Fas inhibition of hypoxic mesenchymal stem cells for myocardial regeneration

Onju Ham

Department of Medical Science

The Graduate School, Yonsei University

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Directed by Professor Yangsoo Jang

The Master's Thesis Submitted to the

Department of Medical Science,

the Graduate School of Yonsei University

in partial fulfillment of the requirements for the

degree of Master of Medical Science

Onju Ham

December 2010

This certifies that the Master's Thesis of Onju Ham is approved.

Thesis Supervisor: Yangsoo Jang

Thesis committee Member #1: Ki-Chul Hwang

Thesis committee Member #2: Jong Sun Kim

The Graduate School

Yonsei University

December 2010

ACKNOWLEDGEMENTS

I would like to deeply thanks the various people how provide us with useful and helpful assistance.

I especially want to thanks my advisor, Dr. Yangsoo Jang and Jong Sun Kim, for insightful discussion of the manuscript and appreciate to Dr. Ki-Chul Hwang for encouragement and support from experimental guidance to discussion.

I would like to express my thank to Chang Yeon Lee, Heesang Song, Eunmi Choi ,Byeong-Wook Song, Sun Ju Lee, Il-kwon Kim, Min Ji Cha, SeYeon Lee, JunHee Park, Eunhye Lee, Who have helped me directly accomplishing this and giving me leaning environment to grow me personally. I wish to thank those colleagues who have worked at SIRIC: Sook Kyung Kim, Pil Sung Yang, Dong Kyu Kim, Hyung-Ho Moon, Ji-Won Choi, Hyun Ju Jeon, Soo-Young Kim, Beom Seob Lee, Da Jeong Lee, Bo Hyun Kim, Hyun Ji Lee, Jin Kyung Lee.

In particular, I would like to express my sincere thank to my father, mother, sister, Hyun Joo, and brothers, TeaWoong and Tea Yo, who constantly provided emotional support and so care of me in many aspects. I wish to thank my friends, Cholong Park, Min Yong Kang, Eun Kyung Yoo, Yun Jin Shin, In Ae Cha, Yeon Seen Kim, Min Son and Song Chan Lee, standing by for a long times. I thanks to HanBit, Muhack and DeungChon church memebers having gone through happiness and difficulty together.

Last but always first to me, my father God, I really thanks to help me. Must your name be exalted, honored and glorified.

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ABSTRACT

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The use of mesenchymal stem cells (MSCs) as a therapy for myocardial disease is limited by poor viability of the transplanted cells. Reactive oxygen species (ROS) are generated in ischemic surroundings after myocardial infarction. Ischemic heart generated Fas ligand (FasL). FasL belongs to the TNF family that induces apoptosis in target cells binding Fas. This study

aimed to determine if Fas-FasL complex mediated the death of MSCs in the ischemic heart. Expression levels of FasL were detected in a myocardial ischemia-reperfusion injury model. Expression of Fas was significantly increased in H_2O_2-treated MSCs *in vitro*. Survival of FasL-treated MSCs was decreased in H_2O_2-treatment compared to non-treated cells. When Fas was blocked using a Fas recombinant Fc chimera (Fas/Fc), caspase-8 and caspase-3 levels were lower than in FasL-treated MSCs. Increased survival was observed in Fas/Fc-treated MSCs compared to FasL-treated cells. These results indicate that Fas-Fas L complex interferes with the survival of MSCs and confirm a major role for the Fas-Fas L cytotoxic pathway in cardiac infarction.

Key words: ischemic heart disease, cardiomyocytes, mesenchymal stem cells, Fas, Fas ligand

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I. INTRODUCTION

Heart disease continues to be the worldwide leading cause of morbidity and mortality in the last half century[1]. Ischemic heart disease, one of the heart diseases, is characterized by reduced blood supply to the heart muscle[2,3]. Ischemia induces a broad range of cell responses including loss of adhesion

and cell death, depending on the cell type and the duration of the ischemic period[4]. Myocardial infarction (MI) is caused by oxygen and nutrients to the cardiac tissue due to blockade of coronary artery. It is major contributor to chronic heart disease, a leading cause of mortality in the modern world[5].

Recently, new therapeutic approaches such as gene-, growth factor-, and cell-based therapies have emerged to improve simple replacement of the myocardium[6]. The main therapeutic goals in MI are to minimize myocardial damage, improve cardiac repair, and reduce myocardial remodeling[7].

Apoptosis is a physiological program of cellular death and may cause many cardiac disorders in a wide range of clinical settings[8]. Two independent pathways lead to the induction of apoptosis, with limited crosstalk between the two. The type I or “extrinsic” apoptotic pathway is mediated by external factors that bind to members of the death receptor superfamily, of which Fas and tumor necrosis factor-1(TNFR1) are prominent examples. The Fas-FasL complex is an important effector of cell death. The second major pathway for apoptosis is the “intrinsic”, or type II, mitochondria-dependent pathway. The mitochondria contain a number of highly lethal substances that can initiate apoptosis when released into the cytosol. One of these is small electron transporter cytochrome *c*. Under conditions that remain mysterious, cytochrome *c* is released from

mitochondria and forms a complex with procaspase-9 and its cofactor apoptotic protease-activating factor (ARAF)-1[9]. The occurrence of apoptosis has been reported to cause the loss of cardiomyocytes in cardiomyopathy, it is recognized as a predictor of adverse outcomes in subjects with cardiac disease or heart failure[10]. Cardiomyocytes apoptosis by Fas-FasL signaling are closely connected to various pathophysiological conditions such as ischemia/reperfusion injury and congestive heart failure[11].The interaction of Fas with FasL can act on many cell types, including lymphocyte, endothelial cell, smooth muscle cells, and cardiomyocyte which is an important trigger for apoptosis[12]. Especially, the expression of FasL is up regulated by ischemia in ischemic heart failure[13].Fas is plays an important role in the regulation of apoptosis under various physiological and pathophysiological conditions[14, 15]. Its main and best known function in signaling is the induction of apoptosis[16]. Upon receptor activate, the death domain undergoes homotypic interaction with a death domain in the adaptor protein FasL and results in the initiation of apoptosis[17]. The specific binding by FasL with Fas induces receptor aggregation, leading to the cleavage and activation of caspase-8 from procaspse-8, which in turn activates caspase-3.The proteases that transmit the apoptotic pathway are members of the caspase family. The name caspase is derived from Cys-dependent Asp specific protease[18].It

signifies that the catalytic activity is governed by a conserved Cys side chain of the protease and by a stringent specificity for cleaving after Asp residues in substrates, which is very unusual for cellular proteases[19].They make a limited number of cuts in cellular proteins (usually only one or two) and they therefore do not destroy protein structure, but instead they modify it[20].

Stem cell transplantation has emerged as a potential treatment to repair the injured heart, due to the inherent characteristics of stem cells such as self-renewal, unlimited capacity for proliferation and ability to differentiate to various cell lineages[21].Especially, born marrow derived mesenchymal stem cells (MSCs) have the greatest potential for use in cell-based therapy of myocardial infarction (MI). Most recent studies on stem-cell therapy for MI utilizing MSCs suggest that understanding the importance of the tissue micro-environment and how it may be manipulated is critical to realize the effective therapeutic potential of these cells[22]. The therapeutic potential of MSCs in myocardial repair is based on the ability of MSCs to directly differentiate into cardiac tissue[23].

However, the major barrier in clinical application of cell-based therapy is the poor viability of the transplanted cells in the infarcted myocardium[24]. The reason for such modest improvements was attributed to the limited survival of the transplanted cells in the infarcted myocardium[25].

Therefore, we demonstrated that main cause of death of MSCs is the Fas-FasL complex into ischemic heart. For prove my hypothesis, it was tested the several effects that FasL-treated on Fas expressed MSCs. Most importantly, when we treated FasL on Fas expressed MSCs, caspase-8 and caspase-3 are increased. Yet after blocked the interaction of the Fas-FasL apoptosis signal is decreased.

II. MATERIALS AND METHODS

1. Materials

Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin- streptomycine were from Gibco (Gibco BRL, Grand Island, NY, USA). Antibodies for immunoblots were: polyclonal caspase-8 antibody from Abcam and polyclonal caspase-3 antibody from Upstate (Chemicon International Inc. Temecular, CA, USA); monoclonal Fas ligand, CD14, CD34, CD71, CD90, CD105, and CD106 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horse-radish peroxidase-conjugated secondary antibodies to mouse or rabbit were obtained from Santa Cruz Biotechnology. Immunoblot detection system was from Amersham Biosciences (Uppsala, Sweden). Recombinant Rat Fas/TNFRSF6/Fc chimera and FasL were purchased from R&D system. PCR oligonucleotides were synthesized by Bioneer (Bioneer comporation, Deajeon, Korea) and RT-&Go was used for reverse transcription and cDNA synthesis (MP biomedicals, OH, USA)

2. Animals

MSCs were isolated from the bone marrow of 4-week-old Sprague-Dawley

male rats. Also, cardio myocytes were isolated from the neonatal Sprague-Dawley rat 1~2days old. All animal procedures were carried out according to a protocol approved by the Yonsei University Animal Care Committee.

3. Cell culture

A. Mesenchymal stem cells

Rat bone-marrow MSCs were harvested from one-month-old (100~150 g) male Sprague-Dawley rats. Following the rats' death by cervical dislocation, the tibia and femur were dissected, and whole bone-marrow plugs were flushed by means of an 18 gauge needle and 10 mL syringe loaded with DMEM-low glucose supplemented with 10% FBS. MSCs were centrifuged, and resuspended in serum-supplemented medium, loaded to Ficoll (Amersham) density gradient centrifugation. After twice washing steps, MSCs were incubated in 7 ml of fresh DMEM (Gibco BRL) by adding 10% FBS, 100 U/ml penicillin/streptomycin (Gibco BRL). Cells were maintained in 95% air /5% CO_2 at 37 ºC for 10 days, with fresh medium change every 3 days. Upon reaching 80-90% confluence, primary MSCs were trypsinized (Gibco BRL), passaged at a density 5-7 x 10^5 cells/100 mm culture plate [26, 27].

B. Neonatal rat ventricular cardiomyocytes

Cardiomyocytes were prepared from Sprague-Dawley neonatal rat hearts as previously described with minor modification. Isolated heart tissues were washed with Dulbecco's PBS (pH 7.4 Gibco BRL) in order to deplete red blood cells. Using micro-dissecting scissors, hearts were minced to approximately 0.5 mm^3 and treated with 4 ml of collagenase II (1.4 mg/ml, 270 units/mg, Gibco BRL) for 5 min at 37 ºC humidified chamber. The supernatant was removed and washed with 10% FBS containing DMEM. Cell pellets were obtained by centrifugation and resuspended in an equal volume of fresh medium containing 10% FBS. The remaining tissue was treated with fresh collagenase II for an additional 5 min. Incubation was repeated until the tissue was completely digested. The resulting supernatant was centrifuged at 2000 rpm for 3 min at room temperature. The cell pellet was resuspended in 5 ml of cell culture medium and preplated in culture dishes for at least 2 hrs at 37 ºC in 5% CO_2 incubator. The adhered cells are fibroblast and non-adherent cells are cardiomyocytes. Unattached cardiomyocytes were replated on 100 mm culture dish ($5x10^5$ cell/ml) and incubated in α-MEM supplemented with 10% FBS. Cells were then cultured in a

CO_2 incubator at 37ºC. To reduce fibroblast contamination, we used α-MEM with 0.1 mM 5-bromo-2'-deoxyuridine (Brd-U) (Sigma, St. Louis, MO, USA) [28, 29].

4. Preparation of conditioned media

A. Conditioned media from cultured hypoxic cardiomyocytes

Conditioned media were generated neonatal rat ventricular cardiomyocytes. Cardiomyocytes were fed with 10% FBS DMEM. Cardiomyocytes were placed in the hypoxic chamber and washed twice by degassed serum-free DMEM. After exchanged with degassed fresh serum-free DMEM, cells were incubated under hypoxia and media collected and stored -80ºC. Hypoxic conditions were created by incubating cells at 37ºC in an anaerobic system (Technomart INC, Seoul, Korea) with an atmosphere of 5% CO_2, 5% H_2, 85% N_2. The oxygen level in the chamber was ~0.5%.

B. Conditioned media from cultured normoxic cardiomyocytes

Neonatal rat ventricular cardiomyocytes were fed with 10% FBS DMEM. The MSCs were washing twice by serum-free DMEM in

normoxic clean bench and exchanged for fresh serum-free DMEM. The cells were incubated under 5% CO_2, 37ºC normoxic chamber. The media was then collected and stored 4ºC for in vitro experiments.

5. MSCs characterization

Immunocytochemical characterization of MSCs was demonstrated below. Cells were cultured in 4-well slide chamber, washed PBS and incubated in 1% paraformaldehyde solution (sigma, USA) for 10minutes. PBS washing twice, then cells has permeated in 0.1% Triton X-100 for 7 min. After PBS washing twice, they have blocked for 1 hr (blocking solution: 2% bovine serum albumin, 10% horse serum in PBS) and adhered with the following antibodies: CD71, CD90, CD105, CD106, and intracellular adhesion molecule (ICAM)-1. FITC-conjugated mouse, rabbit and goat (Jackson Immun. Lab, USA) were used as secondary antibodies. Then, they were detected confocal microscopy (Carl Zeiss, Germany). MSCs were performed according to the procedure of fluorescence-activated cell sorting (FACS) staining described below. Briefly, cells were detached from the plate with 10% trypsin-EDTA (Gibco BRL), washed in PBS and fixed in 70% ethanol at 4^oC for 30 min with agitation. Cells were washed twice in PBS and resuspended at $2x10^6$ cells/ml in blocking buffer (1% BSA, 0.1% FBS)

containing the following antibodies: CD14, CD34, CD71, CD90, CD105, and CD106; In this case of CD14, we used normal rabbit Ig G as a negative isotype control. After staining, cells were washed twice and then labeled with rabbit or mouse-FITC conjugated Ig G for 20 min in the dark area. After two more washes, flow cytometric analysis was performed on a FACS Calibur system (Becton Dickinson, USA) using CellQuest™ software (Becton Dickinson, USA) with 10000 events recorded for each sample.

6. Neonatal rat ventricular cardiomyocytes in hypoxic conditions

After overnight incubation, neonatal rat ventricular cardiomyocytes were placed in a hypoxic chamber and washing twice with fresh serum-free DMEM. Cardiomyocytes were incubated with hypoxic conditioned media or normoxic conditioned media in the hypoxic chamber for 12 hrs.

7. Cell viability

Cell viability was determined by a CCK-8 kit using a WST-8[2-(2-methoxyl-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] assay. MSCs were plated in triplicate wells in 96-well plates at $1x10^4$ per well, and incubated for 24 hrs. Media was changed with 10% FBS DMEM, and samples were pretreated with 250 μM H_2O_2 at 37 ^oC

for 6 hrs. After incubation, cells were incubated at 37 ^{o}C for 2 hrs. Absorbance was measured at 450 nm with a spectrophotometer.

8. RT-PCR analysis

A. Isolation of total RNA

Total RNA was extracted using 1 ml TRIzol reagent (Sigma). Total RNA was extracted by the chloroform, isopropanol, DEPC-treated 75% ethanol procedure. RNA was resuspended in 30 μL nuclease-free water. Quantity and quality of isolated RNA was determined by OD260/OD280 with a DU 640 spectrophotometer (Eppendorf, Hamburg, Germany).

B. RT-PCR assay

Complementary DNA (cDNA) was generated with a Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's instructions and 1 μg of total RNA was reverse-transcribed in 20 μL containing 5 mM MgCl2, 10 mM Tris—HCl (pH9.0 at 25 ^{o}C), 50 mM KCl, 0.1%Triton X-100, 1 mM dNTP, 20 U of RNase inhibitor, 0.5 μg oligo-(dT) 15 primer, 10 U reverse transcriptase

for 15 min at 42 °C and the reaction was terminated by heating at 99 °C for 5 min. To monitor cDNA synthesis, an aliquot of the RT reaction mixture was subjected to PCR for GAPDH with primer sequences were as follows: 5-ACCACAGTCCATGCCATCAC-3 and 5-TCCACCACCCTGTTGCTG-3 (450 bp).Fas primer was: 5-TGCCTCCACTAAGCCCTCTA-3 and 5-CAAGACTGACCCCGGAAGTA-3 (420 bp). PCR conditions were a cycle of denaturing at 95 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 62 °C for 30 sec, extension at 72 °C for 90 sec before a final extension at 72 °C for 10 min. The first round of PCR used 1 μL of cDNA from reverse transcription. PCR mix contained 10 pM of each primer, and 200mM Tris HCl (pH 8.8), 100 mM KCl, 1.5 mM $MgSO_4$, 1% Triton X-100, 0.1 mM dNTP and 1.25 U of Taq polymerase of 25 μL. PCR was carried out in a thermal cycler using the following conditions: 95 °C for 3 min, 95 °C for 1 min and then individual conditions for each gene. All RT-PCR products were separated by electrophoresis in a 1.2% agarose gel (Bio-Rad,Hercules, CA, USA) and Gel-Doc (Bio-Rad, Hercules, CA, USA) visualized using a after staining with ethidium bromide (EtBr; Sigma) [30].

9. Immunoblot assay

Cells were washed once in PBS and lysed in buffer (Cell Signaling Technology) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na_2-EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined using the Bradford Protein Assay Kit (Bio-Rad). Proteins were separated on 12% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels and transferred to polyvinylidenedifluoride membranes (Chemicon). After blocking with Tris-buffered saline-Tween 20 (TBS-T, 0.1% Tween 20) containing 5% nonfat dried milk for 1 hr at room temperature, membranes were washed twice with TBS-T and incubated with primary antibody for 1 hr at room temperature or overnight at 4°C. Membranes were washed three times with TBS-T for 10 min and incubated for 1 hr at room temperature with horseradish peroxidase-conjugated secondary antibodies. After extensive washing, bands were detected by enhanced chemiluminescence reagent (Santa Cruz Biotechnology). Band intensities were quantified using the Photo-Image System (Molecular Dynamics) [31].

10. Trichloro acetic acid protein precipitation

Trichloroacetic acid (TCA) precipitates were generated from chilled samples

by adding 2 volumes of ice cold 20% TCA, vortexing, and incubating 30 min on ice. Precipitates were centrifuged 15 min 4ºC, supernatants removed, and 200 μL acetone added, with centrifugation for 5 min at 4ºC. Pellets were dried at room temperature for 5-10 min before adding 1x SDS-PAGE loading buffer for 12% SDS-PAGE after heating at 65ºC for 3 min.

11. Caspase-3 assay

Relative caspase-3 activity was determined using the Apop-Target™ Capase-3 Colorimetric Protease. This assay is based on the generation of free DEVD-pNA chromophore when the provided substrate is cleaved by caspase-3. Upon substrate, free pNA light absorbance is quantified using a microplate reader at 405 nm. Briefly, after treatments, cultured MSCs (3×10^6 cells) were harvested in lysis buffe, and cell extracts centrifuged to eliminate cellular debris. Protein concentration was determined by the BSA assay (Bio-Rad). Aliquots (50 μL) of cell extracts were incubated at 37°C for 2 hrs with the chromophore substrate. Free DEVD-pNA was determined colormetrically. Comparison of apoptotic samples with uninduced controls allowed determination of fold increase in caspase-3 activity.

12. Statistical analysis

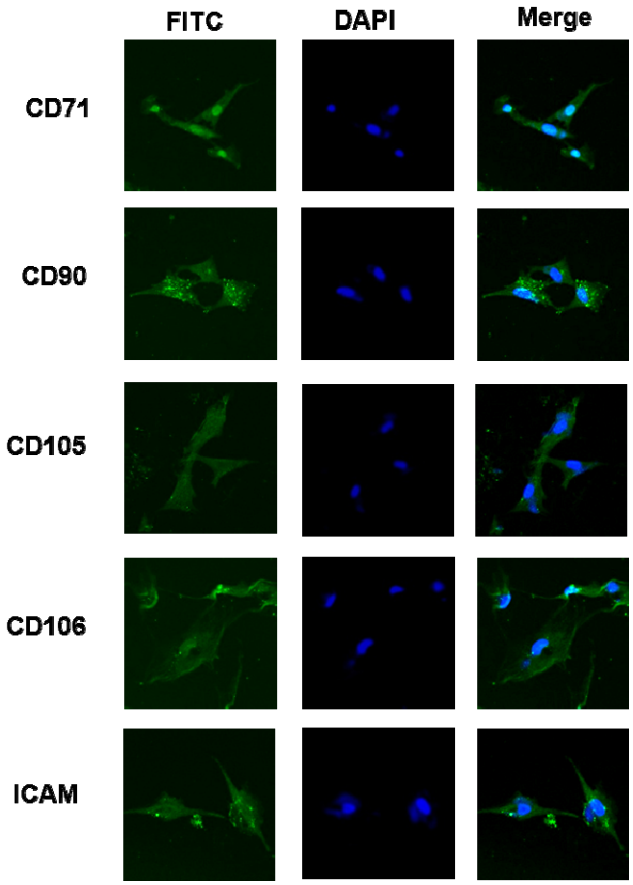
All quantified data are an average of at least triplicate samples. Error bars represent the standard deviation of mean.Statistical significance was determined by Student's *t*-test, with. $p < 0.05$ was considered significant.

III. RESULTS

1. Isolation and characteristics of MSCs

MSCs were first isolated from mixed cultures with hematopoietic cells based on their attachment on the culture plate. 3 days later after mixed cultures have seeded, tapped a plate, old culture medium was discarded, and fresh 10% FBS contained DMEM was poured into a plate. As time goes by, the cells were adherent, elongated and spindle-shaped, and they were yielded $3x10^6$ cells within 2 weeks of culture with 90% purity. The MSCs maintained a fibroblastic morphology through repeated passages, and their identity was confirmed by immunocytochemistry (Figure 1, A) and FACS analysis (Figure 1, B). Cultured MSCs expressed CD71, CD90, CD105, CD106, and ICAM-1.They expressed neither the hematopoietic marker CD14 nor CD14 (Figure 1).

(A)


FITC
DAPI
Merge
CD71
CD90
CD105
CD106
ICAM

(B)

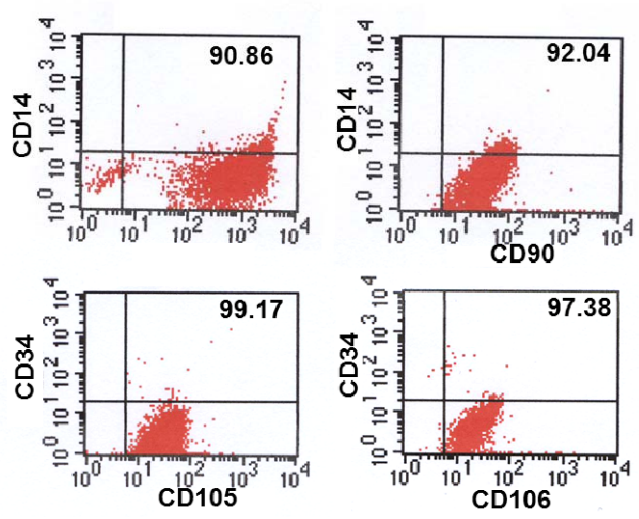

90.86
CD14
92.04
CD14
CD90
99.17
CD34
CD105
97.38
CD34
CD106

Figure 1. The characteristics of MSCs. Most adherent MSCs are practically fibroblastic in morphology and some polygonal cells were contained after a 6 week culture. Magnification, 400x. Cells were cultured from bone marrow after density fractionation and are shown at 10 days after plating. At 14 days, the MSCs were positive for CD71, CD90, CD105, CD106, and ICAM and were negative for CD14 and CD34 by flow cytometry.

2. Hypoxic conditions induced apoptosis of MSCs

In the ischemic heart condition, the amount of oxygen and nutrients were extremely deficiency. It was the reason that why injected MSCs were death. To determine if hypoxic condition-mediated cell death effect *in vitro*, MSCs were washed deoxygenated serum free DMEM media than encountered in hypoxic condition by time-dependent manners. The survival of MSCs was reduced by time-dependent manner. Thus we thought that which factor cause of death of MSCs in ischemic condition.

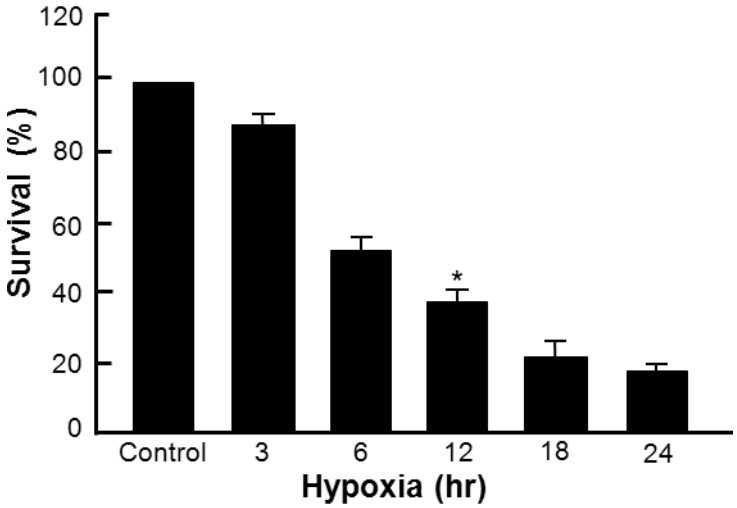


Figure 2. Survival of hypoxic MSCs. Hypoxia induced death of MSCs. MSCs incubated at 37℃ inside an anaerobic system (Technomart INC, Seoul, Korea) with an atmosphere of 5% CO_2, 5% H_2, 85% N_2. The oxygen level in the chamber was ~0.5%.

3. Hypoxic conditions induced expression of Fas in MSCs

The hypoxic condition was caused to death of MSCs. Hypoxia induced apoptosis with enhanced expression of Fas [32].To explore the possibility that Fas plays a role in MSCs apoptosis, RT-PCR was performed after exposure of cells to hypoxia and time dependent increased expression was seen to be greater at 6hrs than in other conditions. Figure 2 and this data were means that Fas is significantly important signal when these were death by hypoxic condition.

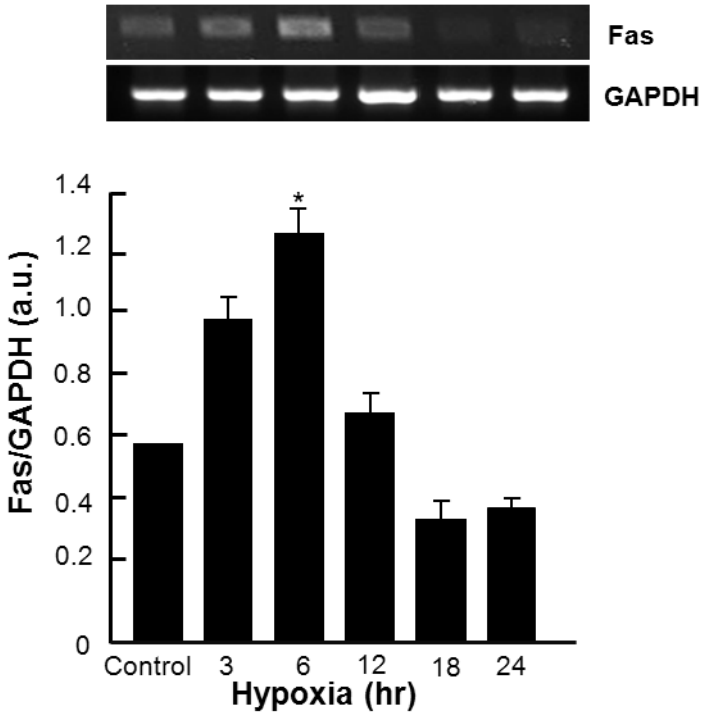


Figure 3. Fas expression on hypoxic MSCs Fas expression was detected by RT PCR. MSCs were incubated at 37℃ inside an anaerobic system (Technomart INC, Seoul, Korea) with an atmosphere of 5% CO_2, 5% H_2, 85% N_2. The oxygen level in the chamber was ~0.5%.

4. The expression of Fas ligand in hypoxic cardiomyocytes media

To determine whether FasL induced cardiomytocytes apoptosis is a critical event, we investigated whether hypoxic cardiomyocytes and normoxic cardiomyocytes secreted FasL by TCA protein precipitation and immunoblotting. Hypoxic cardiomyocytes expressed anaerobic serum free α-MEM media and Normal cardiomyocytes were treated serum free α-MEM media for 12 hrs. Hypoxic cardiomyocytes were expressed 6 times higher FasL than normoxic cardiomyocytes one.

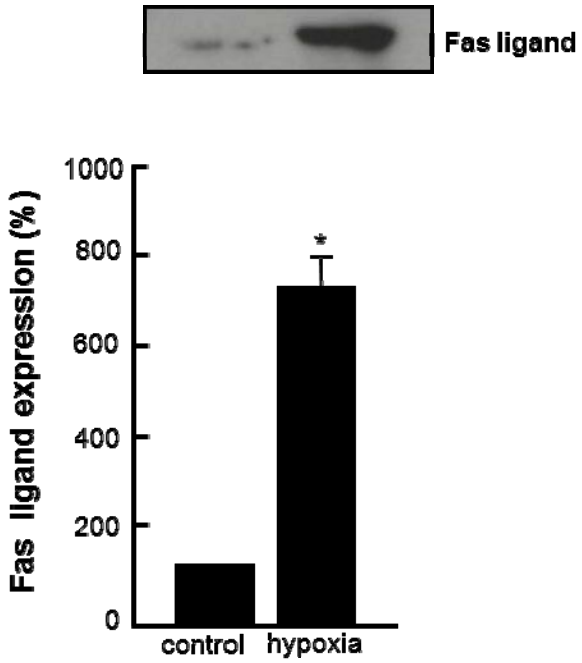


Figure 4.Expression of Fas ligand in hypoxic cardio myocytes media. Western blot for detect FasL in normoxic cardiomyocytes media and hypoxic cardiomyocytes media through protein precipitation by TCA. Expression of FasL was significantly augmented in the hypoxic cardiomyocytes media than normoxic media. Signal was quantified by scanning densitometry.*P<0.05 vs control.

5. Expression of Fas ligand in ischemic heart

In I/R rat heart and normal rat heart, FasL was detected by immunoblotting. Animal model surgured for I/R model, rat heart was tied by 6-0 polybren for 1 hr and reperfused.Then, rat heart was sacrificed especially, LAD. Immunoblot analysis of cardiac tissue showed in 12-fold increase in expression of FasL in I/R rat hearts compared to normal heart.

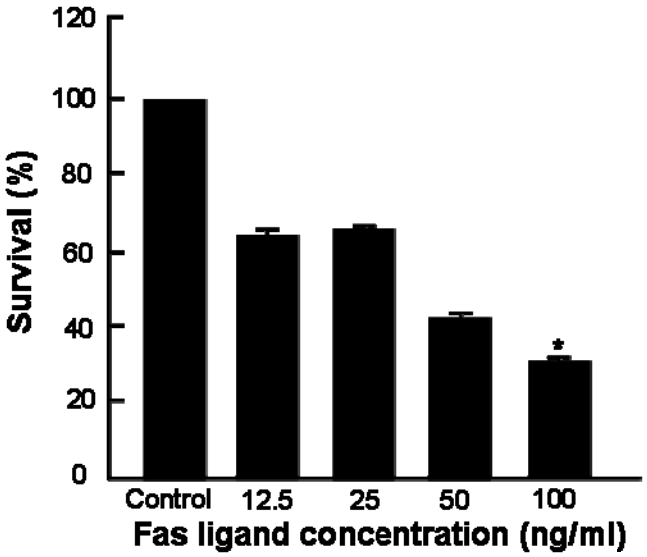


Figure 5. Expression of Fas ligand in ischemic heart. Immunoblot for detect FasL in normal rat heart and I/R rat heart. The protein products of FasL extracted from the left ventricles of excised heart. FasL is significantly increased in I/R rat heart.

6. Survival of hydrogen peroxide-treated MSCs

H_2O_2 and other ROS induced cell death at relatively high concentrations in many cell types[33]. Cultured MSCs exposed to H_2O_2 by dose dependent manners.To test the effect of ROS, MSCs were subjected to H_2O_2 for 6 hrs.The survival of MSCs was similar in normal to 500 μM treated MSCs.

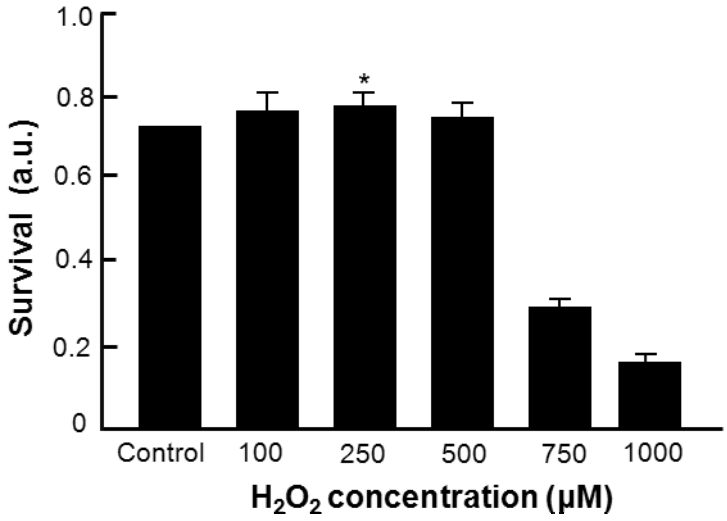


Figure 6. Survival on H_2O_2-treated MSCs. H_2O_2-treated MSCs were shown different survivla. MSCs were incubated at 37℃ for 6 hrs. Cell viability was measured by CCK.

7. Hydrogen peroxide induced Fas expression on MSCs

H_2O_2 induces up-regulation of death receptor Fas in human endothelial cells[34].Therefore, to confirm in MSCs, H_2O_2 was added to cells at different dose and Fas expression was detected by RT-PCR. $5x10^5$ cells seed in the 60 mm dish. After a day, MSCs were treated H_2O_2 in 10% DMEM during 6 hrs. As shown in this figure**,** the highest Fas expression was detected for 250 μM H_2O_2-treated MSCs. Therefore hypoxic conditions induced Fas expression in MSCs.

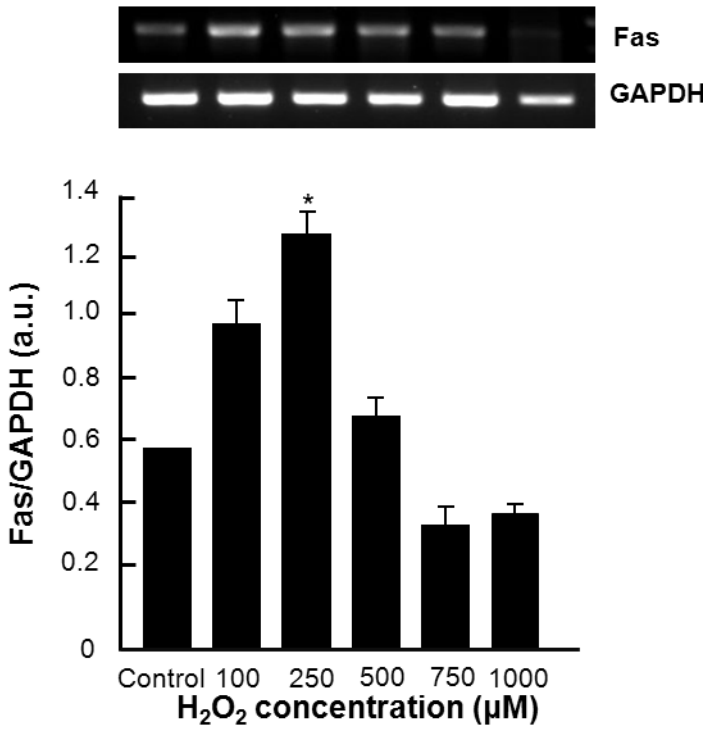


Figure 7. Expression of Fas in H_2O_2 treated MSCs. MSCs were assayed by RT PCR for detection of Fas. MSCs were incubated 37℃, 5% CO_2. RT-PCR was performed using Tri reagent.

8. Fas ligand was not induced death of MSCs without hydrogen peroxide.

To determine FasL-induced apoptosis, FasL was treatment to cultured MSCs in 1% DMEM during 72 hrs. After 72 hrs, cell viability was measured through CCK. Survival of FasL-treated MSCs was similar for both control and FasL had not effect on apoptosis of MSCs.

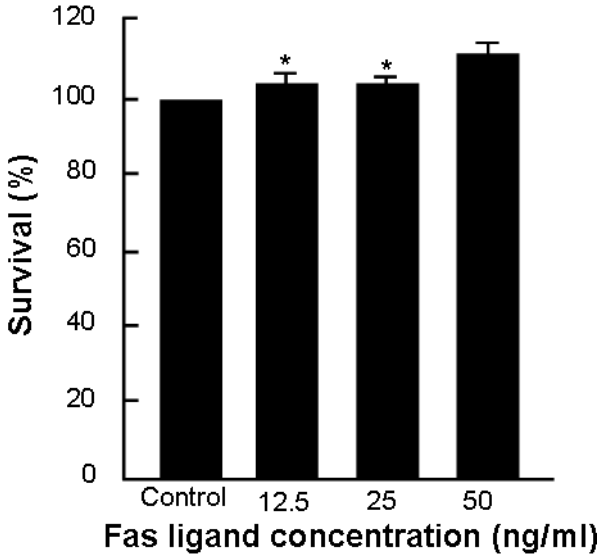


Figure 8. Effects of Fas ligand on cultured MSCs. FasL were treated in cultured MSCs through dose dependent manners. MSCs were incubated in 37℃, 5% CO_2,72 hrs. Signal was quantified by scanning densitometry. **P*<0.1 vs control.

9. Fas-Fas ligand interaction induced apoptosis in MSCs after H_2O_2 pretreatment

As shown the Figure 8, FasL-treated MSCs were not induced apotposis. Therefore we tried pretreatment of H_2O_2 before treatment FasL. In the Figure 7, Fas expression was the highest, cell viability was similar to normal MSCs in 250 μM H_2O_2.Thus MSCs were pretreated with 250 μM H_2O_2 in 10% DMEM for 6 hrs and then, exposed to FasL 100 ng/ml in 1% DMEM, for 72 hrs. After 72 hrs, cell survival was detected by CCK. Survival of FasL-treated MSCs was reduced suggesting that Fas-FasL interaction induced apoptosis.

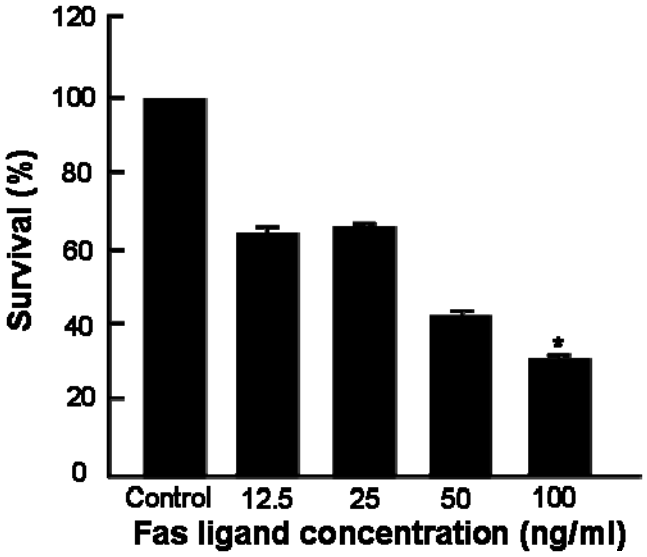
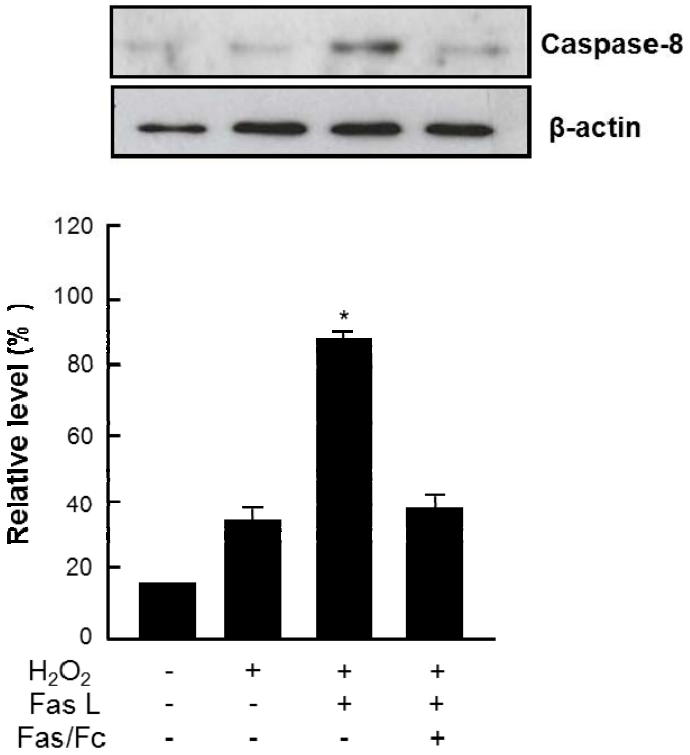


Figure 9. Effect on Fas ligand in H_2O_2-treated MSCs. MSCs were pretreated with H_2O_2 (250 μM) in 10% DMEM for 6 hrs and then, exposed to FasL (100 ng/ml) in 1% DMEM, for 72 hrs.

10. The signal pathway of Fas-Fas ligand blocked MSCs

In apoptosis, activation of the family of caspase cysteine proteases induces the proteolytic cleavage of critical proteins, leading to cell suicide[35, 36]. Fas is a member of the tumor necrosis factor (TNF) superfamily that plays an important role in receptor-mediating apoptosis[37].When FasL binds death receptor Fas, apoptosis signaling is entered. We have explored that block of FasL was involved in the Fas-mediated pathway using western blot to detect caspase-8 and caspase-3 assay to detect caspase-3 activity. First, we processed H_2O_2 250 μM for 6 hrs in 10% DMEM and Fas/Fc was treated in 10% DMEM for 1 hr last FasL was treated in100 ng/ml for 12 hrs at 1% DMEM. As shown figure 10.A, caspase-8 level of Fas/Fc-treated MSCs was lower than FasL-treated MSCs. Figure10. B was shown the caspase-3 activity in Fas blocked MSCs. In the Figure B, caspase-3 activity was tested. Caspase-3 was downstream of caspase-8 and activated by caspase-8 and other signals. Activated caspase-3 induced apoptosis in most cells. Likely figure10.A, caspase-3 level of Fas/Fc-treated MSCs lower than FasL-treated cells. Therefore, we thought that apoptosis of MSCs was caused by Fas-FasL interaction which was up-regulate caspase-8 and caspase-3 and induced apoptosis.

(A)


Caspase-8
β-actin
Relative level (%)
120
100
80
60
40
20
0
*
H2O2 - + + +
Fas L - - + +
Fas/Fc - - - +

(B)

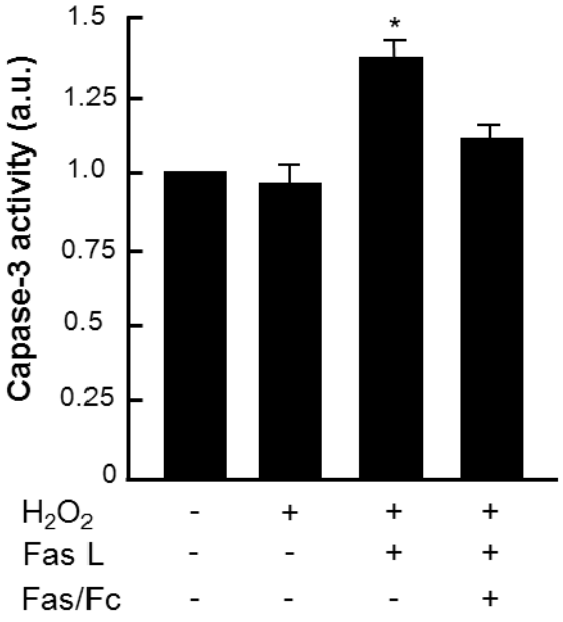

Capase-3 activity (a.u.)
1.5
1.25
1.0
0.75
0.5
0.25
0
*
H2O2 - + + +
Fas L - - + +
Fas/Fc - - - +

Figure 10. The signal pathway of Fas/Fc-treated MSCs. MSCs were cultured in 10% DMEM and treated H_2O_2 for 6 hrs then treatment 1 μg/ml Fas/Fc for 1 hr. After 100 ng/ml FasL were treated 1% DMEM during 12 hrs. (A) western blot to detect caspase-8. (B) caspase-3 assay to detect caspase-3 activity.

11.Survival of Fas blocked MSC

In this figure, The survival of Fas/Fc-treated MSCs was examed by CCK. $3x10^3$ cells/well were seeding in 96 well plate. Then, we tested survival of MSCs using CCK at the same condition in Figure 10. FasL was treated 100 ng/ml for 72 hrs, Fas/Fc was treated 1 mg/ml for 1 hr on cultured MSCs. Fas/Fc-treated cells were similar to control MSCs.This showed that Fas-FasL interactions was important for MSCs apoptosis.

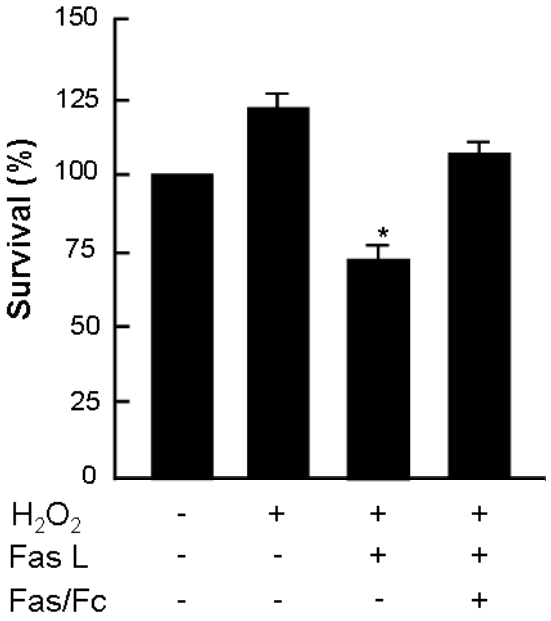


Figure 11. Survival of Fas/Fc-treated MSC. MSCs were cultured in 10% DMEM and treat H_2O_2 for 6 hrs then treatment 1 μg/ml Fas/Fc for 1 hr. After 100 ng/ml Fas ligand were treated 1% DMEM during 12 hrs. Survival was measured by CCK.

IV. DISCUSSION

Over the past decade, born marrow stem cells have been used as therapeutic vectors in the treatment of a wide variety of diseases[38]. However, a high percentage of donor cells die within few hours or days after transplantation, which must be resolved to achive optimal outcomes with this procedure[39]. In spite of various approaches to overcome the limitations improve cardiac function in an I/R model, no solution currently exists for the problem of programmed cell death induced by loss of matrix attachments in the transplanted cells[40]. Indeed, engrafted MSCs first encounter harsh condition coupled with the loss of survival signals because of inadequate interaction between cells and matrix[41].Hypoxic environment induce apoptosis and may be responsible for loss of engrafted MSCs both *in vivo* and *in vitro*[42-44]. In addition, hypoxia derived-ROS are a major cause of injury after I/R, and may hinder the adhesion and spread of MSCs[45].Therefore, this study focused on the reason for death of MSCs in ischemic heart.

Hypoxia, the decline in normal level of tissue oxygen tension, is a well-known cause of cell death[46.47]. We confirmed that hypoxic condition influenced MSCs in a time-dependent manner (Fig. 2), and tested Fas expression in MSCs in hypoxic condition (Fig. 4). Based on the results, we

hypothesized that up regulation of Fas caused death of MSCs.

FasL is a critical mediator of cardiomyocyte death and MI during I/R[48]. Secreated FasL from ischemic heart was detected using precipitation using TCA and immunobloted (Fig. 4) and Hypoxic conditions indyced FasL which binds to Fas in transplanted MSCs to induce apoptosis (Fig.5).

Several studies have suggested a role of H_2O_2 as a second messenger in intracellular signaling pathway and it has been shown to affect apoptosis [49].The survival of MSCs were measured by CCK. It was similar to H_2O_2 treated survival up to 500 μM but in 750 μM and 1000 μM decline rather than control. (Fig. 6) And we tested that H_2O_2-induced Fas expression in MSCs through RT-PCR. (Fig. 7) We decided that Fas expression high in 250 μM H_2O_2 and it was treated on MSCs.

The Fas/FasL is crucial in myocardial I/R injury [48, 50]. For made Ischemic condition like *in vivo*, FasL-treated MSCs were examined with or without H_2O_2 pretreatment. As shown Figure.3, H_2O_2-treated MSCs expressed Fas. Without H_2O_2 pretreatment, apoptosis did not occured in FasL-treated MSCs (Fig. 8). However, with H_2O_2-pretreatment, FasL induced apoptosis (Fig. 9). Therefore, H_2O_2 induced Fas expression in MSCs, and exogenous treatment of FasL, which bound Fas, was suggested as the reason for the apoptosis.

Fas-mediated apoptosis was initiated by interaction with Fas and FasL,

which resulted in the caspase cascade[47]. Caspases are cysteine proteases that are activated by initiator and effector caspases[51, 52]. We tested these signaling of caspase-8 and caspase-3. Caspase-8 was tested by immune blotting (Fig.10, A), and caspase-3 was tested by caspase-3 activity assay (Fig.10, B). We tested the signaling of caspase-8 and caspase-3.MSCs were pretreated with 250 μM H_2O_2, followed by FasL and/or Fas/Fc treatment. Caspase-8 expression increased in FasL treated MSCs but Fas/Fc treated cells were similar to a control (Fig.10.A). Figure 10.B show FasL-induced caspase-3 activity, which was up-regulated in FasL-treated MSCs but down-regulated in Fas/Fc-treated cells. Fas-FasL interaction induced up-regulation of caspase-8 and caspase-3 which resulted in apoptosis. And survival was decreases in FasL-treated MSCs. (Fig. 11). Fas-FasL interaction induced cell death through the caspases cascasde signal pathway, inducing apoptosis in cells. Thus, we determined that Fas-FasL interaction induced apoptosis in MSCs transplantated into ischemic heart.

Ⅴ. CONCLUSION

In this study, we showed that Fas-FasL interaction induced apoptosis in MSCs in the ischemic heart. Hypoxic conditions induced cell death and Fas expression in MSCs. FasL was expressed in ischemic heart and hypoxic neonatal cardiomyocytes. Apoptosis occurred in MSCs not only after injection into ischemic heart, but also in Fas L-treated culture conditions. In addition, caspase-8 and caspase-3 expression levels increased in FasL-treated MSCs and survival decreased. These results suggest that the Fas-FasL pathway is the major cause of MSC death in ischemic hearts.

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ABSTRACT (in Korean)

심근 재생을 위한 허혈성 중간엽 줄기세포의 Fas 억제

<지도교수 장 양 수>

연세대학교 대학원 의과학과

함 온 주

중간엽 줄기 세포 (MSCs) 는 심장 질환 치료 특히 허혈성 심질환 치료 중 세포 치료를 기초로 하는 치료법을 위한 많은 가능성을 가지고 있다. 그러나 줄기세포를 기초로 하는 치료에는 경색이 있는 심장에 이식된 중간엽 줄기세포의 생존율이 낮다는 점이 문제가 되고 있다. Fas 는 세포사멸과 관련된 수용체이며, 종양 괴사 인자 (TNF) 유전자 초 거대 집단의 구성원이다. Fas 리간드 (Fas ligand) 역시 TNF 구성원으로 표적 수용체인 Fas 에 붙어 세포사멸을 유도한다.

Fas 경로는 caspase-8 과 caspase-3 의 활성을 유도하여 결국엔 세포사멸이 일어난다. 따라서 본 연구는 이식된 중간엽 줄기세포의 생존율이 낮은 원인이 허혈성 심장에 이식된 중간엽 줄기세포에 사멸수용체인 Fas 의 발현이 증가하며 허혈성 심질환에서 증가된 Fas 수용체가 중간엽 줄기세포의 Fas 와 결합에서 사멸한다는 것을 알아보고자 하였다. 저 산소 환경에서 자란 신생백서의 심근세포와 동물모형에 허혈성 심질환을 유도 하고 7 일 이후에 분리한 심장 에서 면역 탁본법을 통하여 Fas 리간드의 발현을 확인한 결과 7 배 정도 증가한 것을 확인하였다. 또한 저 산소 환경에서 중간엽 줄기세포에서 Fas 발현이 증가된 것을 확인하였다. 또한 허혈성 심질환에서 유도되는 활성 산소(ROS)가 Fas 의 발현을 증가시키는지 알아보고자 RT-PCR 을 수행한 결과 Fas 의 발현은 250 μM 의 H_2O_2 를 6 시간 처리하였을 때 가장 높은 것으로 확인되었다. 따라서 Fas 리간드와 Fas 의 발현이 증가된 중간엽 줄기세포가 결합하여 세포사멸이 일어나는 것을 확인하고자 H_2O_2 를 6 시간 전 처리 한 후 100 ng/ml 의 Fas ligand 를 72 시간동안 처리한 후 세포사멸 신호인 caspae-8 과 caspase-3 를 확인하였다. 이 중 caspase-8 는 면역

탁본법으로 확인하였으며, caspae-3 의 발현은 caspase-3 활성 측정법을 통하여 확인하였다.

실험을 통하여 Fas 리간드를 처리한 중간엽 줄기세포에서 증가된 caspase-8 의 발현과 caspae-3 의 활성 증가를 확인하였다. Fas 와 Fas 리간드의 결합을 억제하기 위해 1 mg/ml 의 Fas FC chimera 를 처리한 중간엽 줄기세포에서는 caspase-8 의 발현과 caspae-3 의 활성이 감소함을 확인할 수 있었다. 이때의 생존율을 확인한 결과, Fas 리간드를 처리한 중간엽 줄기세포에서 생존율이 가장 낮았으며, Fas FC chimera 를 처리한 중간엽 줄기세포에서 생존율이 증가됨을 확인하였다. 본 연구는 허혈성 심질환에 이식된 중간엽 줄기세포의 손실의 원인이 Fas 와 관련되어 있다는 것을 규명한 논문으로 앞으로 Fas 의 발현을 저해하여 심장에 이식된 줄기세포의 생존을 높여 허혈성 심질환 치료에 기여할 수 있을 것으로 기대된다.

핵심 되는 말: 골수 간엽 줄기 세포, 저 산소 성 심근세포, Fas, Fas 리간드