Gene and cytokine therapy for heart failure: molecular mechanisms in the improvement of cardiac function

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Nagai T, Komuro I. Gene and cytokine therapy for heart failure: molecular mechanisms in the improvement of cardiac function. Am J Physiol Heart Circ Physiol 303: H501–H512, 2012.—Despite significant advances in pharmacological and clinical treatment, heart failure (HF) remains a leading cause of morbidity and mortality worldwide. Many new therapeutic strategies, including cell transplantation, gene delivery, and cytokines or other small molecules, have been explored to treat HF. Recent advancement of our understanding of the molecules that regulate cardiac function uncover many of the therapeutic key molecules to treat HF. Furthermore, a theory of paracrine mechanism, which underlies the beneficial effects of cell therapy, leads us to search novel target molecules for genetic or pharmacological strategy. Gene therapy means delivery of genetic materials into cells to achieve therapeutic effects. Recently, gene transfer technology in the cardiovascular system has been improved and several therapeutic target genes have been started to examine in clinical research, and some of the promising results have been emerged. Among the various bioactive reagents, cytokines such as granulocyte colony-stimulating factor and erythropoietin have been well examined, and a number of clinical trials for acute myocardial infarction and chronic HF have been conducted. Although further research is needed in both preclinical and clinical areas in terms of molecular mechanisms, safety, and efficiency, both gene and cytokine therapy have a great possibility to open the new era of the treatment of HF.

myocardial infarction

Introduction

Despite significant advances in pharmacological and clinical treatment, heart failure (HF) remains a leading cause of morbidity and mortality worldwide. Numerical evidence suggests that HF is a progressive disorder of which pathogenesis is related to many factors such as ischemia, mechanical stress, inflammation, metabolic disorders, and genetic disorders (74). Many new therapeutic strategies, including cell transplantation, gene delivery, and cytokines or other small molecules, have been explored to treat the various pathophysiological status of HF. Functional improvement after cell transplantation in the failing heart after ischemia has been demonstrated in preclinical and clinical studies for different subsets of bone marrow-derived cells and other adult progenitor cells, as well as cardiac stem cells, embryonic or induced pluripotent stem cells, are considered as cellular sources for generating cardiomyocytes (68, 133). Thus cell therapy is a promising approach for the treatment of HF. One of the most recent hypotheses is that the transplanted cells exert their beneficial effect via a paracrine mechanism in which molecules from transplanted cells may promote angiogenesis, cell survival, stem cell homing, myocardial contraction, and cardiomyogenesis (33). A quantitative proteomic approach and secretome analysis of transplanted cells may enable us to uncover the target molecules for pharmacological or genetic strategy. Since the particular drug or gene therapy may enhance the effect of cell therapy on cardiac regeneration, cell and pharmacological or gene therapy are complementary.

HF is characterized by impaired contraction and relaxation of the affected ventricles. Recently, great attention has been paid to two molecular pathophysiological abnormalities in failing myocytes: downregulation and desensitization of β-adrenergic receptors (β-ARs) (10, 26) and alterations of intracellular Ca2+ handling (6, 44). Although treatment with β-blockers improved the prognosis of HF, other pharmacological therapy intended to reverse or bypass β-adrenergic desensitization, such as phosphodiesterase inhibitors, catecholamines, or other positive inotropic agents, showed symptomatic improvement but increased mortality in patients (28). Similarly, transgenic overexpression of β-ARs, G proteins, or protein kinase A (PKA) caused short-term improvements in cardiac function but long-term cardiac dysfunction (23, 26). Ca2+ transients in failing cardiomyocytes are characterized by a lower amplitude and slower decline compared with those of normal cardiomyocytes (5, 50). It has been reported that sarcoplasmic reticulum (SR) and its membrane-anchored proteins, ryanodine receptors (RyRs), sarco(endo)plasmic reticulum...
lum Ca\textsuperscript{2+}-ATPase (SERCA), and phospholamban (PLN), play a pivotal role in Ca\textsuperscript{2+}-handling system (5).

Recently, our understanding of the molecules involved in \( \beta \)-adrenergic systems and Ca\textsuperscript{2+} handling has been advanced. Therefore, more appropriate HF therapies by using newly developed drugs or genes has been proposed. Currently, numerous small molecule chemicals that have ability to improve cardiac function or attenuate cardiac remodeling have been reported. These include G protein \( \beta \)-subunit (G\( \beta \)) inhibitors, cardiac myosin activators, neuregulin-1\( \beta \), thyroid hormone analog, and P2X4 purinergic receptor agonists and so on (112, 115). Although a careful elucidation of dose/regimen is required to prevent the potential risk of systemic adverse effects, some of the chemicals have advanced into clinical trials. A phase Ib study of elective allosteric activator of cardiac myosin omecamtiv mecarbil in patients with left ventricular systolic dysfunction hospitalized for acute HF is now enrolling patients (73). Phase II and phase III studies of a neuregulin-1\( \beta \) fragment and a phase I study of a full-length glycosylated recombinant human neuregulin-1\( \beta \)3 are ongoing (109). Because a complete review of all HF drugs is beyond the scope of this review, the authors recommend recent excellent reviews of key cardiac signaling molecules that are potential drug targets to treat HF (115).

Gene therapy means the delivery of genetic materials into cells to achieve therapeutic effects. As mentioned above, the discovery of new molecular targets for HF therapy facilitate the clinical application of pharmacological manipulations; however, a number of these molecules cannot be intervened systemically, rather suitable for gene therapy. In addition, the heart is easily and selectively accessible by percutaneous catheter approaches so that the myocardium is ready for gene delivery system. Over the past decade, there has been a great advancement in cardiovascular gene transfer technology in terms of the transduction efficiency and safety of viral vectors, methods of vector delivery, and nonviral gene carriers (59, 110, 135). Nonviral vectors such as plasmid DNA, liposome-DNA, and polymer-DNA are less expensive and safe; however, viral vectors are superior to the nonviral vectors in terms of efficiency of myocardial transduction and long-term transgene expression. Currently, the most commonly used viral vector in clinical cardiovascular gene therapy is adenovirus (121). Adeno-associated virus (AAV) and retrovirus are also used in some clinical trials. There are the advantages and limitations in each vector. Recombinant human adenoviruses efficiently deliver and express their genomes in both dividing and nondividing cardiomyocytes. However, the immune response that results in the myocardial inflammation and elimination of infected cells is a large obstacle. AAV can induce stable and long-lasting gene expression compared with adenovirus because of its minimal immunogenicity and particular serotypes, which has excellent tropism for the heart. The major disadvantage of AAV is their limited genome packing capacity. In addition, like adenovirus, the presence of neutralizing antibodies against some serotypes limits the efficiency of gene therapy with AAV. Retrovirus has the advantage in high-transduction efficiency and stable transduction into dividing cells; however, vector genome integration into the host genome raise a concern for the risk of oncogenic transformation. Lentivirus resembles retrovirus in its ability to stably integrate into the target cell genome. In contrast to retrovirus, lentivirus can transduce larger transgenes into nondividing cells. It is possible to minimize the risk of oncogenic transformation by modifying the vector design (75). Because ischemic heart disease is the most common cause of HF, the initial aim of gene therapy was targeted to restore the coronary vasculature. Genetic transfer of angiogenic growth factors or cytokines has been evaluated on animal models, and several controlled clinical trials for ischemic heart disease have been published including VEGF-A165, VEGF-121, VEGF-C, and FGF-4 (66). Along with elucidating multiple steps at which \( \beta \)-AR signaling systems and excitation-contraction coupling are dysregulated, various targets for gene therapy such as G protein-coupled receptor kinase 2 (GRK2), adenyl cyclase type 6 (AC6), SERCA2a, PLN, inhibitor protein (IN) 1 and 2, and S100A1 have been emerged. Preclinical data suggest that these molecules are important to restore the normal cardiac function. In the first part of this review, we will focus on the recent advancement of the gene therapy targeting on the above seven molecules.

Various growth factors and cytokines, such as granulocyte colony-stimulating factor (G-CSF), erythropoietin (EPO), and insulin-like growth factor-1 have been reported to prevent cardiomyocytes from apoptosis as well as promote angiogenesis (12, 40, 89). Recently, particular interest has been focused on mesenchymal stem cells (MSCs) from bone marrow or fat tissue as potential cell therapy candidates (130). It has been reported that MSCs secrete a variety of cytokines, which inhibit apoptosis, inflammatory cascade, and degradation of extracellular matrix, leading to cardiac repair (97). Preclinical studies have shown the improvement in cardiac function after administration of MSC-conditioned medium (33). Secretome analysis of MSCs under stimulated conditions, such as hypoxia or pharmacological and genetic modulation, is expected to unveil novel paracrine factors (97). These findings prompt the clinical trials of cytokine therapy for treating cardiovascular diseases as cell-free therapy alternate to using MSCs. In contrast to newly developed pharmacological chemicals, cytokines are a component of intrinsic factors, and several cytokines have already been used for human hematopoietic or autoimmune diseases, suggesting less concern about safety for clinical application. Therefore, a proper use of cytokines may be a promising therapeutic strategy against HF. In the second part of this review, we focus on the results of recent clinical trials of cytokine therapy, especially of G-CSF and EPO, and introduce the current understanding of their beneficial mechanisms.

**Gene Therapy Targeted on \( \beta \)-Adrenergic Signaling**

\( \beta \)-ARs are seven transmembrane-spanning G protein-coupled receptors. \( \beta \)-ARs are comprised of three subtypes: \( \beta_1 \)-, \( \beta_2 \)-, and \( \beta_3 \)-ARs. In the heart \( \beta_1 \)-ARs are the predominant subtype. Activation of \( \beta \)-ARs in response to sympathetic neurotransmitters such as epinephrine and norepinephrine results in the dissociation of the stimulatory G protein \( \alpha \)-subunit (G\( \alpha \)) from G\( \beta \)\( \gamma \). Gas stimulates AC to produce cAMP from ATP, leading to the activation of cAMP-dependent PKA, locally bound by an A kinase-anchoring protein. PKA regulates different intracellular, sarcolemmal, and myofilibril substrates, resulting in the increasing of Ca\textsuperscript{2+} influx and efflux and actin-myosin interaction. Therefore, \( \beta_1 \)-ARs play a critical role in the regu-
GRK2 phosphorylates GRK2 translocation and binds to its carboxyl-terminal domain. G protein-coupled receptor kinase 2. In the failing heart, one of the biological defects is a significant alteration of β-AR system (11). The molecular mechanism of β-AR dysfunction is clarified as a selective reduction of β1-AR density at the plasma membrane (downregulation) and an uncoupling of β1-ARs from G proteins (desensitization) (9, 72). GRK2 are serine/threonine kinases, which consist of a central catalytic domain (~270 amino acids) flanked by an amino-terminal domain (~185 amino acids) and a variable-length carboxyl-terminal domain (~105–230 amino acids). In the heart, GRK2 is a predominant form. When catecholamine occupies β1-AR and Gαi dissociates from Gβγ, the remaining Gβγ facilitates GRK2 translocation and binds to its carboxyl-terminal domain. GRK2 phosphorylates β1-AR and enhances the affinity for binding to β-arrestins, which prevents further G protein activation and induces the endocytic process of β1-AR (16, 91). It has been reported that the expression and activity of GRK2 are significantly elevated in human HF (126). Therefore, the potential effects of GRK2 inhibition on in vivo model were extensively studied.

Koch et al. (62) developed carboxy-terminus of β-AR kinase (β-ARKct) peptide, which competes with endogenous GRK2 for binding to Gβγ and works as a GRK2 inhibitor. Mice overexpressing the β-ARKct peptide displayed enhanced cardiac contractility in vivo with or without isoproterenol (62). Subsequent studies showed that overexpression of β-ARKct improves cardiac function in a murine model of HF, such as muscle LIM protein-deficient and calsequestrin-overexpressing mice (41, 102). The beneficial effects of β-ARKct in HF were also observed in species other than the mouse. Intracoronary delivery of adeno-virus encoding β-ARKct into rabbit HF model after myocardial infarction (MI) revealed the improvement of systolic function and the increase in β-AR density and AC activity (111). Rengo et al. (100) have reported that long-term suppression of GRK2 by delivery of recombinant AAV serotype 6 (AAV6) encoding β-ARKct improves cardiac contractility and reverses LV remodeling in the rat HF model after MI. Furthermore, adeno-virus-mediated β-ARKct gene transfer in failing human ventricular myocytes increased the contraction and relaxation velocities as well as AC activity in response to β-agonist (131). Recently, Katz et al. (57) have reported successful delivery of self-complementary AAV6 encoding β-ARKct into the normal sheep heart by using molecular cardiac surgery with recirculating delivery system. The heart treated with AAV6 encoding β-ARKct showed a significant increase in the maximum values of the first derivative of LV pressure (dP/dt max) compared with control and moderate increase in cAMP and β-AR density (57). Raake et al. (96) delivered AAV6 encoding β-ARKct by retrograde injection into the anterior interventricular vein of porcine model of HF after MI. Treatment with β-ARKct significantly ameliorated LV hemodynamics and contractile function in HF pigs (96). Although further studies with preclinical large animal models of HF are needed, β-ARKct gene therapy in human HF seems to be promising.

Adenylyl cyclase type 6. AC6 regulates the conversion of ATP to cAMP, leading to the activation of PKA and initiating a variety of intracellular signaling cascades that influence heart function. The general structure of AC consists of two transmembrane regions and two cytosolic loops. Cytosolic loops comprise the catalytic core, a primary site for the regulation of AC activity. A dominant isoform expressed in mammalian cardiac myocytes is AC6 and AC5 (39, 92). Gao et al. (31) have reported that transgenic mice with cardiac-directed expression of AC6 show normal cAMP production, cardiac function, myocardial β-AR number, and G protein content, expect for an increase in GRK2 content. Under the stimulation through the β-AR, cardiac function and cAMP production were increased (31). It is noteworthy that long-term overexpression of AC6 does not alter β-AR signaling except when receptors are activated. This is in contrast to β-AR and G protein overexpression, which cause detrimental effects on cardiac function. The beneficial effect of cardiac-directed expression of AC6 has been reported in Gaq-associated myocardopathy and MI model (105, 118). Roth et al. (106) have reported that indirect intracoronary delivery of adenovirus encoding AC6 produces a significant increase in cardiac contractile responses to β-AR stimulation (106). The same group showed that adenovirus-mediated AC6 expression improved cardiac function in cardiac-directed overexpression of Gaq mice (98). In preclinical studies, by using pig pacing-induced HF model, Lai et al. (65) have reported that intracoronary delivery of adenovirus-encoding AC6 attenuates LV remodeling and increases fractional shortening. LV dP/dt max, the minimum values of the first derivative of LV pressure (dP/dt min),

**Fig. 1.** Cardiomyocyte β-adrenergic receptor (β-AR) signaling during heart failure (A) and after treatment with carboxy-terminus of β-AR kinase (β-ARKct) and adenylyl cyclase type 6 (AC6) gene therapy (B). A: G protein-coupled receptor kinase 2 (GRK2) phosphorylates β-AR and facilitates the recruitment of β-arrestin, resulting in β-AR downregulation and impairment of further G protein activation. B: β-ARKct inhibits the recruitment of GRK2, re-sensitizes β-ARs, and promotes normalization of cAMP signaling. AC6 augments Ca2+ handling via protein kinase A (PKA)-dependent and -independent pathway. ATP, adenosine 5'-triphosphate; cAMP, adenosine 3',5'-cyclic monophosphate; CA, catecholamine; PI3, phosphotidylinositol 3; ATF3, activating transcription factor-3.
and cAMP production were enhanced in response to \( \beta_1 \)-AR agonist, and levels of B-type natriuretic peptide were reduced (65). Currently, a phase-I/II study of human adenovirus-5 encoding human AC6 gene transfer in patients with HF began enrollment (ClinicalTrials.gov, NCT00787059). The study was a randomized, double-blinded, placebo-controlled study. The vector will be delivered by intracoronary injection with dose escalation. Recently, cAMP-independent effects of AC6 on cardiomyocytes have been reported. These included an increase in activating transcription factor-3 expression, leading to reduced PLN transcription and an increase in phosphatidylinositol 3-kinase (PI3K)/Akt activation, resulting in increased PLN phosphorylation and expression of Bcl-2 protein (32). Although the contribution of these mechanisms is uncertain in the failing human heart, the multifunctional roles strengthen the value of AC6 as the target of gene therapy.

**Gene Therapy Targeted on Excitation-Contraction Coupling**

The \( Ca^{2+} \) handling during contraction and relaxation is a prominent feature of cardiomyocytes (5). In brief, membrane depolarization triggers \( Ca^{2+} \) influx through L-type \( Ca^{2+} \) channels, followed by a \( Ca^{2+} \)-induced \( Ca^{2+} \) release through the RyR of SR. The elevated cytosolic \( Ca^{2+} \) binds to troponin C and activates the contraction unit. During the systolic period, SR \( Ca^{2+} \) content is being depleted, followed by an inactivation of RyRs, and cardiomyocytes then turn from a systolic to diastolic mode. During the diastolic period, the cardiac SERCA2a and sarcolemmal \( Na^+/Ca^{2+} \) exchanger are the major mechanisms in \( Ca^{2+} \) extrusion. \( Ca^{2+} \) uptake through the SERCA pump is negatively regulated by PLN. The amplitude and frequency of the \( Ca^{2+} \) transient are regulated by the phosphorylation status of \( Ca^{2+} \) cycling regulators, which depends on the balance between the activity of kinase and phosphatase. PKA and \( Ca^{2+}/calmodulin \)-dependent protein kinase II (CaMK II) are essential kinases in cardiomyocytes. PKA is activated by \( \beta \)-AR signal, which is mediated by AC and cAMP formation. PKA phosphorylates L-type \( Ca^{2+} \) channels and RyR, which enhances \( Ca^{2+} \) influx and promotes SR \( Ca^{2+} \) release, respectively. PKA also phosphorylates and inactivates PLN, resulting in the augmentation of SERCA pump activity. Proteins phosphorylated by PKA or CaMK II are actively dephosphorylated by phosphatases such as protein phosphatase (PP) 1 and 2A. Cytosolic PP1 activity is regulated by IN-1 and IN-2, which are activated by PKA phosphorylation and works as an amplifier of \( \beta \)-AR signaling. Figure 2 shows a schema of \( Ca^{2+} \) signaling during excitation-contraction coupling and current targets of gene therapy in \( Ca^{2+} \) cycling regulators.

**SERCA2a.** Since Hasenfuss et al. (43) reported that a loss of activity of SERCA2a and subsequent decrease in SR \( Ca^{2+} \) uptake are a feature of the failing human heart, the SERCA2a becomes one of the most studied \( Ca^{2+} \) handling proteins.
targeting for gene therapy. Overexpression of SERCA2a gene in human failing cardiomyocytes induced a faster contraction and enhanced relaxation velocity (18). The therapeutic potential of SERCA2a gene transfer to failing heart has been evaluated in small animal model of HF. Intracoronary adenovirus-encoding SERCA2a delivery to rats in HF resulting from pressure overload showed improved systolic and diastolic function (19, 107). A direct injection of adenovirus-encoding SERCA2a into myocardium prevented decrease of $dP/d_{max}$ and $dP/d_{min}$, restored wall thickness, and reduced infarct size in rat ischemia-reperfusion model (20). The studies with preclinical large animal models of HF have also proved the therapeutic potential of SERCA2a gene delivery. In a pig model of volume-overload HF due to mitral regurgitation, intracoronary delivery of recombinant AAV1 carrying SERCA2a improved adjusted $dP/d_{max}$ and LV function (58). In sheep, a HF model induced by MI with a mitral regurgitation model, intracoronary delivery of AAV6 carrying SERCA2a improved $dP/d_{max}$ and LV remodeling (4). There were two reports, which contradict the above findings. In transgenic rats with cardiac SERCA2a overexpression, there was no beneficial effect on LV function after MI and even an increased mortality due to ventricular arrhythmia (14). Gene transfer of SERCA2a to isolated cardiomyocytes from a canine LV pressure-overload diastolic HF model improved diastolic function, however, but abrogated β-adrenergic responsiveness and inotropic support (47). Although it has been subsequently reported that proarrhythmic effect is not observed and rather arrhythmias were attenuated in porcine model of ischemia-reperfusion model after SERCA2a gene therapy (95), caution must be taken when gene therapy is applied to HF with normal ejection fraction and MI.

Recently, the first clinical trial of SERCA2a myocardial gene therapy in patients with HF was launched. Calcium Uptregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease Trial (CUPID) is designed to evaluate the safety profile and the biological effects of gene transfer of the SERCA2a cDNA by intracoronary delivering of AAV1/SERCA2a in patients with advanced HF. This study compares three doses of AAV1/SERCA2a with placebo in patients with advanced HF (New York Heart Administration class III and IV), a LV ejection fraction ≤ 35% of both ischemic and nonischemic dilated cardiomyopathy. In a phase I/II trial, the safety and feasibility of AAV1/SERCA2a administration have been demonstrated (54). Recently, Jessup et al. (55) have reported the results of a phase II trial, in which 39 patients were enrolled. At 6 mo, AAV1/SERCA2a high-dose group showed clinically significant improvements in patients’ symptoms and functional status, as well as a significant reduction of cardiovascular events and hospitalization times. This was supported by a reduction in NH2-terminal prohormone brain natriuretic peptide and improvement of ventricular function/remodeling (55). In this phase II trial, 509 patients were prescreened and 63 patients with undetectable neutralizing antibodies against AAV were considered as candidates because patients who had pre-existing anti-AAV neutralizing antibodies did not respond to AAV1/SERCA2a in earlier phase I/II trial. Although a relatively high incidence of the presence of neutralizing antibodies limits the efficacy of gene therapy, SERCA2a gene therapy seems to be promising and the larger trial is awaited.

**Phospholamban.** In consideration of the good outcomes from a CUPID trial, PLN, a key negative regulator of SERCA2a, also looks like a candidate for therapeutic target of gene therapy for HF. Indeed, transgenic mice overexpressing a mutant PLN superinhibitor of SERCA2a in cardiomyocytes suffered cardiomyopathy (36), whereas PLN knockout mice showed enhanced systolic and diastolic function (71). However, the results of studies, which were designed to rescue HF by ablation of PLN, are variable. Muscle LIM protein-deficient and calsequestrin-overexpressing mice showed the improvement of cardiac function and $Ca^{2+}$ transient when crossbred with PLN knockout mice (77, 108). On the contrary, cardiac ablation of PLN did not rescue the hypertrophic phenotype of β2-AR overexpressing and mutant myosin-binding protein C mice and did not improve the cardiac dysfunction of mice with cardiac-specific overexpression of tumor necrosis factor-α, although cellular contractility showed significant improvement (29, 53, 116). Adenoviral gene transfer of antisense PLN prevented contractile dysfunction in $Ca^{2+}$ overload-induced LV dysfunction (123). Gene transfer of recombinant AAV encoding phosphomimetic mutant (S16E) form of PLN enhanced myocardial SR $Ca^{2+}$ uptake and suppressed progressive impairment of LV systolic function in BIO14.6 cardiomyopathic hamsters (49). Intracoronary delivery of adenovirus expressing PLN-S16E demonstrated improved cardiac function in sheep pacing-induced HF (60). However, cardiac gene transfer of AAV6 expressing short hairpin RNA against PLN resulted in depressed cardiac function along with reduction of PLN protein (7). These findings suggest that endogenous PLN may play a protective role in the heart in some context. Of note, there are several reports of familial human cardiomyopathies, which are caused by mutations and deletions in the PLN gene or its promoter and where the outcome is a loss of PLN inhibition on SERCA2a (37, 109a). Therefore, before human gene therapy targeting on PLN lowering is launched, more studies on PLN might be warranted.

**IN-1 and IN-2.** Expression levels and activity of IN-1 and IN-2 are decreased in HF, which is associated with the increase in global and βAR-associated PKA activity, leading to depressed SR $Ca^{2+}$ pump activity (25, 34, 39). Overexpression of IN-1 in cardiomyocytes augmented $Ca^{2+}$ cycling and cell contraction/relaxation in response to β-AR signaling (24). Adenoviral gene delivery of constitutively active IN-1 has been shown to augment cardiac contractility, attenuate hypertrophy, and prevent HF (90). In addition, cardiac-specific overexpression of IN-2 in mouse and gene delivery of IN-2 in cardiomyopathic hamster increased cardiac contractility by augmenting $Ca^{2+}$ cycling (61, 134). However, Wittköpper et al. (132) have recently reported that conditional cardiomyocyte-restricted expression of constitutively active mutant form of IN-1 increases contractile function in the cost of lethal arrhythmia and exaggeration of cardiomyopathy after adrenergic stress and with aging. The authors (132) confirmed similar phenotypes in another line of transgenic mice, which expressed an active form of IN-1 resistant to PKC-α phosphorylation. The authors attributed their results to RyR2 phosphorylation in both of Ser2809 (PKA site) and Ser2815 (CaMKII site) in their transgenic mice. RyR2 phosphorylation in Ser2809 has been previously reported and related to beneficial effects; however, phosphorylation in the CAMKII site has not been examined so far. Phosphorylation of RyR2 at Ser2815 may increase $Ca^{2+}$ fre-
Review

Cytokine Therapy

Many of the growth factors, cytokines, and receptors have been reported to be potent as a therapeutic regimen for cardiac repair following MI. Most of the factors improve cardiac function and attenuate LV remodeling via angiogenesis, anti-apoptotic effects, and stem cell homing. Among these factors, G-CSF and Epo are extensively studied, and several clinical trials are achieved.

Granulocyte colony-stimulating factor. G-CSF is a 25-kDa glycoprotein cytokine that stimulates the proliferation and survival of granulocytes lineage-derived cells. Orlic et al. (86) reported that G-CSF stimulates bone marrow cells to mobilize into the infarcted area and to differentiate into cardiomyocytes in mice. However, there is still a controversy about G-CSF-mediated cardiac regeneration by bone marrow stem cells (22, 78). The mechanisms other than cardiac regeneration have emerged from the findings that G-CSF receptor is expressed on cardiomyocytes and endothelial cells and that its downstream Janus-activated kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway is essential for the effect of G-CSF in the heart (29). It has been reported that G-CSF reduces the number of apoptotic cells in the ischemic border zone and increases Akt activities in the myocardium in mice, rats, and swines in an acute phase of MI (52, 85, 117). G-CSF also increased the production of nitric oxide (NO) through Akt/endothelial NO synthase pathway and enhanced the proliferation and migration of endothelial cells (88, 124). Neutrophils and macrophages play important roles in accelerating the infarct healing process. G-CSF-mediated infiltration of inflammatory cells is the first step to regulate cardiac healing. Fujita et al. (30) have reported that G-CSF mobilizes monocytes/macrophage, which differentiate into myofibroblasts and enhance cardiac repair. G-CSF treatment improved early postinfarct ventricular expansion through enhancement of expression of transforming growth factor-β, and promotion of reparative collagen synthesis in the infarcted heart (92). Although there was a conflicting report showing that G-CSF reduces ratio of metalloproteinases to their tissue inhibitors and aggravates excessive fibrosis deposition in infarcted areas of the myocardium (15), the more sophisticated protocol of dose and duration of G-CSF treatment may ensure the beneficial effect on the infarct healing process. Recently, several reports suggest that G-CSF exerts pleiotropic effects on cardiac tissue such as improvement of impaired mitochondrial electron transport and oxygen consumption (46). Cardioprotective effects of G-CSF have been reported in chronic myocardial ischemia and HF through angiogenesis (42, 17), reduction of apoptosis (42), prevention of the formation of excessive granulation tissue (17), and reduced expression of the angiotensin II type-1 receptor and tumor necrosis factor-α (69). These results highlight that the effects of G-CSF treatment might also occur in the chronic phase through similar or distinct mechanism in acute phase. Figure 3 showed a diagram of cellular and molecular interaction in cardiac repair after G-CSF therapy after MI.

Many clinical studies using G-CSF for patients of acute MI have been reported. Table 1 shows a summary of clinical studies with G-CSF in infarcted patients. Since the Front-Integrated Revascularization and Stem Cell Liberation in Evolving Acute Myocardial Infarction by Granulocyte Colony-Stimulating Factor Trial (FIRSTLINE-AMI) first reported that patients receiving G-CSF had increased mobilization of CD34+ mononuclear cells, had improved ejection fraction, and had prevented LV remodeling without safety concerns (51), several randomized, controlled trials have been conducted; however, the results of these trials are discordant, and recent published reports of the meta-analysis of the randomized controlled trials are still inconclusive. Kang et al. (56) have reported that G-CSF treatment improves LV ejection fraction in acute MI from 14 randomized controlled trials (56). The result of the meta-analysis by Zollnhofer et al. (137) has shown that G-CSF treatment does not enhance the improvement of ejection fraction from 10 randomized controlled trials. Although clinical trials have shown equivocal results, stem cell
mobilization with G-CSF is still promising because of its feasibility and general applicability. In addition to an elucidation of the underlying mechanisms, additional careful and well-designed protocols in terms of dose of G-CSF, timing of the treatment, and profiles of patients are still necessary to examine because it has been reported that the degree of improvement in LV ejection fraction is inversely associated with the time to G-CSF administration in patients after reperfusion and LV ejection fraction at baseline (1, 120).

Erythropoietin. Erythropoietin (Epo) exerts its hematopoietic effects by stimulating the proliferation of early erythroid precursors and the differentiation of later precursors of the erythroid lineage (64). Independent of erythropoiesis, recently EPO was shown to protect the heart from ischemic injury (77, 118). EPO receptor was expressed in both ventricular myocytes and endothelial cells (21). The interaction between EPO and its receptors leads to dimerization of the receptors and autophosphorylation of JAK-2. JAK-2 then phosphorylates different signal transducers and activates several downstream signaling pathways, including STAT5, PI3K/Akt, and mitogen-activated protein kinase (MAPK). Erythropoietin prevented apoptosis of endothelial cells and cardiomyocytes by activating the protein kinase C and PI3K/Akt pathway (38). In addition, ATP-dependent and Ca2+-activated potassium channels also mediate the protective effects of EPO in ischemic reperfusion injury (13). Recently, it has been reported that EPO induces cardiac mitochondrial biogenesis through EPO receptor-dependent activation of endothelial NO synthase, serving to protect cardiac oxidative phosphorylation during hypoxia (13). EPO promotes angiogenesis by several distinct mechanisms. Westenbrink et al. (129) have reported that EPO stimulates VEGF protein expression through STAT3 pathway predominantly in cardiomyocytes, and secreted VEGF induces the proliferation of endothelial cells and promotes the incorporation of endothelial progenitor cells (129). Ueda et al. (125) have reported that EPO induces angiogenesis by stimulating cardiomyocytes to secrete VEGF and angiopoietin 1 through sonic hedgehog signaling independent of STAT3 pathway. Hochs et al. (48) have reported that STAT3 is crucial for expression of EPO in cardiomyocytes and cardiomyocytes-derived EPO activates blood vessel formation by cardiac progenitor cells through C–C motif ligand-2/C–C motif receptor-2 system (48). Besides protective and angiogenic effects of EPO, it has been recently reported that EPO exhibits direct positive inotropic and lusitropic effects in cardiac myocytes and ventricular muscle preparation through PI3K and PKCε isoform signaling to directly affect both Ca2+ release dynamics and myofilament function (45). Figure 3 shows a

Table 1. Overview of randomized controlled trials with G-CSF in infarcted patients

<table>
<thead>
<tr>
<th>Trial</th>
<th>N</th>
<th>Age, yr</th>
<th>Dose, µg/kg (days)</th>
<th>Cardiac function measurement</th>
<th>Mean follow-up duration, mo</th>
<th>Outcomes</th>
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<tr>
<td>Ince et al. (51)</td>
<td>50</td>
<td>50</td>
<td>10 (6)</td>
<td>ECHO</td>
<td>4</td>
<td>LVEF ↑, MACE →</td>
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<tr>
<td>Zohnhöfer et al. (136)</td>
<td>114</td>
<td>59</td>
<td>10 (5)</td>
<td>MRI</td>
<td>4</td>
<td>LVEF →, MACE →</td>
</tr>
<tr>
<td>Valgimigli et al. (127)</td>
<td>20</td>
<td>60</td>
<td>5 (4)</td>
<td>SPECT</td>
<td>6</td>
<td>LVEF →, MACE →</td>
</tr>
<tr>
<td>Ripa et al. (101)</td>
<td>78</td>
<td>56</td>
<td>10 (6)</td>
<td>MRI</td>
<td>6</td>
<td>LVEF →, MACE →</td>
</tr>
<tr>
<td>Engelmann et al. (27)</td>
<td>44</td>
<td>58</td>
<td>10 (5)</td>
<td>MRI</td>
<td>3</td>
<td>LVEF →, MACE →</td>
</tr>
<tr>
<td>Takano et al. (119)</td>
<td>40</td>
<td>62</td>
<td>2.5 (5)</td>
<td>SPECT</td>
<td>6</td>
<td>LVEF ↑, MACE →</td>
</tr>
<tr>
<td>Leone et al. (67)</td>
<td>41</td>
<td>55</td>
<td>10 (5)</td>
<td>ECHO</td>
<td>6</td>
<td>LVEF ↑, MACE →</td>
</tr>
</tbody>
</table>

N represents the total number of patients. Age is shown as mean. G-CSF, granulocyte colony-stimulating factor; ECHO, echocardiography; LVEF, left ventricular ejection fraction; MACE, major adverse cardiac events; MRI, magnetic resonance imaging; SPECT, single-photon emission computed tomography.
diagram of cellular and molecular interaction in cardiac repair after EPO therapy after MI.

A single bolus of EPO in patients with acute MI showed that EPO was safe and well tolerated (70). EPO stimulated endothelial progenitor cell mobilization; however, LV ejection fraction was similar between the EPO-treated and control groups in this pilot study. Subsequently, A Prospective, Randomized, Clinical Study to Examine the Effects of a Single Bolus Erythropoietin on Left Ventricular Function in Patients with an Acute Myocardial Infarction (HEBE-III) was initiated to assess the effects of EPO in a larger number of acute MI patients. The results showed that a single dose of EPO after successful percutaneous coronary intervention did not improve LV ejection fraction, but the treatment with EPO related less major adverse cardiovascular events and a favorable clinical safety profile (128). No improvement on cardiac function or reduction in infarct size was observed in A Randomized, Double-Blind, Placebo-Controlled Trial of Erythropoietin in Patients with ST-Segment Elevation Myocardial Infarction Undergoing Percutaneous Coronary Intervention (REVIVAL-3), which was another prospective, randomized, double-blind, placebo-controlled trial of EPO in patients with acute MI (87). Currently, the Reduction of Infarct Expansion and Ventricular Remodeling with Erythropoietin after Large Myocardial Infarction Trial (REVEAL) and Exogenous erythropoietin in Acute Myocardial Infarction: New Outlook and Dose Association Study (EPAMONDAS) trials are ongoing (2, 76).

Table 2 shows a summary of clinical studies with EPO in infarcted patients. Despite the lack of beneficial effects of EPO on cardiac function in patients of MI, treatment with erythropoiesis-stimulating agents for chronic HF with anemia showed the improvement of LV ejection fraction along with better exercise tolerance and reduction of symptoms (63). Since it has been reported that there is an ideal dose of EPO for MI (134), the results of further clinical trials may provide us a new insight of the safety and efficacy of EPO in cardiovascular disease.

Summary

HF is a major clinical cause of morbidity and mortality, and the need for better treatments has been recognized for decades. Many new approaches to HF therapy including cells, drugs, and genes have been explored, and it is important to note that these therapies are complementary. Gene therapy means the delivery of genetic materials into cells to achieve therapeutic effects. AAV own attractive characters such as the defective replication, lack of human pathology, and prolonged transgene expression in clinical studies. Because β-adrenergic signaling and excitation-contraction coupling are crucial for cardiac contraction, gene therapy targeting for GRK2, AC6, SERCA2a, PLN, IN-1, IN-2, and S100A1 have shown promising preclinical data. Clinical trials of AC6 and SERCA2a have launched. A growing amount of evidence indicates that G-CSF and EPO promote cardiac repair after MI through mobilization of the stem or progenitor cells from bone marrow and also through direct pleiotropic cardiovascular effects such as antiapoptosis, angiogenesis, regulation of fibrosis, and improved mitochondrial function. The preclinical studies have been actively investigated with promising data, which show that G-CSF and EPO improve cardiac function in various kinds of models of HF.

Future Directions

A recent advance in our understanding of the molecular mechanisms associated with HF clarifies several key molecules, which are promising targets for gene therapy. Gene therapy is considered as a viable adjunctive treatment to mechanical and pharmacological therapies for HF; however, further development of advanced vectors based on elaborated preclinical experiments by using large animals are still needed. The more favorable characters of next generation vectors include the regulated expression pattern by which we can manipulate the on/off system or environment-dependent expression such as tetracycline regulatory system and hypoxia-inducible factor-1α-dependent gene expression. Because of the complexity of the pathophysiology of HF, the more target molecules that are amenable to genetic manipulations have to be considered. These include key molecules that govern the homing and differentiation of stem cells, cell death/survival, and myocyte cell cycle. Further studies along with the development of more selective and noninvasive vector delivery systems will undoubtedly lead to safer and more effective gene therapy for HF.

Although most of the meta-analyses suggest that the administration of G-CSF or EPO does not improve the beneficial effects of standard HF therapy, safety of the usage of G-CSF and EPO has been demonstrated. In addition, some methodological issues, such as patient age, time of onset of symptoms to percutaneous coronary intervention, and time to administration of cytokines, need to be carefully addressed. To clarify the efficacy of G-CSF and EPO treatment, the establishment of better protocols in terms of the appropriate indications, doses, therapeutic window, as well as more mechanistic insight of molecular pathways under G-CSF and EPO are needed.

Table 2. Overview of randomized controlled trials with EPO in infarcted patients

<table>
<thead>
<tr>
<th>Trial</th>
<th>N</th>
<th>Age</th>
<th>Dose</th>
<th>Cardiac function measurement</th>
<th>Mean follow-up duration, mo</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipson et al. (70)</td>
<td>50</td>
<td>50</td>
<td>300 µg (60,000 IU) darbepoetin-α single bolus IV</td>
<td>Radionuclide ventriculography</td>
<td>4</td>
<td>LVEF→, MACE→</td>
</tr>
<tr>
<td>Voors et al. (128)</td>
<td>529</td>
<td>61</td>
<td>60,000 IU epoietin-α single bolus IV</td>
<td>Radionuclide ventriculography</td>
<td>1.5</td>
<td>LVEF→, MACE→</td>
</tr>
<tr>
<td>Ott et al. (87)</td>
<td>138</td>
<td>61</td>
<td>33,300 IU epoietin-β single bolus IV</td>
<td>Angiography</td>
<td>6</td>
<td>LVEF→, MACE→</td>
</tr>
<tr>
<td>Melloni et al. (76)</td>
<td>210</td>
<td></td>
<td>15, 30, or 60 × 10⁵ IU epoietin-α single bolus IV</td>
<td>MRI</td>
<td>3</td>
<td>Ongoing</td>
</tr>
<tr>
<td>Andreotti et al. (2)</td>
<td>102</td>
<td></td>
<td>100 or 200 IU·kg⁻¹·day⁻¹ epoietin-α triple 30 min IV</td>
<td>MRI</td>
<td>12</td>
<td>Ongoing</td>
</tr>
</tbody>
</table>

N represents the total number of patients. Age is shown as mean. EPO, erythropoietin; IU, international units; IV, intravenous.
DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

T.N. and I.K.: conception and design of research; T.N.: drafted manuscript;
I.K.: edited and revised manuscript; I.K.: approved final version of manuscript.

REFERENCES


Review

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