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Current Status of Therapeutic Angiogenesis with Protein, Gene and Cell Therapy

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Abstract: Therapeutic angiogenesis, stimulated growth of new vasculature to compensate for tissue ischemia, has been studied in a number of clinical trials in patients with various ischemic vascular diseases. These clinical trials include growth factor protein and gene therapy, as well as cell therapy. However, almost randomized clinical trials using vascular endothelial growth factor and fibroblast growth factor families, delivered as either recombinant protein or gene therapy, have failed to demonstrate improvement in patients with coronary artery or peripheral artery disease until now. However, randomized clinical trials using bone marrow-derived cells demonstrated modest but some significant benefit in patients with myocardial infarction. This report reviews the current status of randomized clinical trials and some non-randomized clinical trials using these therapies, plus related potential problems.

Key Words: VEGF, FGF, bone marrow-derived cell, peripheral arterial disease, myocardial infarction, clinical review.

INTRODUCTION

Despite substantial advances in medical therapy and revascularization techniques, vascular diseases combine to lead all cases of mortality especially in Western societies. In the United States, nearly a third of the annual mortality is related to vascular disease, with coronary heart disease accounting for approximately 480,000 deaths in 2003 [1]. Due to peripheral circulation disease, it has been estimated that nearly 25 major amputations are performed per 100,000 patients annually [2]. Symptomatic patients with extensive coronary artery disease (CAD) not amenable to conventional treatment, such as percutaneous transluminal angioplasty or coronary artery bypass grafting, have been estimated to account for 6-12% of the patients referred to interventional centers [3], resulting in poorer 5-year survival and angina-free survival rates in these patients [4]. Similarly, limited clinical options exist for a large proportion of patients with atherosclerotic peripheral arterial disease (PAD) and critical limb ischemia [5,6], resulting in debilitating symptoms and limb loss in these patients [2,5,6]. Advances over the last two decades in understanding of the molecular mechanisms that govern collateral artery growth and capillary neovascularization offer several approaches based on the generation of new vessels in ischemic tissues by angiogenic factor delivery, either as a recombinant protein or by gene transfer. In addition to this approach, novel therapeutic approaches aimed at regeneration of cardiomyocytes (cellular cardiomyoplasty) and/or promoting blood vessel formation (angiogenesis) using cell transplantation have become an attractive alternative treatment for CAD. Bone marrow (BM)-derived cells have recently gained attention for myocardial regeneration due to their easy accessibility from patients for autologous transplantation [7].

This review will focus on: (1) summarizing the results from the latest randomized clinical trials and some non-randomized trials using these approaches for ischemic cardiovascular disease; (2) describing potential related problems with these approaches. In this review, the term "angiogenesis" refers not only to narrow angiogenesis, but also to vasculogenesis and arteriogenesis.

1. CLINICALLY UTILISED GROWTH FACTORS FOR PROTEIN AND GENE THERAPY

Vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), granulocyte macrophage colony-stimulating factor (GM-CSF) and hepatocyte growth factor (HGF) have been used in clinical trials. For these growth factors, clinical trials have mainly focused on VEGF and FGF. Members of the VEGF family are highly specific ligands for endothelial cells and play pivotal roles in regulation of vascular and lymphatic growth, vascular permeability and influx of inflammatory cells [8]. VEGF-A is the most potent angiogenic growth factor known and plays a central role during the angiogenic response in tissue growth and repair [9-11]. VEGF-C, another member of the VEGF family, can stimulate growth of lymphatic vessels, a process called lymphangiogenesis [12]. Members of the FGF family are multifunctional growth factors that stimulate proliferation of many cells, including endothelial cells and smooth muscle cells [13]. HGF and GM-CSF also have angiogenic effects [14,15].

2. CLINICAL TRIALS UTILIZING RECOMBINANT PROTEIN AND GENE THERAPY

2.1. Coronary and Peripheral Vascular Disease

The randomized clinical trials described below are listed in Table **1**.

2.1.1. Recombinant Protein Therapy

In the TRAFFIC trial, 190 patients with moderate-to-severe intermittent claudication caused by infra-inguinal

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Table 1. Randomized Clinical Trials Using Protein and Gene Therapy for Coronary and Peripheral Vascular Disease

	Disease	Treatment	No. of Patients
TRAFFIC [16]	Intermittent claudication	Recombinant FGF-2	190
Seiler *et al.* [17]	CAD	Recombinant GM-CSF	21
START [18]	Intermittent claudication	Recombinant GM-CSF	40
Makinen *et al.* [19]	CLI, Critical ischemia from infra-inguinal artery disease	Adenovirus VEGF-A_{165}	54
RAVE [21]	Intermittent claudication	Adenovirus VEGF-A_{121}	105
GRONINGEN [22]	CLI	VEGF-A_{165} plasmid	54
TALISMAN 201 [23]	CLI	NV1FGF	125
VIVA [24]	CAD	Recombinant VEGF-A_{121}	178
Latine *et al.* [25]	CAD	Plasmid/liposome VEGF-A_{165}	15
Kupio Angioplasty [26]	CAD	Adenoviral VEGF, plasmid/liposome VEGF complex	103
REVASC [27]	CAD	Adenovirus VEGF-A_{121}	67
Powell *et al.* [28]	CLI	HGF plasmid	28

atherosclerosis were enrolled [16]. In this double-blinded, placebo-controlled trial, patients were randomized to bilateral intra-arterial infusion of placebo, single bolus or double bolus recombinant FGF-2. The primary endpoint was peak walking time (PWT). Patients in all three groups had significant differences in PWT compared to baseline. There was a statistically significant increase in PWT in the single bolus group compared to placebo, but not in the double bolus group compared to placebo at 90 days. However, the effect on PWT was not sustained at 180 days as a result of improvement in the placebo group PWT. Secondary endpoints were ankle-to-brachial indexes (ABIs), onset of claudication and quality of life, that did not differ between groups. Seiler *et al.* demonstrated in a pilot trial in 21 patients with extensive CAD, a significant positive effect on collateral artery flow from a single intracoronary bolus of GM-CSF, followed by a two week subcutaneous treatment period [17]. Although small in size, this was the first randomized placebo-controlled trial aiming specifically at stimulation of collateral artery growth. However, similar efficacy was not reproduced in other trials described below.

In the START trial, 40 patients with moderate or severe intermittent claudication were enrolled [18]. In this double-blinded, placebo-controlled trial, patients were randomized to subcutaneous application of placebo or GM-CSF. Walking distance increased in both groups at days 14 and 90, and no difference was observed between groups. Change in walking time, the primary endpoint, was not different between groups.

2.1.2. Gene Therapy

The first randomized, placebo-controlled, double-blinded study in patients with critical limb ischemia was reported in 2002 [19]. Fifty-four patients with intermittent claudication or critical ischemia from infra-inguinal arterial disease (severe stenosis or occlusion) suitable for angioplasty were enrolled. In this study, patients were randomized to receive adenovirus VEGF-A_{165} vector, VEGF-A_{165} plasmid/liposome complex or placebo at the angioplasty site. Primary endpoint was vascularity at angiography at 3-months follow-up. Angiography indicated significantly increased vascularity in both VEGF-A_{165}-treated groups. However, the consensus paper on suitable tools and endpoints for therapeutic angiogenesis investigation, pointed out that angiographic endpoints, such as vascularity measured by digital subtraction angiography used in this trial, are not reliable and therefore not recommended for this kind of trial, unless used during the screening process [20]. Secondary endpoints were restenosis rate, Rutherford class, and ABI at 3-months follow-up. Mean Rutherford class and ABI showed statistically significant improvement in both VEGF-treated groups, but similar improvements were also observed in control patients; furthermore, intergroup comparison between Rutherford class and ABI did not reach statistical significance.

In the RAVE trial, 105 patients with unilateral exercise-limiting intermittent claudication were enrolled [21]. In this double-blinded, placebo-controlled trial, patients were randomized to receive intramuscular injections of low-dose or high-dose adenovirus VEGF-A_{121} vector or placebo. The primary endpoint was PWT at 12 weeks, however no difference was observed between the three groups. Secondary endpoints included onset of PWT at 26 weeks, claudication onset time, ABI and quality of life at 12 and 26 weeks. These secondary endpoints were also similar between the three groups. Adenovirus VEGF-A_{121} vector administration was associated with peripheral edema.

In the GRONINGEN trial, 54 adult diabetic patients with critical limb ischemia (CLI) were enrolled [22]. In this double-blinded trial, patients were randomized to receive intra-

muscular administration of VEGF-A_{165} plasmid or placebo. The primary endpoint was the amputation rate at 100 days. However, no significant difference was observed between groups. Secondary endpoints were a 15% increase in pressure indices (ABI and toe-to-brachial index) and clinical improvement (skin, pain and Quality of Life score (QOL)). Statistical improvement of the pressure parameters and skin ulcers were observed for the VEGF-A_{165} plasmid group compared to the placebo group. Decrease of pain was not significantly different between the groups and there was no improvement in QOL with VEGF-A_{165} plasmid. However, clinical and/or hemodynamics responders, but not nonresponders, showed improved physical functioning, social functioning, and health changes in the VEGF plasmid group compared to the placebo group.

In the TALISMAN 201 trial, 125 patients with CLI were enrolled [23]. In this double-blinded, placebo-controlled trial, patients were randomized to receive intramuscular injections of non-viral FGF-1 (NV1FGF) or placebo. The primary endpoint was incidence of complete healing of at least one ulcer at week 25. Improvement in ulcer healing was the same between the NV1FGF and placebo groups. Secondary endpoints were amputation, death, ABI and transcutaneous oxygen pressure. Treatment with NV1FGF significantly reduced risk of and time to all and major amputations versus placebo. Clinical trials of NV1FGF therapy are ongoing as large-scale international joint studies.

In the VIVA trial, 178 patients with CAD were enrolled [24]. In this double-blinded, placebo-controlled trial, patients were randomized to receive intracoronary infusion, then intravenous infusion of placebo, low- or high-dose recombinant VEGF-A_{121}. The primary endpoint was a change in exercise treadmill test (ETT) time from baseline to 60 days, with no difference between groups. Secondary endpoints were angina frequency, myocardial infusion and quality of life at 60 days, with no difference between groups. The only benefit of VEGF-A_{121} therapy was an improvement in angina class from high-dose VEGF treatment at day 120, but not at day 60. Laitine *et al.* performed the first randomized, placebo-controlled trial investigating efficacy of plasmid/liposome VEGF-A_{165} gene transfer to coronary arteries guided by a perfusion-infusion catheter in 15 patients with stable CAD undergoing percutaneous coronary intervention (PCI) [25]. No differences were detected in the degree of coronary stenosis between treatment and control groups.

In the Kuopio Angioplasty Trial, 103 patients with CAD were enrolled [26]. In this placebo-controlled, double-blinded trial, patients were randomized to receive adenoviral VEGF, a plasmid/liposome VEGF complex or placebo using a perfusion-infusion catheter just after coronary angioplasty and stenting. The primary endpoint was restenosis rate, with no difference between groups. The only benefit was an improvement in myocardial infusion at 6 months from adenovirus VEGF treatment.

In the REVASC trial, 67 patients with CAD (Canadian Cardiovascular Society (CCS) class 2 to 4) were enrolled [27]. In this trial, patients were randomized to either continue best standard medical therapy or to receive adenovirus VEGF-A_{121} into the myocardium *via* thoracotomy. The primary endpoint, time to 1-mm ST-segment depression during an exercise test, was significantly improved at 26 weeks but not at 12 weeks in the adenoviral groups. Secondary endpoints, CCS class, and total exercise tolerance also improved at 12 weeks. No difference was observed in nuclear myocardial perfusion between the two groups. However, a significant contribution of placebo effect in these results cannot be ruled out because of the thoracotomy technique. This trial was also not blinded towards the treatment group, presenting a potential bias.

Powell *et al.* determined the efficacy of intramuscular injection of HGF plasmid at a low dose, middle dose and high dose in 104 patients with CLI in a double-blinded, placebo controlled trial [28]. Limb tissue perfusion, the primary endpoint, increased in the high-dose group compared to placebo, low-dose and middle-dose groups. However, there was no difference between groups in secondary endpoints, including ABI, toe-brachial index, pain relief, wound healing or major amputation. On the other hand, the sponsor claims that clinical trials in Japan have shown HGF plasmid therapy to be effective, although the results have not been published yet. Furthermore, an application for HGF plasmid as a new drug is now under review for approval in Japan.

2.2. Myocardial Ischemia

The randomized clinical trials described below are listed in Table 2.

2.2.1. Recombinant Protein Therapy

In the FIRST trial, 337 patients with coronary artery disease were enrolled [29]. In this placebo-controlled, open-label trial, patients were randomized to intracoronary infusion of placebo or three different concentrations of a single intracoronary infusion of recombinant FGF-2. The primary endpoint was ETT time from base line. Exercise tolerance increased in all groups at 90 and 180 days, with no differences observed between groups. Secondary endpoints were an angina questionnaire and nuclear perfusion imaging. Treatment with FGF-2 did not change nuclear perfusion compared to the placebo group at either 90 or 180 days. Although the angina questionnaire favored treatment with FGF-2, the effect was lost at 180 days.

2.2.2. Gene Therapy

Losordo *et al.* performed a randomized, double-blinded, placebo-controlled trial investigating the efficacy of naked plasmid VEGF-C gene transfer to left ventricular myocardium guided by NOGA mapping in 19 patients with chronic myocardial ischemia [30]. The only benefit of VEGF-C therapy was a significant reduction in CCS angina class. In the Angiogenic GENe-1 Trial, 79 patients with chronic angina CCS class 2 or 3 were enrolled [31]. In this double-blinded, placebo-controlled trial, patients were randomized to intracoronary infusion of placebo or 5 increasing doses of a single intracoronary infusion of adenoviral FGF-4. The endpoint was ETT. Exercise tolerance increased in all groups at 4 and 12 weeks, with no differences observed between groups. Censoring data only to include patients with a baseline ETT of 10 min. or less indicated a significant improvement in ETT time to angina in treated compared to placebo groups at both 4 and 12 weeks.

Table 2. Randomized Clinical Trials Using Protein and Gene Therapy for Myocardial Ischemia

	Disease	Treatment	No. of Patients
FIRST [29]	Coronary artery disease	Recombinant FGF-2	337
Losordo *et al.* [30]	Chronic myocardial ischemia	Naked plasmid VEGF-C	19
Angiogenic GENe-1 [31]	Chronic angina	Adenoviral FGF-4	79
AGENT-2 [32]	Stable angina, Reversible ischemia	Adenovirus FGF-4	52
AGENT-3, AGENT-4 [33]	Chronic angina	Adenovirus FGF-4	532
EUROINJECT-One [34]	Ischemic heart disease	Naked plasmid VEGF-A_{165}	80

On the basis of suggested partial anti-ischemia effects from the AGENT-1 trial, the AGENT-2 trial was performed [32]. In the AGENT-2 trial, 52 patients with stable angina and reversible ischemia comprising >9% of the left ventricle were enrolled. In this double-blinded, placebo controlled trial, patients were randomized to intracoronary infusion of placebo or single intracoronary infusion of adenovirus FGF-4. Adenosine single-photon emission computed tomography was performed to assess improvement of myocardial perfusion after 8 weeks. Treatment with adenovirus FGF-4 significantly reduced ischemia defect size, with placebo treated patients showing no improvement. However, the change in reversible perfusion defect size between groups was not significant.

In the AGENT-3 and AGENT-4 trials, 532 patients with chronic angina were enrolled. In this double-blinded, placebo-controlled trial, patients were randomized to intracoronary infusion of placebo or intracoronary infusion of low- or high-dose adenovirus FGF-4. Unexpectedly, these larger trials were stopped when an interim analysis of the AGENT-3 trial indicated that the primary endpoint change from baseline in total ETT time at 12 weeks would not reach significance [33]. Analysis of pooled data showed that the effect of placebo was large and not significantly different to active treatment in males. The placebo effect in women was negligible, but ETT time, time to 1 mm ST-segment depression and time to angina and CCS class were significantly improved from active treatment compared to placebo, suggesting a gender-specific angiogenic response from adenovirus FGF-4 in these trials.

In the EUROINJECT-One study, 80 patients with severe stable ischemia heart disease (CCS class 3 or 4) were enrolled. In this double-blinded trial, patients were randomized to receive intramyocardial injections of naked plasmid VEGF-A_{165} or placebo with a NOGA catheter [34]. After a 3 month follow-up, no significant differences were observed between groups in CCS class or size of perfusion defect. The only benefit of VEGF-A_{165} therapy was a significant improvement in local wall motion disturbances.

3. CLINICALLY USED CELLS

Currently, a variety of autologous adult progenitor cells are undergoing clinical evaluation. The use of allogenic human adult progenitor cells may potentially avoid the poor quality of autologous progenitor cell resources in patients with severe diseases and/or many risk factors. However, these cells may provoke immune responses as foreign antigens, resulting in rapid elimination of these cells from the body and occurrence of severe clinical complications such as anaphylaxis in some cases. Therefore, clinical trials using these cells have not been performed.

Adult BM is composed of a multitude of cell types: hematopoietic cells, mesenchymal cells, endothelial progenitor cells and stroma cells, as well as other cell types yet to be characterized. The mononuclear cell fraction of BM from density gradient centrifugation is used in most studies. To date, isolated BM-derived cells have been injected into the heart without further *ex vivo* expansion.

Endothelial progenitor cells (EPCs) have been defined by their cell surface expression of the hematopoietic stem cell markers CD133 and CD34 and the endothelial marker VEGF receptor-2, plus capacity to differentiate into endothelial cells. EPCs are readily isolated from blood and BM mononuclear cells on the basis of the CD34 cell-surface receptor, then expanded *in vitro*.

Skeletal myoblasts are progenitor cells that normally lie in a quiescent state under the basal membrane of mature muscle fibers. They are easily obtained and have an exceptional capacity to proliferate *in vitro*. In addition, skeletal myoblasts graft into ischemic myocardium and are highly resistant to hypoxia. These cells are committed to a myogenic lineage.

A specific subfraction, the hematopoietic progenitor CD34+ cells, can be enriched from whole blood after granulocyte colony-stimulating factor (G-CSF)-mediated mobilization from the BM into the blood [35].

Although there are several other stem cell types, including embryonic stem cells, adult cardiac stem cells, fetal cardiomyocytes and multipotent adult germline stem cells, that are candidates for stem cell therapy [36,37], these stem cell types are not currently being investigated in clinical trials.

4. CLINICAL TRIALS USING CELL THERAPY

4.1. Acute Myocardial Infarction

4.1.1. Clinical Trials Using BM-Derived Cell Therapy

The randomized clinical trials described below are listed in Table 3.

Table 3. Randomized Clinical Trials Using BM-Derived Cell Therapy for Acute MI

	No. of Patients
BOOST [38, 39]	60
Janssens *et al.* [40]	67
Lunde *et al.* [41]	100
REPAIR-AMI [43, 44]	204
ASTAMI [41, 45]	100
Melzin *et al.* [46]	66
Ge *et al.* [47]	20
Panovsky *et al.* [48]	82
Meluzin *et al.* [49]	60
Erbs *et al.* [50]	58

In the BOOST trial, 60 patients with acute myocardial infarction (MI) were enrolled. In this trial, patients were randomized to receive conventional medical therapy or intracoronary infusion of BM-derived cells plus medical therapy after successful percutaneous coronary intervention [38]. At 6 months follow-up, there was greater improvement in global left ventricular ejection fraction (LVEF) in BM-derived cell-treated patients. However, no significant benefit from BM cell therapy on LVEF was observed at 16 months [39]. Janssens *et al.* performed a double-blinded, randomized controlled trial in 67 patients with acute MI that included a sham control group [40]. They demonstrated that intracoronary transfer of BM-derived cells at one day after successful percutaneous coronary intervention was associated with a reduction of infarct size over 4 months, but did not show any significant improvement in LVEF myocardial flow, metabolism in infarcted segments or clinical outcome.

Lunde *et al.* performed a randomized control trial in 100 patients with acute MI that underwent percutaneous coronary intervention [41]. They demonstrated that intracoronary transfer of BM-derived cells did not improve LV function at 6 months. Seeger *et al.* suggested that the cell isolation procedure and the composition of the cell storage buffer used were not as effective for recovering cell and ensuring proper cell function in comparison with that of BOOST trial [42], respectively. In the REPAIR-AMI trial, 204 patients with acute MI were enrolled. In this double-blinded, placebo controlled trial, patients were randomized to receive either placebo medium or BM-derived cells [43,44]. REPAIR-AMI trial showed that BM cell therapy was associated with a significant 2.5% improvement in LVEF [44]. There was also a significant reduction of the occurrence of major adverse cardiovascular events, such as the pre-specified cumulative endpoint of death, myocardial infarction and requirement for revascularization, or combined endpoint of death, recurrence of myocardial infarction and rehospitalization at 12 months [43].

REPAIR-AMI trial also showed that three-fold LVEF improvement in those with baseline LVEF<49% (train medium LVEF) compared to subjects with LVEF above median 49% [44], suggesting that further clinical trials will focus on patients with higher risks for morbidity and mortality (i.e. lower LVEF) after acute MI. In the ASTAMI trial, 100 patients with acute MI were enrolled [41,45]. In this controlled trial, patients were randomized to receive either intracoronary injection of BM-derived cells or control. The ASTAMI trial indicated improvement in exercise time and heart response to exercise from intracoronary BM cell therapy [45]. However, there was no improvement in LV infarct area, LV size, and function at 6 months follow-up [41]. Meluzin *et al.* performed a randomized control trial in 66 patients with acute MI [46]. They demonstrated that intracoronary transfer of BM-derived cells was associated with improvement of regional myocardial function of the infarcted wall in a dose-dependent manner at 6 months. Ge *et al.* performed a randomized control trial that included BM supernatant in 20 patients with acute MI [47]. They demonstrated that intracoronary transplantation of BM-derived cells after primary percutaneous coronary intervention improved LVEF, left ventricular end diastolic internal diameter and myocardial perfusion defect scores. Panovsky *et al.* performed a randomized trial in 82 patients with acute MI treated with stent implantation [48]. They did not demonstrate improvement of myocardial function with transplantation of BM-derived cells. Although the reasons the clinical trial failed to demonstrate the improvement are unknown, the cell isolation procedure may have adversely affected the cell component, or the effect of the composition of the cell storage buffer on the cell stability may not have been appropriate for ensuring proper cell function. Meluzin *et al.* performed a randomized control trial in 60 patients with acute MI [49]. They demonstrated that intracoronary transplantation of BM-derived cells improved global left ventricular systolic function. However, improvement of regional systolic function of the infarcted wall was partially lost at 12 months. Erbs *et al.* performed a randomized, double-blinded, placebo-controlled trial in 58 patients with reperfused acute MI [50]. They demonstrated that intracoronary transplantation of BM-derived cells improved coronary flow reserve in the infarct artery, which is associated with an improvement in maximal vascular conductance capacity at 6-month follow-up.

4.1.2. Clinical Trials Using Mobilization of Hematopoietic Progenitor Cells by G-CSF

The randomized clinical trials described below are listed in Table **4**.

Non-randomized and randomized clinical studies have shown that granulocyte colony stimulating factor (G-CSF) therapy after acute MI reduces LV remodeling and improves LV ejection fraction [35,51,52]. However, recently two randomized, blinded trials after MI have not shown any beneficial effect on left ventricular function with G-CSF after 3-6 months follow-up [53,54]. The difference in outcomes between the trials may be a result of the specific targeting ability of the mobilized stem cells to the infarcted myocardium, the time of G-CSF delivery and the effect of

Table 4. Randomized Clinical Trials Using Mobilization of Hematopoietic Progenitor Cells by G-CSF for MI

	No. of Patients
Kang *et al.* [51]	27
Valgimigli *et al.* [52]	14
Zohlnhofer *et al.* [53]	114
Engelmann *et al.* [54]	44

pro-inflammatory cells. Large-scale clinical research studies using G-CSF are ongoing in Japan.

4.2. Chronic MI

4.2.1. Clinical Trials Using BM-Derived Cell Therapy

The randomized clinical trials described below are listed in Table 5.

Table 5. Randomized Clinical Trials Using BM-Derived Cell Therapy for Chronic MI

	Disease	No. of Patients
Hendrikx *et al.* [55]	Chronic MI	20
Kang *et al.* [56]	Old MI	50
Assumus *et al.* [57]	Stable MI	75
Tse *et al.* [58]	CAD	28
Yao *et al.* [59]	Chronic MI	24

Hendrikx *et al.* performed a randomized control trial in 20 patients with chronic MI [55]. They demonstrated that intramyocardial injection of BM-derived cells during coronary artery bypass graft did not improve global LVEF, but did recover regional contraction function in areas with a previously nonviable score. Kang *et al.* performed a randomized control trial in 50 patients with old MI [56]. Assumus *et al.* performed a randomized, controlled crossover trial in 75 patients with stable MI either previously receiving no cell infusion or intracoronary infusion of BM-derived cells [57]. They demonstrated that intracoronary infusion of BM-derived cells improved global and regional LVEF compared to EPCs or no infusion at 3 months follow-up. Tse *et al.* performed a randomized, blinded, and placebo-controlled trial that included plasma injection in 28 patients with severe CAD [58]. They demonstrated that endomyocardial implantation of BM-derived cells improved exercise treadmill time, LVEF and New York Heart Association class at 6 months, but did not improve CCS class. Yao *et al.* performed a randomized control trial that included saline infusion in 24 patients with chronic MI [59]. They demonstrated that intracoronary transfer of BM-derived cells after successful PCI did not improve cardiac systolic function, infarct size or myocardial infusion, but did improve diastolic function.

4.2.2. Clinical Trials Using EPC Therapy

The randomized clinical trials using EPC therapy described below are listed in Table 6.

TOPCARE-AMI clinical trials involving patients with AMI demonstrated that circulating progenitor cell therapy improved myocardial function to the same extent as BM cell therapy [60,61], although these clinical trials were not randomized or controlled. Erbs *et al.* performed a randomized, placebo-controlled trial in 26 patients with chronic MI [62]. They demonstrated that intracoronary injection of blood-derived circulating progenitor cells after recanalization of chronic coronary total occlusion results in increase of coronary flow reverse in response to adenosine, decline of the number of hibernating segments in the target region, reduction of infarcted size and increase of LVEF. However, Assumus *et al.* could not demonstrate improvement of global and regional LVEF by intracoronary infusion of blood-derived circulating progenitor cells in the randomized controlled crossover trial described above [57]. No adverse effects were reported in these clinical trials. It may be interesting to speculate why blood-derived circulating cell-derived therapy failed to demonstrate the efficacy in the randomized controlled crossover trial, whereas BM-derived cell-derived therapy demonstrated modest but some significant efficacy in various clinical trials as described above. Although adult BM is composed of multiple types of cells other than EPC, blood-derived circulating cells are composed of EPC only. Therefore, blood-derived circulating cells may be not sufficient to stimulate angiogenesis to the extent of demonstrating efficacy. Currently, a limited number of clinical trials of cultured EPC are ongoing in Thailand, China, and Canada [63]. In addition, many clinical research studies using cultured peripheral mononuclear cells as highly advanced medicine are ongoing in Japan.

4.2.3. Clinical Trials Using Skeletal Myoblast Therapy

Several small scale Phase 1 clinical trials involving patients with chronic ischemic cardiomyopathy indicate that administration of skeletal myoblasts improves myocardial function [64-69]. At the same time, a high incidence of monomorphic ventricular tachycardia was found in some of these clinical trials [64,69]. In the MAGIC trial, 97 patients with left ventricular dysfunctioning, myocardial infarction, and indication for coronary surgery were enrolled [70]. In this placebo-controlled trial, patients were randomized to receive skeletal myoblasts or placebo, combined with coronary surgery. Skeletal myoblast transfer did not improve regional and global LV function. A higher number of arrhythmic events happened in the skeletal myoblast-treated patients. The MAGIC trial is listed in Table 6.

5. SAFETY

5.1. Clinical Trials Using Recombinant Protein and Gene Therapy

To date, no evidence of increased tumorigenesis, neovascularization in non-target organs, vascular malformation, increased atherogenesis, or plaque destabilization has been observed in the clinical trials described above. Edema was reported in two early clinical trials of VEGF gene therapy

Table 6. Randomized Clinical Trials Using EPC and Skeletal Myoblast Therapy for MI

	Disease	Treatment	No. of Patients
Erbs *et al.* [62]	Chronic MI	Blood-derived circulating progenitor cells	26
Assumus *et al.* [57]	Stable MI	Blood-derived circulating progenitor cells	75
MAGIC [70]	MI, left ventricular dysfunctioning	Skeletal myoblasts	97

and the RAVE trial. Lower extremity edema was also found to be associated with naked plasmid VEGF gene therapy in 34% of patients with critical limb ischemia, as a result of increased permeability of endothelium [4]. Edema was less common for patients with claudication than in those with pain at rest or ischemia ulcers for naked plasmid VEGF gene therapy [71]. Treatment was typically limited to a brief course of oral diuretics without serious sequelae. Peripheral edema was found to be associated with adenoviral VEGF-A_{121} vector therapy in the RAVE trial [21]. However, edema was not reported in other clinical trials described above. Mild transient fever and development of anti-adenovirus antibodies have been reported after intra-arterial administration of adenoviral vectors [19,24]. Forty-four diabetic patients were treated with naked plasmid VEGF gene, however there was no evidence of progression of retinopathic change to proliferative disease [72], although the type of VEGF isn't certain. In summary, according to current experience from clinical studies, treatment with VEGF and FGF protein, and gene therapies has been well tolerated, and no therapy-related serious adverse effects have been reported.

5.2. Clinical Trials Using BM-Derived Cell Therapy

To date, no evidence of several potential issues (electrical stability, tumorgenesis, increased restenosis, or progression of atherosclerosis) has been reported in the clinical trials described above.

Intramyocardial calcification was reported to occur in murine models of myocardial infarction after direct injection of unpurified bone marrow cells [73,74]. However, the occurrence of calcification has not been reported in the clinical trials described above. This may be explained by the enrichment of mononuclear cells by density gradient used in the majority of the clinical trials. Injection of unfractionated BM cells or mesenchymal stem cells, but not purified hematopoietic progenitor cells induces development of encapsulated structures containing calcification and/or ossification in the murine model. Therefore, the mesenchymal stem cell fraction in BM cells is likely to be the origin of extended bone formation in the infarct myocardium [74].

In summary, according to current experience from clinical studies, treatment with BM-derived cells has been well tolerated, and no therapy-related serious adverse effects have been reported.

6. CHOICE OF ANGIOGENIC FACTORS IN CLINICAL TRIALS USING RECOMBINANT PROTEIN AND GENE THERAPY

The failure of expected benefit in almost of the clinical trials describe above suggests that single growth factors delivered for a short duration, are not sufficient to sustain angiogenic response. Indeed, angiogenesis is a complex process that requires concerned, sequential, and sustained action of multiple growth factors, angiogenesis inhibitors and modulators. For example, robust expression of VEGF in animal hearts after viral delivery resulted in the formation of an unstable and permeable vascular network, that undergoes regression relatively quickly [75-77]. Early clinical studies using plasmid VEGF-A_{165} administered to patients with ischemic limbs showed angiogenic evidence of new vessel formation, however these vessels did not persist and regressed within three months [78]. These findings indicate that VEGF alone may not be sufficient to form stable, mature vessels characterized by recruitment of perivascular mural cells, such as pericytes or smooth muscle cells [79]. Therefore, precise harmonized interplay of different angiogenic factors may result in proper vascular network formation. However, arteriogenesis, which can compensate for arterial occlusion and maintain distal tissue perfusion, is the preferred type of neovascularization purpose of restoring myocardial infusion. A variety of growth factors appear to have important effects enhancing arteriogenesis, including monocyte chemoattracting protein 1 [80,81], transforming growth factor beta [2], GM-CSF [17], FGF-1, FGF-2 [82] and platelet-derived growth factor-BB (PDGF-BB) [82]. Several combinations of growth factors enhancing angiogenesis and arteriogenesis, including VEGF-A_{165} plus angiopoietin-1 [83], VEGF plus PDGF-BB [84], and VEGF plus FGF-2 [85] have been shown to stimulate angiogenesis and/or arteriogenesis synergistically in animal models. However, many additional investigations need to be performed to elucidate the real cross-talk between all of those factors in order to establish their optimal combinations for clinical use.

7. MODE AND METHOD OF DELIVERY

7.1. Clinical Trials Using Recombinant Protein and Gene Therapies

The most likely explanation for the negative clinical results is that growth factor concentration in human tissues has not reached sufficient levels and/or has not persisted long enough for triggering relevant vascular growth. This, in turn, can result from several factors, such as the short half-life of recombinant growth factors, insufficient dose of adenovirus, too short a time for gene expression or a compromised delivery route. Serum half-life of human recombinant FGF-2 was reported to be 50 min. in mongrel dogs [86]. Basic science studies suggest that efficient transduction and expression of the introduced gene in small animal models rarely results in the same effect in humans. For example, adenovirus LacZ transfection has been estimated to be up to 10 times more efficient in mice than humans [87]. Of the few studies in

primates, naked DNA delivery was far more less efficacious and far more variable than in rodents [88]. Intramyocardial NOGA catheter-mediated injections of naked VEGF-A_{165} or a mature, soluble form of VEGF-D plasmid did not induce significant protein production or any vascular effects [89]. This result agrees with the findings in the Eurojoint One trial, suggesting intramyocardial injections of naked VEGF-A_{165} or –C plasmid likely yielded insufficient transfection efficiency [34]. In the RAVE trial, the adenoviral VEGF-A_{121} dose was approximately 2 logs less per kilogram than that used in recent pre-clinical experiments showing very high angiogenic efficacy, with also approximately 2 logs lower biological efficacy of VEGF-A_{121} than VEGF-A_{165} [21,90-92]. Intravenous and intracoronary delivery of protein has limited myocardial tissue distribution and retention in most studied strategies. The majority of the growth factor delivered intravenously is taken up by the liver [93]. Intrapericardial delivery of protein resulted in improved myocardial distribution and retention, however endocardial penetration was poor [86,94]. Intramyocardial delivery resulted in the best myocardial deposition and retention (still less than 20%), although myocardial distribution is limited to areas immediately surrounding the injection sites. Although it is also important to develop more suitable vectors with specificity targeting the injury site, high penetration capability into the injured site and high expression capability for the gene product, that discussion is beyond the scope of this chapter. Please refer to the review by Jazda *et al.* for further discussion of this topic [95].

7.2. Clinical Trials Using BM-Derived Cell Therapy

The main task of delivery is to transplant a sufficient number of cells into the myocardium and achieve maximum survival and retention of those cells within the infarcted area.

7.2.1. Intracoronary Injection

Intracoronary transplantation is a catheter-based technique. Cells are aspirated into an over-the-wire intracoronary balloon catheter placed at the target lesion and delivered *via* the distal lumen. This method of delivery is attractive as cells are delivered directly to the infarcted myocardium without having to go through the systematic circulation. It is relatively easy and rapid, and also does not require surgical intervention to provide access to the target tissue. In the BOOST trial using this method, 1% to 2% of infused BM-derived cells were detected in the infarcted myocardium [39]. Hofmann *et al.* also showed that 1.3% to 2.6% of infused BM-derived cells were detected in infarcted myocardium using this method [96]. Most of the clinical trials described above have used intracoronary transplantation, demonstrating efficacy of this approach.

7.2.2. Intramyocardial Injection

Intramyocardial injection of cells for cardiomyoplasty involves direct injection into the myocardium of the infarcted region. The advantage of intramyocardial injection is that it provides a direct route of administration to the affected myocardium. The major disadvantage is the need for concomitant open-heart surgery. Two of the clinical trials described have used intramyocardial injection [55,58], with efficacy of this approach demonstrated in one clinical trial [55].

7.2.3. Transendocardial/Transepicardial Injection

Transendocardial injection is performed under the guidance of electromechanical mapping [97]. Several non-randomized clinical trials have demonstrated the feasibility of transendocardial injection of BM-derived cells [98-100]. Transepicardial injection can be performed as an adjunct to CABG. Injection during open heart surgery allows for direct visualization of the myocardium. Drawbacks of this approach remain its invasiveness and difficulty to document results of cellular cardioplasty [101]. This approach has not been used for clinical trials of stem cell therapy.

7.2.4. Intravenous Infusion

Intravenous delivery of BM-derived cells did not result in the homing of these cells to infarcted myocardium in one clinical trial [96]. One possible explanation is that intravenously administered cells will often home to non-cardiac organs [102].

8. PATIENT SELECTION

8.1. Clinical Trials Using Recombinant Protein and Gene Therapy

Most clinical trials to date have opted to study patients with disease or comorbid conditions so advanced that they have no other therapeutic options. This patient population may be a difficult one to demonstrate clinical efficacy in, as the pathophysiology may be too far advanced to be amenable to change with proangiogenic therapy. Animal studies have demonstrated efficacy of various growth factors in stimulating new blood vessel formation and improvement of perfusion in models of chronic myocardial and perivascular ischemia utilizing young, healthy animals free of co-morbid cardiovascular disease risk factors. This is likely the reason that angiogenic factors have been demonstrated as effective in preclinical studies, yet not effective in clinical studies. Indeed, diabetes, elevated cholesterol, and their resultant endothelial dysfunction (contributors to coronary artery disease) result in an impaired angiogenic response [103-105]. For example, diabetic mice were shown to have impaired limb revascularization compared to healthy animals, related to an alteration in the angiogenic regulatory network [106]. Hypercholesterolemia has also been shown to have a detrimental effect on collateral artery growth in mice [107]. In a porcine model of chronic myocardial ischemia, hypercholesterolemia showed significant endothelial dysfunction and impaired angiogenesis compared to a control, normal diet [105]. Many similar studies in diseased animal models suggest that biological response to ischemia is likely to be very different between healthy subjects and patients with comorbid disease.

Although *in vitro*, proangiogenic factors can promote microvessel growth, there is a less clear understanding of the angiogenesis microenvironment in patients with limb or cardiac ischemia. In one study, patients with CLI were found to have normal VEGF levels, elevated FGF levels, but decreased VEGF receptor levels [108]. In another study, circu-

lating VEGF levels were found to increase following peripheral angioplasty [109]. It is uncertain whether an increase in VEGF to supraphysiological levels acts to stimulate and patient-specific or disease-specific lack of responsiveness to VEGF is a fundamental issue.

However, a limited number of individual patients appear to respond much better to treatment than others. The TRAFIC study suggests that outcomes may be better for current smokers, but a dedicated study has not been completed. In a recent small, randomized, placebo-controlled trial of diabetics with CLI treated with plasmid VEGF-A_{165}, a significant improvement of some clinically important outcomes was observed after treatment, although no significant change in amputation rates was observed [22]. In the AGENT-3 and AGENT-4 trials, adenoviral FGF-4 vector treatment improved ETT time, time to 1 mm ST-segment depression, time to angina and CCS class in female, but not male, patients with angina [33].

Standard medical therapy and current revascularization methods improve prognosis, relieve symptoms and can be applied to most patients with CAD or PAD. Therefore, a novel approach would be the combination of conventional revascularization procedures with adjuvant VEGF gene therapy. Recent findings on blood flow as a modulator of vascular growth support this kind of novel concept in which peripheral angiogenesis could improve the "run-off" of grafts and possibly lead to better outcomes [92,110]. In this setting, patients with less severe forms of disease would also become eligible for VEGF trials.

8.2. Clinical Trials Using Cell Therapy

Aging and various degrees of cardiovascular risk factors strongly influence the mobilization and functions of BM-derived cells. Hill *et al.* showed that there was a highly significant negative correlation between the number of circulating progenitor cells and subject combined Framingham risk factor score [111]. In addition, EPCs from subjects at high risk for cardiovascular events had higher rates of *in vitro* senescence than cells from subjects at low risk [111]. Therefore, patients with these risk factors may not be suitable for stem cell therapy.

9. ENDPOINT

Several endpoints, including ETT, prolonged survival and improvement in health-related QOL, are used for the assessment of clinical efficacy in the cardiovascular therapies described above. In addition to these endpoints, rate of patient survival, rehospitalization, and revascularization, as well as frequency of angina and patient functional status need be closely monitored. However, there are several problems about the validity of some endpoints and their measurement techniques. Please refer to the detailed review by Simon *et al.* [20] for a discussion of these problems.

10. DOSE OF CELL THERAPY

The number of BM-derived cells transplanted is highly variable between trials, ranging from approximately 1×10^8 cells in ASTAMI to approximately 3×10^9 cells in the BOOST trial. It is interesting to compare the correlation between efficacy and cell dose. However, meta-analysis could not demonstrate a statistical association between number of cells injected and LVEF change [112]. This may be because a wide range of cell numbers infused are needed to show any correlation, or because of differences in the functional capacity of cells, such as the ability to specifically home to and engraft in the infarcted area, plus ability to produce paracrine factors.

11. TIME POINT FOR CELL TRANPLANTATION IN ACUTE MI

The peak of inflammatory response in myocardial infarction is observed in the first days, with excessive production of cytokines, growth factors and extracellular matrix proteins mediating myocardial repair [113]. Transplantation of active progenitors in this period may exacerbate undesirable effects of inflammation on regenerative processes in the myocardium. Several animal and clinical studies support this concept. In one animal study, fetal rat cardiomyocytes were implanted into cryoinjured adult rat hearts immediately, 2 weeks and 4 weeks after injury [114]. Negative results for immediate cell transplantation were reported, with the best results obtained when progenitor cells were implanted after 2 weeks. In one negative clinical study [40], cells were delivered at 24 hours after coronary reperfusion. However, positive results were obtained in patients that received the intervention several days later [38,44,56,57,97].

12. MECHANISM OF ACTION OF CELL THERAPY

A great deal of earlier evidence indicates that haematopoietic progenitor cells differentiate into cardiomyocytes, leading to improvement in cardiac function in cardiomyoplasty [115-117]. However, other studies in animals have not demonstrated differentiation of haematopoietic progenitor cells into cardiomyocytes [118-120]. This suggests that the positive results shown in earlier studies were due to background signals from fluorescently tagged antibodies that track cell fate [121]. Even if differentiation does happen, the number of differentiated and functionally integrated myocytes derived from transplanted stem cells is too low to explain the observed improvements in cardiac function [122,123]. Cell fusion is another mechanism for describing stem cell plasticity. Although stem cells fused with myocytes [124,125], fusion events are not frequent, therefore not likely to be clinically relevant. Another mechanism by which cell therapy may exert a beneficial influence is a paracrine factor effect. Several studies suggest that cardiac function could be improved by a paracrine factor effect, whereby growth factors, cytokines, and signaling molecules produced by the infused stem cells induce neoangiogenesis, proliferation of endogenous cardiac stem cells, and favor the viability of myocytes because of the inhibition of apoptotic signaling [126-128]. However, the exact mechanism remains to be elucidated.

13. *EX VIVO* MANUPLATION OF CELLS

Therapeutic potency of cell therapy products may be enhanced by genetic modification with expression vectors to overexpress pro-survival factors, angiogenic factors, growth factors, or stem-cell homing factors. For example, *ex vivo* transfection of BM-derived cells or skeletal myoblasts with adenovirus VEGF-A_{165} vector was shown to improve their

capacity to augment neovascularization in a hind limb ischemia model [129,130]. *Ex vivo* transplantation of skeletal myoblasts and BM-derived cells with adenovirus VEGF-A_{165} vector or liposome based plasmid VEGF-A_{165} was shown to improve their capacity to augment neovascularization and cardiac function in acute MI and ischemic cardiomyopathy models [131-133]. *Ex vivo* transfection of BM-derived cells with adenovirus or retrovirus Akt vector improved their capacity to augment cardiac function in a myocardial infarction model [122,134-136]. *Ex vivo* non-viral transfection of skeletal myoblasts with stromal cell derived factor (SDF-1α) gene was shown to enhance angiomyogenesis in a myocardial infarction model [137].

Pre-conditioning of cells with cell survival and migration factor is another strategy. Priming of endothelial progenitor cells with SDF-1α augmented their therapeutic potential in an ischemic hind limb model [138]. Pretreatment of BM mononuclear cells with endothelial NO synthase enhancer AVE9488 was shown to improve neovascularization and exercise capacity in an ischemic hind limb model [139].

14. FUTURE PERSPECTIVES

There are several issues to be overcome before consideration of protein and gene therapy and/or cell therapy as future angiogenic therapies. The main issues are summarized below. It is anticipated that appropriate endpoints will be closely monitored at long-term follow-up in larger randomized clinical trials.

14.1. Protein and Gene Therapy

To date, almost randomized clinical trials using VEGF and FGF growth factor families, delivered as either recombinant protein or gene-based therapy, have not provided convincing evidence of clinical efficacy in patients with CAD and PAD. Certain issues need to be overcome: (1) optimal trial design, (2) optimal delivery of growth factor and gene, (3) optimal dosage of growth factor and gene, (4) optimal selection of growth factors and (5) proper patient selection.

14.2. Cell Therapy

At present, BM-derived cell therapy is the most promising strategy for MI because of the modest but some significant benefit demonstrated in several randomized clinical trials. Certain issues need to be overcome: (1) optimal dosage and composition of cells, (2) optimal method to increase engraftment and survival of transplanted cells, (3) optimal method for enhancing cell function and (4) optimal timing for transplanting cells.

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Freeze-Drying of Proteins in Glass Solids Formed by Basic Amino Acids and Dicarboxylic Acids

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The purpose of this study was to produce and characterize glass-state amorphous solids containing amino acids and organic acids that protect co-lyophilized proteins. Thermal analysis of frozen solutions containing a basic amino acid (*e.g.*, L-arginine, L-lysine, L-histidine) and a hydroxy di- or tricarboxylic acid (*e.g.*, citric acid, L-tartaric acid, DL-malic acid) showed glass transition of maximally freeze-concentrated solute at temperatures (T'_g) significantly higher than those of the individual solute solutions. Mixing of the amino acid with some dicarboxylic acids (*e.g.*, oxalic acid) also suggested an upward shift of the transition temperature. Contrarily, combinations of the amino acid with monocarboxylic acids (*e.g.*, acetic acid) had T'_gs between those of the individual solute solutions. Co-lyophilization of the basic amino acids and citric acid or L-tartaric acid resulted in amorphous solids that have glass transition temperatures (T_g) higher than the individual components. Mid- and near-infrared analysis indicated altered environment around the functional groups of the consisting molecules. Some of the glass-state excipient combinations protected an enzyme (lactate dehydrogenase, LDH) from inactivation during freeze-drying. The glass-state excipient combinations formed by hydrogen-bonding and electrostatic interaction network would be potent alternative to stabilize therapeutic proteins in freeze-dried formulations.

Key words freeze-drying; protein formulation; amorphous; stabilization; glass

Freeze-drying is a popular method of ensuring the stability of proteins that are not stable enough in aqueous solutions during the period required for storage and distribution.[1,2] Various freeze-dried protein formulations contain excipients (*e.g.*, sugars, polymers, and amino acids) that protect proteins from physical and chemical changes. Disaccharides (*e.g.*, sucrose, trehalose) are the most popular among them because they stabilize proteins both thermodynamically and kinetically in aqueous solutions and freeze-dried solids.[3—5]

The development of freeze-dried protein formulations containing amino acids is often more challenging than the development of formulations with saccharides because of the varied physical and chemical properties (*e.g.*, crystallinity, glass transition temperature) of the freeze-dried amino acids, as well as their tendency to form complexes with other ingredients.[6] Many amino acids are considered to protect proteins basically in similar mechanisms with disaccharides. They thermodynamically stabilize protein conformation in aqueous solutions and probably in frozen solutions by being preferentially excluded from the immediate surface of proteins.[7] Glass-state amorphous solids formed by freeze-drying of the disaccharides or some amino acids protect proteins from structural changes thermodynamically by substituting surrounding water molecules.[8] They also reduce chemical degradation of freeze-dried proteins kinetically by reducing the molecular mobility.[2,8] In addition, some amino acids (*e.g.*, L-arginine) also prevent protein aggregation in aqueous solutions prior to the drying process and after reconstitution.[9] Choosing appropriate counterions that form glass-state solid should be one of the key factors in designing amino acid-based amorphous freeze-dried formulations.[10,11] For example, glass transition temperatures (T_g) of freeze-dried L-histidine salts depend largely on the counterions.[12] Co-lyophilization of L-arginine and multivalent inorganic acids (*e.g.*, H_3PO_4, H_2SO_4) results in glass-state amorphous solids that protect proteins during the process and storage (*e.g.*, tissue plasminogen activator formulation, PDR 2003).[13] Some organic acid and inorganic cation combinations (*e.g.*, sodium citrates) also form high glass transition temperature amorphous solids.[14] Various functional groups (*e.g.*, amino, carboxyl, hydroxyl) in the constituting molecules contributes significantly to form the glass-state amorphous salt solids.[15] Producing glass-state amorphous solids by freeze-drying of amino acid and organic acid combinations, and their application in pharmaceutical formulations are interesting topics to explore.[15]

The purpose of this study was to produce stable amorphous solids that protect proteins by freeze-drying combinations of amino acids and organic acids. The physical properties of frozen solutions and freeze-dried solids containing the popular excipients and model chemicals were studied. The effect of the excipient combinations on the freeze-drying of lactate dehydrogenase (LDH) was also examined.

Experimental

Materials LDH (rabbit muscle) was obtained from Sigma Chemical (St. Louis, MO, U.S.A.). Succinic acid was produced by Kanto Chemical Co. (Tokyo, Japan). L-(+)-Tartaric acid, DL-malic acid, and other chemicals were of analytical grade and were purchased from Wako Pure Chemical (Osaka, Japan). The protein solutions were dialyzed against 50 mM sodium phosphate buffer (pH 7.0), and then centrifuged (1500 g×5 min) and filtered (0.45 μm, polyvinyliden difluoride (PVDF), Millipore) to remove insoluble aggregates before the freeze-drying study.

Freeze-Drying A pH meter (HM-60G, TOA-DKK Co., Tokyo, Japan) was used to determine the pH of the aqueous solutions at 25 °C. A freeze-drier (Freezvac 1C, Tozai-Tsusho, Tokyo, Japan) was used to lyophilize the aqueous solutions. Aliquots of aqueous solutions (250 μl) in flat-bottom glass vials (10 mm diameter) were frozen by immersion in liquid nitrogen. The solutions were freeze-dried without shelf temperature control (20 h), and then at 35 °C (8 h). Solid samples for diffuse-reflection near-infrared analysis were prepared by freeze-drying the aqueous solutions (2 ml) in glass vials (21 mm diameter).

Thermal Analysis Thermal analysis of frozen solutions and dried solids

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was performed using a differential scanning calorimeter (DSC) (Q-10, TA Instruments, New Castle, DE, U.S.A.) and software (Universal Analysis 2000, TA Instruments). Aliquots of aqueous solutions (10 μl) in aluminum cells were cooled from room temperature at 10 °C/min, and then scanned from −70 °C at 5 °C/min. The effect of heat-treatment (annealing) on the thermal properties of the frozen solutions was studied after the initial heating scan paused at −10 °C, then the samples were maintained at this temperature for 10 min. Thermal data were acquired in the subsequent heating from −70 °C at 5 °C/min. Freeze-dried solids (1—2 mg) in hermetic aluminum cells were subjected to the thermal analysis from −20 °C at 5 °C/min under nitrogen gas flow. Melted organic acids (approx. 5 mg, 200 °C) in aluminum cells were rapidly cooled to −50 °C, and then scanned at 5 °C/min to obtain the glass transition temperatures. Glass transition temperatures were determined as the midpoint (maximum inflection) of the discontinuities in the heat flow curves.

Powder-X-Ray Diffraction (XRD) The powder X-ray diffraction patterns were measured at various temperatures by using a Rint-Altima diffractometer (Rigaku, Tokyo, Japan) with Cu*K*α radiation at 40 kV/40 mA. The samples were scanned in the area of $5° < 2\theta < 35°$ at an angle speed of 15°/min by heating at 2 °C/min from room temperature.

Mid- and Near-Infrared Analysis A Fourier-transform infrared spectrophotometer (MB-104, Bomen, Quebec, Canada) with a gas generator (Balston, Haverhill, MA, U.S.A.) and Grams/32 software were used to obtain mid-infrared spectra of freeze-fried solids. Approximately 0.5 mg of the solid was mixed with dried KBr powder (250 mg) and made into tablets by compression. The KBr tablets were scanned 128 times to obtain the spectra in the 400—4000 cm^{-1} region. Near-infrared spectroscopy was performed by using a Bruker MPA system with a diffuse-reflectance integrating-sphere probe (PbS detector) and OPUS software (Ettlingen, Germany). Near-infrared light was directed upward from the bottom of the glass vials containing freeze-dried solids to obtain the reflected signal over a range of 4000—12000 cm^{-1} with a resolution of 4 cm^{-1} in 128 scans. The freeze-dried solids were measured twice by rotating the sample vials between measurements.

Activity of Lactate Dehydrogenase in Freeze-Dried Solids Aqueous solutions (250 μl) containing LDH (0.05 mg/ml) and excipients were freeze-dried in flat-bottom glass vials (10 mm diameter). One of the enzyme solutions was freeze-dried at a higher sodium phosphate buffer concentration (50 mM, pH 7.0). Other enzyme solutions contained the added excipients and lower concentration buffer components (<1 mM) diluted from the dialyzed protein solutions. Activity of LDH was obtained spectrophotometrically at 25 °C. Each 1.0 ml of assay mixture contained 0.35 mM pyruvic acid and 0.07 mM reduced nicotinamide-adenine dinucleotide (NADH) in 50 mM sodium phosphate buffer (pH 7.5). The enzyme reaction was started by the addition of LDH solution (50 μl), and the decrease in the absorbance at 340 nm was monitored. The enzyme activity (%) relative to that before freezing was shown.

Results

Physical Property of Frozen Solutions The thermal profiles of frozen solutions containing L-histidine and citric acid at various concentration ratios (total 200 mM) are shown in Fig. 1. The single-solute frozen L-histidine solution (200 mM) showed a T_g' (glass transition temperature of maximally freeze-concentrated solute) at −33.5 °C, and an exotherm peak that suggests eutectic crystallization at around −8 °C.[12] Freeze-drying of solutions at above their T_g' often induces physical collapse because of the significantly reduced local viscosity in the freeze-concentrated phase.[1] The second scan of the 200 mM L-histidine solutions after the heat-treatment (−10 °C, 10 min) gave flat thermograms that indicate crystallized solute up to the ice melting temperature (data not shown). The citric acid solution (200 mM) had a T_g' at −55.1 °C, indicating that the solute remained amorphous in the freeze-concentrated phase surrounding ice crystals. The L-histidine crystallization peak disappeared in the presence of citric acid. The two-solute frozen solutions showed transitions (T_g's) at temperatures as high as −22.8 °C at the equal (100 mM) L-histidine and citric acid concentrations.

Figure 2 shows transition temperatures (T_g') of frozen solutions containing amino acids and organic acids at various concentration ratios. Some single-solute frozen amino acid or organic acid solutions (200 mM) had apparent T_g' transitions at −44.2 °C (L-arginine), −55.1 °C (citric acid), and

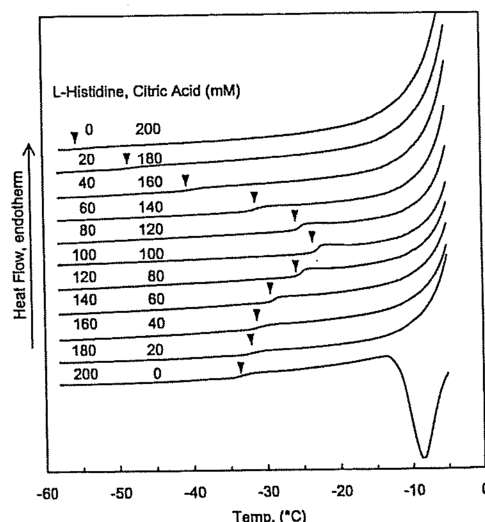

Fig. 1. Thermal Profiles of Frozen Solutions Containing L-Histidine and Citric Acid

Aliquots (10 μl) of solutions in hermetic aluminum cells were scanned from −70 °C at 5 °C/min. Glass transition temperatures of maximally freeze-concentrated solutes (T_g') are indicated by inverted triangles (▼).

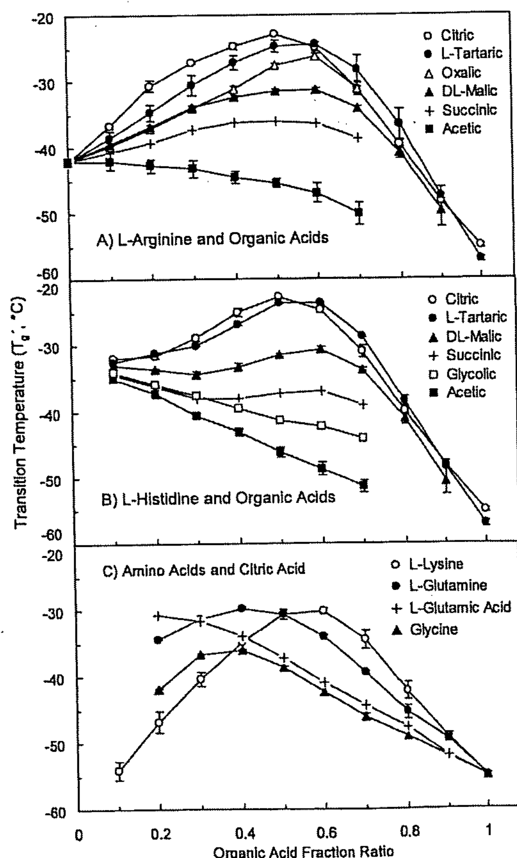

Fig. 2. Glass Transition Temperatures of Maximally Freeze-Concentrated Solute (T_g') in Frozen Solutions Containing an Amino Acid and an Organic Acid at Varied Concentration Ratios (Total 200 mM, Average±S.D., $n=3$)

$-57.1\,°C$ (L-tartaric acid). The frozen L-glutamine solution showed both T_g' ($-42.8\,°C$) and the subsequent eutectic crystallization peak (approx. $-25\,°C$) in the heating scan (data not shown). Thermograms of the frozen L-lysine and DL-malic acid solutions inclined gradually without apparent transition up to the ice melting endotherm, which suggested T_g's lower than $-60\,°C$. Exotherm peaks either in the cooling process (glycine, acetic acid) or in the heating scan (oxalic acid) indicated eutectic crystallization in the frozen solution.[16] Potential T_g' transitions of some frozen solutions that also showed eutectic crystallization peaks (*e.g.*, 200 mM L-histidine or L-glutamine) were not included in the figure. The limited solubility of some amino acids and organic acids (*e.g.*, L-glutamic acid, fumaric acid, maleic acid) prevented them from undergoing thermal analysis at 200 mM. A lower concentration glutamic acid solution (100 mM) showed a T_g' at $-32.2\,°C$ and an exotherm peak that suggests eutectic crystallization at around $-11.0\,°C$ (data not shown).

Mixing of the solutes induced some unique physical properties in the frozen solutions that depend on the number of functional groups in the consisting molecules. The transition temperatures (T_g's) of frozen solutions containing a basic or neutral amino acid (L-histidine, L-arginine, L-lysine, L-glutamine, glycine) and a hydroxy di- or tricarboxylic acid (citric acid, L-tartaric acid, DL-malic acid) showed bell-shaped profiles. The frozen solutions containing a hydroxy di- or tricarboxylic acid (citric acid, L-tartaric acid) and an acidic amino acid (L-glutamic acid) did not show the mixing-induced upward T_g' shift. Citric acid also effectively prevented the crystallization of glycine in the frozen solutions. Dicarboxylic acids (succinic acid, maleic acid, fumaric acid, oxalic acid) showed a high tendency to crystallize in the single-solute frozen solutions and in some mixture frozen solutions.[15,17] The frozen solutions containing L-arginine and oxalic acid or succinic acid also presented the high transition temperature (T_g') by mixing. A mono-carboxylic acid (acetic acid), a hydroxy mono-carboxylic acid (glycolic acid), and HCl did not show the upward T_g' shift in the mixture with the basic amino acids.[13]

Physical Property of Freeze-Dried Solids Freeze-drying of the single-solute amino acid solutions resulted in cylindrical cakes that showed varied crystallinity in the powder X-ray diffraction (XRD) and thermal analyses (Figs. 3, 4). Freeze-dried L-arginine showed the typical harrow XRD pattern of amorphous solids. Thermal scan of the solid showed the glass transition (52.6 °C) and subsequent crystallization exotherm (105—110 °C). Freeze-dried L-histidine showed largely amorphous XRD pattern (30 °C) with the broad glass transition (65—100 °C) and crystallization at varied temperatures (120—150 °C). The L-arginine and L-histidine solids showed apparent crystallization peaks in the XRD patterns at the elevated temperature (150 °C). The dried L-glutamine (200 mM) solids showed features of both crystalline (*e.g.*, peaks in the XRD pattern) and amorphous (*e.g.*, glass transitions and heat-induced crystallization exotherm) solids. The solute concentration in the initial solution and thermal history in the freeze-drying process should determine the crystallinity of the freeze-dried L-histidine and L-glutamine.[12] Glycine was freeze-dried as β polymorph crystal.[18] Freeze-drying of citric acid or L-tartaric acid solutions (200 mM) resulted in unstructured or particulate solids that indicate physical collapse in the primary during process. Amorphous solids of the organic acids prepared by rapid-cooling of the melt liquid showed glass transition at 9.2 °C (citric acid) and 68.1 °C (L-tartaric acid) in the thermal scan ($n=3$).[19]

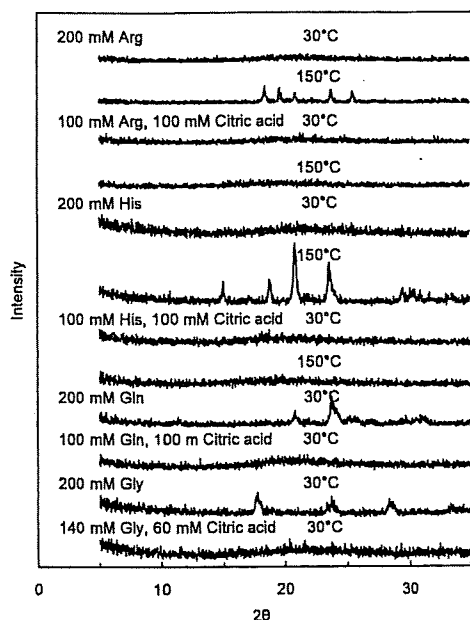

Fig. 3. Powder X-Ray Diffraction Patterns of Freeze-Dried Solids Containing Amino Acids and Citric Acid

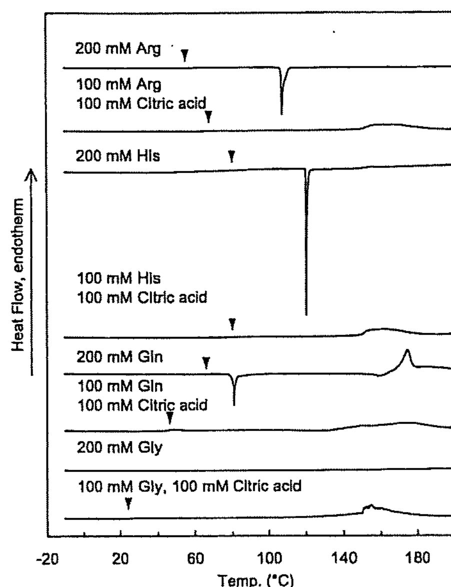

Fig. 4. DSC Thermograms of Freeze-Dried Solids Containing Amino Acids and Citric Acid

Freeze-dried solids (1—2 mg) in hermetic aluminum cells were scanned from −20 °C at 5 °C/min.

Co-lyophilizing the basic or neutral amino acids (L-arginine, L-histidine, L-glutamine, glycine) and the organic acid (citric acid, L-tartaric acid) produced cylindrical non-crystalline cake solids at wide initial concentration ratios (Figs. 3—5). The solids obtained by freeze-drying the basic amino acids (L-arginine, L-histidine) with citric or L-tartaric acid showed glass transition at temperatures (T_gs) much higher

than those of the individual components. The transitions were observed at temperatures as high as 89.5 °C (140 mM L-arginine, 60 mM citric acid) or 98.5 °C (160 mM L-histidine, 40 mM citric acid). Shrinking of some solids containing higher ratio of organic acid during the freeze-drying process suggested their low glass transition temperatures. The XRD and thermal analysis also indicated that the co-lyophilized solids remained amorphous up to 150 °C. Some binary freeze-dried solids showed a broad endotherm that suggests component decomposition at the elevated temperatures. The mixing of L-arginine with citric acid and with L-tartaric acid showed similar T_g profiles, in spite of the large difference in their transition temperatures of the cooled-melt solids. The bell-shaped profiles of the transition temperatures were significantly different from the reported transitions of binary nonionic molecule systems that follow Gordon–Taylor equation.[20] Glass transition temperatures of amorphous solids containing ideally mixed nonionic molecules without particular attractive or repulsive interactions shift between those of the individual components. Contrarily, the glass transition temperatures of co-lyophilized L-glutamine and citric acid combination solids shifted linearly between those of the individual components, which suggested absence of the particular attractive interactions between the heterogeneous molecules in the solids. Co-lyophilization of glycine and citric acid resulted in amorphous cake solids only at limited molar ratios.

Transition temperatures (T'_g, T_g) of the excipient combinations obtained at a fixed (0.1) molar ratio interval were plotted against the pH of the initial solutions (25 °C, Fig. 6). Some mixtures (*e.g.*, L-arginine and citric acid, L-histidine and citric acid) yielded high T'_g frozen solutions and high T_g freeze-dried solids from weakly acidic initial solutions ($-35\,°C<T'_g$, $80\,°C<T_g$, pH 4—6), which are preferable in parenteral protein formulations. Small changes in the L-arginine and organic acid compositions (0.1 molar fraction) significantly shifted pH at the neutral region.

The mid- and near-infrared spectra of the freeze-dried L-arginine and citric acid combinations showed broad absorption bands that are typical of amorphous solids (Figs. 7, 8).[21] Co-lyophilization with citric acid reduced an amino group absorption band of L-arginine at 1550 cm^{-1} in the mid-IR spectra (KBr method), indicating altered environment of the functional group. Similar reduction of the amino group band has been reported in L-arginine–HCl salt crystal and L-argi-

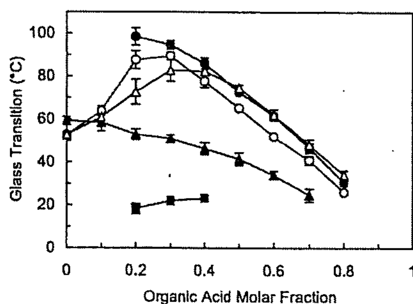

Fig. 5. Glass Transition Temperatures of Freeze-Dried Binary Solids

Each symbol denotes transition of solids containing L-arginine and citric acid (○), L-arginine and tartaric acid (△), L-histidine and citric acid (●), L-glutamine and citric acid (▲), or glycine and citric acid (■) (total: 200 mM, average±S.D., n=3).

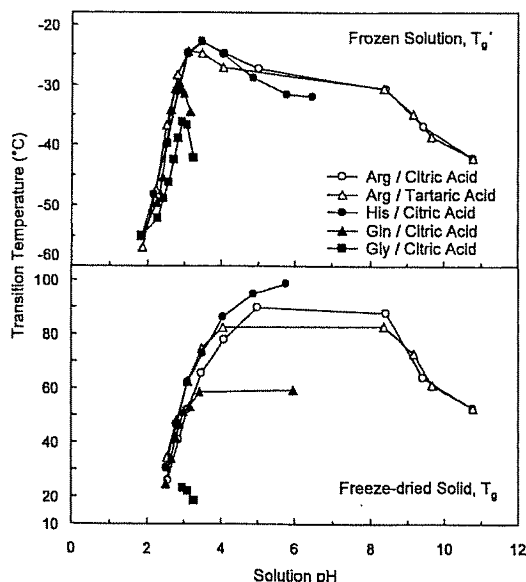

Fig. 6. Effect of Initial Solution pH (25 °C) on the Transition Temperatures of Frozen Solutions (T'_g) and Freeze-Dried Solids (T_g) Containing an Amino Acid and an Organic Acid at a Fixed (0.1) Molar Concentration Ratio Intervals (200 mM Total, n=3)

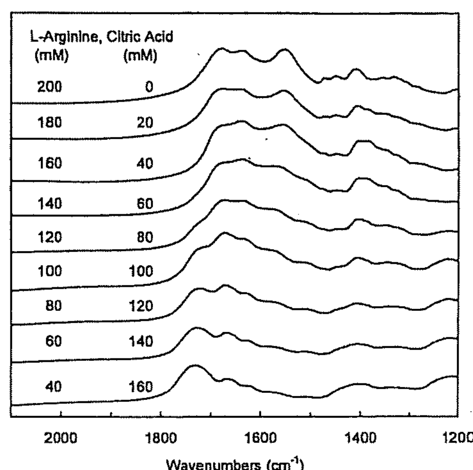

Fig. 7. Mid-Infrared Spectra of Freeze-Dried L-Arginine and Citric Acid Combinations Obtained by a KBr Tablet Method (128 Scans)

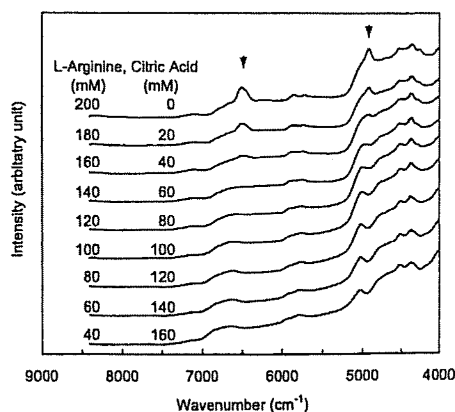

Fig. 8. Diffuse-Reflection Near-Infrared Spectra of Freeze-Dried L-Arginine and Citric Acid Combinations Obtained at the Bottom of the Glass Vials (128 Scans)