Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;45:1003-1012

DOI: 10.1159/000487295

Published online: February 09, 2018

Accepted: January 16, 2018

Original Paper

Clusterin Reduces Cold Ischemia-Reperfusion Injury in Heart Transplantation Through Regulation of NF-kB Signaling and Bax/Bcl-xL Expression

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Key Words

Ischemia reperfusion injury • Heart transplantation • Clusterin • NF-κB • Bax • Bcl-xL

Abstract

Background/Aims: Ischemia-reperfusion (I/R) injury is an unavoidable event occurring during heart transplantation and is a key factor in graft failure and the long-term survival rate of recipients. Therefore, there is an urgent need for the development of new therapies to prevent I/R injury. Clusterin is a hetero-dimeric glycoprotein with an antiapoptotic function. In this study, we investigated whether clusterin was cardioprotective in heart transplantation against I/R injury using an *in vivo* rat model and an *in vitro* cell culture system, and examined the underlying mechanisms of I/R injury. ***Methods:*** Heart grafts from wild-type C57BL/6 mice were preserved in UW solution (control) or UW solution containing recombinant human apolipoprotein-J (hr clusterin) for 24 h. The preserved hearts were implanted into recipient mice of the same strain as the donors for 72 h, and the heart grafts were then taken for histopathological and gene expression analyses. An *in vitro* ischemia reperfusion model using H9C2 cells or H9C2/clusterin cDNA cells was constructed. The expression of clusterin, p65, Bax, Bcl-xL, IL-1β, and TNF-α protein and mRNA in heart tissue and H9C2 cells was detected by western blot, reverse transcription-polymerase chain reaction (RT-PCR), and quantitative RT-PCR assays; IL-1β and TNF-α protein was detected by enzyme-linked immunosorbent assays; NF-kB activity was detected by an electrophoretic mobility shift assay; cell apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling and flow cytometric analyses. ***Results:*** Cold I/R caused severe morphologic myocardial injury to heart grafts from wild-type C57BL/6 mice, whereas grafts from hr clusterin preservation showed less damage, as demonstrated by decreased cell apoptosis/death, decreased neutrophil infiltration, and the preservation of the normal structure of the heart. Clusterin reduced the expression of p65, pre-inflammatory IL-1β, and TNF-α, and the pro-apoptotic gene Bax, while it enhanced

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the expression of the anti-apoptotic gene Bcl-xL *in vitro* and *in vivo*. Clusterin inhibited cell apoptosis/death and reduced pre-inflammatory. ***Conclusion:*** Clusterin is a promising target for preventing cold I/R injury in heart transplantation. This study also shows that the resultant protective effects of clusterin are mediated by NF-κB signaling and Bax/Bcl-xL expression.

Introduction

Cardiac transplantation is the last resort for patients with end-stage heart failure. Ischemia-reperfusion (I/R) injury is a major issue in cardiac transplantation. I/R injury is associated with increased primary organ dysfunction and subsequent delayed organ function after cardiac transplantation. To improve the rate of successful heart transplantations, organ preservation should be optimized. However, the functional depression of cardiac grafts in postoperative recovery is not exceptional and the vitality of the transplanted tissue depends considerably on cardioplegic and storage conditions. At present, heart preservation is limited to 4–6 h of cold ischemic storage [1]. Reperfusion injury occurs when there has been inadequate myocardial protection during the preceding ischemic period.

Extended cold ischemic times during heart transplantation have been associated with an increased risk of developing graft vasculopathy and failure in animal models [2, 3] and humans [4]. Moreover, prolonged times between donor brain death and organ retrieval have been associated with increased mortality in cardiac transplant recipients [5]. Graft coronary microvascular dysfunction after ischemia and reperfusion can culminate in primary graft failure or untreatable chronic rejection [6]. Cold ischemia stimulates the expression of inflammatory mediators acting as "danger signals" and amplifying tissue injury and graft rejection.

In recent years, maintaining organ viability has become more challenging because the shortage of donors has led to broader criteria for donor acceptability and consequently to organs with greater compromise [7]. RNA interference can be used to inhibit the expression of specific genes *in vitro* and *in vivo*, thereby providing an extremely useful tool for investigating gene function; however, limited success has been reported in animal studies [8]. Thus, there is an urgent need for an increased understanding of cold I/R injury and to identify new targets.

Clusterin is a hetero-dimeric glycoprotein secreted by a number of cell types. It has a cytoprotective effect in response to diverse stresses. Previous studies suggest that clusterin plays important roles in cell adhesion, spermatogenesis, tumor metastasis, and lipid transportation [9]. In addition to its chaperone activity, clusterin may have an antiapoptotic function. With regard to the role of clusterin in apoptotic cell death, multiple lines of evidence have demonstrated its cytoprotective effects in several tumors, epithelial cells, endothelial cells, and so on [10]. Moreover, clusterin protects cells from heat shock and the action of TNF-α, suggesting that clusterin could be a survival factor related to the apoptotic pathway [11]. The anti-apoptotic effect of clusterin was also reported in the cardiac system based on its increased expression in the injured heart [12]. Clusterin reportedly protects cardiomyocytes from ischemia-induced cell death [13] or ethanol-induced cardiac injury [14].

However, the specific impact of clusterin on cold I/R injury in heart transplantation remains unknown and its underlying mechanism has not been identified. In this study, we determined that clusterin has a protective effect on cold I/R injury in heart transplantation *in vivo* and *in vitro*. We discovered that clusterin exerts its protective effect through an interaction with NF-κB signaling.

Materials and Methods

Animals

C57BL/6 WT mice were purchased from Shanghai Laboratory Animal Research Center. The animals were housed under conventional conditions at the Animal Care Facility at the affiliated hospital of Qingdao

University, and were cared for in accordance with the guidelines established by the China Council on Animal Care.

Cell line and cell culture

The rat heart cell line H9C2 was purchased from ATCC (Shanghai, China) and cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Hangzhou, China), which was supplemented with 10% fetal bovine serum (FBS; Sigma, Hangzhou, China) and 100 U/mL penicillin and streptomycin.

Heterotopic heart transplantation with prolonged ischemia reperfusion

Heterotypic cardiac transplantation was performed as described previously [15]. The heart was removed immediately from the donor and placed in chilled Euro-Collins solution on ice. The heart was perfused with a recombinant human apolipoprotein-J (hr clusterin) solution through the inferior vena cava and aorta until the vessels of the heart turned clear. The tube was kept there to support the lumen of the inferior vena cava and tied with silk sutures. The donor heart was then excised and immersed in a 10 μg hr clusterin solution at 4°C for 24 h. Before anastomosis to the recipient was performed, the donor heart was flushed with fresh hr clusterin solution through the tube to wash out potentially harmful cellular metabolites that accumulate during a period of cold ischemia. Then, the tube was removed and the inferior vena cava was ligated permanently. The preserved heart was implanted into a recipient mouse of the same strain as the donor. On day 3 post-transplantation, the transplant mice were sacrificed and heart grafts were taken for histopathological and gene expression analyses.

Adenovirus infection

H9C2 cells (80, 000 cells/well) were plated in a 6-well plate and cultured overnight and then infected with a human clusterin cDNA expression adenovirus (SignaGen Laboratories, Rockville, MD, USA) at a multiplicity of infection of 100 in 600 μL FBS-free DMEM for 6 h. Then, 600 μL culture medium containing 20% FBS was added to the infected cells and the cells were cultured overnight.

In vitro ischemia reperfusion model

H9C2 cells or H9C2/clusterin cDNA cells were plated in a 6-well plate (80, 000 cells/well) and cultured in DMEM supplemented with 10% FBS and 100 U/mL penicillin and streptomycin overnight. The medium was replaced with deoxygenized phosphate-buffered saline (PBS) and then placed in an Invivo$_2$ Hypoxia Workstation (Baker Ruskinn, Sanford, MA, USA) with 0% O_2 at 10°C for 16 h. After 24 h of hypoxia treatment, PBS was removed and new complete culture medium was added to the cells. The cells were moved to a normal culture environment with 5% CO_2 and 28% O_2 at 37°C for 24 h.

Western blot assay

Proteins were isolated from total cells using 14, 000 × *g* centrifugation for 10 min at 4°C and the supernatant was collected; nuclear fractions were isolated using a Nuclear/Cytosol Fractionation Kit (BioVision, Inc., Mountain View, CA, USA). Proteins quantified using a Lowry assay were separated by 10% SDS-PAGE, transferred onto a nitrocellulose membrane, and blocked overnight in blocking solution (10% of 10× TBS pH 7.6, 0.1% Tween 20, and 5% w/v of nonfat dry milk). The membrane was then incubated with a 1:200 dilution of rabbit polyclonal anti-NF-κB/p65, anti-Bax, anti-Bcl-xL, or anti-clusterin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, Shanghai, China) and 10 μL rabbit polyclonal anti-β-actin antibody (Abcam, Shanghai, China) as a loading control, in blocking solution for 1 h at room temperature. The nitrocellulose membrane was then incubated with a 1:1000 dilution of HRP-conjugated anti-rabbit secondary antibody in blocking solution for 1 h at room temperature. Proteins were detected by chemiluminescence and autoradiography. Band density was measured using a ChemiDoc XRES (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed using Quantity One software (Bio-Rad).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted using a TRIzol® Plus RNA Purification Kit (Invitrogen, Guangzhou, China). The concentration of RNA was measured by an ND2000 spectrophotometer (Thermo Scientific, MA, USA). First-strand cDNA synthesis was performed using 2.5 μg total RNA with Invitrogen SuperScript Reverse Transcriptase (Thermo Fisher Scientific, Shanghai, China). cDNA was amplified in a 25-μL PCR mixture containing 1 μL

deoxynucleotide triphosphates, 1× PCR buffer, 2.5 mM $MgCl_2$, and 1 U DNA Taq polymerase (Promega, Madison, WI, USA) with 25 pmol of primers specific for clusterin (Integrated DNA Technologies, Shanghai, China), Bcl-xL (sense: 5′-TATTGGTGAGTCGGATTGCA-3′, antisense: 5′-GCTCTCGGGTGCTGTATTGT-3′), Bax (sense: 5′- AGGCCTCCTCTCCTACTTCG-3′, antisense: 5′-AAATGCCTTTCCCCtTTCCCC-3′), IL-1β (sense: 5′-GTCTTCCGCCTCTCGGTAAT-3′, antisense: 5′-AGAGATACGGATCGCACAGG-3′), or TNF-α (sense: 5′-CTTTTGGAGTTTGAGGTAGTATACCTA-3′, antisense: 5′-GCTGCGCAGAATGAGATGAGTTGTC-3′). PCR was performed according to the manufacturer's protocol for Applied Biosystems Power SYBR® Green PCR Master Mix and RT-PCR as follows: enzyme activation step: 10-min hold at 95°C for AmpliTaq Gold® enzyme activation, followed by 35 cycles of PCR (15 s at 95°C and 60 s at 60°C). GAPDH was used as an internal control (sense: 5′-TCACAGCGGCCCTCCTGACACCTA-3′, antisense: 5′-GCGAGTATACTCCTTCTCGATCCT-3′). GAPDH PCR conditions consisted of 17 cycles (94°C for 30 s, 52°C for 30 s, and 72°C for 1 min). After cycling, the samples were incubated at 72°C for 10 min. The reproducibility of the quantitative measurements was evaluated by three independent cDNA syntheses and PCR amplification from each preparation of mRNA. The relative mRNA expression level was determined as a ratio of the signal intensity to that of GAPDH.

Quantitative PCR (qPCR)

qPCR was conducted in a 10 μL PCR with 1× SYBR Green mixture (Bio-Rad), 100 nM primers, and 1 μL cDNA, with the following thermal profiling: an initial activation step at 95°C for 2 min, followed by 35 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 20 s. Expression levels between I/R and non-I/R were quantitatively compared using the ΔΔCt method with GAPDH as the endogenous control for mRNA expression.

Tissue preparation for TNF-α and IL-1β measurements

The basal side of heart tissue was rinsed with ice-cold saline to remove any red blood cells or clots and then homogenized with a high-intensity ultrasonic liquid processor in 1:10 (w/v) PBS that contained 1% Triton X-100 and a protease inhibitor cocktail. The homogenate was centrifuged at 2, 500 × *g* for 20 min at 4°C. The supernatant was collected for determination of TNF-α and IL-1β according to the manufacturer's instructions using enzyme-linked immunosorbent assay (ELISA) kits (RayBiotech, Inc., Norcross, GA, USA).

Electrophoretic mobility shift assay (EMSA)

Nuclear and cytosolic proteins were prepared from cells and tissues [16]. The oligonucleotides for NF-kB (5′-AGTTGAGGGGACTTTCCCAGGCC-3′) were synthesized by Applied Biosystems, and annealed oligonucleotides were labeled with [γ-^{32}P] ATP (3000 Ci/mmol; Shanghai, China) using T4 polynucleotide kinase. Commercially available antibodies for p65 RelA (sc-372) were used for EMSA. EMSAs were carried out utilizing an infrared dye 700 labeled oligonucleotide (IR oligo) consisting of the NF-kB consensus binding sequence. The binding reaction consisted of 1× binding buffer (10 mM Tris, 50 mM KCl, 1 mM DTT, pH 7.5), sterile water, 1 μg poly(dI-dC) (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in 10 mM Tris and 1 mM EDTA pH 7.5, 2.5 mM DTT/0.25% Tween 20, and 1 μL NF-kB IR oligo. Five micrograms of nuclear protein was added to the mixture and incubated at room temperature for 30 min in darkness. For supershift analysis, the above mixture was incubated with 5 μg anti-NF-kB p50 (SC-7178X; Santa Cruz) or anti-p65 antibody (SC-109X; Santa Cruz) for an additional 30 min. The nuclear protein-DNA complexes were separated in native 4% polyacrylamide gels and scanned in an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

Blood sampling for measurement of plasma cardiac troponin I (cTnI)

At the end of the experiment, approximately 2 mL blood was collected from the heart. The blood sample was placed in a tube containing disodium EDTA (22 mg/mL) as an anticoagulant and mixed thoroughly then centrifuged at 3000 rpm for 15 min. The supernatant was used to determine plasma cTnI according to the manufacturer's instructions using an ELISA kit (Life Diagnostics, Fort Lauderdale, FL, USA).

Histological analysis

Heart grafts were collected from mice and tissue slices and harvested into 4% formalin before being processed for paraffin wax embedding. Once embedded, tissue sections were cut at 2 μm using a sledge microtome (Leitz, Wetzlar, Germany) and sections were stained with hematoxylin and eosin (H&E).

H&E slides were prepared and morphological assessment of cardiac injury was performed using a semi-quantitative scale of 0 to 4 (4 being the most severe) [17]. Hearts were assigned a score of 5 if they failed to function immediately. In functioning cardiac grafts, the myocardium was assessed for the following: myocytolysis (dissolution of myocytes), myocardial necrosis, and inflammatory infiltrate. The degree of injury was measured by the extent of myocardium involved in the biventricular section: 1, <10%; 2, ≥10% and ≤30%; 3, ≥30% and <60%; and 4, ≥60%.

Apoptosis in I/R injured hearts in vivo and cells in vitro

A fluorescein *in situ* cell death detection kit was used according to the manufacturer's instructions for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (Roche Applied Science, Mannheim, Germany). Positive cells were counted in 10 non-overlapping fields of the outer medulla in each sample (magnification, ×200). Caspase-3 activity in the heart was measured using a Caspase-3 Colorimetric Assay Kit, following the manufacturer's protocol (Beyotime, Shanghai, China) [18].

Flow cytometric analysis

Cell apoptosis was determined using a FITC Annexin V Apoptosis Kit (BD Pharmingen, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. In brief, the cells were washed with ice-cold PBS and resuspended in binding buffer (10 mmol/L HEPES, pH 7.4, 140 mmol/L NaCl, and 2.5 mmol/L $CaCl_2$) at a concentration of 1.0×10^6 cells/mL. The cells were stained with annexin V-FITC and propidium (PI) for 15 min in the dark before analysis with a flow cytometer (Beckman Coulter, Inc., Miami, FL, USA).

Statistical analysis

Results are presented as the mean ± standard error of the mean. Statistical analysis was performed by a two-tailed t-test or Mann-Whitney test using GraphPad Prism 4 software. Differences associated with probability values of $p < 0.05$ were considered statistically significant.

Results

Clusterin protects the heart from I/R injury in heart transplantation

We excised donor hearts and preserved donor organs with a chilled Euro-Collins solution and/or hr clusterin solution for 24 h, followed by syngeneic heterotopic heart transplantation. At 24 h later, the implanted hearts were harvested for protein expression by western blot analysis. The results showed that 24 h cold I/R increased the expression of clusterin by 1.44 ± 0.18-fold compared with that in the control grafts without I/R injury; however, cold hr clusterin/I/R increased the expression of clusterin by 5.7 ± 0.48-fold compared with that in the control grafts without I/R injury (Fig. 1A). Perfusion of the heart with hr clusterin resulted in a significant increase in clusterin levels.

We next examined whether hr clusterin induces cardioprotection during cold I/R injury. We observed that transplanted hearts from wild-type mice did not start to beat after implantation,

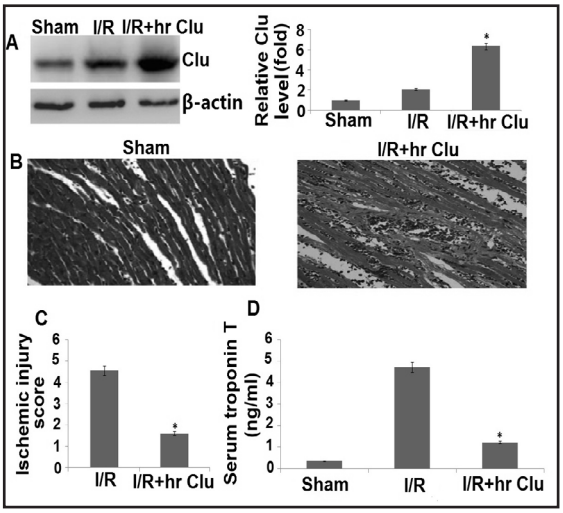

Fig. 1. Clusterin protects the heart from I/R injury in heart transplantation. Preserved donor organs with a chilled Euro-Collins solution and/or hr clusterin solution for 24 h, followed by syngeneic heterotopic heart transplantation. A, Clusterin protein expression in heart tissue was detected by western blot assay. B, H&E staining. At 3 days after transplantation, heart grafts were harvested for H&E staining. C, The ischemic scores in both groups. D, Serum TnT was measured in sham, I/R, and I/R+hr clusterin treated recipients at 72 h post-transplantation versus control. *p<0.01.

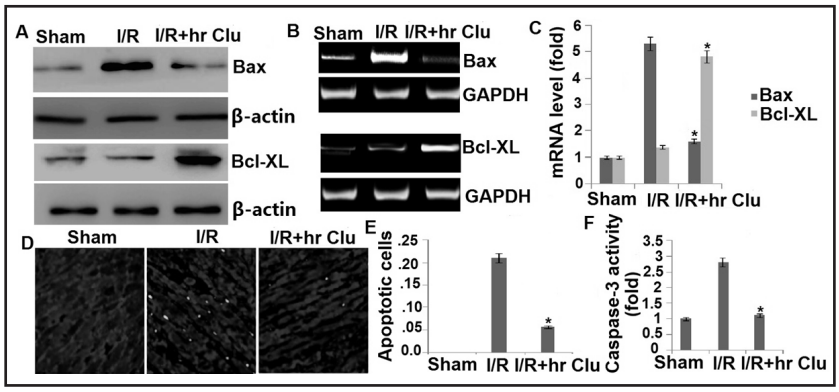

Fig. 2. Clusterin decreases the expression of pro- and anti-apoptotic genes and reduces apoptotic cells in heart grafts. A, Expression of Bax and Bcl-xL was detected by western blot assay. B, The expression of Bax and Bcl-xL was determined using RT-PCR. C, The expression of Bax and Bcl-xL was determined using qRT-PCR. D, Cell apoptosis was detected by TUNEL assay. Caspase-3 activity in heart tissue was detected with a Caspase-3 Colorimetric Assay Kit, quantitatively estimated as fold-change relative to the I/R group. Versus I/R, $^{*}p<0.01$.

while the hearts from hr clusterin mice began to beat immediately once they were revascularized and continued to beat strongly at a steady speed. At 3 days after transplantation, we harvested heart grafts to assess I/R injury by examining histopathological changes with H&E staining. Fig. 1B shows the histopathological changes in heart grafts including cold I/R and cold I/R+hr clusterin. The ischemia score was significantly higher in the cold I/R groups than in the cold I/R+hr clusterin groups, indicating that hr clusterin provided protection against heart I/R injury (Fig. 1C, $p < 0.01$). We detected cardiac troponin T (TnT) production, and found that at 72 h post-transplantation, TnT production was reduced by hr clusterin+cold I/R in comparison with the cold I/R alone group (Fig. 1D, $p < 0.01$).

Clusterin inhibits cell apoptosis and reduces the expression of pro-apoptotic proteins in vivo

The induction of cell apoptosis is one of the mechanisms by which I/R causes organ damage. We found that the pro-apoptotic gene Bax was over-expressed, while the anti-apoptotic gene Bcl-xL was decreased in I/R injury heart grafts compared with that in non-I/R injured tissues. This change in gene expression was attenuated by hr clusterin treatment according to western blot (Fig. 2A), RT-PCR (Fig. 2B), and qualitative RT-PCR (qRT-PCR) analyses (Fig. 2C).

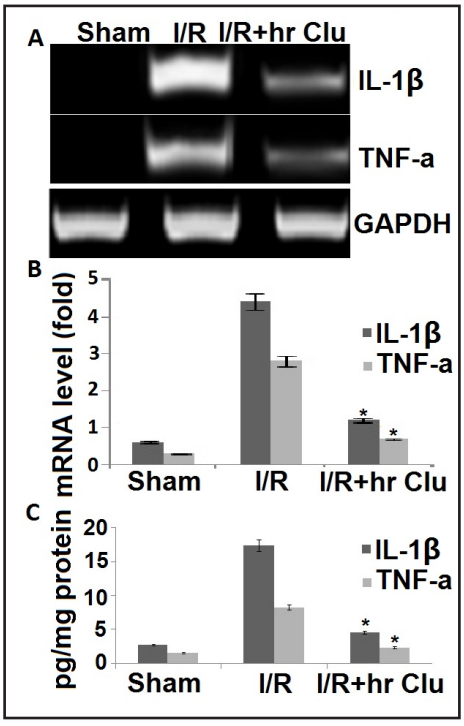

Fig. 3. Inflammatory cytokine expression. A, Expression of TNF-α and IL-1β in I/R injured hearts was detected by RT-PCR. B, Expression of TNF-α and IL-1β was determined using qRT-PCR. C, TNF-α and IL-1β in I/R injured hearts was detected by ELISA. versus I/R, $^{*}p<0.01$.

The representative photographs in Fig. 2D show that TUNEL-positive cardiomyocytes were observed more frequently in the wild-type heart graft group compared with those in the hr clusterin group. As an executioner caspase, caspase-3 is activated in apoptotic cells by both extrinsic (death ligand) and intrinsic (mitochondrial) pathways. The caspase-3 zymogen exhibits virtually no activity until it is cleaved by an initiator caspase following apoptotic signaling events. As shown in Fig. 2E, caspase-3 activity was significantly reduced in the hr

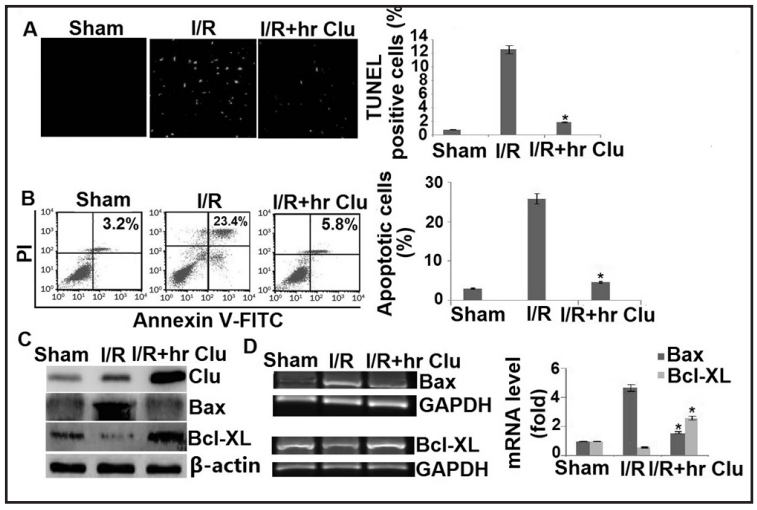

Fig. 4. Clusterin prevents cell apoptosis and death induced by I/R injury in vitro. H9C2 cells (80,000 cells/well) were plated in 6-well plates and cultured overnight at 37°C, 5% CO_2. The cells were infected with a human clusterin expression adenovirus or control null virus for 24 h. The cells were then subjected to a hypoxia chamber with 0% O_2 and 15% CO_2 at 10°C for 16 h, followed by 24 h reperfusion at 5% CO_2 and 28% O_2 at 37°C. A, Cell apoptosis was detected by TUNEL staining. B, Cell apoptosis was detected by double staining with FITC-labeled annexin-V and PI and flow cytometry. C, Protein expression of clusterin, Bax, and Bcl-xL was detected by western blot assay. D, Gene expression of clusterin, Bax, and Bcl-xL was detected by RT-PCR. E, Gene expression of clusterin, Bax, and Bcl-xL was detected by qRT-PCR. Data are the summary of three independent experiments. Versus I/R, *p<0.01.

clusterin group compared with that in the wild-type heart graft group.

Clusterin reduces proinflammatory cytokine production in heart grafts in vivo

In response to I/R injury, myocardial cells reportedly up-regulate the expression of pro-inflammatory cytokines, such as TNF-α and IL-1β in I/R injured hearts. To investigate whether inflammatory cytokines are involved in TNF-α and IL-1β expression in I/R injured hearts, we first assessed the mRNA levels of TNF-α and IL-1β in I/R injured hearts by RT-PCR and qRT-PCR. In the hr clusterin groups, the levels of TNF-α and IL-1β were significantly decreased compared with those in the I/R groups by RT-PCR (Fig. 3A) and qRT-PCR (Fig. 3B). The protein levels of TNF-α and IL-1β were also significantly decreased in the hr clusterin groups compared with those in the I/R groups by ELISA (Fig. 3C).

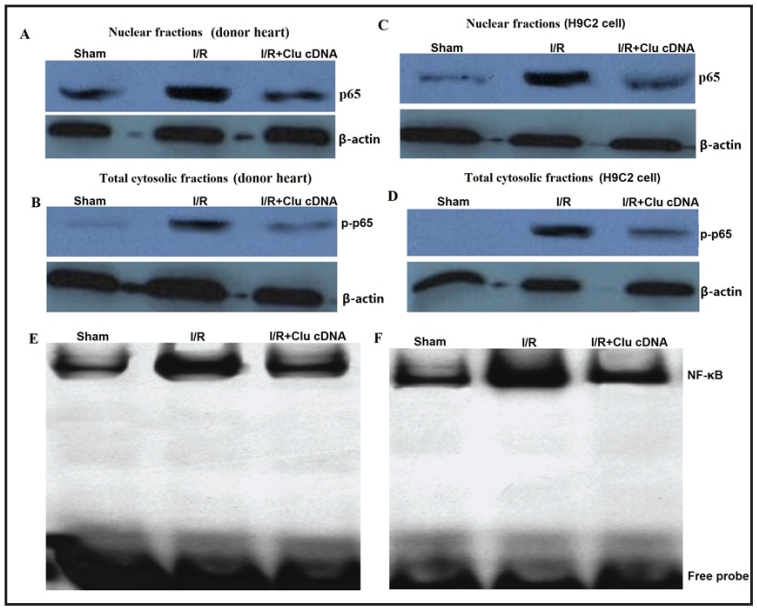

Fig. 5. Expression of p65. The cells were treated and protein was extracted from the cells as in Fig. 4. A, The expression of p65 was detected by western blotting in heart grafts in vivo. B, The expression of p65 was detected by western blotting in H9C2 cells induced by I/R in vitro. C, NF-κB activity was detected by EMSA in heart grafts in vivo. D, NF-κB activity was detected by EMSA in H9C2 cells induced by I/R in vitro. Versus I/R, *p<0.01.

Clusterin protects cardiomyocytes from cell apoptosis induced by I/R injury in vitro

To verify further that the effect of clusterin on cell death induced by cold I/R injury is protective, not causative, we cultured rat H9C2 cells, the most commonly used heart cell line for *in vitro* studies of I/R [19]. H9C2 cells were transfected with a clusterin expression adenovirus prior to exposure to 16 h cold hypoxia at 10°C followed by 24 h reperfusion at 37°C *in vitro*. Apoptosis in H9C2 cells was assessed by TUNEL staining (Fig. 4A) and annexin V-PI double staining (Fig. 4B). We confirmed that I/R induced cell apoptosis and it was significantly inhibited by clusterin transfection. We also confirmed that clusterin and Bax expression was up-regulated at both the mRNA and protein levels in clusterin adenovirus-infected cells, and Bcl-xL was down-regulated at both the mRNA and protein levels in clusterin adenovirus-infected cells (Fig. 4C–4E).

Clusterin protects heart cells from I/R injury through the NF-κB signaling pathway

As shown in Fig. 5, I/R injury increased NF-κB/p65 (p65) translocation by western blot assay (Fig. 5A) and increased NF-κB activity by EMSA (Fig. 5B), whereas the expression of p65 in the hr clusterin I/R injured grafts was significantly decreased compared with that in the wild-type I/R injured grafts and almost recovered to the levels seen in hearts without I/R injury. These data indicate that clusterin inhibits p65 nuclear translocation.

As shown in Fig. 5C–5D, we found that *in vitro* cold hypoxia/reperfusion also increased p65 translocation and NF-κB activity as compared with control cells maintained in normal culture conditions, and that transfection with a clusterin expression adenovirus (clusterin cDNA) decreased p65 translocation and NF-κB activity.

Discussion

Cardiac transplantation is the last resort for patients with end-stage heart failure; however, I/R injury is a major issue in cardiac transplantation. I/R injury is associated with increased primary organ dysfunction and subsequent delayed organ function after cardiac transplantation. In the long term, this correlates with increased episodes of acute and chronic rejection. Thus, the development of more effective drugs or interventions to protect the myocardium from reperfusion injury is required to provide greater clinical benefits for patients with ischemic heart disease.

Clusterin, also known as testosterone-repressed message-2, is overexpressed in the rat prostate during castration-induced programmed cell death [20]. As clusterin is usually present in resistant cells [21, 22], it has been described as an anti-apoptotic factor. In cultures of renal tubular epithelial cells, downregulation of clusterin expression results in an increase of proinflammatory cytokine-induced cell apoptosis. In a mouse model of renal I/R injury, the overexpression of clusterin is associated with a reduction of renal I/R injury and dysfunction of the kidneys and also contributes to the promotion of renal repair [23]. These results suggest that the local expression of clusterin may protect renal cells from cell death during kidney transplant rejection.

Although clusterin has long been proposed to participate in cell survival and it has been studied extensively to inhibit the pro-inflammatory cytokine TNF-α, no studies have been carried out to investigate the link between clusterin and survival signaling pathways. Clusterin expression renders donor hearts resistance to cold I/R injury in transplantation, suggesting that upregulation of clusterin expression in donor hearts may have the potential to protect heart grafts from cold I/R injury [24, 25]. In this study, we found that the expression of pro-inflammatory cytokines (IL-1β and TNF-α) was impeded by hr clusterin treatment in a cold I/R model. Furthermore, we observed that hr clusterin inhibited p65, a member of the NF-κB family, and NF-κB activity. Our data suggest that the attenuation of inflammation by clusterin is mediated by inhibition of the NF-κB signaling pathway. Our study shows a new circumstance in which clusterin protects against inflammation, and further supports a role

for clusterin as a cardio-protective agent against inflammation under ischemic conditions.

Apoptosis is a mechanism of programmed cell death. This process usually involves an increased ratio of pro-apoptotic (*i.e.*, Bax and Bim) *versus* anti-apoptotic (*i.e.*, Bcl-2 and Bcl-xL) molecules. NF-κB antagonizes apoptosis and promotes cell survival by inducing the expression of pro-survival genes (Bcl-2, Bcl-xL, and IEX1) and by repressing the pro-apoptotic genes Bax and Bim [26, 27]. Zhang *et al* [28]. reported the I/R-induced up-regulation of the pro-apoptotic protein and mRNA levels of Bax, Cyto-c, Apaf-1, and caspase-9/3, while it increased the ischemia/reperfusion-induced decrease of the anti-apoptotic factor Bcl-2. Clusterin is a stress-associated cytoprotective chaperone expressed in many cancers that is upregulated in an adaptive cell survival manner by various apoptotic triggers, such as Bax and Bcl-2/Bcl-xL and confers treatment resistance [29, 30].

In this study, we found that the number of apoptotic cells was decreased in hr clusterin-treated grafts, as detected by TUNEL assays. The expression of the apoptotic gene Bax was reduced and the anti-apoptotic gene Bcl-xL was increased in heart grafts with overexpression of clusterin compared with those in WT grafts. Additionally, our *in vitro* results with H9C2 cells showed that hr clusterin treatment reduced H9C2 cell apoptosis under cold hypoxia/reperfusion stress by downregulating Bax and upregulating Bcl-xL. Therefore, clusterin is an anti-apoptotic protein that protects heart cells against apoptosis induced by cold I/R injury in a heart transplant model. The ability of clusterin to prevent cell apoptosis suggests that it has a potential therapeutic value in preventing I/R injury in heart transplantation.

In conclusion, this study is the first to demonstrate that clusterin is a promising target to prevent cold I/R injury in heart transplantation and to show the association of clusterin with Bax. We also demonstrated that the protective effect of clusterin is mediated by Bax and NF-κB signaling.

Acknowledgements

Funding: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Disclosure Statement

No Disclosure Statements exists.

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