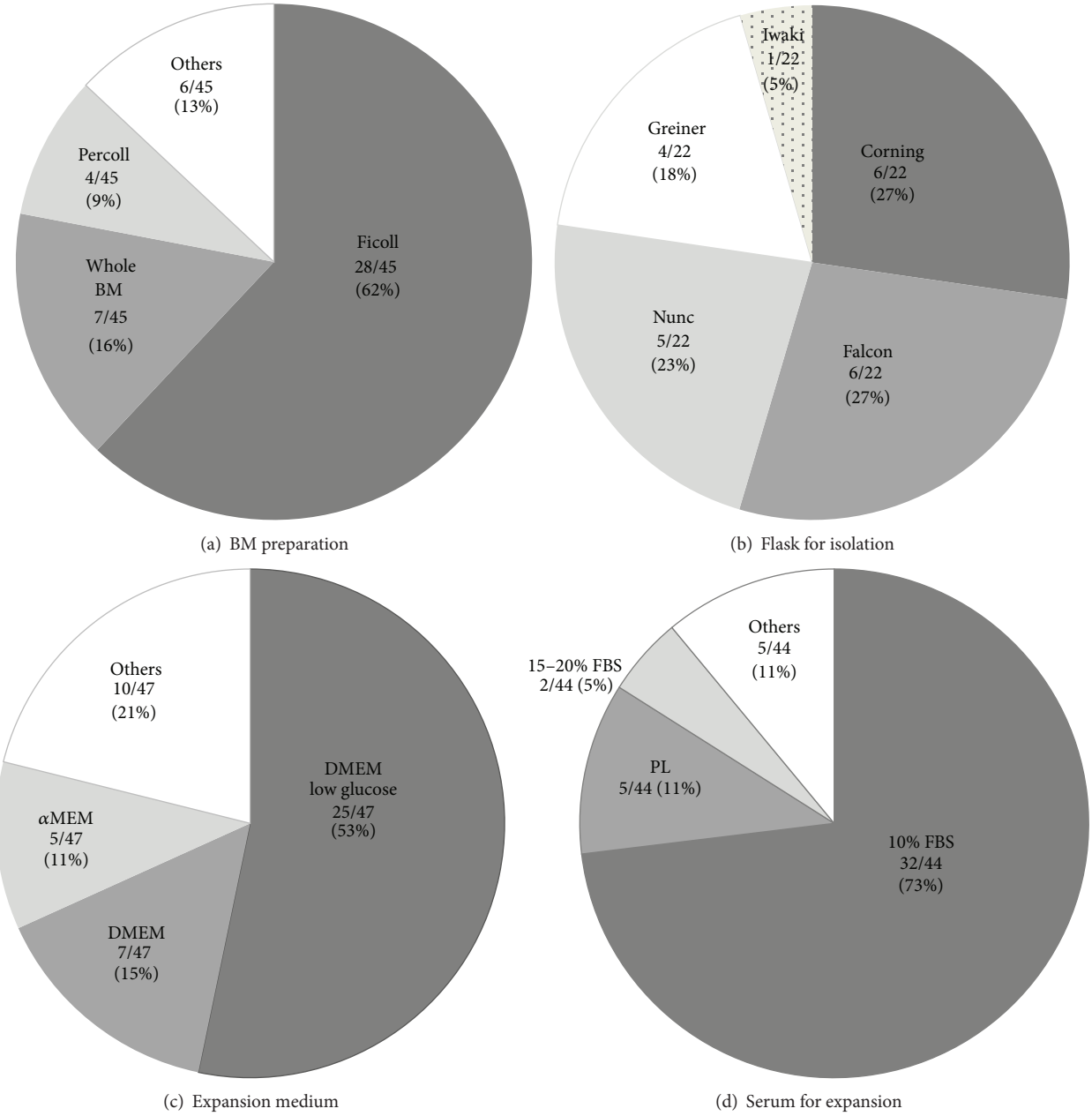

Figure 2: Protocols and materials used for MSC isolation and expansion. Different methods and materials were used in the recent MSC-therapy clinical trials, in terms of method for bone marrow (BM) preparation for MSC isolation (a), culture flask used for MSC isolation (b), culture medium used for MSCs expansion (c), and serum used for MSC expansion (d). PL: platelet lysate; FBS: fetal bovine serum.

subjected to day-to-day environmental changes. In the real world, the most commonly used flask was Corning (27%) and Falcon (27%) equally, followed by Nunc (23%), Greiner (18%), and Iwaki (5%) in our analysis of current clinical trials (Figure 2(b)).

3.3. Cell Seeding Density for MSC Isolation. Cell seeding density of BM mononuclear cells or whole BM cells is another important factor to determine the efficiency of MSC yield as this affects adherence of MSCs, contamination by other cell types, and initial growth of adhered MSCs. Sotiropoulou et al. reported that, between the range from 1×10^3 to 2×10^5 BM mononuclear cells/cm^2, the lower initial seeding densities achieved increasingly larger numbers of adherent cells at Passage 0 [16]. Both et al. also reported that MSCs seeded at lower densities had a faster proliferation than those seeded at higher densities, with MSCs plated at 100 cells/cm^2 reaching their target of 2×10^8 cells 4 days faster than cells that were seeded at 5×10^3 cells/cm^2 [18]. Further decrease in the seeding density below 100 cell/cm^2 showed a further increase in proliferation rate; however, there is a lower limit in the plating density in the clinical settings. Given that 1×10^7–1×10^8 BM mononuclear cells or a larger number of whole BM cells are commonly obtained,

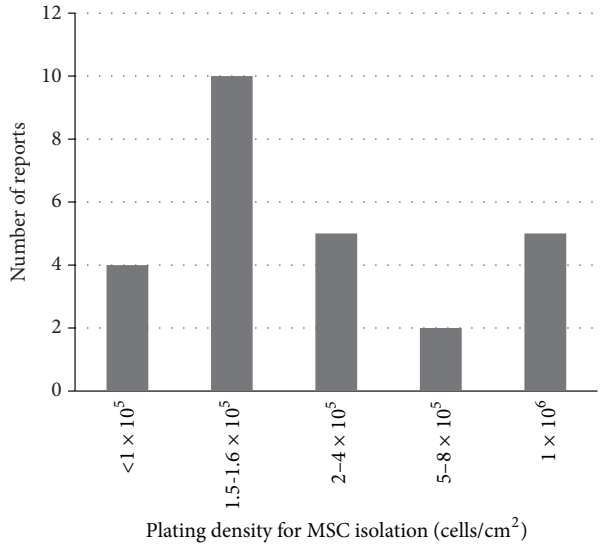

Figure 3: Plating density for MSC isolation. Plating cell densities of BM mononuclear cells for MSC isolation used in 26 clinical trial reports are presented. 1.5-1.6 × 10^5 cells/cm^2 was most frequently used.

it is not practical to seed such a large number of cells at below 1 × 10^4. Seeding 1 × 10^8 BM mononuclear cells at 1 × 10^3 cells/cm^2 would require approximately 600 × 175 cm^2 flasks, which is too high a cost in terms of materials and labor. As a matter of fact, the cell seeding density used in current clinical trials is quite high, with extreme variability ranging from 1.1 × 10^3 to 1.0 × 10^6 mononuclear cells/cm^2 (Supplementary Table 1). The most commonly used seeding density of BM mononuclear cells is 1.5-1.6 × 10^5 cells/cm^2, followed by 1.0×10^6 cells/cm^2 and 2–4×10^5 cells/cm^2 (Figure 3). For the future clinical application, it is suggested that BM-mononuclear cells should be plated at as a low density as far as the cost, facility, and labor allow. There is very limited experimental evidence to discuss the optimal plating dose of whole BM cells but a pre-clinical study has shown that 10,000 cells/cm^2 would be the most advantageous condition [15].

3.4. Medium and Supplement. Optimal medium and culture supplements for MSC isolation remain much less unstudied, compared to those for MSC expansion (see Section 4 for detailed information). In the vast majority of current clinical trials, the same medium and serum/supplement appeared to be simply used for both MSC isolation and expansion (very few reports described this particular method). This may be convenient and economical in practice; however, the most effective conditions for isolation and expansion of MSCs could be different, requiring further research to elucidate the optimal culture medium for MSC isolation.

4. Expansion of MSCs

4.1. Flask for MSCs Expansion. A comprehensive laboratory investigation of the proliferation efficacy of MSCs cultured on 4 major types of culture flasks has indicated that the most improved expansion of cultured MSCs was acquired in the flasks from Falcon, followed by those from Corning, Nunc, and Greiner, although the quality and functions of produced MSCs did not differ among the different types of flasks examined [16]. In contrast, the most commonly used flask in previous clinical trials we investigated here was Corning flasks (35%), followed by Nunc flasks (25%), Falcon flasks (20%), and Greiner flasks (20%) (Supplementary Table 1). In the majority of the reports, we found that the same manufacturer's flasks are preferably used for both isolation and expansion of MSCs. The use of the same manufacturer's flasks may be more convenient and economical; however, it should be noted that the optimal flask surface for initial isolation of MSCs could be different from that for MSC expansion as the scientific evidence indicates [16].

A wide surface area is required to obtain a sufficient number of MSCs for clinical application. To reduce the number of culture flasks used, manufacturing companies such as Nunc and Corning offer large, multilayered culture systems that can fit to usual cell culture incubators. Decreasing the number of flasks will improve the microbiological safety and traceability and also reduce staff workload and cost. The CellStacks (Corning, USA) and CellFactory (Nunc, Denmark) systems, which start from a unit surface area of 635 cm^2, offer the possibility of 2, 5, 10, and 40 stages per container. In addition, these devices can be connected by tubes, allowing for convenient, sterile, GMP-compliant operations (e.g., culture initiation, medium exchange, and cell harvesting).

4.2. Basal Culture Medium. Basal culture medium consists of amino acids, glucose, and ions including calcium, magnesium, potassium, sodium, and phosphate. There is no doubt that the types of culture medium used affect proliferation and differentiation of MSCs. There is a preclinical report showing that DMEM is preferable to IMDM (Iscove's modified Dulbecco's medium) with respect to preservation of MSC stemness [19]. It has also been experimentally demonstrated that αMEM (minimal essential medium) better preserves osteogenic properties of MSCs and achieves higher CFU-F retrieval than DMEM [20]. Figure 2(c) shows that the basal culture media used for MSC expansion in current clinical trial includes DMEM-low glucose (53%), DMEM (15%), and αMEM (11%).

L-Glutamine is an essential nutrient for energy production as well as protein and nucleic acid synthesis in cell culture, and thus this is commonly supplemented into culture media. However, this spontaneously degrades in culture media, and its chemical breakdown and cellular metabolism lead to ammonia formation, possibly inhibiting cell growth [21]. To solve this issue, Glutamax is recently used as substitute for L-glutamine, as this is more stable in aqueous solutions and does not spontaneously degrade. Sotiropoulou et al. systemically compared the expansion efficacy of MSCs among 8 different basal media (IMDM, Optimem, αMEM with L-glutamine, αMEM with Glutamax, DMEM with low glucose and L-glutamine, DMEM with low glucose and Glutamax, DMEM with high glucose and L-glutamine, and DMEM

with high glucose and Glutamax) [16]. The authors have found significant differences: among the 8 types of medium studied, αMEM containing Glutamax achieved the greatest expansion of cultured MSCs, followed by αMEM containing L-glutamine. Unfortunately many previous clinical trial papers did not clearly describe the type of glutamine used (Supplementary Table 1).

4.3. Growth Factor Supplement for MSC Expansion. It is known that growth factor supplement to culture medium enhances proliferation with maintenance of important properties of MSCs. In particular, fibroblast growth factor-2 (FGF2) [22], platelet-derived growth factor (PDGF) [23], epidermal growth factor (EGF) [24], transforming growth factor (TGF)-β [23], and insulin-like growth factor (IGF) [25, 26] play a role. Previously, fetal bovine serum (FBS) has been most frequently used (10% FBS in 73% and 15–20% FBS in 5%) to supply growth factors to MSC culture medium (Figure 2(d)), because FBS contains all these factors and is relatively readily available at clinical grade. However, it should be noted that FBS shows considerable variation in growth factor activity from batch to batch, and therefore large amounts of batch-tested FBS will need to be reserved for a clinical application. Furthermore, FBS remains associated with safety issues including transmission of prion or viral disease, anaphylatoxic reactions, and production of anti-FBS antibodies [27, 28]. Regulatory authorities in an increasing number of countries, including Paul-Ehrlich-Institute in Germany, now prohibit the clinical use of FBS, while in contrast the Australian Therapeutic Goods Authority allows the use of FBS for the production of clinical grade materials as long as it is sourced from cattle in a country free of bovine spongiform encephalitis such as Australia or New Zealand.

To avoid such a risk related to the use of animal materials, the use of human products, including human serum and platelet lysate, has been proposed. The effect of human allogeneic serum from adult donors to enhance proliferation of MSCs with preservation of important cellular properties is controversial [29, 30]. On the other hand, it will be problematic to acquire a large amount of autologous serum sufficient to generate clinically relevant numbers of MSCs. Moreover, autologous serum from elderly patients may have deteriorated capacity to support cell growth. Allogeneic human serum from umbilical cord blood [31] and placenta [32] has also been proposed as a potential alternative to replace FBS because these primitive tissues are a rich source of growth factors.

Platelet lysate has recently been a more preferred human product; more than 10% of previous clinical trials between 2007 and 2013 used Platelet lysate (Figure 2(d)). Platelet lysate can be easily obtained from apheresis products [26], as well as from buffy coats [33] of healthy volunteers. Immediately after collection, platelet products are frozen at −80°C and subsequently thawed to obtain the release of growth factors included in platelets with centrifugation to eliminate platelet bodies. The obtained growth factors include PDGFs, b-FGF, VEGF, IGF-1, and TGF-β [25, 26], which improve proliferative capacity of MSCs. Platelet lysate from several healthy donors may be pooled for the use [26]. Doucet et al. first demonstrated that growth factors contained in platelet lysate are able to promote MSC expansion in a dose-dependent manner [25]. This was further substantiated by the data showing that a culture medium supplemented with 5% platelet lysate is superior to 10% FBS in clonogenic efficiency and proliferative capacity of MSCs, therefore providing more efficient expansion, together with a significant time saving [26]. However, several studies have shown limitations of platelet lysate, including reduction of osteogenic or adipogenic differentiation potential [33, 34] and decreased immunosuppressive capacity with altered surface marker expression [35]. In addition, there is a risk that any allogeneic human product may be contaminated with human pathogens that might not be detected by routine screening. Moreover, crude blood derivatives are poorly defined and also suffer from batch-to-batch variation, and thus their ability to maintain MSC growth and therapeutic potentials could be variable. Further studies are need for platelet lysate to be part of a standard protocol.

Issues associated with human products encourage the use of serum-free and animal component-free MSC culture media. StemPro MSC SFM from Invitrogen is the first FDA-approved commercial product of this type. Agata et al. showed an enhanced effect of StemPro MSC SFM to improve rapid proliferation at early (<5) passage stages compared to FBS [36]. Of note, important characteristics of MSCs, including surface antigen expression, stemness, and differentiation potential, are different between MSCs cultured with FBS and MSCs with serum-free medium [36]. Although the formulations of these commercial media are not disclosed, it is important to evaluate each commercial media and select the most suitable product for each type of treatment.

4.4. Direct Addition of Growth Factors for MSC Culture. Although ideal growth factor supplements for MSC culture are still undefined, administration of several types of growth factors with or without serum or platelet lysate has been tested if it could increase MSC expansion with maintenance of important cellular properties. These include at least b-FGF [22], PDGF [23], TGF-β, EGF [24], and IGF1 [25, 26]. FGF2 induces excellent expansion efficiency of MSCs with maintenance of their differentiation potential and has been used for clinical trials of MSC-based therapy [37]. However, recent data suggests that b-FGF upregulates HLA-DR and Stro-1 and downregulates CD44 in a dose-dependent fashion [16, 38]. PDGFs were first found in platelets and they might be responsible for some of the platelet lysate activity in MSC growth. PDGFs have a role in osteogenic, adipogenic, and chondrogenic differentiation of MSCs; however, the primary effect is likely to be mitogenic action with inhibition of differentiation [39]. The PDGF-BB isoform can activate all PDGF receptors and therefore may be the best choice as a culture supplement. A recent report found that a combination of b-FGF, PDGF, and TGF-β could replace the serum component in cell culture medium to expand human MSCs without compromising differentiation potential, at least up

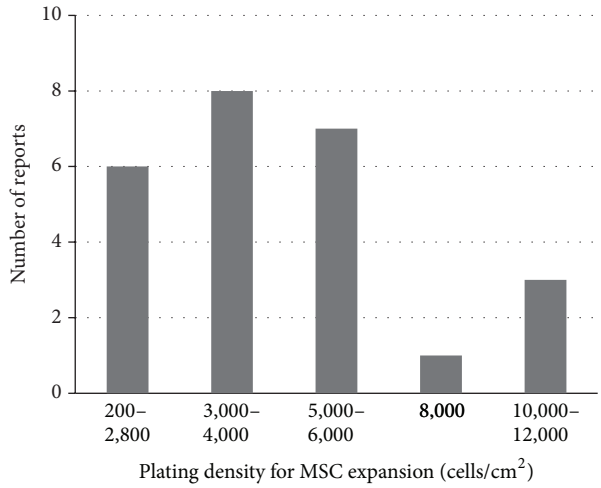

Figure 4: Plating density for MSC expansion. Plating cell densities for MSC expansion used in 25 clinical trial reports are presented. 3,000–6,000 cells/cm^2 was commonly used.

to 5 passages [23]. Further evidence is required for this promising approach to become a widespread protocol.

4.5. Cell Plating Density for MSCs Expansion. Plating cell density is influential not only on initial isolation but on expansion of cultured MSCs. Generally, a higher plating density results in a reduced proliferation ability, probably due to contact inhibition and/or less availability of nutrients per cell [40]. The log phase lasts for a longer duration in cells plated at lower densities, and hence more population doublings occur due to a longer exponential growth phase [41]. A comparable study showed that, after 10 days in culture, BM-derived MSCs seeded at 2,500, 250, 25, and 2.5 cells/cm^2 resulted in 2.7 ± 0.5, 4.8 ± 0.4, 6.7 ± 0.5, and 7.6 ± 1.0 population doublings [42]. This study also showed that the seeding density does not affect cellular properties of MSCs including cell surface marker expression.

However, for clinical-scale production (1×10^6/kg body weight or more) of MSCs, use of a very low plating density is unrealistic due to demanding cost, facility, and labor; a plating density of 1,000 cells/cm^2 is suggested as a reasonable, evidence-based compromise [43]. In the clinical arena, however, more compromise for the cost/labor is usually taken; over 75% of current clinical trials used plating densities of over 3,000 cells/cm^2 (Figure 4).

5. Passaging and Storing of MSC

5.1. Dissociation of Adherent MSCs. For the purpose of passaging for expansion or collection for administration, adhered MSCs on plastic flasks need to be dissociated. In our search, administered MSCs were received in less than 1 passage in 23%, 1–5 passages in 71%, and over 5 passages in 6% of reported clinical trials (Figure 5(a)). To this end, the majority of current clinical trials used enzymatic digestion using trypsin-EDTA solution (Supplementary Table 1). Of note, the concentration of trypsin-EDTA used was widely ranging; 0.25, 0.05, and 0.025% trypsin-EDTA was used in 58, 26, and 16% of previous trials, respectively (Figure 5(b)). Excessive trypsinisation can damage cells, while on the other hand insufficient trypsin-treatment will reduce the yield of cells. Optimal trypsinisation condition may be different among flask types used and cell density/confluence. Thus, the choice of trypsinisation conditions (not only concentration but also duration and temperature) should be carefully decided case-by-case based on scientific evidence. In addition, many previous trials appeared to utilise porcine-derived trypsin, which should be replaced with human trypsin or other alternatives [44, 45] to reduce safety concerns [46].

5.2. Storing of MSCs. Isolated and expanded BM-derived MSCs were sometimes stored until the time of treatment.

In our search 17 out of 49 (35%) trials used cryopreserved MSCs (Figure 5(c); two out of 47 clinical trials used both fresh cells and cryopreserved cells, making the total number 49). This allows for great flexibility in the clinical setting, but extreme caution is needed on possible adverse effects on MSCs. Although there are many preclinical studies showing that cryopreservation does not change the biological behavior of MSCs such as differentiation, growth, and/or surface marker expression [47, 48], on the other hand, there are reports warning hazardous effects by cryopreservation [49]. Further refinement of the protocol is warrantied. Important ingredients in current freezing solution include dimethylsulfoxide (DMSO) and serum. DMSO has been extensively used at 5–10% as a cryoprotectant with its high membrane permeability. However, DMSO can be damaging to cells when used in high concentration, especially during the thawing procedure. Also, if it remains in MSC suspension for administration, DMSO can cause adverse reactions in patients, including nausea, vomiting, tachycardia, bradycardia, and hypotension. Haack-Sørensen et al. [46, 50] advocate the use of 5% concentrations of DMSO together with 95% FBS. However, the use of animal sera will have a risk in the use for patients as discussed above. Defined, serum-free and animal component-free freezing media, such as Cryostor CS10 StemCell Technologies [51] or Plasmalyte-A [52], may be possible alternatives. Cell concentration during cryopreservation was proposed to be optimal with 0.5–1 × 10^6/mL [53]. A controlled rate freezing method (freezing at rate of 1°C per minute) will achieve superior outcome than uncontrolled freezing [54].

5.3. Injection Vehicle. It is important to optimise the vehicle of MSCs for injection, as this will affect donor cell viability and loss before and after injection. In previous clinical trials, 66% used saline and 17% used PBS (Figure 5(d)). There were attempts to supplement human serum albumin to protect cells from environmental stress and prevent adherence to the walls of tubes and needles. Further systematic comparisons between injection vehicles are warrantied.

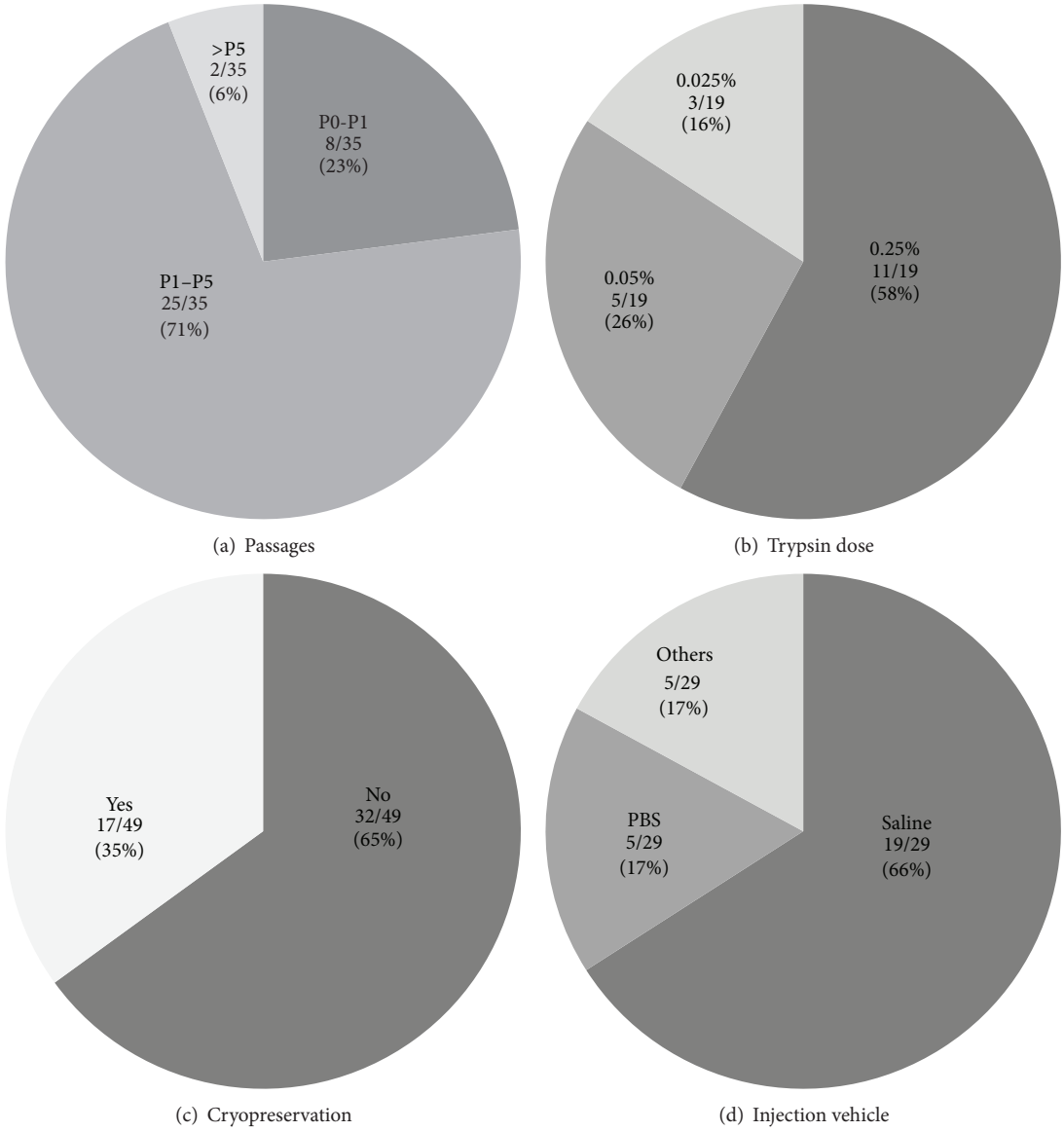

Figure 5: Preparations of MSCs. (a) Over 70% clinical trials used MSCs that received 1–5 passages. (b) Doses of trypsin used for passaging were widely varied. (c) Cryopreserved MSCs were used in 35% of clinical trials reviewed. In 47 clinical trials studied, two trials used both fresh cells and cryopreserved cells, thus the total number of reports shown in the graph is 49 (see Supplementary Table 1). (d) It was common to use saline as injection vehicle.

6. Conclusion and Future Perspective

Recent advance in basic and medical science and technologies has realised the employment of BM-derived MSCs for a variety of therapeutic indications including regenerative therapies. A sufficient sum of initial clinical trials has shown feasibility and safety of this approach at least and also suggested the therapeutic effect (though preliminary), encouraging further study for this approach to become an established generic treatment. One of the major hurdles for this development will be the establishment of optimised and standardized GMP-compliant protocols for isolation and expansion of MSCs. This review demonstrates how various the current protocols were. Many protocols lack scientific validation and appear to be suboptimal.

It is now urgently important to solve this issue of the lack of conformity between MSC manufacturing protocols, which is considered as potential threat to further development of MSC-based therapy. As summarised in this review, a range of relevant scientific evidence is available for this purpose. Active cooperation between academics, clinicians, companies, and regulatory authorities is encouraged in order to develop international standards for BM-derived MSC production, which should be evidence-based, regulatory

authority-compliant, of good medical practice grade, cost-effective, and clinically practical, for the future success of MSC-based therapy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Regenerative Medicine for the Cornea

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Regenerative medicine for the cornea provides a novel treatment strategy for patients with corneal diseases instead of conventional keratoplasty. Limbal transplantation has been performed in patients with a limbal stem cell deficiency. This procedure requires long-term immunosuppression that involves high risks of serious eye and systemic complications, including infection, glaucoma, and liver dysfunction. To solve these problems, ocular surface reconstruction using cultured limbal or oral mucosal epithelial stem cells has been successfully applied to patients. However, cell sheets must be fabricated in a cell processing center (CPC) under good manufacturing practice conditions for clinical use, and the expenses of maintaining a CPC are too high for all hospitals to cover. Therefore, several hospitals should share one CPC to standardize and spread the application of regenerative therapy using tissue-engineered oral mucosal epithelial cell sheets. Consequently, we developed a cell transportation technique for clinical trial to bridge hospitals. This paper reviews the current status of regenerative medicine for the cornea.

1. The Structure of the Cornea

The cornea is an avascular and transparent tissue that forms part of the anterior ocular segment. Together with the sclera, it forms the outer shell of the eyeball. The cornea serves as the transparent window of the eye that allows light to enter, whereas the sclera provides a dark box that allows an image to form on the retina. The cornea is exposed to the outer environment, whereas the white sclera is covered with the semitransparent conjunctiva and is not directly exposed to the outside.

The central cornea is 515 μm thick [1]. It comprises an outer stratified squamous nonkeratinized epithelium, an inner connective tissue stroma, and the innermost layer, a cuboidal endothelium (Figure 1). Disorders in any of these layers can cause corneal opacity and visual disturbance: epithelial disorder (e.g., limbal stem cell deficiency), stromal disorder (e.g., corneal dystrophy), and endothelial disorder (e.g., bullous keratopathy).

2. Limbal Stem Cells

The corneal epithelium has five to seven cell layers and is 50–52 μm thick. It is composed of small basal cells, flattened middle cells (wing cells), and polygonal flattened superficial cells. Corneal epithelial stem cells (limbal stem cells) are thought to reside in the basal layer of the limbus, the transitional zone between the cornea and the conjunctiva (Figure 2) [2, 3]. Transient amplifying (TA) cells are generated by stem cells and then migrate into the central cornea. Although stem cells are low-cycling, TA cells proliferate rapidly.

Thoft and Friend hypothesized that corneal epithelial maintenance can be defined by the equation $X + Y = Z$, with X being the proliferation of basal epithelial cells; Y being the contribution to the cell mass by the centripetal movement of peripheral cells; and Z being the epithelial cell loss from the surface [4]. Therefore, the proliferation and migration of TA cells differentiated from stem cells play very important roles in the maintenance of corneal epithelium.

p63, ATP-binding cassette subfamily G member 2 (ABCG2), N-cadherin, K19, NGF receptors (TrkA), and integrin a6 have been reported as candidate markers of limbal stem cells. However, a specific marker has not yet been identified [4–9].

The palisades of Vogt are distinctive normal features of the human corneoscleral limbus [10] (Figure 3). They are more discrete in younger and more heavily pigmented

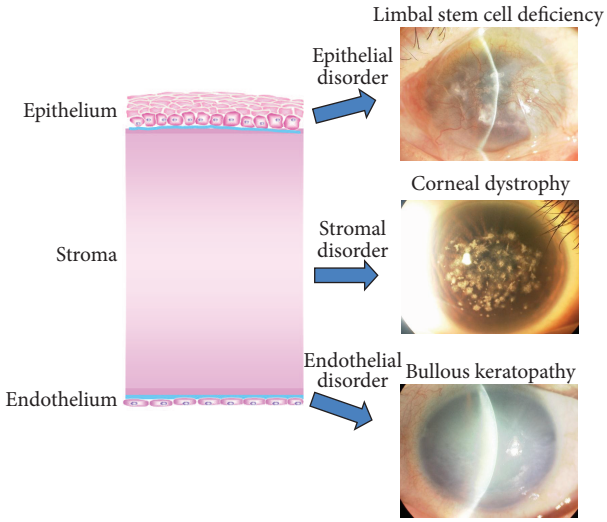


Figure 1: The structure and disorders of the cornea. The cornea consists of three layers: epithelium, stroma, and endothelium. Visual acuity can be affected by disorders of any of these layers, including limbal stem cell deficiency, corneal dystrophy, and bullous keratopathy.

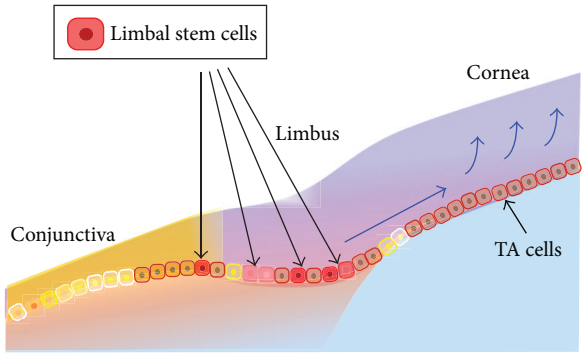


Figure 2: Limbal stem cells. Limbal stem cells are believed to be located within the basal layer of the limbus. Transient amplifying (TA) cells are progenitor cells that differentiate from limbal stem cells and then migrate into the central cornea.

individuals, and they appear more regular and prominent at the lower limbus than at the upper limbus. They are observed infrequently along the horizontal meridian.

Lately, a new phenomenon, "limbal epithelial crypts (LEC)," has been reported as a putative limbal stem cell niche [11, 12]. Cells within LEC have the phenotype of CK3−/CK19+/CD34−/Vimentin+/p63+/Connexin43+/MIB (Ki67)−.

3. Limbal Stem Cell Deficiency

If limbal stem cells are completely absent, vascularized conjunctival epithelium invades into the cornea. The condition is called limbal stem cell deficiency (LSCD) (Figure 4). It results in a corneal neovascularization and opacification that disturbs visual acuity. The causative diseases can be classified

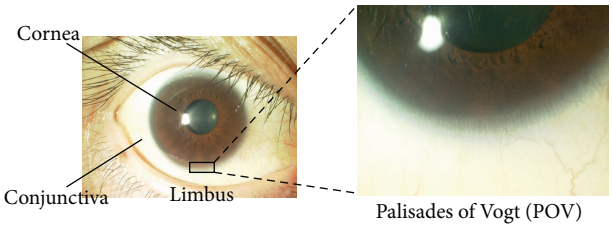


Figure 3: Palisades of Vogt (POV). A slit lamp examination reveals the limbus-specific feature, the "palisades of Vogt" (POV).

into four groups: (1) congenital diseases caused by congenital aplasia of stem cells (e.g., aniridia, sclerocornea); (2) diseases with an external cause, involving the loss of stem cells due to trauma (e.g., thermal, alkali, and acid burns); (3) diseases involving internal, stem cell exhaustion, such as Stevens-Johnson syndrome and ocular cicatricial pemphigoid; and (4) idiopathic diseases of unknown cause [13].

4. Limbal Transplantation

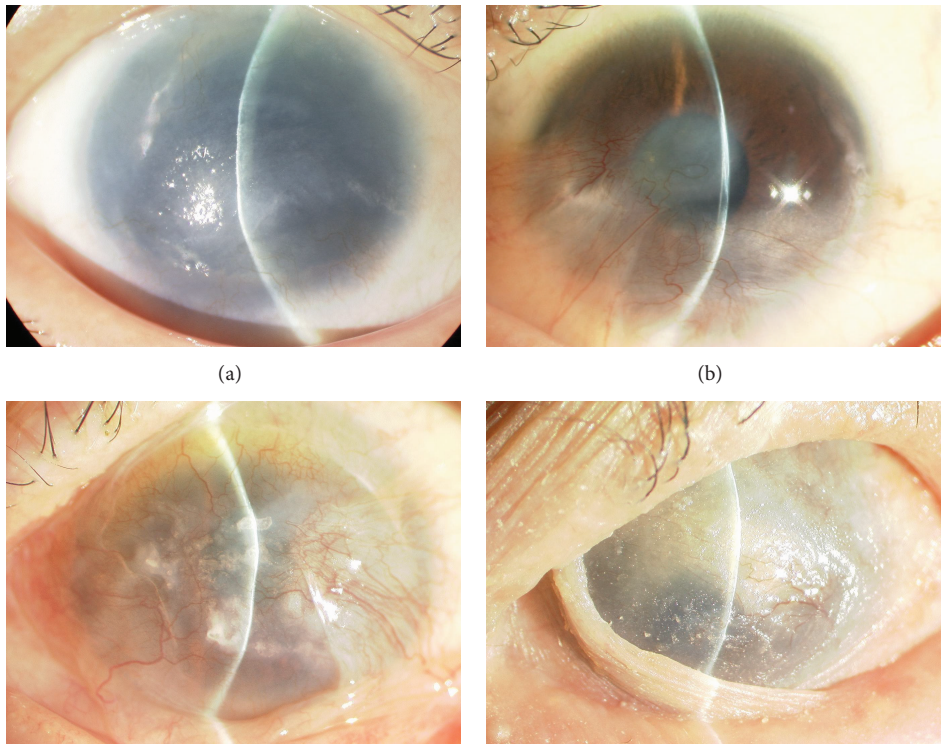
In patients with unilateral limbal stem cell deficiency, autologous limbal transplantation can be used to achieve surface reconstruction of the cornea [14]. However, this procedure requires a large limbal graft from the healthy eye (incurring a risk of causing limbal stem cell deficiency in the healthy eye [15]) and is not applicable to bilaterally affected patients [16]. Limbal-allograft transplantation can be performed in patients with unilateral or bilateral deficiencies [17, 18]. However, it has two main problems: postoperative complications and a donor shortage.

Postoperative complications include rejection and bacterial or fungal keratitis [19–22]. Limbal transplantation requires long-term immunosuppression, which involves high risks of serious eye and systemic complications, including infection and liver and kidney dysfunction. Even with immunosuppression, graft failure is common in patients with Stevens-Johnson syndrome or ocular pemphigoid due to serious preoperative conditions, such as persistent inflammation of the ocular surface, abnormal epithelial differentiation of the ocular surface, severe dry eye, and lid-related abnormalities.

Donor shortage is also a major problem in many countries, including Japan. The Japan Eye Bank Association reported that the number of patients waiting for keratoplasty was 2,286 but that the number of donors was 891 in Japan in 2012.

5. Regenerative Medicine and Tissue Engineering

Langer and Vacanti established the field of "tissue engineering" [23, 24]. Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function. This technology has been applied to various tissues and organs, including the



(a) (b) (c) (d)

Figure 4: Slit lamp photographs of patients with a limbal stem cell deficiency. (a) Aniridia; (b) alkali burn; (c) ocular cicatricial pemphigoid; (d): Stevens-Johnson syndrome.

skin, cornea, cartilage, and heart [25–34]. Enormous promise has been ascribed to this technology because it offers a paradigm shift from conventional organ transplantation to a novel treatment strategy that relies on cultured stem cells. Furthermore, this technique could potentially solve the two main problems of corneal transplantation.

6. Cultivated Limbal Epithelial Cell Transplantation (CLET)

Autologous cultivated limbal epithelial cell transplantation was initially reported by Pellegrini and colleagues [26]. They reported that two patients with LSCD caused by alkali burns were restored using autologous cultivated corneal epithelium, and the outcome persisted for more than two years after grafting. This report is the first of a clinical application in the field of regenerative medicine for the cornea.

Following this report, many investigators have reported the effectiveness and safety of CLET [35–39]. The amniotic membrane and fibrin glue have been mainly used as substrates for cultivated cells. The amniotic membrane has been used as a natural substrate because it can expand into the stem cell niche [40]. Various cytokines released from the amniotic membrane, such as epidermal growth factor, keratinocyte growth factor, hepatocyte growth factor, nerve growth factor, and basic fibroblast growth factor, have been reported to play important roles within the niche of limbal stem cells. The amniotic basement membrane offers a basement membrane for corneal epithelial cell adhesion.

Rama et al. recently reported long-term corneal regeneration using autologous cultivated limbal stem cells [29]. They showed that permanent restoration and a renewal of the corneal epithelium were achieved in 76.6% of 107 eyes with LSCD caused by chemical and thermal burns, and the success of the ocular surface reconstruction was significantly associated with the percentage of p63-bright, holoclone-forming stem cells in culture. No severe adverse events were observed. Their results demonstrated the effectiveness and safety of CLET and the importance of the stem cell population within the cultured cells.

7. Cultivated Oral Mucosal Epithelial Cell Transplantation (COMET)

Although CLET can be applied to patients with unilateral LSCD, it cannot be applied to patients with bilateral disease because they have completely lost their own limbal stem cells as a cell source. Consequently, autologous cultivated oral mucosal epithelial cell transplantation (COMET) has been developed for patients with bilateral LSCD [28, 41]. Even in unilateral cases, some patients wish to avoid tampering with the limbus of their unaffected eye.

We use a temperature-responsive culture dish to fabricate oral mucosal cells [42]. Cell sheets are cultivated on the dishes coated with a temperature-responsive polymer, poly(N-isopropylacrylamide) (PIPAAm), which is hydrophobic below 32°C and hydrophilic above 32°C (Figure 5).

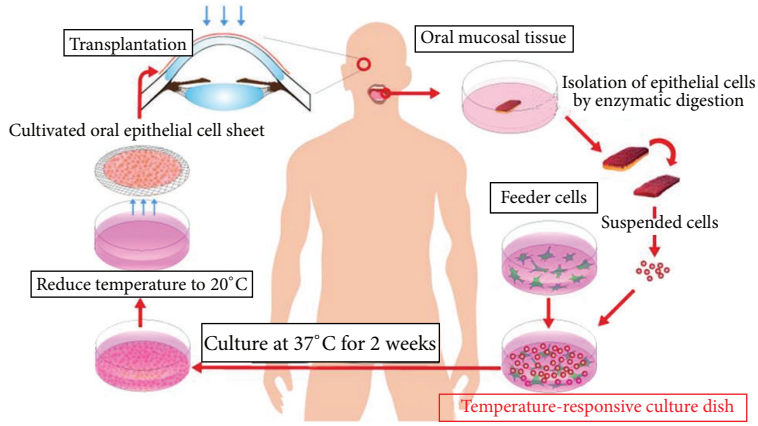


Figure 5: Ocular surface reconstruction via the autologous transplantation of tissue-engineered cell sheets fabricated from oral mucosal epithelial cells. Oral mucosal tissue containing whole epithelial cell layers was excised from the oral cavities of the patient. The cells were then seeded onto a temperature-responsive culture dish. The cultured cells were harvested as a cell sheet by reducing the culture temperature. The cells were then transplanted onto the corneal surfaces of the patient.

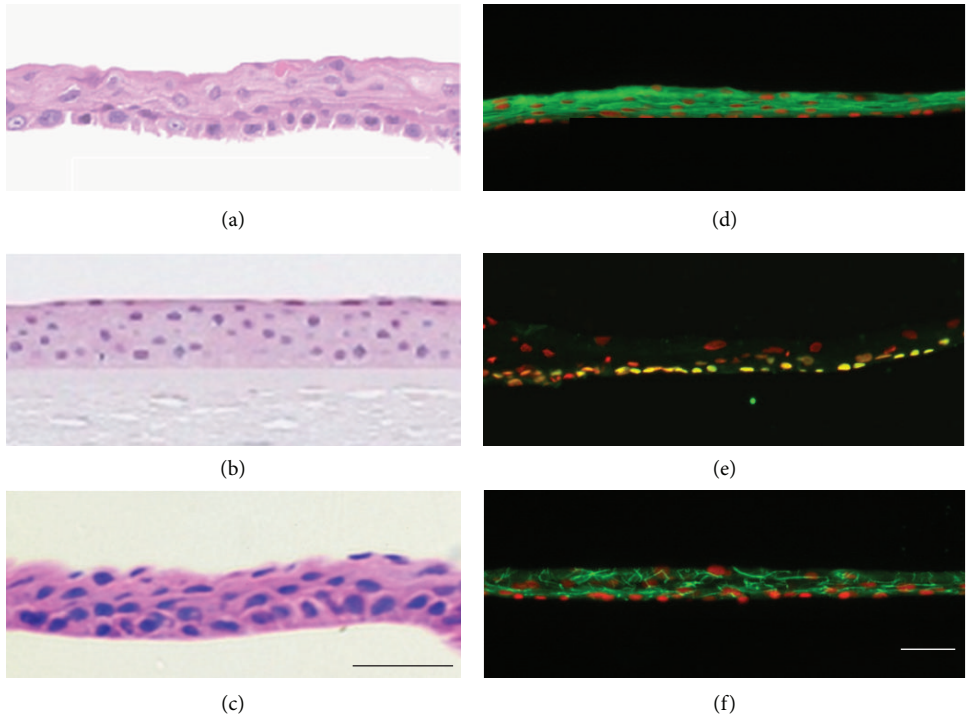
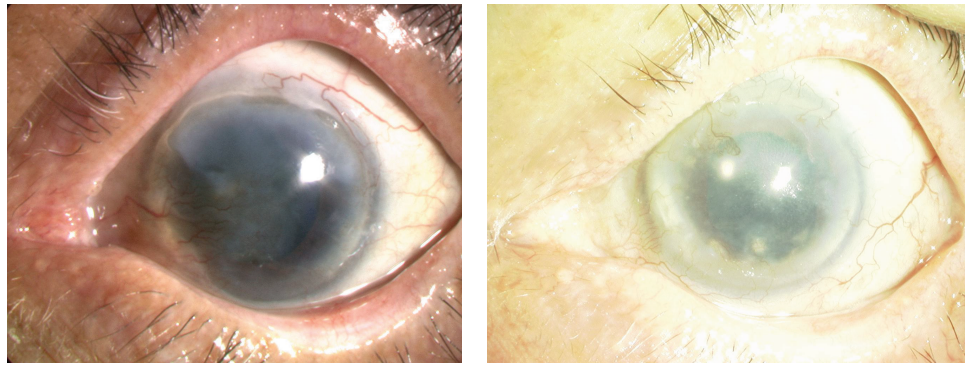


Figure 6: Histological and immunohistochemical analyses of cell sheets. HE staining was performed for an oral mucosal epithelial cell sheet (a), a normal cornea (b), and a corneal epithelial cell sheet (c). Human oral mucosal epithelial cell sheets were stained with antikeratin 3/76 (d), anti-p63 (e), and anti-ZO-1 (f) antibodies. Nuclei were costained with Hoechst 33342. Scale bars: 50 μm.

This change releases the cell sheet, allowing it to be removed without destroying the cell-cell or the cell-extracellular matrix interactions within the cell sheet. Therefore, cultivated oral mucosal epithelial cells can be harvested using a temperature reduction without the use of enzymes.

The cell morphology of an oral mucosal epithelial cell sheet fabricated on a temperature-responsive culture dish is similar to that of the normal cornea or a corneal epithelial cell sheet (Figure 6). It consists of approximately four epithelial layers, flattened superficial cells, and small basal cells with a high C/D ratio. Keratin 3/76, a marker of corneal and oral mucosal epithelium, is positive in the oral mucosal epithelial cell sheet. p63, a putative stem cell marker, is positive for basal cells. ZO-1, a marker of tight junctions, is positive, particularly between superficial cells. The oral mucosal epithelial cell sheet is assumed to have a similar phenotype to that of corneal epithelium as well as enough stem cells and a barrier capability for ocular surface reconstruction. We have already



(a) (b)

Figure 7: Slit lamp photographs of patients before and after cultivated oral mucosal epithelial cell transplantation. (a) The right eye has a total limbal stem cell deficiency caused by ocular cicatricial pemphigoid. The VA is 20/2000. (b) Eight years postoperatively, the corneal epithelial clarity was well maintained. The VA is 20/222 despite macular degeneration.

begun a clinical study using this technique and achieved favorable results (Figure 7) [28].

There has been no report on severe adverse events including development of malignant tumor following COMET. However, if there is a problem, we can easily observe and remove transplanted oral mucosal cells from ocular surface. From that point of view, ocular surface would be the ideal environment for application of this kind of new treatment.

Longstanding survival of transplanted oral mucosal epithelial cells remains unclear. Because autologous oral mucosal cells are used as a cell source, there is no method to distinguish transplanted cells and native cells from host tissue. Even if phenotype of transplanted oral mucosal epithelial cells is maintained, there remains the possibility that host conjunctival cells invaded into cornea and changed the phenotype. However, analyses on phenotype of corneal epithelium excised during keratoplasty following COMET suggest longstanding survival of transplanted oral mucosal epithelial cells. Nakamura et al. reported that phenotype of transplanted cultivated oral epithelial cells (keratin 3[+], Muc5ac[−]) was maintained in clinically successful COMET grafts, and the phenotype was not maintained in failed grafts [43]. Chen et al. showed that all specimens were unanimously positive for K3, -4, and -13 but negative for K8 and MUC5AC, suggesting that the keratinocytes were oral-mucosa-derived [44].

Ohki et al. also applied tissue-engineered oral mucosal epithelial cell sheets to prevent esophageal stricture after endoscopic submucosal dissection [45]. Oral mucosal epithelial cells can be applied for other diseases in the future.

8. Transportation Technique for Regenerative Medicine

The cell sheets must be fabricated in a cell processing center (CPC) under good manufacturing practice (GMP) conditions for clinical use. However, the expenses for a CPC are extremely high, and it is impossible for all hospitals to cover the cost. Therefore, multiple hospitals should share one CPC to standardize and spread regenerative therapy using tissue-engineered oral mucosal epithelial cell sheets. Therefore, the development of cell transportation techniques is necessary for bridging many hospitals.

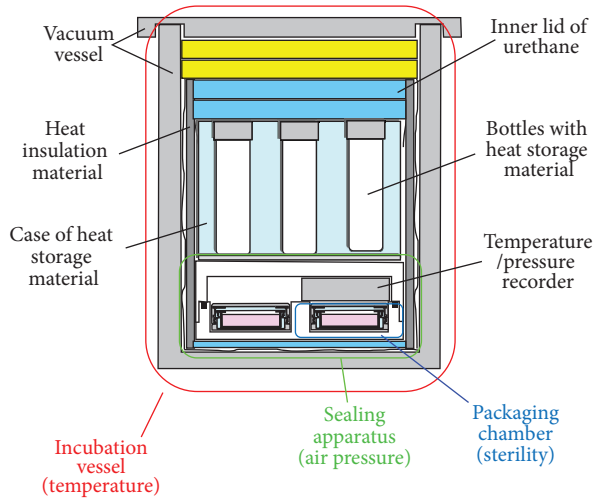


Figure 8: Cross-sectional view of a cell transportation container for cell sheets, consisting of an incubation vessel for temperature, a sealing apparatus for air pressure, and four packaging chambers for sterility. Bottles with heat storage material are set inside the incubation vessel.

We developed a transportation container with three basic functions: maintaining a constant interior temperature, air pressure, and sterility (Figure 8) [46]. The interior temperature and air pressure were monitored by a sensor. Human oral mucosal epithelial cells obtained from two healthy volunteers were cultured on temperature-responsive culture dishes. The epithelial cell sheets were transported via airplane between Osaka University and Tohoku University using the developed cell transportation container. Histological and immunohistochemical analyses and flow cytometric analyses for cell viability and cell purity were performed on the cell sheets before and 12 h after transportation to determine the

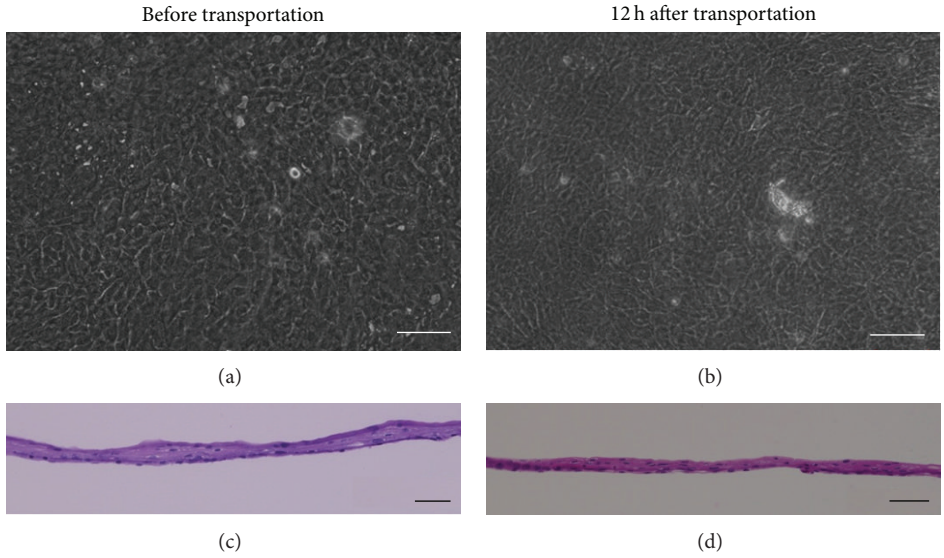


FIGURE 9: Human oral mucosal epithelial cell sheets before and 12 h after transportation. The cell morphology was examined using phase-contrast microscopy (a, b) and HE staining (c, d). Scale bars: 100 μm (a, b), 50 μm (c, d).

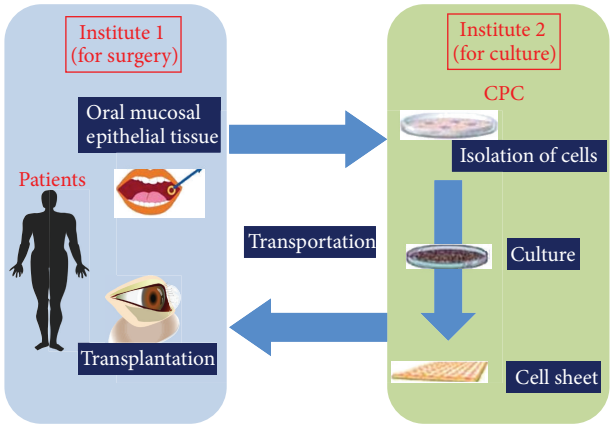


FIGURE 10: Multicenter clinical study of a cell sheet transportation system. Oral surgery for mucosal tissue and cell sheet transplantation are performed at institute 1; the cell sheets are cultured in the CPC at institute 2.

effect of transportation on the cell sheets. Sterility tests and screening for endotoxins and mycoplasma in the cell sheets were performed before and after transportation.

During transportation via airplane, the temperature inside the container was maintained above 32°C, and the air pressure did not fluctuate more than 10 hPa. The cell sheets were well stratified and successfully harvested before and after transportation (Figure 9). The expression patterns of keratin 3/76, p63, ZO-1, and MUC16 remained consistent before and after transportation. The cell viability was 72.0% before transportation and 77.3% after transportation. The epithelial purity was 94.6% before transportation and 87.9% after transportation. Sterility tests and screening for endotoxins and mycoplasma were negative for all cell sheets.

We are conducting a multicenter clinical study using the transportation technique described herein (Figure 10). In this clinical study, we will harvest oral mucosal epithelial tissues from patients at institute 1 and transport them to institute 2. At institute 2, a cell sheet will be fabricated at the CPC. Next, the cell sheet will be sent to institute 1 for transplantation. We will culture oral mucosal epithelial cells from two other institutes in Osaka University. Culturing at a single CPC enables better control of the quality of the tissue-engineered human oral mucosal epithelial cell sheet. If this effort is successful, we will be able to treat many patients in many hospitals all over the world without the need for a CPC.

The newly developed transportation technique for air travel is an essential technology for regenerative medicine and promotes the standardization and spread of regenerative therapies.

9. Conclusion

Many researchers have engaged in basic and clinical study in the fields of stem cell and regenerative medicine for the cornea. However, there are no government-approved tissue-engineered medical products for the cornea so far. Although first-in human clinical study using a novel cell source or technology is both sensational and impressive, efforts to turn this established technique into a standardized therapy should be a continuous process. We are struggling to make oral mucosal epithelial cell sheets an approved medical device. We believe that, once approved, this medical product will help visually impaired patients all over the world and that this goal can be achieved in the near future.

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Review Article

Present and Future Perspectives on Cell Sheet-Based Myocardial Regeneration Therapy

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Heart failure is a life-threatening disorder worldwide and many papers reported about myocardial regeneration through surgical method induced by LVAD, cellular cardiomyoplasty (cell injection), tissue cardiomyoplasty (bioengineered cardiac graft implantation), *in situ* engineering (scaffold implantation), and LV restrictive devices. Some of these innovated technologies have been introduced to clinical settings. Especially, cell sheet technology has been developed and has already been introduced to clinical situation. As the first step in development of cell sheet, neonatal cardiomyocyte sheets were established and these sheets showed electrical and histological homogeneous heart-like tissue with contractile ability *in vitro* and worked as functional heart muscle which has electrical communication with recipient myocardium in small animal heart failure model. Next, as a preclinical study, noncontractile myoblast sheets have been established and these sheets have proved to secrete multiple cytokines such as HGF or VEGF *in vitro* study. Moreover, *in vivo* studies using large and small animal heart failure model have been done and myoblast sheets could improve diastolic and systolic performance by cytokine paracrine effect such as angiogenesis, antifibrosis, and stem cell migration. Recently evidenced by these preclinical results, clinical trials using autologous myoblast sheets have been started in ICM and DCM patients and some patients showed LV reverse remodelling, improved symptoms, and exercise tolerance. Recent works demonstrated that iPS cell-derived cardiomyocyte sheet were developed and showed electrical and microstructural homogeneity of heart tissue *in vitro*, leading to the establishment of proof of concept in small and large animal heart failure model.

1. Introduction

Therapeutic treatments using cells or cell-based tissues have been developed to regenerate the damaged myocardium associated with ischemic heart disease. This technique has already been evaluated in the clinical setting, using myoblasts [1] or bone marrow mononuclear cells (BM-MNCs) [2]. Although these studies demonstrated the feasibility and safety of this approach, the efficacy associated with this technology was generally insufficient to repair severe myocardial damage. Thus, a second generation of myocardial regenerative therapy, tissue-engineered cardiomyoplasty, is currently being developed. A large number of achievements concerning basic, preclinical, and clinical works about cell sheet technology have been done and this review summarizes recent advances in myocardial regeneration emerging from the development of cell sheet technology.

2. Development of Cell Sheet Technology

Cell-sheet techniques have been applied to several diseased organs, such as the heart [3], eye [4], and kidney [5], in the laboratory and the clinic. Cell sheets can be prepared on special dishes that are coated with a temperature-responsive polymer, poly(N-isopropylacrylamide) (PIPAAm), that changes from being hydrophobic to hydrophilic when the temperature is lowered. This change allows cells to be removed without EDTA or enzymatic treatment and without destroying the cell-cell or cell-extracellular matrix (ECM) interactions within the cell sheet.

Shimizu et al. used such temperature-sensitive culture dishes to develop a contractile chick cardiomyocyte sheet that exhibited a recognizable heart tissue-like structure and showed electrical pulsatile amplitude [6]. Next, they layered single-cell sheets to generate bilayer-cell sheets, forming an electrically communicative three-dimensional cardiac construct, which exhibited spontaneous and synchronous pulsation with electrical communication between the cell sheets, mediated by connexin 43. Furthermore, the cell sheets adhered together rapidly, as indicated by the presence of desmosomes and intercalated disks between them [7]. When the pulsatile cardiac tissue was implanted subcutaneously, it was found to assume a heart tissue-like structure and exhibited neovascularization and spontaneous beating for up to one year. The size, conduction velocity, and contractile force of the engrafted sheets increased in proportion to the host growth [8, 9].

Miyagawa et al. demonstrated that a neonatal cardiomyocyte sheet could communicate electrically with the host myocardium, as indicated by the presence of connexin 43, and changes in the QRS wave and action potential amplitude, leading to improved cardiac performance in a rat model of ischemic heart disease [3]. This study clearly showed electrical and morphological coupling between the cell sheet and host myocardium and that the cell sheet could contract synchronously with the beating of the host heart and improve the regional systolic function.

A detailed analysis of the vascularization process following cell sheet implantation was undertaken by Sekiya et al. These authors reported that the cardiomyocyte sheet expresses angiogenesis-associated genes and forms an endothelial cell network. Evidence was also presented suggesting that the vessels arising in the engrafted sheet migrate to connect with the host vasculature [10].

Myocardial tissue grafts engineered with cell sheet technology represent a promising therapy for repairing the damaged myocardium, but there may be some inherent limitations. For example, cellular treatment for heart failure may be not suitable for emergency situations. Another issue is that wide therapeutic use will require improvement in the uniformity in the quality of the cultured cells.

Recently, new medications that imitate the paracrine effects of cytokines in cell sheets have been reported, and the addition of such medications could improve the regenerative treatment for heart failure. It was reported that the direct introduction of a prostacyclin agonist into the damaged myocardium induced significant functional recovery in a canine model of dilated cardiomyopathy, via the upregulation of multiple cytokines, including HGF, VEGF, and SDF-1 [11]. Similarly, the implantation of an atelocollagen sheet containing a prostacyclin analogue induced improved cardiac function and a prolonged survival rate in a mouse model of acute myocardial infarction, accompanied by an enhanced expression of SDF-1 [12]. Recent work has also revealed that prostacyclin may be upregulated in the implanted myoblast sheet in the early phases after implantation in response to ischemic conditions and may in turn stimulate endothelial or smooth muscle cells to secrete multiple cytokines including HGF, VEGF, and SDF-1 (data not shown).

3. Experimental and Clinical Work on Myoblast Sheets

In the clinical setting, cellular cardiomyoplasty is reported to have potential regenerative capability, and a method using skeletal myoblasts has been evaluated in clinical trials and found to be relatively feasible and safe [13]. For tissue cardiomyoplasty, skeletal myoblasts are the cell source closest to being ready for clinical application at this time. Memon et al. demonstrated that the nonligature implantation of a skeletal myoblast sheet into a rat cardiac ligation model regenerated the damaged myocardium and improved global cardiac function, by attenuating cardiac remodeling via hematopoietic stem-cell recruitment and growth-factor release, with better restoration of the implanted cells than that obtained using needle injection [14]. In another study, the application of a skeletal myoblast sheet into a 27-week dilated cardiomyopathy hamster model resulted in the attenuated deterioration of cardiac performance accompanied by the preservation of alpha-sarcoglycan and beta-sarcoglycan expression in the host myocytes, and an inhibition of fibrosis, leading to prolonged survival rates [15]. In addition, the grafting of skeletal myoblast sheets attenuated cardiac remodeling and improved cardiac performance in a pacing-induced canine heart failure model [16]. Studies from our group have shown that myoblast sheets may improve cardiac performance via cytokines such as HGF or VEGF (XX).

The mechanism of recovery in the damaged myocardium has not been completely elucidated and may be very complicated. As mentioned above, cytokine release and hematopoietic stem-cell recruitment are possible mechanisms of regeneration; however, other regenerative mechanisms are likely to be involved as well. Skeletal myoblasts cannot beat synchronously with the host myocardium *in vitro* [17] or *in vivo* [18], and, thus, they do not appear to be functionally integrated. However, data from our human and porcine studies suggested that after myoblast sheet implantation, the diastolic dysfunction in the distressed region of the myocardium was significantly recovered compared with controls, leading to improved systolic function in the same region, without contraction of the implanted myoblasts (data not shown). Massive angiogenesis in the implanted region was detected histologically and appeared to be a critical feature associated with the improvement. Thus, we speculate that angiogenesis and the recovery of diastolic function are both major components of the regenerative mechanism in myoblast sheet implantation [19].

On the other hand, immunohistochemical analysis has indicated that the myoblast sheet may only survive for a few months after implantation. We speculate that in the early phases after implantation of the myoblast sheet, the ischemic conditions induce the upregulated expression of several cytokines by the myoblasts that promote their own survival. These cytokines then in turn enhance angiogenesis and the recruitment of stem cells, leading to improved blood perfusion to reactivate the damaged myocardium. The system may continue to be effective in spite of the short-lived myoblast sheet, due to long-term maintenance of the newly developed vasculature.

We recently initiated a clinical evaluation of autologous myoblast sheet implantation. We tested the technology in four patients who were using left ventricular assist devises (LVADs); three of the four patients showed functional recovery, and in two of the patients, the treatment provided a bridge to recovery [20]. Six years later, these two patients have no symptoms of heart failure. We have also implanted myoblast sheets into eight patients with ischemic cardiomyopathy and seven with dilated cardiomyopathy (who were not using LVADs). In that study, some of the patients exhibited left ventricle reverse remodeling and improvements in exercise tolerance and symptoms, with no major adverse cardiac events (MACEs) (data not shown). This clinical research program is ongoing, as we continue to evaluate patients with dilated cardiomyopathy and ischemic cardiomyopathy with and without the use of LVADs.

4. Other Types of Cell Sheets

In addition to cardiomyocytes and myoblasts, other types of cell sheets have been used effectively to improve cardiac performance. The transplantation of a mesenchymal stem cell (MSC) sheet onto the infarcted myocardium of rats resulted in increased anterior wall thickness and new vessel formation, accompanied by a low incidence of differentiation of the implanted cells to cardiomyocytes [21]. While the small number of differentiated cardiomyocytes may not have contributed to the observed improvement in systolic function in this study, the cell sheet exhibited self-propagating properties that promoted the generation of a thick-layered sheet. Although the MSC sheet exhibited a maximum thickness of approximately 600 μm, which would not be strong enough to correct human end-stage heart failure [22], this method of self-propagation is a potential strategy for creating a thick-layered sheet *in vivo*, with the potential for cardiac tissue regeneration.

A further development in cell sheet technology is the creation of a cell sheet composed of two types of cocultured cells; this type of cell sheet was developed to enhance angiogenesis [23, 24]. The cocultured cell sheet, which combined fibroblasts and endothelial progenitor cells, enhanced blood vessel formation and led to functional improvement in a rat myocardial infarction model [24]. Cocultured cell sheets combining fibroblasts and human smooth muscle cells were found to accelerate the secretion of angiogenic factors *in vitro* and to increase blood perfusion *in vivo* by the formation of new vessels [25]. This enhanced effectiveness attained by coculturing two cell types is supported by another study in which the coimplantation of BMCs and myoblasts showed improved results compared to the transplantation of a single cell type in a canine model of ischemic cardiomyopathy [26].

Cell sheets composed of stem cell antigen-1- (sca-1-) positive, or kit-positive cells may represent additional promising approaches. Matsuura et al. demonstrated that sca-1-positive cell sheets could differentiate into cardiomyocytes *in vivo* and produce VCAM-1, leading to improved cardiac performance in a mouse model of myocardial infarction [27]. The administration of c-kit-positive stem cells has shown efficacy in animal models of cardiac dysfunction, and this approach is currently being tested in clinical trials in combination with coronary artery bypass grafting, with encouraging preliminary results [28]. In another study, a c-kit-positive cell sheet combined with endothelial progenitor cell injection was found to induce better functional recovery of endocardial scar tissue than that induced by the cell sheet alone, despite the poor transdifferentiation ability of the c-kit-positive cells into cardiomyocytes [29].

Many of the cell sources mentioned above demonstrate regenerative ability based on the paracrine effect of secreted cytokines; however, newly differentiated cardiomyocytes may be the best candidate cells to regenerate the damaged myocardium. In 2006, Takahashi and Yamanaka reported the development of induced pluripotent stem (iPS) cells that can differentiate into various types of cells, such as cardiomyocytes, cartilage, and nerve cells [30]. Since then, there have been many reports showing that cardiomyocytes derived from iPS cells demonstrate electrophysiological, functional, and microstructural similarities to native cardiomyocytes [31]. Cardiomyocyte sheets derived from human or mouse iPS cells that contract synchronously *in vitro* have been developed, and studies indicate that these cardiomyocyte sheets can contract *in vivo* as analyzed by X-ray diffraction with synchrotron radiation. The transplantation of these sheets leads to functional recovery with upregulated electrical potential in the scarred areas in large [32] and small animal myocardial infarction models [33].

Although preclinical studies appear promising, the safety of these artificially generated cells must be evaluated thoroughly before they can be used in the clinic. In addition, a potential limitation of iPS cell-derived cardiomyocytes may be the loss of cardiomyocytes due to ischemia after implantation. Recent studies have proposed supplemental strategies to avoid ischemia. In one study, the combination of an iPS-derived cardiomyocyte sheet with omentum, which has a rich vasculature network, resulted in retention of the implanted cardiomyocytes and enhanced functional recovery compared with the cardiomyocyte sheet alone [34]. In another study, the transplantation of a cardiomyocyte sheet containing iPS cell-derived endothelial cells led to enhanced functional recovery in a rat myocardial infarction model and increased survival of the implanted cardiomyocytes [35]. Thus, to successfully treat the severely damaged myocardium using iPS cell-derived cardiomyocyte sheets, additional strategies to increase angiogenesis and reduce ischemia may be required.

5. Advantages of Cell Sheet Technology

Studies on the original myoblast cell therapy, in which cells were directly injected into the myocardium, indicated that the proportion of injected cells surviving to engraft the infarcted myocardium was too low to be effective. This low level of engraftment may have been caused by the injected cells leaking out of the injected region and being carried to other organs, or due to mechanical stress resulting in cellular loss of function. The resulting rapid cell loss [14] limited the usefulness of the original myoblast cell therapy.

To overcome the problems associated with the intramyocardial injection of cells, many investigators have combined cell transplantation with protein or gene therapy [36], or with tissue-engineered techniques [3]. We have also developed a new cell delivery system that uses tissue-engineered myoblast grafts grown as cell sheets and have utilized animal studies to guide clinical trials. These studies showed that the viability of the transplanted cells was higher than that of injected cells, and that the transplanted myoblasts survived for at least 3 months in the cardiac tissue of a porcine model of heart failure treated with autologous myoblast sheets. Using tissue-engineered temperature responsive techniques, we found that the implanted cells could be applied in larger numbers, were viable during transplantation, and were not lost from the applied region. Furthermore, we showed that cell sheets could be engrafted onto the failed myocardium and contribute to the attenuation of cardiac dysfunction and remodeling [14].

In cell therapy for cardiac disease, life-threatening adverse events involving arrhythmogenicity are a potential risk in both animal models and human clinical trials [37]; however, life-threatening arrhythmias have not been observed during the clinical course of patients who have received autologous cell sheet transplants. In any case, arrhythmias can occur during the natural clinical course of severe heart failure, so their cause may not be easily determined. Procedures using needle injection may cause scars in the myocardium that could in turn induce arrhythmias. Our cell delivery techniques using cell sheets prepared on temperature-responsive culture dishes may carry less risk for the induction of arrhythmias. Myoblasts have a weak electrical potential, and it may be possible for these cells to induce arrhythmia if they survive in the myocardium. However, cell sheets may not be able to induce arrhythmia, since they are attached to the epicardium.

Another potential problem is the limited blood perfusion to the implanted cell sheets. Although the survival of implanted cells using the cell sheet technique has already been shown to exceed the cell survival using other delivery routes, the survival rate was still found to be relatively low when the cells were implanted on the epicardium with this technique [38]. Although we have reported that improved cardiac performance depends on the dose of implanted myoblast sheets, the use of too many cell sheets results in a reduced blood supply. Thus, additional strategies, such as combining myoblasts with angiogenic factors [36] or other types of cells [23] to establish a vasculature network, may be needed to solve this problem. One strategy discussed above, is the combination of a myoblast sheet with omentum tissue that has a rich vasculature network. One report recently demonstrated the effectiveness of this approach for retention of the implanted cell sheets [39]. This report also suggested that the implanted myoblast sheet might induce vasculature connections between arteries of the transplanted omentum and the native coronary arteries, suggesting the possibility of biocoronary artery bypass grafting. This method may also be used in conjunction with iPS cell-derived cardiomyocytes to generate an artificial thick cardiac structure with increased vascular connections.

6. Conclusions

In this review, we surveyed many exciting topics in the area of cell sheet technology for cardiac repair. Owing to these studies, some techniques have already been tested in clinical applications, but the mechanisms by which they improve cardiac function are only partially understood, and much of the technology is still in the early stages of development, both experimentally and in the clinic. Nevertheless, the field of clinical myocardial regenerative therapy holds much promise, and we expect to witness more progress in this innovative technology in the near future.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Virosome Presents Multimodel Cancer Therapy without Viral Replication

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A virosome is an artificial envelope that includes viral surface proteins and lacks the ability to produce progeny virus. Virosomes are able to introduce an encapsulated macromolecule into the cytoplasm of cells using their viral envelope fusion ability. Moreover, virus-derived factors have an adjuvant effect for immune stimulation. Therefore, many virosomes have been utilized as drug delivery vectors and adjuvants for cancer therapy. This paper introduces the application of virosomes for cancer treatment. In Particular, we focus on virosomes derived from the influenza and Sendai viruses which have been widely used for cancer therapy. Influenza virosomes have been mainly applied as drug delivery vectors and adjuvants. By contrast, the Sendai virosomes have been mainly applied as anticancer immune activators and apoptosis inducers.

1. Introduction

Currently, general cancer therapies include surgery, chemotherapy, and radiation therapy, but all three have limitations. Applications of surgical and radiation therapy are limited to localized cancer. Chemotherapy is used for a wide range of cancers, including distant metastases, via the systemic administration of anti-cancer drugs; however, it also kills normal cells and induces severe side effects. Therefore, many groups are investigating ways to improve conventional treatments and to develop novel treatments for more effective cancer elimination with fewer side effects.

In recent years, much attention has been paid to cancer immunotherapy, which stimulates anti-cancer immunity, and several cancer immunotherapy systems (Provenge, Ipilimumab and anti-PD1 antibody) have been developed [1–5]. When anti-cancer immunity is systemically activated, it is expected that the primary cancer cells and distant metastases will be eliminated by immune cells. Various tumor-associated antigens (TAAs) have been identified [6–9], for example, HER2/nu, CEA, MAGE, and WT1. TAAs are expressed in cancer cells and are targeted by immune cells, especially cytotoxic T lymphocytes (CTL) [10–13]. Therefore, immunostimulation by TAAs can be applied to cancer immunotherapy. To activate anti-cancer immunity by TAAs, fragments of TAAs should be presented on antigen-presenting cells (APCs) by forming a complex with major histocompatibility complex class I (MHC-I) and II molecules [14]. Generally, cytoplasmic foreign proteins, such as viral proteins expressed in the cytoplasm during viral infection, complex with MHC-I and stimulate $CD8^+$ T cells (CTLs) [14, 15]. However, endocytosed foreign proteins also complex with MHC-II and stimulate $CD4^+$ T cells [14, 16]. Moreover, APCs have a cross-presentation system that presents endocytosed foreign proteins with MHC-I to activate CTLs [17]. Previous reports have shown that the administration of TAA alone does not induce an effective CTL response [18]. Therefore, it is believed that an endocytosed antigen is not sufficient for the activation of MHC-I-restricted CTLs, and, to activate an effective CTL response by TAAs, they should be introduced to the cytoplasm directly.

A new technology, gene therapy, has been developed and applied to cancer treatment. Various cancer gene therapy methods have been reported, such as adoptive immunotherapy using *ex vivo* gene transfer to immune cells [19], intratumoral injection of cytokine genes [20], suicide gene therapy using the herpes virus thymidine kinase gene [21], and intratumoral injection of the p53 gene [22]. To achieve

high gene expression, viral vectors such as retrovirus and adenovirus vectors have been utilized. However, in general, cancer gene therapy has not had satisfactory therapeutic effects. Therefore, to enhance the cancer-cell-killing effect, viruses that replicate mainly in cancer cells have been used for treatment [23]. Various types of oncolytic viruses have been developed by isolating viruses with inherent tumor selectivity [24, 25] and by engineering recombinant viruses [26, 27]. Furthermore, the combination of an oncolytic virus and gene therapy has been applied for cancer treatment, such as vaccinia virus including the GM-CSF gene [28]. Although these oncolytic viral treatments exhibited a strong therapeutic effect, safety might be a problem because the virus with an intact genome still exists in noncancerous cells [29].

An inactive virus that did not have the ability to amplify its progeny virus in host cells has also been used as a high-safety delivery vector for drugs and plasmids in cancer therapy. In particular, enveloped-virus-derived vectors have attracted attention because enveloped-vector-delivered molecules can escape endosomal degradation by direct introduction to the cytoplasm via membrane fusion [30]. A vector derived from an inactive enveloped virus is called a virosome, which is now an all-inclusive term for a reconstituted envelope that contains viral envelope proteins (Figure 1(a)) or viral envelope particles (Figure 1(b)) [31]. Several types of virosomes have been generated, for example, virosomes based on influenza virus [32], hepatitis B virus [33], human immunodeficiency virus [34], Newcastle disease virus [35], and Sendai virus [36, 37]. In many studies, virosomes have been used as vectors for drug delivery, with the inclusion of various therapeutic molecules, such as DNA, RNA, proteins, and drugs [38, 39]. Moreover, virosomes function as adjuvants to induce the activation of the immune system [40]; therefore, many groups are studying virosomes as tools for cancer therapy.

In this review, we introduce the previous research on virosomes, especially virosomes derived from the influenza (influenza virosome) and Sendai viruses (Sendai virosome) for the use in cancer therapy. The influenza virosome has been applied mainly as a delivery vector for TAAs and TAA-expressing plasmids. Sendai virosomes have been used as anti-cancer immune activators and apoptosis inducers.

2. Influenza Virosomes

Influenza virus is an Orthomyxovirus that has a nucleocapsid with a segmented single-stranded RNA genome and is covered with a viral envelope [41, 42]. Two types of membrane proteins, hemagglutinin (HA) and neuraminidase (NA), are present on the surface of the envelope. HA binds to sialic acid, which is its receptor, on the surface of host cells and is used for the adhesion of viral particles [43]. HA is responsible for membrane fusion of the viral envelope with the host cell membrane [44]. However, HA does not induce membrane fusion in neutral conditions, and it acquires its fusion activity through conformational change in acidic conditions [45, 46]. Viral particles are taken into the endosomes of host cells by endocytosis after HN-receptor binding, thereby exposing the particles to acidic conditions. Next, membrane fusion of the viral envelope with the endosomal membrane is induced by the conformational change of HA, and the viral genome is induced into the cytoplasm of host cells.

An influenza virosome is an artificial liposome that includes influenza membrane proteins [31] and is prepared by reconstituting influenza virus surface proteins and phospholipids [47]. The influenza viral envelope is first collapsed to phospholipids by the treatment with detergent, and the nucleocapsid is eliminated from the mixture. Then, the influenza virosome, including surface proteins and virus-derived phospholipids, is reconstituted from the mixture. An influenza virosome maintains its membrane fusion ability because it has HA on its surface [48]. Therefore, it works as a delivery vector to introduce macromolecules into the cytoplasm by including them in the virosome [38, 49]. Influenza virosomes have powerful immunogenicity. Vaccination with influenza virosomes induces protective levels of influenza-specific antibodies [50], and an influenza virosome is already licensed as an influenza vaccine [51]. Influenza virosomes also exhibit an adjuvant effect when they are coadministered with other antigens [52–54]; therefore, many groups have studied the application of influenza virosomes in the activation of antitumor immunity.

2.1. CTL Activation by Plasmid DNA Encapsulation in Influenza Virosomes. Correale et al. reported that TAA-specific CTLs were induced by the administration of an influenza virosome containing TAA plasmids in mice [55]. In this study, a plasmid expressing parathyroid hormone-related peptide (PTH-rP), which is a TAA expressed in prostate and spinocellular lung carcinomas, was included in an influenza virosome, which was administered intranasally. As a result, PTH-rP-specific CTL activity was significantly induced in mice, and this activity was also shown in human PBMCs activated by human DCs treated with the PTH-rP virosome. In addition, Cusi et al. demonstrated that TAA-specific CTLs were enhanced by the stimulation with an influenza virosome containing a CD40L-expressing plasmid [56]. CD40L binds to CD40 on APCs and upregulates the expression of its costimulatory molecules, B7.1 and B7.2, in the cells, which are important factors for the activation and amplification of naïve T cells [57, 58]. In this study, plasmids expressing carcinoembryonic antigen (CEA), which is a marker of colon cancer, and CD40L were encapsulated in influenza virosomes, and these virosomes were administered intranasally. Coadministration of CEA- and CD40L-virosomes resulted in a CEA-specific CTL response that was stronger than that in the CEA-virosome alone, by upregulating B7.1 and B7.2 expression on APCs.

2.2. CTL Activation by Peptide Encapsulation in Influenza Virosomes. Antigen presentation of TAAs by APCs is important for the activation of anti-cancer immunity. To activate CTLs, TAAs should be presented with MHC-I, which complexes with cytoplasmic antigens. Therefore, TAAs should be introduced to the cytoplasm for the effective activation

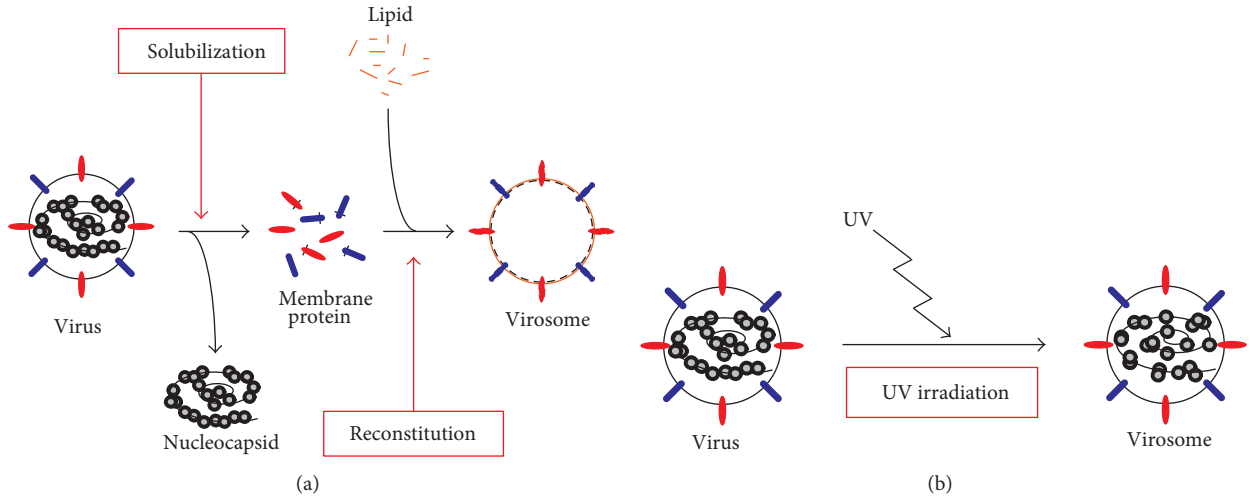

FIGURE 1: Concept of virosomes. (a) Reconstituted envelope containing viral envelope proteins. Viral membrane proteins are solubilized from viral particles, and artificial envelope is reconstituted with the viral proteins and exogenous lipids. (b) Viral envelope particles. Virus is inactivated with UV irradiation leading the fragmentation of viral genome.

of CTLs. Bungener et al. demonstrated influenza virosome-mediated OVA delivery to DCs [59] and that the delivery leads to OVA presentation on MHC-I and -II. Fusion-inactive virosomes presented OVA on MHC-II but not on -I. Therefore, it is suggested that influenza virosomes introduce encapsulated TAAs to the cytoplasm through membrane fusion and that TAA introduction is needed for the presentation of TAAs on MHC-I. Angel et al. reported influenza virosome-mediated delivery of TAAs to DCs [60]. The authors encapsulated the Melan-A peptide, which is a TAA from melanoma, in an influenza virosome and introduced the Melan-A peptide into plasmacytoid DCs (PDCs). Melan-A-containing, virosome-treated PDCs activated CD8 T cells more effectively than did free Melan-A peptide-pulsed PDCs. In addition, Correale et al. reported that PTH-rP-derived peptide (PTR)-4-encapsulated influenza virosomes significantly suppressed tumor growth [61]. In this study, PTR-4/virosome treatment effectively activated CTL activity, and the treatment inhibited the angiogenesis of tumors. The findings therefore suggest a new function of influenza virosomes in cancer therapy.

2.3. Modification of the Influenza Virus. To make influenza virosome-mediated cancer therapy more effective, modifications of the influenza virosome have been attempted. HA has an important function in influenza virosome-mediated delivery and immunostimulation. However, the HA receptor is ubiquitously expressed on nearly all cells. Therefore, the influenza virosome does not have affinity for specific cells. Mastrobattista et al. generated an influenza virosome that could target ovarian carcinoma (OVCAR-3) *in vitro* [62]. They coated influenza virosomes with polyethylene glycol (PEG) to inhibit HA-mediated binding, and then Fab' fragments of antiepithelial glycoprotein-2 (EGP-2) antibody (323/A3) were conjugated to the PEG on the virosomes. 323/A3-PEG-coated influenza virosomes exhibited low HA-mediated binding to sialic acid because of the PEG coating and gained specific binding for EGP-2-expressing ovarian cancer cells by 323/A3 conjugation. As a result, although the binding function of HA was depleted, the 323/A3-PEG virosomes were able to fuse with OVCAR-3 membranes. Because HA induced membrane fusion without binding to its receptor [63], it is thought that the 323/A3-PEG virosomes maintained their membrane fusion ability. Waelti et al. used the same strategy to demonstrate targeted delivery of doxorubicin (Doxo) to HER-2/neu-overexpressing breast cancer cells *in vivo* [64]. In this study, influenza virosomes were coated with anti-Neu mAb Fab' (7.16.4)-conjugated PEG (7.16.4/PEG), and Doxo was encapsulated in the 7.16.4/PEG-virosomes. Intravenous administration of Doxo-containing 7.16.4/PEG-virosomes significantly inhibited subcutaneous Neu+, but not Neu-, breast cancer. Jamali et al. recently reported the enhancement of the efficacy of influenza virosome-mediated delivery *in vitro* by reconstituting the virosome with cationic lipids [65].

As described above, influenza virosomes are useful for the cancer therapy. Recently, phase I clinical trial of influenza virosomes was carried out for the patients with metastatic breast cancer (MBC) [66]. In this trial, MBC patients were intramuscularly administrated influenza virosomes including three individual peptides of the extracellular domain of Her-2/neu protein. The trial tested the safety and Her-2/neu-specific immune responses. As a result, specific antibodies against naïve Her-2/neu protein were detected in serum. IL-2 production was significantly increased and Treg population was significantly decreased in PBMC. Although local erythema at the infection site has appeared in four patients, other serious side effects were not detected. Therefore, there is a possibility that influenza virosomes are used for future cancer therapy.

3. Sendai Virosomes

Sendai virus (hemagglutinating virus of Japan; HVJ) is a paramyxovirus that has a nucleocapsid with a single-stranded RNA genome and is covered with a viral envelope [67]. Two types of glycoproteins, hemagglutinin-neuraminidase (HN) and fusion protein (F), are present on the surface of the viral envelope [68]. HN enables the viral particle to adhere to the host-cell surface by binding to sialic acid [69], and then F induces membrane fusion of the viral envelope with the host-cell membrane [70]. F fuses these membranes under neutral conditions [71]; therefore, HVJ particles do not require uptake into the endosome for membrane fusion.

Previously, HVJ-liposomes were generated via reconstitution from HVJ surface proteins and phospholipids, similar to the influenza virosome [36]. Because HVJ-liposomes have membrane fusion ability, they have been used as a vector for DNA delivery [72]. However, because the membrane fusion efficiency of HVJ-liposomes is not high (approximately 2% of native HVJ) [73], an increase in the fusion activity of the vector is needed.

Kaneda et al. generated a new type of Sendai virosomes called HVJ-envelope (HVJ-E) [37]. HVJ-E is an inactivated HVJ particle that has been irradiated by UV light. The viral RNA genome is cleaved into many fragments; therefore, HVJ-E does not have the ability to produce progeny virus in infected cells. However, HVJ-E maintains its membrane fusion ability, which is dramatically higher than that of HVJ-liposomes [37]. HVJ-E has been used as a vector for plasmid DNA delivery to various cells and tissues [74–76]. In addition, plasmid DNA, anti-cancer drugs, and siRNAs have been delivered by HVJ-E, and there have been reports of cancer therapy using HVJ-E-mediated drug delivery [77, 78].

Cancer suppression by viral infection has also been reported [79]. Since that study, various viruses have been used for cancer therapy, and, in particular, the development of oncolytic viruses has attracted attention [80–83]. Oncolytic viruses function by inducing the lysis of cancer cells by infection [84]. Because the oncolytic activity is decreased by UV irradiation, it has been suggested that the viral amplification in cancer cells is responsible for oncolysis [85]. However, it is also possible that the virus's components contribute to the suppression of cancer. Recently, it was indicated that HVJ-E itself has an inhibitory effect against cancer growth [86, 87], and it was revealed that the viral components, in the absence of viral amplification, contribute to the anti-cancer effects. Since then, the HVJ-E-mediated anti-cancer effect has been studied.

3.1. HVJ-E for the Activation of Anticancer Immunity. Kurooka and Kaneda demonstrated that the intratumoral administration of HVJ-E dramatically eradicated intradermal cancer (Figure 2) [86]. They found that HVJ-E stimulated DCs to release various types of cytokines, such as interferon (IFN)-α, and -β, tumor-necrosis factor (TNF)-α and interleukin (IL)-6, and that IL-6 inhibited the proliferation of regulatory T cells (Tregs). Tregs negatively control effector T cells [88, 89] and interfere with the activation of anti-cancer immunity [90]. Therefore, HVJ-E-mediated eradication

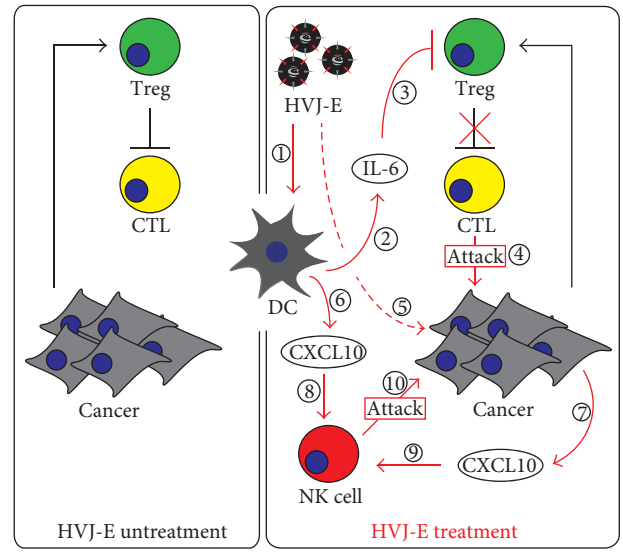

Figure 2: HVJ-E-mediated stimulation of anticancer immunity. Various modes of stimulations of the immune system that are induced by HVJ-E treatment. Black lines indicate the original reactions in cancer. Red lines indicate the reactions induced by HVJ-E treatment. *CTL Activation*. 1: HVJ-E stimulates dendritic cells (DCs). 2: DC secretes IL-6. 3: IL-6 suppresses regulatory T cell (Treg) function, which inhibits cytotoxic T cell (CTL) activity. 4: CTLs attack cancer cells. *NK Cell Activation*. 1, 5: HVJ-E stimulates DCs and cancer cells. 6, 7: DCs and cancer cells secrete CXCL10. 8, 9: CXCL10 attracts natural killer (NK) cells to cancer cells. 10: NK cells effectively attack the cancer cells.

of cancer results from the activation of anti-cancer immunity by IL-6-mediated suppression of Tregs. It is known that RNA viruses stimulate DCs via the recognition of the viral RNA genome by Toll-like receptor (TLR)-7 and -8 and Rig-I [91–93]. However, Suzuki et al. showed that the sugar chain of the F protein is important for HVJ-E-mediated, DC activation of IL-6 secretion [94]. Therefore, they suggested that DCs possess an unknown receptor for F that is involved in maturation.

In addition, HVJ-E suppressed tumor growth in the intradermal renal carcinoma SCID mouse model, in spite of their deficient T and B cells [95], which suggests that HVJ-E undergoes another stimulation mechanism that activates anti-cancer immunity. Fujihara et al. indicated that the anti-cancer activity of NK cells was led by the intratumoral administration of HVJ-E into the intradermal renal carcinoma SCID mouse model (Figure 1) [95]. In addition, HVJ-E directly stimulated cancer cells and induced their secretion of CXCL10. CXCL10 is a chemokine for monocytes/macrophages, T cells, NK cells, and DCs; therefore, it is suggested that active NK cells were attracted to the tumor by CXCL10.

Taken together, these reports demonstrate that HVJ-E is a powerful activator of anti-cancer immunity.

3.2. Direct Cancer Killing Activity of HVJ-E. Recently, it was reported that HVJ-E has a direct killing effect against cancer

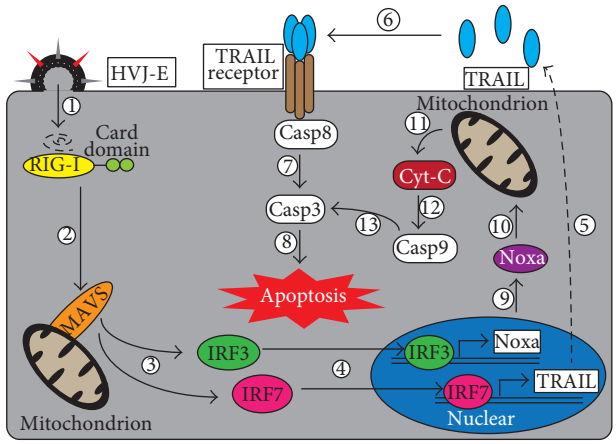

Figure 3: HVJ-E-mediated apoptosis pathway in cancer cells. HVJ-E-mediated signal transduction in cancer cells. 1: RNA fragments derived from the HVJ genome are introduced into the cytoplasm by membrane fusion, and RIG-I recognizes these RNAs. 2: RIG-I conveys the signal to the mitochondrial antiviral signaling adaptor (MAVS). 3: MAVS activates IRF-7 and -3. 4: activated IRF-7 and -3 localize to the nucleus. 5, 9: IRF-7 and -3 induce the expression of TRAIL and Noxa. 6: expressed TRAILs are recognized by the TRAIL receptor, and TRAIL receptors activate caspase-8 (Casp-8). 7: activated Casp-8 activates Casp-3. 8: activated Casp-3 induces apoptosis. 10, 11: Noxa induces the secretion of cytochrome-C (Cyt-C) into the cytoplasm from the mitochondria. 12: Cyt-C activates Casp-9. 13: Casp-9 activates Casp-3.

cells (Figure 3). Kawaguchi et al. showed that the viability of two castration-resistant human prostate cancer cell lines (PC3 and DU145) was remarkably decreased by the treatment with HVJ-E *in vitro* [96]. HVJ-E-treated PC3 cells exhibited some apoptotic phenotypes, namely, increases in the number of TUNEL-stained cells and in the expression levels of caspase-3 and caspase-8. However, HVJ-E-mediated inhibition of cell viability was not observed in normal prostate epithelium (PNT2), suggesting that HVJ-E-mediated apoptosis is specifically induced in cancer cells. HVJ-E contains many fragments of its RNA genome, and these RNA fragments are introduced to the cytoplasm by the fusion of HVJ-E and the cell membrane. Matsushima-Miyagi et al. revealed that the viability of prostate cancer cells (PC3 and DU145), but not normal prostate epithelium (PNT1 and PNT2), was significantly decreased by viral RNA introduction (Figure 2) [87]. The RNA fragments were recognized by RIG-I in the cytoplasm, and the signal was transduced to MAVS [97]. HVJ-E-mediated cell growth inhibition of PC3 was suppressed by the knockdown of RIG-I and MAVS, indicating that the RIG-I/MAVS signaling pathway is important for this process. Moreover, HVJ-E treatment induced the expression of TRAIL and Noxa (known as apoptosis inducers [98, 99]) in PC3 and DU145 cells, but not in PNT2 cells, via RIG-I/MAVS signaling. The fact that the knockdown of TRAIL and Noxa suppressed the HVJ-E sensitivity of PC3 and DU145, respectively, indicates that these apoptosis inducers are responsible for HVJ-E-induced cancer cell apoptosis. Furthermore, the knockdown of IRF7 and 3—transcription factors of TRAIL and Noxa, respectively [100, 101]—also suppressed the HVJ-E sensitivity of prostate cancer cells, suggesting that RIG-I/MAVS signaling regulates the expression of TRAIL and Noxa via IRF7 and 3 in cancer cells. Matsushima-Miyagi et al. [87] elucidates the mechanism of HVJ-E-induced cancer cell apoptosis. However, it is still unknown why the expression of these apoptosis inducers is induced in cancer cells by HVJ-E stimulation.

3.3. Combination Therapy with HVJ-E and Modification of HVJ-E. In attempts to enhance the strength and decrease the side effects of HVJ-E-mediated antitumor treatment, various combination therapies that include HVJ-E and modifications of HVJ-E have been used. Eg5 is an important factor in the early stages of mitosis [102] and its inhibition leads to mitotic arrest and results in apoptosis [103]. Matsuda et al. demonstrated that HVJ-E-mediated apoptosis in human glioblastoma cell lines (A-172, T98G and U-118MG) was effectively enhanced by the encapsulation of siRNAs against Eg5 in HVJ-E *in vitro* and *in vivo* [104]. The authors also observed that HVJ-E-mediated anti-cancer immunity was enhanced by the encapsulation of the IL-2 plasmid and that the astrocytoma cell line (RSV-M) was effectively eradicated when using this method *in vivo* [105].

HVJ-E adheres to the cell surface via HN binding to sialic acid (e.g., GD1a and SPG) [106]. Therefore, cancer cells with mild expression of these sialic acids exhibit low sensitivity to HVJ-E-mediated apoptosis because of their weak affinity for HVJ-E. To induce HVJ-E-mediated apoptosis in less sensitive cancer cells, Nomura et al. used the combination therapy of HVJ-E and 13-cis retinoic acid (13cRA) against human neuroblastoma cells (NB1), which are less sensitive to HVJ-E [107]. NB1 cells barely express GD1a and SPG and exhibit low sensitivity to HVJ-E-mediated apoptosis. 13cRA treatment induced the expression of GD1a in NB1 cells, and the HVJ-E sensitivity of NB1 cells was increased *in vitro*. Moreover, NB1 tumor volume in mice was significantly decreased and their survival rate was increased by the combination of HVJ-E and 13cRA *in vivo*.

Improvements to HVJ-E were made to enhance its performance. Sialic acids, such as GD1a and SPG, to which HN bind, are ubiquitously expressed in nearly all cells, and they are highly expressed in red blood cells. Therefore, HVJ-E does not have an affinity for a specific cell type, and it induces hemagglutination by intravenous administration. For the systemic administration of HVJ-E to treat cancer effectively, it must have high affinity for cancer cells and low affinity for sialic acids. Transferrin (Tf) is a protein in blood plasma that is responsible for ferric ion delivery, and the Tf receptor is highly expressed in various cancer cells. Shimbo et al. generated a cancer-targeting HVJ-E using Tf [108]. The HN on HVJ-E was depleted by siRNA [109], and Tf was presented on the surface of HVJ-E via the expression of a Tf/F recombinant fusion protein on HVJ-E. Tf-presented HVJ-E (Tf-HVJ-E) exhibited affinity for the human uterocervical cancer cell (Hela) line, which expressed the Tf receptor, and Tf-HVJ-E accumulated at tumor masses in mice after their systemic administration.

In addition, HVJ-E-mediated antitumor immunity was enhanced by HVJ-E modification. HVJ-E activates anti-cancer immunity; however, HVJ-E does not directly induce IFN-γ secretion. IFN-γ is an important factor for various anti-cancer activities [110], and IL-12 is a robust inducer of IFN-γ from immune cells [111, 112]. Saga et al. revealed that HVJ-E dramatically enhanced IL-12 activity for IFN-γ secretion from splenocytes; however, HVJ-E alone did not induce IFN-γ secretion [113]. The authors generated IL-12-conjugated- and HN-depleted HVJ-E (IL-12-HVJ-E) to enhance HVJ-E-mediated anti-cancer immunity. IL-12-HVJ-E induced secretion of IFN-γ from splenocytes *in vitro*. In addition, upon intratumoral injection, scIL12-HVJ-E activated antitumor immunity against mouse malignant melanomas (F10 melanoma) and suppressed tumor growth more effectively than the wild-type (wt) HVJ-E. Furthermore, upon intravenous injection, IL-12-HVJ-E, but not wt-HVJ-E, was especially localized to the lungs, where it induced IFN-γ expression and reduced the lung metastatic foci of F10 melanomas.

As described above, HVJ-E has the ability to induce anti-cancer effects in several types of cancers. Now, clinical trials of HVJ-E are ongoing to test its safety and anti-cancer immunity against melanoma and prostate cancer. Moreover, there is a possibility that the combination therapy of HVJ-E and other immune therapies, such as CTLA-4 antibody, exhibits a more effective activation of antitumor immunity, and it will be performed in the near future.

4. Conclusion

We have documented the utility of virosomes for cancer treatment. However, we believe that no omnipotent therapeutic technologies are currently available to completely eradicate various types of cancers. Cancers are heterogeneous and can transform themselves to be resistant to the treatment that they have received and to escape from the environment of cancer treatment [114]. In this scientific research field, it is absolutely necessary to identify the genes that direct tumorigenesis. However, in the clinical field, it is very important to prepare cancer treatments using a variety of therapeutic principles. Clinicians should provide cancer patients with the appropriate therapeutic tools according to the patient's condition. Thus, from a practical standpoint, virosome-mediated cancer therapy may have an important role in cancer treatment.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Review Article

Gene Therapy and Cell-Based Therapies for Therapeutic Angiogenesis in Peripheral Artery Disease

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Gene therapy and cell-based therapy have emerged as novel therapies to promote therapeutic angiogenesis in critical limb ischemia (CLI) caused by peripheral artery disease (PAD). Although researchers initially focused on gene therapy using proangiogenic factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and hepatocyte growth factors (HGF), cell therapy using bone marrow mononuclear cells (BMMNCs), mesenchymal stem cells (BMMSCs), G-CSF-mobilized peripheral blood mononuclear cells (M-PBMNCs), and endothelial progenitor cells (EPCs) have also been extensively studied. Based on the elaborate studies and favorable results of basic research, some clinical phase I/II trials have been performed, and the results demonstrate the safety of these approaches and their potential for symptomatic improvement in CLI. However, the phase 3 clinical trials have thus far been limited to gene therapy using the HGF gene. Further studies using well-designed larger placebo-controlled and long-term randomized control trials (RCTs) will clarify the effectiveness of gene therapy and cell-based therapy for the treatment of CLI. Furthermore, the development of efficient gene transfer systems and effective methods for keeping transplanted cells healthy will make these novel therapies more effective and ease the symptoms of CLI.

1. Introduction

Peripheral artery diseases (PAD), ischemic stroke, and coronary artery diseases refer to arterial stenosis caused by atherosclerosis and thrombosis. Critical limb ischemia (CLI) is a complication of PAD and causes pain on walking (claudication), pain at rest, and nonhealing ulcers. Although patients with CLI are treated with a combination of risk factor modification, such as statins, antiplatelet drugs, and angioplasty, these treatments are occasionally insufficient to recover sufficient blood flow to maintain normal tissue function. To overcome this limitation, therapeutic angiogenesis has emerged as a potential strategy to promote the growth of new vessels and thereby to supply sufficient blood flow. To date, researchers have focused on gene therapy using proangiogenic factors and/or cell-based therapy using several types of cells, including bone marrow cells (BMCs) and endothelial progenitor cells (EPCs), to achieve therapeutic angiogenesis. In gene therapy, the development of efficient gene transfer systems and investigation of suitable pro-angiogenic genes, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and hepatocyte growth factor (HGF), have been extensively studied in preclinical studies, whereas researchers in cell-based medicine have tried to find the most relevant cells and efficient methods for transplantation. Based on these results, clinical trials have been performed, and promising results have been reported.

This review summarizes the basic aspects and clinical trials of therapeutic angiogenesis in PAD and discusses future directions.

2. Gene Therapy Using Proangiogenic Genes

Among pro-angiogenic genes, VEGF, a 45-kDa basic heparin that binds homodimeric glycoprotein, has been the most

Table 1: Clinical trials of gene therapy in peripheral artery diseases.

Authors	Year	Gene	Vector	Delivery route	n	Reference number
Baumgartner et al.	1998	$VEGF_{165}$	naked pDNA	IM	9	[4]
Isner et al.	1998	$VEGF_{165}$	naked pDNA	IM	6	[5]
Rajagopalan et al.	2001	$VEGF_{121}$	adenovirus	IM	5	[7]
Mäkinen et al.	2002	$VEGF_{165}$	adenovirus, plasmid/liposome	IA	54	[6]
Comerota et al.	2002	FGF-1	naked pDNA	IA	51	[11]
Rajagopalan et al.	2003	$VEGF_{121}$	adenovirus	IM	1 : 1 : 1 fashion to low dose, high dose, or placebo arms (35-36 patients in each group)	[9]
Kusumanto et al.	2006	$VEGF_{165}$	naked pDNA	IM	54	[8]
Nikol et al.	2008	FGF-1	naked pDNA	IM	125	[12]
Shigematsu et al.	2010	HGF	naked pDNA	IM	44	[13]
Belch et al.	2011	FGF-1	naked pDNA	IM	525	[14]
Morishita et al.	2011	HGF	naked pDNA	IM	22	[15]

VEGF: vascular endothelial growth factor; FGF: fibroblast growth factor (FGF); HGF: hepatocyte growth factors.

extensively studied. VEGF has 4 main isoforms: VEGF A, B, C, and D. There are additional isoforms in VEGF A: VEGF121, VEGF165, which is the most biologically active [1], VEGF189, and VEGF206. The receptors for VEGF are FLT-1 and FLK-1, which activate intracellular tyrosine kinase. Neuropilin 1 (NP-1) is another receptor for VEGF and is bound by VEGF165 [2]. NP-1 and FLK-1 are key mediators of the phosphoinositide-3-kinase and Akt (PI3K/Akt) and mitogen-activated protein kinase (MAPK) kinase pathways. The efficacy of therapeutic angiogenesis was initially reported using VEGF plasmid DNA gene transfer in human patients [3–5] (Table 1). An initial trial in 1994 used a hydrogel catheter with naked VEGF165 plasmid DNA and seemed to effectively stimulate collateral formation of blood vessels [3]. Intra-arterial administration into the site of percutaneous transluminal angioplasty (PTA) with adenoviruses or liposomes containing the VEGF165 gene was also reported to exhibit beneficial effects in increasing vascularity [6]. However, intramuscular injection of naked plasmids encoding the VEGF165 gene has also been attempted and reported to have beneficial effects in patients with peripheral arterial disease [4, 5] since many patients lack an appropriate target vascular lesion for catheter delivery. Adenovirus-mediated gene delivery of VEGF121 has also been reported to be effective in improving lower-extremity endothelial function and flow reserve [7]. Thus, gene therapy using the VEGF gene appears to be promising, but its efficacy remains controversial because two later randomized clinical trials (phase II) failed to meet the primary endpoint of significant amputation reduction [8] or a change in peak walking time (Delta PWT) at 12 weeks [9]. Although the former clinical trial exhibited benefits in the secondary endpoints of hemodynamic improvement, improvement in skin ulcers, and decreased pain [8], the latter clinical trial reported increased peripheral edema as well as no benefits in secondary endpoints such as DeltaPWT, the ankle-brachial index, claudication onset time, and quality-of-life measures [9]. Recently, Muona et al. reported a 10-year safety followup in patients that had undergone local VEGF gene transfer to ischemic lower limbs [10]. In the study, there were no differences in the causes of death or in the incidence of cancer or diabetic retinopathy between the control patients and the VEGF-treated patients. Furthermore, no significant differences were demonstrated in the number of amputations. From the viewpoint of the authors, treatment with VEGF gene transfer might not induce serious side effects but requires additional development to achieve further therapeutic effects.

FGF is another angiogenic factor that has been studied in PAD. There are at least 23 structurally related FGF proteins. Among them, FGF-1 (aFGF) and FGF-2 (bFGF) have been extensively studied. The safety and efficacy of increasing single and repeated doses of intramuscular naked plasmid DNA encoding FGF type 1 administered to patients with unreconstructable end-stage PAD was first shown in a phase I study [11]. In that study, a significant reduction in pain and aggregate ulcer size was detected after FGF gene transfer associated with an increased transcutaneous oxygen pressure (TcO2) and ankle pressure index (ABI) compared with baseline pretreatment values [11]. Furthermore, phase II trials demonstrated that NV1FGF-treated patients exhibited a significantly reduced risk of all amputations and major amputations and a trend towards a reduced risk of death, although improvements in ulcer healing were similar between the NV1FGF-treated group and the control group [12]. These results were promising, but recent large randomized phase III trials including 525 patients demonstrated no beneficial effects on either the secondary or primary endpoints, including reduction in time to amputation or death [14]. Thus, gene therapy using the FGF gene has been controversial thus far.

Another promising pro-angiogenic factor is HGF, the efficacy of which has been reported in a phase III clinical trial [13]. HGF was first discovered as the most potent mitogen of hepatocytes, but it has been shown to possess multiple effects, including cell proliferation, angiogenesis, morphogenesis, anti-inflammation, and motility [16]. HGF exerts its angiogenic activity through tyrosine phosphorylation of its

specific receptor, c-Met, which is expressed in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) [17]. Compared to bFGF, HGF can induce angiogenesis without the induction of vascular inflammation by nuclear factor-κB (NFκB)-induced interleukin-1 (IL-1) and monocyte chemotactic protein-1 (MCP-1) or vascular permeability through increased expression of aquaporin 1 (AQP1) [17]. Furthermore, gene transfer using naked plasmid HGF DNA was shown to induce therapeutic angiogenesis in animal models [18–23]. Based upon these findings, a human clinical trial (phase I/IIa) was started using intramuscular injection of naked human HGF plasmids [15]. Twenty-two patients with peripheral arterial disease or Buerger's disease staged as Fontaine IIb ($n = 7$), III ($n = 4$), or IV ($n = 11$) were treated with two injections of either 2 mg or 4 mg of HGF plasmid. No serious adverse events caused by gene transfer were detected over a followup of 6 months, and no peripheral edema was observed. Two months after gene transfer, the ankle-brachial index was increased. Additionally, the size of the largest ischemic ulcers and the visual analog scale score were decreased [15]. Recently, the long-term followup of this study was reported. An ankle-branchial pressure index >0.1 was observed in 11 of 14 patients (79%) at 2 years after gene therapy (11 of the 17 patients (65%) at 2 months). Reduction in rest pain (>2 cm in visual analog scale) was observed in 9 of 9 patients (100%) at 2 years (in 8 of 13 (62%) patients at 2 months). A reduction in ischemic ulcers accompanied by a decrease in the size of ulcers was observed in 9 of 10 patients (90%) at 2 years. Severe complications and adverse effects were not detected [24]. Powell et al. performed another double-blind placebo-controlled study with an HGF plasmid [25]. TcPO2 increased at 6 months in the high-dose group (4.0 mg at day 0, 14, 28) compared with the placebo, low-dose (0.4 mg at day 0, 14, 28), and middle-dose (4 mg at day 0, 28) groups, but there were no differences in the ankle-brachial index, toe-brachial index, pain relief, wound healing, or incidence of major amputation [25]. Finally, Shigematsu et al. performed a randomized, double-blind, placebo-controlled clinical trial of HGF plasmids in patients with PAD (phase III) [13]. The primary endpoint was improvement of rest pain in patients without ulcers (Rutherford 4) or a reduction of ulcer size in patients with ulcers (Rutherford 5). Secondary endpoints included ABI, amputation, and quality of life (QOL). Forty-four patients were recruited, and a significant difference in the primary endpoint was noted; improvement was observed in 70.4% of the HGF group and in 30.8% of the placebo group. When the analysis was limited to Rutherford 5 patients, HGF-treated patients exhibited a significantly higher improvement rate (100%) than did the placebo group (40%). QOL also improved in the HGF-treated group, and there were no major safety problems, although this trial failed to demonstrate an improvement of ABI or amputation rate. Following these favorable outcomes in HGF-treated patients, a global multicenter phase III clinical trial, which will recruit over 500 PAD patients, is scheduled.

Recently, one clinical trial (phase I) has reported the safety and possible efficacy of a plasmid HGF gene, VM202 [26], that encodes two isoforms of HGF, one consisting of 728 amino acids (known as HGF) and the other consisting of 723 amino acids (known as deleted HGF) [27]. In this trial, the median ABI and toe brachial pressure index (TBI) in the HGF-treated group were significantly increased at 12 months of followup, and the median visual analogue scale (VAS) decreased from 57.5 to 16.0 mm at 6 months of followup without significant differences in side effects. Based on these results, a phase 2 trial is ongoing (ClinicalTrials.gov NCT01064440).

Thus, the effectiveness of gene therapy using pro-angiogenic factors remains controversial, but some clinical trials have shown promising results. Further large-scale clinical trials will clarify their efficacy.

3. Cell-Based Therapy in PAD

While the development of gene therapy has been ongoing, researchers have attempted to develop more effective treatments (Table 2). On such potential treatment is the transplantation of stem or progenitor cells, which possess the capability to self-renew and to differentiate into organ-specific cell types as well as to mediate paracrine effects through the release of pro-angiogenic growth factors. The cells that have been used in these studies include bone marrow mononuclear cells (BMMNCs), bone marrow mesenchymal stem cells (BMMSCs), G-CSF-mobilized peripheral blood mononuclear cells (M-PBMNCs), endothelial progenitor cells (EPCs), and G-CSF monotherapy.

Broadly, bone marrow cells (BMCs) are harvested from bone marrow and can be identified as crude, unfractionated, or mononuclear cells (BMMNCs) by density centrifugation [42]. Although BMCs contain MSCs and EPCs as well as hematopoietic stem cells and hemangioblasts, EPCs can also be isolated from the peripheral blood. G-CSF can mobilize peripheral blood $CD34^+$, $CD133^+$, and/or KDR^+ cells with the capacity to differentiate into EPCs [30].

3.1. Clinical Trials of Therapy Using Bone Marrow Mononuclear Cells, Mesenchymal Stem Cells, or Peripheral Blood Mononuclear Cells. The Angiogenesis using cell transplantation (TACT) study demonstrated that intramuscular injection of the mononuclear fraction of autologous BMCs into affected areas in patients with unilateral ischemia of the leg (25 patients) and one leg in bilateral leg ischemia (22 patients) increased perfusion with significant improvements in the ankle-brachial indices, TcO2, and rest pain in injected legs at 4 weeks and that recovery was sustained for 24 weeks [28]. Following this study, some phase I clinical trials have explored the efficacy of BMMNCs in improving limb salvage in patients with CLI [29, 31, 32]. Thus, initial studies have extensively examined the effectiveness of transplantation of BMCs and the best methodology, such as the source of cells and delivery route.

Regarding the association between the subpopulations of BMMNCs and outcomes, a phase I open-label, nonrandomized trial reported a significant difference in counts of KDR^+ cells between treatment responders and nonresponders, although no correlation was observed between total mononuclear cell count and changes in ABI [37]. Additionally, improvements in limb perfusion were associated with KDR^+

Table 2: Clinical trials of cell-based therapy in peripheral artery diseases.

Authors	Year	Cell	Delivery route	n	Reference number
Tateishi-Yuyama et al.	2002	BMMNC or PBMNC	IM	45	[28]
Esato et al.	2002	BMC	IM	8	[29]
Huang et al.	2005	PBMNC	IM	28	[30]
Miyamoto et al.	2006	BMMNC	IM	8	[31]
Durdu et al.	2006	BMMNC	IM	28	[32]
Arai et al.	2006	G-CSF	SC	39	[33]
Huang et al.	2007	BMMNC or PBMNC	IM	150	[34]
Kawamoto et al.	2009	EPC	IM	17	[35]
Procházka et al.	2010	BMC	IM	96	[36]
Murphy et al.	2011	BMMNC	IM	29	[37]
Lu et al.	2011	BMMNC or BMMSC	IM	41	[38]
Walter et al.	2011	BMMNC	IA	40	[39]
Powell et al.	2012	Ixmyelocel-T	IM	72	[40]
Losordo et al.	2012	EPC	IM	28	[41]

PBMNC: peripheral blood mononuclear cells; BMC: bone marrow cell; BMMNC: bone marrow-derived mononuclear cell; BMMSC: bone marrow mesenchymal stem cell; G-CSF: granulocyte colony-stimulating factor; EPC: endothelial progenitor cell.

but not $CD34^+$ or $CD133^+$ subpopulations of BMMNCs [37]. In this trial, the amputation-free survival at 1 year was 86.3% with significant recovery in first toe pressure, TBI, perfusion index, rest pain, and QOL and a trend towards improvement in ABI [37]. Another study using BMMNCs reported that bone marrow lymphopenia in the initial bone marrow concentrates in patients might be potential causative factors for failure of BMMNC therapy, suggesting that at least partial correction with platelet supplementation may be beneficial [36]. In this trial, a total of 96 patients were randomized into a BMMNC treatment group or a standard medical care group. The frequency of major limb amputation was 21% in the BMMNC group and 44% in the control group within the 120 days of followup [36].

To identify better cells for the treatment of diabetic CLI and foot ulcers in a pilot trial, a double-blind, randomized, controlled trial compared the effectiveness of BMMSCs and BMMNCs [38]. The ulcer healing rate of the BMMSC group was significantly higher than that of the BMMNC group at 6 weeks after injection and reached 100% four weeks earlier than the BMMNC group. After 24 weeks of followup, the improvements in limb perfusion induced by the BMMSC transplantation were more significant than those induced by BMMNC transplantation in terms of painless walking time, ABI, TcO2, and magnetic resonance angiography (MRA) analysis [38]. The authors concluded that BMMSC therapy might be better tolerated and more effective than BMMNC therapy for increasing lower limb perfusion and promoting foot ulcer healing in diabetic patients with CLI [38]. Another randomized trial, which recruited 150 patients, was performed to compare the effectiveness of M-PBMNCs and BMMNCs [34]. Seventy-six patients received M-PBMNCs, and 74 patients received BMMNCs; the groups were followed up for 12 weeks. Twelve weeks after cell implantation, ABI, skin temperature, and rest pain were significantly better in M-PBMNC-treated patients than in BMMNC-treated patients. However, no significant differences were observed in pain-free walking distance, TcO2, ulcers, or the rate of lower limb amputation between the two groups [34]. The authors concluded that M-PBMNC treatment would be more practical than treatment with BMMNCs [34].

Regarding the administration route, cells were injected i.m. in most studies, but the efficacy of intra-arterial injection was also examined in the intra-arterial progenitor cell transplantation of bone marrow mononuclear cells for induction of neovascularization in patients with peripheral arterial occlusive disease (PROVASA) study [39]. In this multicenter, phase II, double-blind, randomized-start trial, forty patients with CLI were included and received intra-arterial administration of either BM-MNC or placebo followed by active treatment with BM-MNC (open label) after 3 months. As a result, intra-arterial administration of BMMNCs did not achieve the primary endpoint, which was an increase in ABI. However, cell therapy resulted in improved ulcer healing versus placebo within 3 months, although limb salvage and amputation-free survival rates did not differ between the groups. Repeated BMMNC administration correlated significantly with limb salvage [39].

To minimize the invasiveness of BM absorption, cellular therapy with Ixmyelocel-T and treatment with commercial preexpanded cells obtained from a small amount of a subject's own bone marrow under conscious sedation were evaluated in a prospective, randomized, double-blind, placebo-controlled, multicenter study (RESTORE-CLI) [40]. Patients with lower extremity CLI with no options for revascularization received single injections into one leg and were followed for 12 months. Ixmyelocel-T treatment resulted in a significantly prolonged time to the first occurrence of treatment failure (major amputation of injected leg, all-cause mortality, doubling of the total wound surface area from baseline, or de novo gangrene). There was a trend towards increased amputation-free survival after Ixmyelocel-T treatment, but

the trend was not statistically significant. The treatment effect in the post hoc analyses of patients with baseline wounds was more pronounced [40].

G-CSF monotherapy is one treatment that can avoid the invasiveness of bone marrow transplantation [33]. Thirty-nine patients were randomly assigned to conventional drug therapy, conventional drug therapy plus bone marrow transplantation (BMT), or conventional therapy plus G-CSF. Subjective symptoms, ABI, and TcO2 were significantly improved in the G-CSF and BMT groups to the same degree, whereas such improvements were not observed in the conventional therapy group [33].

3.2. Meta-Analysis in BMC- and M-PBMNC-Based Clinical Trials. Based on these clinical trials, meta-analyses or systematic reviews have been reported recently [43–45]. One meta-analysis of 37 controlled and noncontrolled, randomized and nonrandomized trials demonstrated that autologous BMCs or M-PBMNCs were effective in improving surrogate indexes of ischemia, subjective symptoms, and ulcer healing and amputations, whereas G-CSF monotherapy did not result in significant improvements in the same endpoints [43]. Furthermore, this study demonstrated that cell-based treatment was more effective in patients with Buerger's disease than in those with atherosclerotic PAD. The intramuscular route was better than the intra-arterial route, and the use of BMCs was better than the use of M-PBMNCs. Another recent systematic analysis of 45 cell-based clinical trials and seven non-placebo-controlled and placebo-controlled RCTs reported that cell therapy using BMMNCs or M-PBMNCs resulted in a favorable safety profile with a low adverse event rate, no increase in severe events such as mortality and cancer, and that cell therapy decreased the risk of amputation [44]. No difference in the amputation rate between BMMNC therapy and M-PBMNC therapy was observed [44]. Thus, these meta-analyses demonstrated the feasibility of cell-based therapy, but there are some discrepancies in their findings and those of individual clinical trials regarding the best source of cells and the best route of delivery.

Most recently, a meta-analysis of 12 randomized controlled clinical trials was reported [45]. This meta-analysis studied BM-derived cell therapy compared with standard care with or without placebo in 510 CLI subjects, including 7 trials using BMMNC, 3 trials using BMMSC, 2 trials using M-PBMNC, and 1 trial using Ixmyelocel-T. When major amputation and amputation-free survival were considered as the primary endpoints, beneficial effects of BM-derived cell therapy were observed for both subjective and surrogate objective endpoints, including pain score, pain-free walking distance, ankle-brachial index, and TcO2 measurements. However, when the analysis was limited to the 7 placebo-controlled RCTs, the beneficial effect on major amputation rates and amputation-free survival was reduced and not significant, indicating that a placebo in the control arms is necessary. This result indicates that well-designed larger placebo-controlled RCTs including long-term followup data are needed to confirm the effects of BMC and M-PBMNC treatments [45].

3.3. EPCs. EPCs were first described by Asahara et al. as circulating $CD34^+$ cells that could differentiate into endothelial cells (ECs) and incorporate into foci of neovascularization [46]. Recent studies have described 4 sources of EPCs: hematopoietic stem cells; myeloid cells; other circulating cells, termed "side population cells"; and circulating mature endothelial cells that have sheared off from vessel walls [47]. Although Asahara et al. first used CD34 and VEGF receptor-2 to discriminate EPCs [46], subsequent studies have shown that specific cell markers or functions of EPCs remain controversial [47] because hematopoietic stem cells also express CD34, CD133, and VEGF receptor-2 [48]. However, recent studies have used $CD34^+$ or $CD133^+$ cells in preclinical and clinical studies in PAD and have reported their effectiveness.

One such clinical study is a phase I/IIa, multicenter, single-blinded, dose-escalation clinical trial of autologous $CD34^+$ cells, which include the endothelial and hematopoietic progenitor-enriched fraction, in no-option patients with atherosclerotic peripheral artery disease or Buerger's disease with CLI [35]. $CD34^+$ cells isolated from the G-CSF-mobilized apheresis product were injected i.m. into the leg with more severe ischemia. $CD34^+$ cell-treated patients demonstrated significant recovery in the primary endpoints, including the efficacy score, representing changes in the toe brachial pressure index (TBPI), the Wong-Baker FACES pain rating scale, and the total walking distance 12 weeks after cell transplantation without a significant dose-response relationship. During the 12-week followup, no death or major amputation occurred [35]. Recently, the long-term outcome for these patients was analyzed. The incidence of major clinical events and physiological parameters of limb ischemia were evaluated up to 208 weeks after the therapy [49]. Three patients with PAD died by week 156, and 1 patient with Buerger's disease died by week 208, due to cardiac complications. No patients underwent major amputation, although 1 patient with Buerger's disease underwent minor amputation by week 104. The toe brachial pressure index versus the baseline was sustained up to week 208 and that of transcutaneous partial oxygen pressure was maintained up to week 156. Measures of functional recovery, such as the Wong-Baker FACES pain rating scale, ulcer size, and exercise tolerance, were significantly improved compared with baseline [49].

Most recently, a randomized, double-blind, placebo-controlled pilot study was performed to examine the safety and efficacy of intramuscular injections of autologous $CD34^+$ cells in 28 patients with moderate- or high-risk CLI [41]. There was a trend towards a reduction in amputation rates at 6 and 12 months after treatment without adverse safety signals associated with cell administration, although several surrogate markers, such as ABI, toe brachial index, leg pain, walking distance, and wound healing, did not exhibit differences because of low statistical power [41]. No adverse events associated with cell transplantation were observed [41].

The effect of autologous peripheral blood $CD133^+$ cell implantation was also reported in small cohort study, including 7 patients suffering from ASO, one with Buerger's disease, and one with thromboembolic disorder [50]. $CD133^+$ cells, which were selected from autologous PBSCs collected after the administration of G-CSF, were administered i.m. After

1 year, seven of nine patients were free from leg amputation, and there was a trend towards improvement in pain-free treadmill walking time and exercise capacity [50].

Thus, EPC-based treatment seems also beneficial, but further large-scale studies will clarify the efficacy of EPC transplantation.

4. Conclusion

Overall, clinical trials have demonstrated that gene therapy and cell-based therapy may be safe and effective in the treatment of CLI, although gene therapy using HGF remains a phase III-proven therapy, where the number of recruited patients is not large. A recent meta-analysis in BMCs and M-PBMNCs demonstrated that the beneficial effect on major amputation rates and amputation-free survival was reduced and nonsignificant in the placebo-controlled RCTs [45]; well-designed larger placebo-controlled RCTs are required to establish the efficacies of these novel therapies. Furthermore, the long-term effects of these therapies should be verified.

Also, additional improvement should be pursued to achieve more efficient therapy. Recently, the proliferative and migratory function in EPCs in diabetic patients has been report to be reduced [51]. One of the mechanisms in EPCs dysfunction is associated with defective NO signaling [52]. To overcome the dysfunction, inhibition of NADPH oxidase was reported to restore NO availability and migratory function in diabetic CD34 cells [53]. Thus, adjuvant therapy to promote BMC and EPC health is one of solutions to make the cell-based therapy more effective. In the field of gene therapy, improvements in efficient gene transfer systems are required. These include improvement of the development of devices as well as the structure of vectors. For example, ultrasound-microbubbles-mediated gene transfer is one of such devices that could increase the transfection efficiency of naked plasmid DNA and is shown to enhance angiogenesis in ischemic limb in rodent [54].

We believe that these basic, translational, and clinical studies will lead to improvements in QOL for PAD patients.

Conflict of Interests

Ryuichi Morishita is a founder of AnGes MG and a stockholder. Ryuichi Morishita also has IP of HGF gene therapy drug. Department of clinical gene therapy is financially supported by AnGes MG, Novartis, Shionogi, Boeringher and Rohto. Division of Vascular Medicine and Epigenetics is financially supported by Bayer.

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Review Article

Lymphedema and Therapeutic Lymphangiogenesis

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Lymphedema is a disorder of the lymphatic vascular system characterized by impaired lymphatic return and swelling of the extremities. Lymphedema is divided into primary and secondary forms based on the underlying etiology. Despite substantial advances in both surgical and conservative techniques, therapeutic options for the management of lymphedema are limited. Although rarely lethal, lymphedema is a disfiguring and disabling condition with an associated decrease in the quality of life. The recent impressive expansion of knowledge on the molecular mechanisms governing lymphangiogenesis provides new possibilities for the treatment of lymphedema. This review highlights the lymphatic biology, the pathophysiology of lymphedema, and the therapeutic lymphangiogenesis using hepatocyte growth factor.

1. Introduction

The lymphatic vascular system maintains tissue fluid homeostasis, plays a role in the afferent immune response, and carries proteins and large particulate matter away from the tissue spaces [1, 2]. Lymph stasis can accompany lymphatic anatomical or functional disorders as a result of both congenital and postnatal abnormalities. Because the lymphatic circulation provides the normal conduit for the return of interstitial fluid and protein to the blood circulation, abnormal lymph stasis creates an accumulation of protein and cellular metabolites in the extracellular space, resulting in an ensuing increase in tissue colloid osmotic pressure, water accumulation, and elevation of the interstitial hydraulic pressure. The lymphatic vascular system is a unidirectional transport system arising from blind ends. Fluid, cells, and macromolecules present in the interstitial space first enter blind-ended lymphatic capillaries. The lymphatic network permeates most organs in the body, as only the cornea, cartilage, epidermis, and central nervous system are devoid of lymphatic vessels. In addition to mammals, birds, fish, and amphibians also have a secondary lymphatic or lymphatic-like vascular system [3–6].

In addition to draining and transporting fluid, the lymphatic vascular system also plays an important role in the immune response by transporting leukocytes, antigens, and dendritic cells. Lymphatic vessels have many lymph nodes, which act as checkpoints for the immune response. The lymphatic system is also responsible for the absorption of dietary fats and fat-soluble vitamins from the digestive tract, in which specialized lymphatic capillaries (lacteals) in the intestinal villi absorb the lipid particles (chylomicrons) released by enterocytes.

Anatomic or functional obstruction of the lymphatic system can result in the progressive accumulation of protein-rich fluid in the interstitial spaces (lymphedema) [7, 8]. Lymphedema is divided into primary and secondary forms based on the underlying etiology. Primary (hereditary) lymphedema results from genetic damage, whereas secondary

(acquired) lymphedema is a consequence of lymphatic failure resulting from trauma, surgery, radiotherapy, or parasitic infection. Primary lymphedema is thought to occur in approximately 1–3 out of every 10,000 live births [9], irrespective of race or geographic area, and the female-male ratio is 3.5 : 1 [10]. The vast majority of lymphedema worldwide is secondary lymphedema. The most common cause of secondary lymphedema is filariasis. According to a 2013 report from the World Health Organization (http://www.who.int/mediacentre/factsheets/fs102/en/), lymphatic filariasis afflicts more than 25 million men with genital disease and more than 15 million people with lymphedema in Southeast Asia and African regions. In industrialized countries, cancer therapy is the leading cause of secondary lymphedema. Advanced malignancies frequently require radical surgery, including lymph node removal with or without radiotherapy, resulting in the destruction of the lymphatic vessel network. Approximately 30% of patients who have undergone breast cancer surgery develop lymphedema of the upper limb [11]. Even among patients treated with sentinel navigation surgery, approximately 6% develop lymphedema [11]. Furthermore, 10%–30% of patients with gynecological cancer develop lymphedema [12–14], as do approximately 15% of other lymphedema-related malignant tumor patients (16% for melanoma, 10% for genitourinary, 4% for head/neck tumors, and 30% for sarcoma) [15].

Despite substantial advances in surgical and conservative techniques, therapeutic options for the management of lymphedema are limited [8, 16]. Although rarely lethal, lymphedema is a disfiguring and disabling condition which decreases the quality of life [17]. There is no cure for lymphedema at this time, but treatments to manage and reduce the swelling include physiotherapy, massage, and compression bandages, known as complex physical therapy [18].

2. Pathophysiology of Lymphedema

The pathophysiology of lymphedema is generally divided into two periods. During the first period, the pathological changes occur mainly only in the lymphatics and in the soft tissues lymphedema symptoms are not apparent (occult lymphedema = Stage 0). After this stage, the pathological changes occur in the soft tissue (fat, connective tissue, skin, etc.) of the limbs, resulting in the progressive swelling caused by systematic and combined pathologic factors. This clinical state is characterized not only by progressive swelling but also by fat and scar deposition, immunosuppression, a propensity for cellulitis, and microvascular proliferation (Figure 1).

2.1. Variable Period (Occult Lymphedema). Congenitally deficient or obstructed lymphatics promote lymph stasis, which is accompanied by deranged truncal contractility, progressive valve incompetence, destruction of contractile elements (lymphangioparalysis), and gradual ectasia of lymphatic collectors. After a variable period (occult lymphedema), a series of events is initiated, culminating in chronic lymphedema. Because of difficulties in diagnosis, the pathophysiology of occult lymphedema is almost unknown for most patients.

Table 1: Staging according to the "consensus document" of the International Society of Lymphology.

Clinical stage	Evidence
0	Subclinical with possible clinical evolution
I	Edema regressing with treatments with positive pitting test
II	Edema partially regressing with treatments with negative pitting test
III	Elephantiasis with cutaneous complications and recurrent infections

2.2. Progress of Lymphedema and Exacerbation Factors. When lymphedema is apparent via the pathological changes in the lymphatic system, some findings may be confirmed by images [18]: (1) obstruction of the lymphatic main route; (2) dermal back flow; (3) lack of lymph nodes; (4) existence of collateral lymphatic flow. As lymphedema worsens (Table 1), a decrease of swelling after limb elevation of the limb (Stage 1) will be not seen (Stage 2), and subsequently, the edema changes from pitting edema into nonpitting edema (late Stage 2). In many cases, the symptoms of lymphedema may be resistant to most of the therapies during this late Stage 2. Furthermore, edema is irreversible, and sclerosis of the skin and subcutaneous tissue (elephantiasis) may be remarkable (Stage 3) [18].

These pathological changes in soft tissues are induced by fibrosis and metabolic disorder. At this time, chronic inflammation is recognized as the important mechanism, involving lymphocytes, monocyte/macrophages, and dendritic cells. As previously reported, these inflammatory cells produce many inflammatory cytokines related to fibrosis, such as CTGF, TGF-β, and PDGF. Cellular proliferation and migration of fibroblasts are upregulated [19].

Infection and adipogenesis are exacerbation factors of lymphedema. The propensity for recurrent soft-tissue infection is one of the most troublesome aspects of long-standing lymphedema. Accumulated fluid and proteins provide a good substrate for bacterial growth. Lymphatic dysfunction impairs the local immune response, which plays a permissive role in the propagation of bacterial and fungal invasion. Furthermore, once established, soft tissue infection exacerbates the existing lymphatic dysfunction, sometimes irreversibly.

Although the connection between the lymphatic system and fat absorption/deposition has been recognized by clinicians for well over a hundred years, the subject received relatively little interest until recent publications raised theories regarding the mechanism of this association. It has long been recognized that a lymphedematous limb accumulates fat at an increased rate when compared to the rest of the body and that, conversely, when weight loss is undertaken, the lymphedematous limb loses fat at a slower rate than the rest of the body. The underlying cause of these observations is not well understood. A recent publication studying Prox1 haploinsufficient mice proposed that the lymph itself is stimulatory to fat cells [20]. According to recent reports, adipocytes recruit monocyte/macrophages via activation of

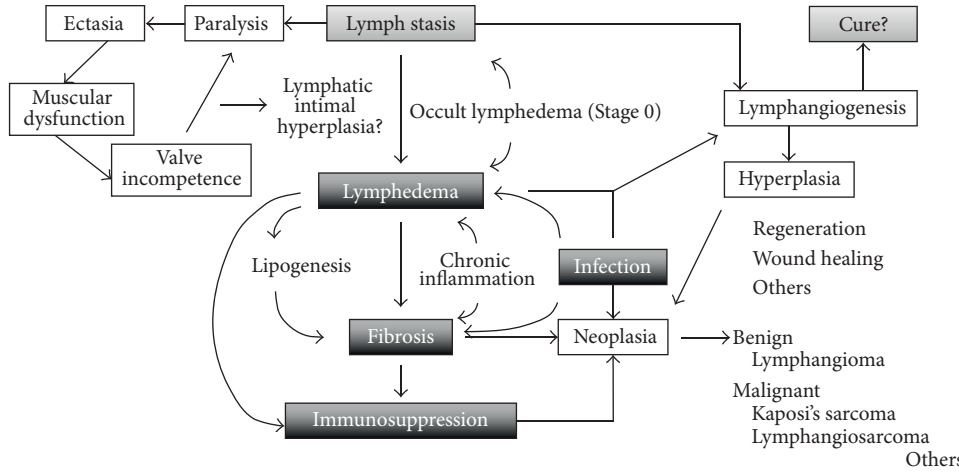

Figure 1: Schematic diagram of the pathophysiology of lymphedema.

NFκB and TNF-α [21]. Furthermore, it was reported that adipocytokines participate in fat absorption [22].

2.3. Primary Lymphedema Related Genes. Three genes were confirmed as the cause of lymphedema [23, 24]: (1) VEGFR-3 (familial Milroy lymphedema); (2) FOXC2 (lymphedema-distichiasis syndrome); (3) SOX18 (hypotrichosis-lymphedema-telangiectasia). In addition to these genes, the following genetic changes are associated with lymphedema: Aagenaes syndrome (chromosome 15), Noonan's syndrome (chromosome 12), trisomy disorders (chromosome 13, 18, 21, and 22), Klinefelter's syndrome (XXY), Turner's syndrome (XO), and chromosomal abnormalities of additions at 11p and deletions at 11q and 13q. Klippel-Trenaunay syndrome is recognized as a nonhereditary disease.

2.4. Malignancy in Patients with Lymphedema. Although lymphedema is recognized to generally not affect prognosis of mortality, in rare cases, chronic lymphedema may be complicated by the development of malignant tumors. One of these malignancies is lymphangiosarcoma (Stewart-Treves syndrome) [25]. The incidence rate of this syndrome in patients with lymphedema 5 years after breast cancer surgery is 0.07–0.45%, according to previous reports [26]. Other malignant tumors that appear with increased frequency in lymphedematous limbs include Kaposi's sarcoma, squamous cell carcinoma, malignant lymphoma, and melanoma. These malignancies frequently result in limb loss or even death. The mechanism of development of malignancy is unclear, but because these malignancies occur in the chronic lymphedema patients of any cause, the lymphedema state is thought to affect the development of these malignancies.

3. Therapeutic Lymphangiogenesis

3.1. Development on Lymphangiogenesis. Many reports regarding the molecular biology of lymphatics and lymphangiogenesis were published during the 1990s, in accordance with progress in the field of vascular biology. These developments were supported by the identification of lymphatic specific markers, such as LYVE-1 and Prox1, and subsequent improved ability to easily observe the lymphatics or lymphatic endothelial cells (LEC).

Recent studies suggest that lymphangiogenesis can be stimulated by various cytokines. For example, vascular endothelial growth factors (VEGF)-C and -D promote lymphangiogenesis by activating the VEGF receptor-3 (VEGFR-3), which is expressed on lymphatic endothelial cells (LEC) [27]. As further evidence, VEGF-C-deficient mice fail to develop a functional lymphatic system [28], transgenic expression of soluble VEGFR-3 results in pronounced lymphedema [29], and gene transfer of VEGF-C effectively reduces lymphedema in an animal model [30]. Another study reported that angiopoietin-1 also promotes lymphatic vessel formation through Tie2 [31] and that fibroblast growth factor 2 stimulates the growth of lymphatic vessels [32].

3.2. HGF Gene Therapy for Lymphedema. Hepatocyte growth factor (HGF) was originally identified as a potent mitogen for hepatocytes, and HGF is currently recognized as a mesenchyme-derived pleiotropic factor that regulates growth, motility, and morphogenesis of various types of cells [33–37]. Furthermore, HGF plasmid DNA is utilized for gene therapies targeting the heart [38], vascular system [39], brain [40], and lung [41]. HGF activates its tyrosine kinase receptor, c-Met [33, 42], and various c-Met-linked intracellular signaling pathways, such as the Ras-mitogen-activated protein kinase cascade (MAPK) or the phosphatidylinositol-3-OH kinase (PI3 K)-Akt cascade [43, 44].

We have previously investigated and reported the lymphangiogenic potency of HGF [45]. Canine lymphatic endothelial cells (cLEC) express c-Met as demonstrated by immunofluorescent staining, suggesting that cLEC are responsive to HGF. Indeed, the treatment of cLEC with HGF resulted in a dose-dependent increase in cellular proliferation and migration (Figure 2(a)). Furthermore, the extracellular signal-regulated protein kinase (ERK) or Akt was

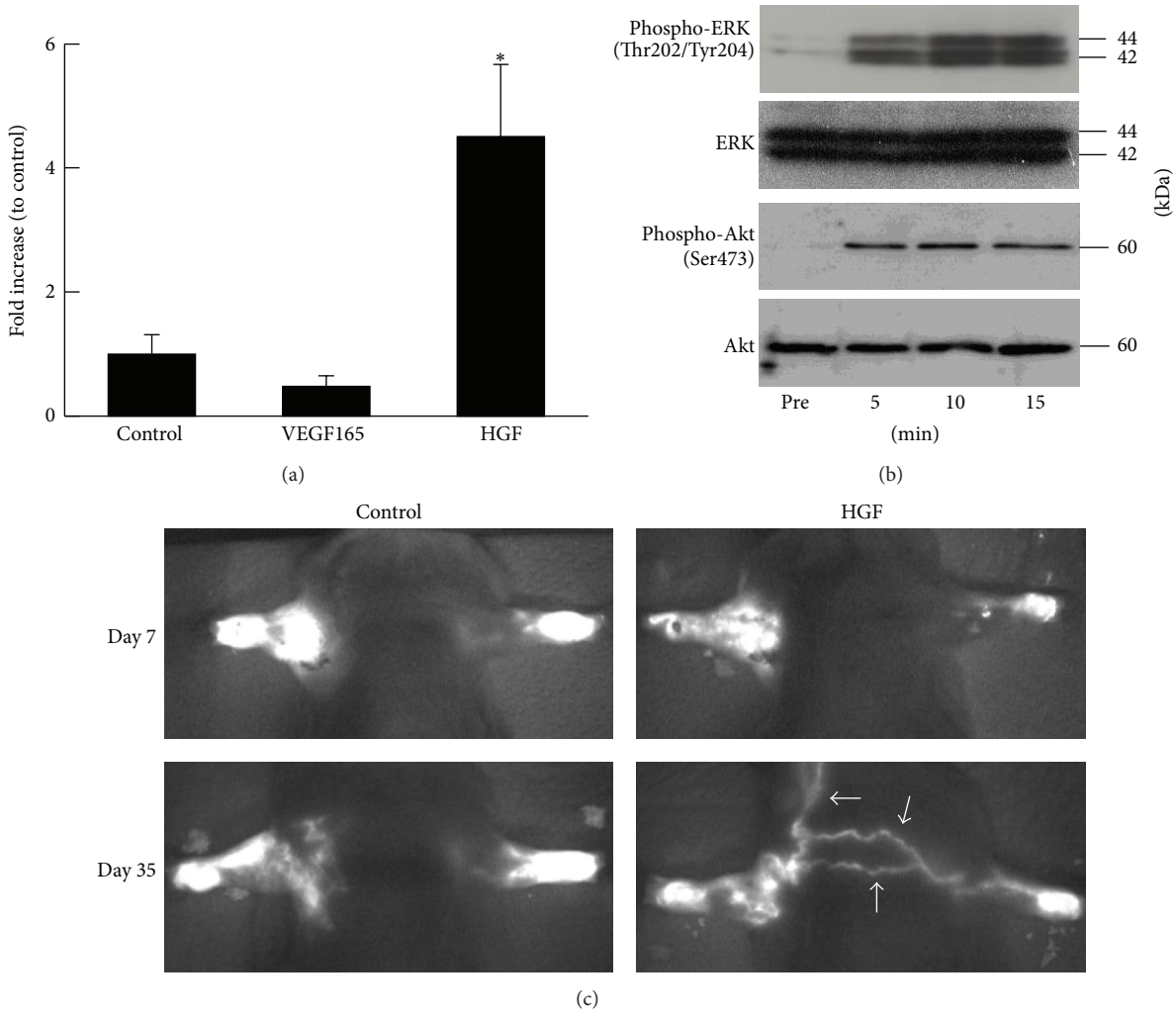

Figure 2: HGF lymphangiogenesis and gene therapy for lymphedema. (a) Effect of HGF plasmid on c-fos promoter activity in LEC. $n = 4$, $^{*}P < 0.05$ versus control, VEGF165. (b) Typical western blot of ERK or Akt and phosphorylated ERK or Akt in cLEC before and 5, 10, and 15 minutes after treatment with human recombinant HGF (50 ng/mL). (c) Representative pictures of the fluorescent lymphography using PDE at day 7 and day 35 after surgery. "HGF" indicates human HGF plasmid (200 μg/0.1 mL) and "control" indicates GFP plasmid injection (200 μg/0.1 mL).

phosphorylated 5–15 minutes after the addition of HGF to cLEC, whereas total ERK or Akt protein levels were not altered by treatment with recombinant HGF (Figure 2(b)).

In accordance with *in vitro* data, we confirm the effect of HGF gene transfer for *in vivo* models. Using the mouse upper limb lymphedema model in a simulation of breast cancer related lymphedema, the operated arm volume began to increase 1 day after the operation and was stable at 7 days after operation in all animal groups. The arm volume of the HGF injected group was significantly decreased on postoperative day 7, compared with the arm volumes of the control group. This significant difference between the HGF injected group and the control group continued to postoperative day 35. Of note, new extra-anatomical lymphatic flow was observed only in the HGF injected group, as detected by the fluorescent lymphography system (PDE; Hamamatsu Photonics K.K. Hamamatsu, Japan, Figure 2(c)). We hypothesized that these lymphatic flows are induced by HGF lymphangiogenic potency.

In view of these results, we hypothesized that HGF gene therapy would stimulate the growth of the lymphatic vascular system and alter the course of lymphedema. In terms of HGF gene therapy, the safety of the use of HGF plasmid DNA in patients with critical limb ischemia has been investigated in a prospective open-labeled clinical trial [46]. There were no signs of systemic or local inflammatory reactions and no development of tumors or progression of diabetic retinopathy in this population. Of note, no edema was observed in this trial, in contrast to the transient lower-extremity edema that was reported after the use of clinical gene therapy using the VEGF-A gene [47]. We are currently preparing to start a clinical trial involving lymphedema patients and expect to observe successful therapeutic lymphangiogenesis management by HGF gene therapy.

4. Conclusion

The lymphatic vessels have three specific functions for maintenance of homeostasis: (1) drainage of tissue fluid; (2) immunosurveillance; and (3) the uptake of dietary fats. Furthermore, they play an important role in the pathogenesis of several diseases, including cancer, lymphedema, and various inflammatory conditions. Consequently, administration of lymphatic growth factors or related molecules provides the potential to target lymphatic vessels in human disease. In particular, therapeutic lymphangiogenesis is a promising gene therapy strategy for the treatment of lymphedema.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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