Review

Anti-death strategies against oxidative stress in grafted mesenchymal stem cells

Woochul Chang[1]*, Byeong-Wook Song[2]*, Jae-Youn Moon[3]*, Min-Ji Cha[4],
Onju Ham[5,6], Se-Yeon Lee[5,6], Eunmi Choi[7], Eunhyun Choi[8] and Ki-Chul Hwang[5,7,8]

[1]Department of Biology Education, Pusan National University, Busan, Republic of Korea, [2]Institute of Catholic Integrative Medicine, Incheon St. Mary's Hospital, The Catholic University of Korea College of Medicine, Incheon, Republic of Korea, [3]Department of Cardiology, CHA Bundang Medical Center, CHA University, Sungnam, Republic of Korea, [4]Deparment of Pathology and Inmunology, Washington University College of Medicine, St. Louis, USA, [5]Cardiovascular Research Institute, Yonsei University College of Medicine, Seoul, Republic of Korea, [6]Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul, Republic of Korea, [7]Severance Biomedical Science Institute, Yonsei University College of Medicine, Seoul, Republic of Korea and [8]Severance Integrative Research Institute for Cerebral & Cardiovascular Disease, Yonsei University Health System, Seoul, Republic of Korea

*These authors have contributed equally to this work

Summary. Mesenchymal stem cells (MSCs) possess the potential for use in cell-based therapy for repair of myocardial injury. The therapeutic potential of MSCs is based on the capacity of MSCs to differentiate into cardiac tissue and release paracrine factors. However, a major problem in the clinical application of MSC-based therapy is the poor viability of transplanted MSCs at the site of graft due to harsh microenvironment conditions, such as ischemia and/or anoikis. Ischemia after myocardial infarction (MI) and interaction of MSCs with their niche is associated with increased production of reactive oxygen species (ROS). ROS hinder cell adhesion and induce detachment of cells, which induces anoikis signals in implanted MSCs. Therefore, strategies to regulate oxidative stress following the implantation of MSCs are therapeutically attractive. In this review, we first describe ROS as a major obstacle in MSC-based therapy and focus on manipulation of implanted MSCs to reduce ROS-mediated anoikis.

Key words: Mesenchymal stem cells, Reactive oxygen species, Anoikis, Myocardial infarction

Offprint requests to: Ki-Chul Hwang, Severance Biomedical Science Institute, Yonsei University College of Medicine, Seoul 120-752, Republic of Korea. e-mail: kchwang@yuhs.ac

Introduction

Despite significant advances in the medical management of heart failure, ischemia/reperfusion injury is still a major cause of death throughout the world (Ferdinandy et al., 2007). Because the adult human heart has limited intrinsic regenerative capacity, myocardial loss after a myocardial infarct is irreversible and the tissue is subsequently replaced by non-contractile scar tissue (Fonarow, 2000; Lloyd-Jones, 2001). The resultant scar formation causes mechanical dysfunction, electrical uncoupling, loss of cardiac function, and ventricular remodeling (Segers and Lee, 2008). Contemporary pharmacologic and interventional strategies are commonly used for myocardial repair; however, these treatments cannot improve the function of a heart with end-stage disease. Recently, new therapeutic approaches such as gene-, growth-factor-, and cell-based therapies to improve simple replacement of the myocardium have emerged (Penn and Mangi, 2008). Cell-based therapies have been intensively studied over the last few years because of the potential benefits in patients who have heart diseases such as acute myocardial injury, stable coronary artery disease, and heart failure (Melo et al., 2004). In particular, stem cells have been investigated due to their therapeutic potential to improve cardiac function through proliferation of host cells and differentiation of injected cells, which possess plasticity and the ability to transdifferentiate into multiple lineages, self-renew, and

fuse with resident cardiomyocytes (Haider and Ashraf, 2005).

Among the different types of stem cells, mesenchymal stem cells (MSCs) are recognized as the best potential candidates for cell-based therapy for heart diseases because MSCs are easily isolated, show rapid expansion *in vitro*, and rarely form teratoma (Kumar et al., 2008). Indeed, many studies have shown a marked recovery in ventricular function from myocardial infarction (MI) after transplantation of MSCs in human patients compared with controls, which demonstrates the safety and feasibility of intra-coronary MSC infusion in post-MI patients (Katritsis et al., 2005). However, the poor survival of engrafted MSCs is a major obstacle in MSC-based therapy. One of the reasons for MSC death following cell transplantation into the infarcted area is the ischemic condition of the injection site, which can lead to the formation of reactive oxygen species (ROS). Adherent cells bind to their support through connections between the cell and the extracellular matrix, and focal adhesion kinase (FAK) is the main tyrosine kinase involved in cell-matrix interactions. FAK activity and cell mortality induced by ROS seem to be correlated, and this correlation could explain the poor survival of transplanted cells. To overcome this limitation, various anti-death strategies have been developed to improve grafted stem cell survival/numbers in infarcted hearts. Here, we review the strategies that have been used to enhance the survival of MSCs against oxidative stress, and we discuss future directions with respect to the cardiac therapeutic potential of modified MSCs in such microenvironment conditions.

Stem cell-based therapy for cardiac repair

Stem cell-based therapy is a relatively new strategy against myocardial ischemia, cardiac dysfunction, or a combination of both. Stem cell transplantation might be an effective therapy if it can improve cardiac function by providing a renewable source of proliferating, functional cardiomyocytes and/or enhancing angiogenesis to nourish not only newly formed cardiomyocytes, but also the environment of the ischemic region (Psaltis et al., 2008; Vertesaljai et al., 2008). Various stem cell types, including bone marrow cells (BMCs), bone marrow-derived mononuclear cells (BMMNCs), hematopoietic stem cells (HSCs), endothelial progenitor cells (EPCs), and embryonic stem cells (ESCs), have been used for cardiac repair, and each cell type has its own advantages and disadvantages for cell-based therapy (Fraser et al., 2004).

ESCs, which are derived from the inner mass of the blastocyst, have unique properties, including pluripotency, self-renewal capacity, and high cell number expansion (Gulati and Simari, 2007). Recently, it was demonstrated that ESCs can be differentiated into cardiomyocytes or cardiac precursor cells, providing an opportunity to induce myocyte development (Min et al., 2003; Swijnenburg et al., 2005). However, the clinical use of ESCs is limited by teratoma formation, immunological incompatibility, and considerable ethical concerns. Recently, in order to overcome the ethical problems, inducible pluripotent stem (iPS) cells, which are derived from adult somatic cells by inducing forced expression of specific genes, have been studied. However, their clinical usage has been restricted by unregulated tumor formation, inadequate conversion, and persistent ectopic gene expression (Nelson et al., 2010).

It has been demonstrated that bone marrow cells, a heterogeneous population composed of HSCs, MSCs, EPCs, and other cell types, can be used to replace cardiac myocytes in infarcted hearts in an animal model (Orlic et al., 2001). When BMCs or BMMNCs were transplanted directly or injected by intracoronary infusion, both a demonstrated improvement in left ventricular function as well as an increase in cell engraftment and cardiac differentiation were observed. These findings led to clinical trials of autologous BMCs or BMMNCs. However, another investigation reported some limitations, including an undefined functional fate and electrophysiology (Haider and Ashraf, 2005). EPCs derived from BM or peripheral blood have been shown to mobilize into peripheral blood for improvement of cardiovascular diseases. Although EPCs have the advantage of being able to induce neovasculogenesis through vessel regeneration and remodeling, their clinical utility is limited because they are a rare heterogeneous population that decreases with atherosclerosis and age (Schmidt-Lucke et al., 2005).

MSCs have been isolated from a variety of human tissues, including umbilical cord, umbilical cord blood, adipose tissue, dental pulp, periosteum, tendon, skin, synovial membrane, amniotic fluid, limbal tissue, and menstrual blood (Zhu et al., 2008; Nekanti et al., 2010). The origin of MSCs is very important in determining biological activity, and thus clinical applications might differ according to the stem cell niche. Recent reports have demonstrated that MSCs have multipotentiality, and can differentiate into chondrocytes, osteocytes, adipocytes, astrocytes, and cardiac myocytes both i*n vivo* and *in vitro* (Bartholomew et al., 2002). This multilineage transdifferentiation potential suggests that MSCs would be a good source of cells to treat different diseases. In addition, MSCs also show less immune rejection because they express low levels of MHC II compared with MHC I (Schuleri et al., 2007). These capabilities make MSCs attractive for use in various cell-based therapies.

Many studies have suggested that MSCs are an attractive source for gene delivery, cell transplantation, and tissue engineering applications. Despite the benefits of MSCs, MSC-based therapy is limited in clinical practice because of negative influences, i.e., anaerobic conditions, an increase in apoptotic factors, and inflammatory responses resulting from oxidative stress, in ischemic myocardium (Segers and Lee, 2008). These factors result in poor viability and/or lower cell adhesion

strength. Toma et al. reported that ~ 0.5% of transplanted human MSCs continued to survive 4 days after cell transplantation in ischemic murine hearts (Toma et al., 2002). Therefore, cell viability is likely to be the major barrier to a therapeutic regenerative stem cell-based approach to cardiac infarction.

Causes of transplanted cell death in infarcted regions

The death of transplanted MSCs may begin during the preparation step. The death of transplanted MSCs in ischemic hearts may begin when cells are detached from the culture dish. Anoikis, which is defined as the process of programmed cell death induced by the loss of matrix attachment, occurs during the engraftment process (Michel, 2003). Anoikis is a potentially major contributor to graft cell death in cell-based cardiac repair. At the engrafted site, MSCs encounter harsh conditions coupled with the loss of survival signals due to inadequate interaction between the implanted cells and the matrix (e.g., the deprivation of nutrients and oxygen (Mylotte et al., 2008) and inflammation (Guo et al., 2007)). In general, adhesion to the structural glycoproteins of the extracellular matrix (ECM) by cross-linking is necessary for the survival of adherent cells, and the adhesion of cells to the matrix, predominantly via integrin molecules, generates an endogenous tensile stress within the cells, called tensegrity (Gartner and Kaplan, 1980; Frisch and Francis, 1994; Memon et al., 2005; Song et al., 2007; Chiarugi and Giannoni, 2008). This physiological cellular process plays an important role in the differentiation, survival and growth of MSCs. In this respect, ROS may intensify the anoikis signals in transplanted MSCs.

When MSCs are transplanted into infarcted areas, they may experience ischemic conditions such as deprivation of nutrients and oxygen and inflammation (Sáenz-Morales et al., 2009). These conditions affect MSCs engrafted in the ischemic area, following ATP depletion, activation of anaerobic glycolysis, and the dysfunction of calcium and other ionic homeostasis (Robey et al., 2008). Ischemia can lead to ROS formation. Even though ROS are formed as a natural part of oxygen respiration, ROS are also dramatically increased in ischemic hearts. Indeed, ROS hinder cell adhesion and induce detachment of cells, which intensifies the anoikis signals in implanted MSCs (Song et al., 2010).

Reactive oxygen species (ROS)-induced apoptosis

ROS are generated from numerous sources, including NADPH oxidase, the mitochondrial electron-transport system, xanthine oxidase, cytochrome p480, and myeloperoxidase (Turrens, 1997; Griendling et al., 2000; Li and Shah, 2004). Among the subcellular organelles, mitochondria are considered to be the main source of ROS, and the respiratory chain reaction in the mitochondrial wall induces the production of superoxide anion (Carriere et al., 2009). ROS such as superoxide ($^•O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^•$) are common byproducts of many oxidative biochemical and physiological processes. ROS generation is a double-edged sword in that oxidative stress caused by the cellular accumulation of ROS induces cellular apoptosis or death (Simon et al., 2000; Xia et al., 2004; Kizaki et al., 2006). Although ROS exhibit negative effects on cellular homeostasis, there is conflicting evidence that ROS at low and nontoxic levels can actually promote cell proliferation and survival (Chiarugi and Fiaschi, 2007; Blanchetot and Boonstra, 2008; Leslie, 2006). Low and nontoxic levels of ROS can activate several mitogen-activated protein serine/threonine kinases (MAPKs), which transduce diverse extracellular stimuli (e.g., mitogen growth factors, environmental stresses, and proapoptotic agents) to the nucleus via kinase cascades in order to regulate a wide array of cellular processes (Kamata et al., 2005) (Fig. 1).

Hypoxic/ischemic conditions have been recognized as important mediators or modulators of apoptosis since these conditions induce excessive ROS production (Wang et al., 2000; Cao et al., 2001; Robey et al., 2008). Indeed, it has been reported that the generation of ROS is dramatically increased by over three-fold in the risk region in ischemia/reperfusion-injured animal hearts compared to uninjured animal hearts (Oshima et al., 2005; Angelos et al., 2006), even though ROS are formed as a natural component of oxygen respiration (Yao et al., 2006). When excessive ROS and oxidative stress are generated in ischemic myocardia, the inner mitochondrial membrane potential is damaged, which may induce apoptosis. In recent years, mitochondria have been recognized as the fate modulator of cell death and survival. In myocardial ischemia-reperfusion injury, intracellular Ca^{2+} and ROS are increased within the cytoplasm and mitochondria. Under these conditions of Ca^{2+} overload due to oxidative stress and ATP depletion, mitochondria undergo a permeability transition associated with the formation of a non-specific permeability transition pore (PTP) in the mitochondrial membrane. Under continued ischemic-reperfusion conditions, the depolarization of the mitochondrial membrane induces further ROS production and ATP hydrolysis, and cell death then occurs by caspase activation via the production of pro-apoptotic protein (Andreadou et al., 2009). ROS can also induce an inflammatory response and prevent cell adhesion, leading to cell death (Pelegrin and Surprenant, 2009).

In cell adhesions, focal adhesion sites are specific areas on the cell membrane where cells attach to the ECM. These are complexes of structural and signaling proteins, anchoring actin filaments and microtubules to the plasma membrane where integrins localize (Frisch and Screaton, 2001). Most integrin ß-subunits interact with proteins such as paxillin, talin, vinculin, and other

focal adhesion proteins that act as linkers between integrins and the actin cytoskeleton (Brown and Turner, 2004; Gilmore, 2005; Mitra et al., 2005). It is known that the key players in integrin-mediated signal transduction are a group of integrin-associated nonreceptor kinases, two of which are focal adhesion kinase (FAK) and integrin-linked kinase (ILK) (Attwell et al., 2000; Gilmore, 2005; Ziegler et al., 2006). Integrins are essential for cell migration and invasion because they mediate the adhesion of cells to the ECM and regulate the intracellular signaling pathways that control cytoskeletal organization, force generation, and survival (Parson, 2003). Activated integrins bind to the ECM, cluster at the binding site, and initiate focal adhesions by recruiting cytoplasmic proteins such as FAK, Src, and paxillin (Huang et al., 2006; Legate et al., 2006). Integrins also activate small GTPase, which in turn activates downstream effector molecules, thereby leading to the rearrangement of actin stress fibers and activation of cell adhesion and spreading (Salsmann et al., 2005).

Moreover, recent evidence suggests that the disruption of integrin contacts in fibroblasts can lead to a rise in intracellular ROS levels and subsequent cell detachment (Chiarugi and Buricchi, 2007) (Fig. 2). Sonoda et al. reported that the incubation of human glioblastoma cells with both herbimycin, a tyrosine kinase inhibitor, and a FAK antisense peptide induced a decrease in the tyrosine phosphorylation of FAK and an increase in apoptosis, suggesting a suppressive role of tyrosine phosphorylation of FAK in hydrogen peroxide-induced apoptosis (Sonoda et al., 1997). They also demonstrated in both HL60 monocytes and glioblastoma cells that overexpression of FAK protects these cells against oxidative stress-induced apoptosis, activates the PI3-kinase/Akt survival pathway, and inhibits the activation of caspase-3 protease by H_2O_2 (Sonoda et al., 1997, 2000). In human endothelial cells subjected to oxidative stress, the cleavage of FAK before endothelial cells became apoptotic was identified (Gervais et al., 1998). Thus, an adequate level of FAK tyrosine phosphorylation should be maintained using antioxidants in order to preserve physiological redox status and to promote cell survival.

Anti-apoptotic strategies for MSC-based therapy against oxidative stress

Based on the strong evidence that ROS are involved in hypoxia-induced cell apoptosis (Kulkarni et al., 2007), numerous antioxidants have been used to enhance cell survival. Generally, ROS accumulation is limited by numerous endogenous antioxidant defense systems, including both enzymatic and nonenzymatic antioxidant mechanisms that either scavenge ROS or prevent their formation. Enzymatic antioxidants include the superoxide dismutase (SOD) family, glutathione peroxidases (GPxs), peroxiredoxins (Prxs), and catalases (CATs) (Bonomini et al., 2008). Other nonenzymatic antioxidants, such as L-r-glutamyl-L-cysteinyl glycine (glutathione) (GSH), NAD(P)H, vitamin C, vitamin E, uric acid, and bilirubin play important roles in the scavenging of ROS (Powers and Jackson, 2008).

Song et al. reported that the elimination of ROS in the transplanted region of an infarcted heart can enhance the adhesiveness of MSCs, leading to improved cardiac repair (Song et al., 2010). Cultured MSCs exposed to H_2O_2 underwent dose-dependent cell detachment and a substantial decrease in adhesiveness. Although H_2O_2 treatment produces harsh conditions, the scavenging of ROS by N-acetyl-L-cysteine (NAC), an antioxidant, recovered MSC adhesion and spreading. Furthermore, a
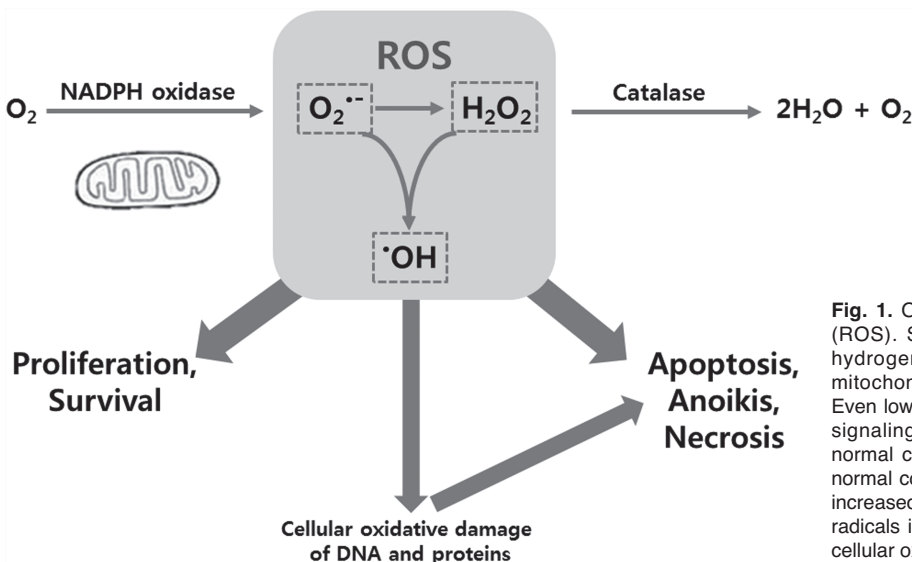

Fig. 1. Overview of the role of reactive oxygen species (ROS). Superoxide ($O^{2\cdot}$), hydroxyl ions (OH), and/or hydrogen peroxide (H_2O_2) are normally produced by mitochondria or NADPH oxidase on the cellular level. Even lower levels of ROS may have protective effects via signaling for preconditioning and may be required for normal cell homeostasis. In unusual circumstances, the normal condition is lost and hydroxyl radical generation is increased via the Fenton reaction. An increase in hydroxyl radicals induces apoptosis, anoikis, and necrosis through cellular oxidative damage of DNA, lipids, and proteins.

decrease in ROS with NAC treatment improved the adhesion and survival of engrafted MSCs and improved heart function after MI. These findings demonstrate that even though the exact mechanism of stem cell therapy is not known, the cell death induced by ischemic conditions is regulated at an early stage. Rodrigues et al. also found that multipotent stromal cells/MSCs exposed to Fas ligand (FasL) produce ROS, and that NAC can survive the harsh conditions induced by FasL-induced ROS by EGF determinatively (Rodrigues et al., 2012). Furthermore, co-injection of stem cells with antioxidants may positively affect engrafted stem cell therapy for cardiac repair of ischemic hearts. Another cell line, muscle-derived stem cells (MDSCs), has also shown promising results with treatment with diethyl maleate (DEM) or NAC (Drowley et al., 2010). Pretreatment with an antioxidant induced myogenic differentiation and cell proliferation, survival, and VEGF secretion. Furthermore, transplantation of pretreated MDSCs into infarcted myocardia significantly improved cardiac function and regenerated injured skeletal muscle. In another study using NAC, Wang et al. demonstrated that human embryonic MSCs have a positive effect on antioxidant capacity to defend against severe redox imbalances (Wang et al., 2013). The administration of NAC-pretreated human embryonic MSCs decreased

pathological factors, including lung inflammation, fibrosis, and apoptosis, in nude mice with bleomycin-induced lung injury.

Through the stimulation of melatonin receptors, Mias et al. found that MSCs have survival and proangiogenic/mitogenic activity (Mias et al., 2008). Melatonin induced the expression of the antioxidant enzymes catalase and Cu/Zn SOD-1 compared to the melatonin receptor antagonist luzindole in MSCs. In a renal ischemia-reperfusion model, the injection of melatonin-treated MSCs increased angiogenesis and vascular organization. This effect was further examined by *in vitro* experiments that involve melatonin pretreatment on paracrine activity stimulated with secretion of proangiogenic and mitogenic factors by MSCs. It has been shown that pretreatment with antioxidants strengthens the survival and angiogenic activity of MSCs. As a plant-derived antioxidant, berberine (BBR) can induce the protection of MSCs under serum deprivation- and hypoxia-induced apoptosis (Zhang et al., 2009). It had been found that MSC-treated BBR have beneficial effects via the activation of PI3K/Akt, the inhibition of ROS-dependent JNK activation, and the destruction of the mitochondrial apoptotic pathway. Furthermore, 14S,21Rdihydroxy-docosa4Z,7Z,10Z,12E,16Z,19Z-hexaenoic acid
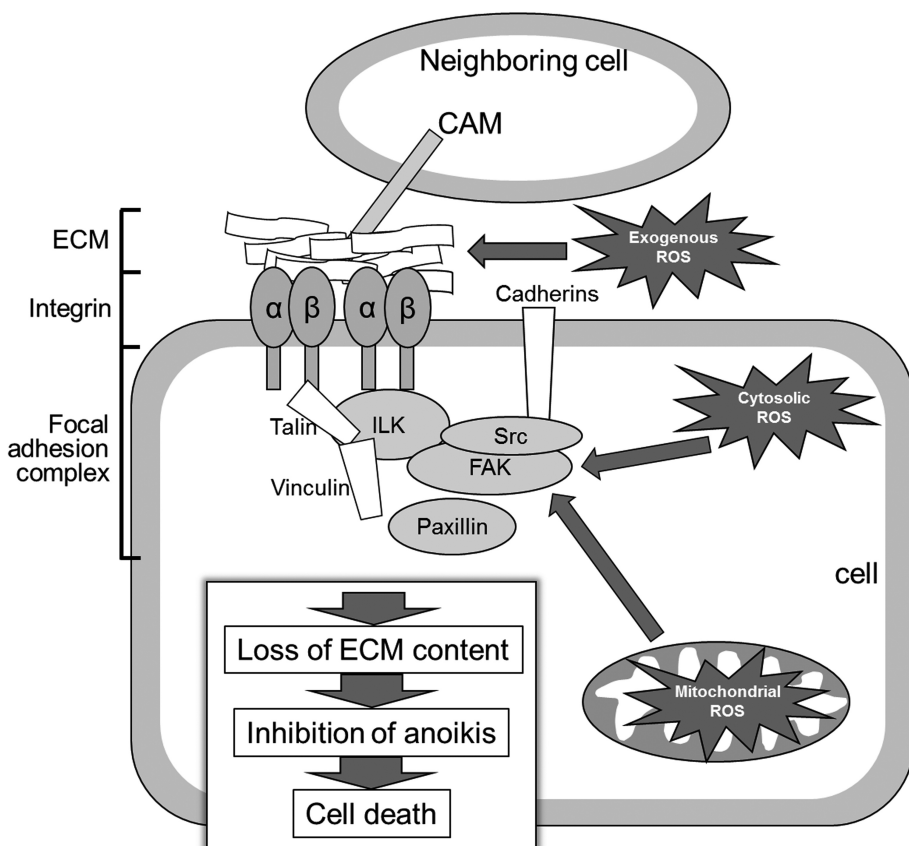

Fig. 2. Schematic diagram of ROS-dependent cell adhesion. Integrin, which connects neighboring cells, mediates cell-to-cell and cell-to-extracellular matrix (ECM) adhesion events via ECM binding and/or transmembrane proteins such as cell adhesion molecules (CAMs). ROS generated in the exogenous, cytosolic, and mitochondrial regions activates the integrin-dependent mediated apoptosis, also known as anoikis, which damage cells. Focal adhesion complex, which includes focal adhesion kinase (FAK), src, paxillin, talin, vinculin, and integrin-linked kinase (ILK), is affected by ROS. These factors have an influence on ECM content, integrin-mediated proteins, and/or focal adhesion complex. Constant activation of ROS causes cell death by inhibiting anoikis signaling.

(14S,21R-diHDHA), a new docosahexaenoic acid (DHA)-derived lipid mediator, was used for treatment of renal ischemic/reperfusion injury (Tian et al., 2012). Conditioned medium from pretreated MSCs induced the inhibition of necrosis factor-α and ROS, and secreted renotrophic hepatocyte growth factor and insulin growth factor-1 via the PI3K/Akt pathway. Using gene transfer, Hagiwara et al. found that kallikrein-overexpressing MSCs (TK-MSC) effectively inhibit oxidative-stress-induced apoptosis through both the pleiotropic effects of kallikrein and the paracrine effects of MSCs (Hagiwara et al., 2008). Apoptosis was significantly downregulated in TK-MSCs during treatment with hydrogen peroxidase. Implantation of TK-MSCs further inhibited apoptosis and inflammatory cell infiltration in acute renal failure.

Conclusion

Although transplantation of stem cells has therapeutic effects on the myocardial regeneration of adult tissue under ischemic conditions, the exact mechanism of stem cell-based therapies is not well known. However, many studies have demonstrated that MSCs are the major cell type for ischemic heart which induce ROS-mediated anoikis. To solve a major obstacle in MSC therapy, this review discusses our current understanding of the anti-death strategy of MSCs under ischemic or hypoxic conditions. ROS caused by ischemic conditions influences the adhesion and survival of transplanted MSCs. Therefore, strengthening cell adhesion and survival and inhibiting ROS production should be major goals in MSC therapy. In order to maximize this phenomenon, the following techniques should be investigated: 1) Pretreatment with antioxidants or specific factors affecting survival, and 2) gene enhancement using plasmids, viruses, and other molecular biological structures. Furthermore, the regulation of the focal adhesion complex, which is localized between cells and the matrix under ROS-stimulated conditions, needs to be addressed in a rigorously controlled mechanism-based study. Due to the current challenges to regenerating ischemic myocardia through stem cell therapy, anti-death and/or pro-survival strategies might be powerful methods to regulate MSC activity under oxidative stress to achieve ischemic myocardial regeneration.

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