Original Paper

Necroptosis Induced by Ad-HGF Activates Endogenous C-Kit⁺ Cardiac Stem Cells and Promotes Cardiomyocyte Proliferation and Angiogenesis in the Infarcted Aged Heart

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Key Words

Ad-HGF • Necroptosis • c-kit⁺ cardiac stem cell • Myocardial infarction

Abstract

Background/Aims: The discovery of c-kit⁺ cardiac stem cells (CSCs) provided us with new therapeutic targets to repair the damaged heart. However, the precise mechanisms regulating CSC proliferation and differentiation in the aged heart remained elusive. Necroptosis, a type of regulated cell death, has recently been shown to occur following myocardial infarction (MI); however, its effect on c-kit⁺ CSCs remains unknown. We investigated the effects of hepatocyte growth factor (HGF) and necroptosis on the proliferation and differentiation of endogenous c-kit⁺ CSCs in aged rat hearts following MI. **Methods:** The c-kit⁺ CSCs and HGF/p-Met expression levels in neonatal, adult and aged rats were compared using immunofluorescence and Western blotting. Immediately after MI, adenovirus carrying the HGF gene (Ad-HGF) was injected into the left ventricular wall surrounding the infarct areas of the aged rat heart. The proliferation and differentiation of the endogenous c-kit⁺ CSCs were studied using immunofluorescence. The signalling pathways were analysed via Western blotting and ELISA. **Results:** HGF/p-Met expression levels and c-kit⁺ CSC abundance gradually decreased with age. Ad-HGF promoted c-kit⁺ CSC differentiation into precursor cells of cardiomyocyte, endothelial and smooth muscle cell lineages and enhanced cardiomyocyte proliferation and angiogenesis in aged rats; these effects were reversed by the inhibition of necroptosis. Ad-HGF administration induced necroptosis by increasing the expression of receptor interacting protein kinase (RIP) 1 and receptor interacting protein kinase (RIP) 3 proteins in the infarcted heart. Moreover, Ad-HGF-induced necroptosis increased high-mobility group box 1 protein (HMGB1) levels and enhanced the abundance of c-kit⁺ cells in the bone marrow, which may partly account for the beneficial effect of necroptosis on the c-kit⁺ CSCs. **Conclusion:** Ad-HGF-induced necroptosis facilitated aged heart repair after MI by promoting c-kit⁺ CSC proliferation and differentiation. These findings may lead to the development of new methods for the treatment of ischaemic heart disease in aged populations.

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Introduction

The loss of cardiomyocytes following myocardial infarction (MI) results in myocardial fibrosis, myocardial stiffness and cardiac systolic and diastolic function disorder and culminates in heart failure and death. Myocardial cells are considered non-renewable and terminally differentiated [1, 2]. Although cardiac regeneration occurs in neonatal mice in response to injury, this potential is lost after the first week of life [3]. It has been reported that the number of c-kit⁺ cardiac stem cells (CSCs) is significantly higher in the neonatal heart than in the adult heart [4]. In addition, these cells are particularly interesting due to their quantity and capacity to differentiate into myocardium [5, 6]. However, the isolation and expansion of the endogenous CSCs used for autologous cell transplantation is a slow and expensive process that yields cells of uneven quality [7, 8]. Therefore, promoting endogenous CSC proliferation and differentiation has become a new research area of interest. Clinically, myocardial infarction occurs primarily in aged individuals [9]. Therefore, studies of myocardial regeneration after MI in the elderly are urgently needed. However, c-kit⁺ CSC proliferation and differentiation in the aged heart remain poorly understood.

Since apoptosis was regarded as the only form of regulated cell death for decades, earlier investigations on cardiomyocyte cell death mainly studied this process. However, recent studies have revealed that necroptosis, another pathway of regulated cell death, occurs in various diseases, including MI [10]. Necroptosis is a well-characterized form of regulated necrosis that depends on the activation of necrosome, a protein complex in which receptor interacting protein kinase 1 (RIP1) and RIP3 are activated [11]. This mode of cell death, characterized by disrupted plasma membranes, has been observed in a variety of physiological and pathological processes, including mammalian development and tissue damage [12]. From an evolutionary perspective, necroptosis is believed to contribute to innate immunity in humans and mice by alerting the immune system through the release of danger signals [13]. However, its role in endogenous c-kit⁺ CSC proliferation and differentiation remains unknown.

Hepatocyte growth factor (HGF) is a secreted cytokine whose plasma level differs with age, gender, pregnancy and disease state. An elevated HGF level is observed at the onset of MI symptoms (including chest pain <60 minutes), peaking on the first day of MI and eventually declining after twenty-four hours and returning to normal three days later. The HGF level in patients with MI is ten-fold above that in a healthy control population [14]. The HGF receptor C-Met is expressed in a variety of cells, including cardiomyocytes, vascular smooth muscle cells and endothelial cells. Studies have found that c-kit⁺ CSCs also express c-Met [8, 15]. By binding to c-Met and leading to the phosphorylation of the receptor, HGF plays key roles in a variety of biological processes, including but not limited to, morphogenesis, inhibition of apoptosis and myocardial protection [16]. However, it remains unclear whether and how HGF promotes endogenous c-kit⁺ CSC proliferation and differentiation in the aged heart following MI.

The present study aimed to investigate the effects of HGF and necroptosis on endogenous c-kit⁺ CSC proliferation and differentiation in aged rats. Our findings may lead to the development of new strategies for treating ischaemic heart disease in the elderly.

Materials and Methods

Animals and Reagents

Sprague-Dawley (SD) rats (male, aged 2–3 days, 6–10 months and 18–22 months) were purchased from Qinglong Shan animal breeding farm (Nanjing, China). Necrostatin-1 (Nec-1) and propidium iodide (PI) were purchased from Sigma (USA). Anti-HGF antibody (ab83760), anti-c-kit antibody (ab25022), and anti-RIP1 antibody (ab72139) were purchased from Abcam; RIP3 antibody (R4277) was purchased from Sigma; Phospho-Met (Tyr1234/1235) antibody (#3077) and HMGB1 (D3E5) antibody (#6893) were obtained from Cell Signaling (USA); c-kit antibody (sc-5535), Troponin I antibody (sc-133117), CD45 antibody (sc-53047), GATA-6 antibody (sc-9055), Nkx-2.5 antibody (sc-376565) and Ets-1 antibody (sc-55581) were

purchased from Santa Cruz Biotechnology. Fluorescein, CY3 and rhodamine-labelled secondary antibodies were purchased from Jackson ImmunoResearch (USA). The replication-deficient adenovirus carrying the HGF gene (Ad-HGF) was constructed according to reported methods [17, 18].

Rat model of MI and in vivo injection of Ad-HGF

All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH publication No. 85-23, revised 1996) and were approved by the Animal Care and Use Committee of Nanjing Medical University. Briefly, rats aged 18–22 months were anaesthetized by intraperitoneal administration of pentobarbital sodium (50 mg/kg). Following the anaesthesia, rats were placed on a heating pad to maintain normothermia (approximately 35°C) and were immobilized in the supine position. The rats were subsequently intubated and ventilated such that a left thoracotomy could be performed to expose the heart. Electrocardiogram, heart rate and respiratory rate were continuously monitored. MI was induced by permanent ligation of the left anterior descending (LAD) coronary artery using a 6-0 polypropylene suture placed approximately 2 mm from the inferior margin of the left auricle. Ischaemia was confirmed by myocardial blanching and electrocardiogram ST-segment elevation. A total of 50 µL of the Ad-HGF (1.0×10^9 PFU) or Ad-null (1.0×10^9 PFU) was injected into the myocardium in the vicinity of the ischaemic region (pale area) of the left ventricular wall immediately following the ligation. Sham operations were performed using the same method, but without tying the suture on the LAD coronary artery. Rats were given either phosphate-buffered saline (PBS) or 3.5 mg/kg Necrostatin-1 dissolved in PBS via a tail vein injection 5 minutes before MI and once daily for 7 days after MI. The rats were subsequently given an intramuscular injection of penicillin and subcutaneous injection of analgesic. After the restoration of spontaneous breathing, rats were placed on an electric blanket until they revived. The animals were sacrificed using carbon dioxide (CO₂) on days 7 and 28.

Immunohistochemistry

For immunohistochemistry, the hearts were harvested, fixed using 4% paraformaldehyde, and embedded in paraffin. The sections were stained with CD31 and Masson's trichrome to identify the vessel density and collagen content. The staining was performed according to the manufacturer's instructions. For the quantification of vessel densities and collagen content in the myocardium, three equivalent sections from each group were randomly selected, and six visual fields from each section were observed. Sections were visualized and photographed under a microscope (Nikon, Tokyo, Japan). Two investigators blind to the experimental groups counted the CD31⁺ microvessels. The collagen content in the myocardium was analysed by computerized planimetry (Image J, version 1.44, NIH, Bethesda, MD).

Immunofluorescence

The hearts were embedded in OCT (Sakura, Japan) and cut into 8-µm sections. Then, the sections were fixed using cold acetone at -20°C and placed in a fume hood to ventilate for 20 minutes. The slides were gently rinsed twice with PBS, permeabilized with 0.1% Triton X-100 for 10 minutes, rinsed again twice with PBS and blocked using 2% bovine serum albumin (BSA) for 30 minutes. The sections were then incubated at 4°C overnight with the following antibodies diluted in the blocking buffer: c-kit (1/200), Ki67 (1/400), p-Met (1/200), cTnI (1/200), CD45 (1/100), Gata6 (1/200), Ets-1 (1/200) and Nkx2.5 (1/200). Then, the sections were incubated for 1 hour in dark with the secondary antibodies diluted in the same blocking buffer (green, 1/200 dilution of fluorescein; red, 1/400 dilution of CY3 or rhodamine-labelled secondary antibodies), followed by three washes with PBS. The sections were then mounted with VECTASHIELD mounting medium containing diamidino-2-phenylindole (DAPI), (Vector Laboratories, Burlingame, CA, USA), and images were acquired using an LSM 5 Live DuoScan Laser Scanning Microscope (Zeiss, Oberkochen, Germany).

Echocardiography

Cardiac function was analysed using a high-frequency ultrasound system Vevo2100 (VisuaSonic Inc., Toronto, ON, Canada) with a 30 MHz central frequency scan head. Before and at 4 weeks following MI, anaesthesia was performed with 1–2% isoflurane vapour. Echocardiographic images in short and long axes were acquired on the condition of monitoring respiration rate, heart rate and electrocardiogram by 2 experienced and independent operators blind to the experiments. The left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were calculated using estimated parameters, including the left ventricular end-systolic diameter (LVESd), left ventricular end-diastolic diameter (LVEDd),

left ventricular posterior wall end-systolic thickness (LVPWTs), left ventricular posterior wall end-diastolic thickness (LVPWTd), interventricular septum end-systolic thickness (IVSTs) and interventricular septum end-diastolic thickness (IVSTd). For all parameters, values from three measurements were averaged.

TUNEL assay

The TUNEL Cell Death Detection kit (Cat. No.11684795910, Sigma, USA) for in situ apoptosis detection was used for labelling the DNA strand breaks; the terminal deoxynucleotidyl transferase (TdT) catalyses polymerization of labelled nucleotides to free 3'-OH DNA ends in a template-independent manner (TUNEL reaction). The sections of the heart samples were treated according to the manufacturer's instructions and co-stained with cardiac troponin I antibody and DAPI. A fluorescence microscope (Nikon, Tokyo, Japan) was used to visualize the TUNEL-positive cardiomyocytes, and Image J (NIH) software was used to analyse the images. Values presented are the averages of the measurements from five different fields.

ELISA

For ELISAs, rat serum was isolated from angular vein blood before and 7 days after surgery. For this purpose, the blood samples were allowed to clot for 2 hours at room temperature, and the serum was removed and stored at -80°C for later use. A rat high mobility group box 1 protein (HMGB1) ELISA kit (Catalogue Number: MBS729203, MyBioSource, USA) was used to measure the HMGB1 protein levels in the rat sera according to the manufacturer's instructions.

Western blot

The Western blotting analysis was performed according to reported protocols [19].

Statistical analysis

Data from at least three independent experiments were used to calculate the means±standard deviations (SD) using SPSS 18.0 statistical software. Statistical analysis was performed employing one-way analysis of variance (ANOVA) with the Student-Newman-Keuls post hoc test for multiple group comparisons. P<0.05 was considered statistically significant.

Results

HGF/p-Met expression levels positively correlated with c-kit⁺ CSCs in rats of three different age groups

We first evaluated the HGF/p-Met expression levels in c-kit⁺ CSCs in the myocardial tissue in the different age groups. Immunofluorescence studies revealed that the cardiac tissue of neonatal rats aged 2–3 days had significantly higher levels of c-kit⁺ CSCs than that of adult rats aged 6–10 months (9.9±1.1% vs. 1.6±0.4%). Ki67 is commonly used to label proliferating cells. The c-kit and ki67 co-staining results revealed a greater abundance of proliferating c-kit⁺ cells in the neonatal cardiac tissue than in the tissue of adult rats (5.1±0.9% vs. 1.1±0.4%). Cardiac troponin I (cTnI) is a molecular marker of cardiomyocytes. The cTnI and ki67 co-staining results revealed that the number of proliferating cardiomyocytes in the heart tissues of neonatal rats was significantly higher than the number in the hearts of adult rats (4.1±0.7% vs. 0.8±0.2%) (Fig. 1A). Further, Western blot revealed that HGF, p-Met and c-kit expression levels in the cardiac tissues gradually decreased from the perinatal stage (2–3 days) to the adult (6–10 months) and aged (18–22 months) stages (Fig. 1B). Notably, a positive correlation between c-kit and HGF/p-Met expression levels was observed with age (Fig. 1C). However, whether exogenous HGF expression in aged rats following MI could promote the proliferation and differentiation of c-kit⁺ CSCs required further characterization.

Ad-HGF administration increased the number of c-kit⁺ CSCs on day 7 in aged rat hearts following MI

We investigated whether Ad-HGF treatment was able to activate endogenous c-kit⁺ CSCs in aged rats following MI. For this purpose, Ad-HGF and Ad-null vectors were injected into three myocardial sites near the ischaemic area immediately after ligation of the LAD coronary artery. One week after the injection, HGF and p-Met expression levels were analysed

Fig. 1. Abundance of c-kit⁺ CSCs and HGF/p-Met expression in myocardial tissues at different ages. (A) Immunofluorescence of frozen cardiac sections from neonatal and adult SD rats [c-kit (green)/DAPI (blue), c-kit (green)/Ki67 (red) /DAPI (blue) and Ki67 (red)/c-TnI (green)/DAPI (blue) staining]. Bar=40 μm, n=4. (B) HGF, p-Met and c-kit protein expression levels in myocardial tissue at various time points (2–3 days, 6–10 months and 18–22 months) in SD rats. Beta-actin served as the loading control. (C) Line chart showing HGF and c-kit expression levels at various time points; the correlation coefficient was 0.97. Data are expressed as the means±SD, *P<0.05, n=4.

Fig. 2. Ad-HGF administration increased the number of c-kit⁺ CSCs on the 7th day in the hearts of aged rats following MI. (A) Representative Western blots demonstrating HGF and p-Met expression in the indicated groups. The bar graph shows the quantitative analysis of the protein expression levels. (B) Representative images of c-kit (green) and DAPI (blue) co-staining in the indicated groups. Bar=40 μm. The bar graph shows the statistical analysis of c-kit cells. (C) Representative images of sections of Ad-HGF-treated cardiac tissue stained for c-kit (green), p-Met (red), and DAPI (blue). Bar=40 μm. (D) Representative images of sections of Ad-HGF-treated cardiac tissue stained for c-kit (green), CD45 (red), and DAPI (blue). Bar=40 μm. I, infarct zone; P, peri-infarct zone; N, normal zone. Data are expressed as the means±SD, n=4, *P<0.05.

Fig. 3. Ad-HGF promoted c-kit⁺ CSC proliferation and differentiation and enhanced cardiomyocyte regeneration and angiogenesis in aged rats following MI. (A) Immunofluorescence analysis of Ki67⁺/c-kit⁺ cells in each group is shown. Bar=20 μ m. (B)-(D) Representative images of the c-kit (green) and Nkx2.5 (red) co-staining, c-kit (green) and Ets-1 (red) co-staining, and c-kit (green) and Gata6 (red) co-staining in the indicated groups. Bar=60 μ m. (E) Bar graph showing the quantification of the c-kit/Nkx2.5, c-kit/Ets-1 and c-kit/Gata6 co-stained cells in the indicated groups. (F) Representative images of sections stained with cTnI (green), Ki67 (red), and DAPI (blue) in the indicated groups. Tissues were harvested 4 weeks after MI. Bar=60 μ m. (G) Representative pictures showing CD31⁺ microvessels in the indicated groups. Bar=50 μ m. The bar graph shows the quantification of CD31⁺ microvessels among the three groups. Data are expressed as the means $\pm$ SD, n=4, *P<0.05.

by Western blot using proteins isolated from the infarct, peri-infarct, and normal myocardial tissues. HGF and p-Met expression was detected in all three zones of the myocardium, with the lowest expression levels observed in the unaffected areas. Moreover, the Ad-HGF treatment group showed significantly higher HGF and p-Met expression in the infarct and peri-infarct zones than the Ad-null group (Fig. 2A). Immunofluorescence analysis of the frozen cardiac section was used to identify the c-kit⁺ CSCs. Seven days after acute MI, Ad-HGF-treated infarcted hearts showed more c-kit⁺ cells in the border region ($8.7\pm1.2\%$ vs. $1.8\pm0.5\%$) and even more in the infarcted area ($11.4\pm1.3\%$ vs. $2.6\pm0.8\%$) than the Ad-null-treated hearts (Fig. 2B). A lower abundance of c-kit⁺ cells was noted in the normal zone. Nearly 90% of the c-kit⁺ cells in the Ad-HGF-treated post-MI hearts expressed the p-Met receptor (Fig. 2C). Cardiac mast cells also express c-kit [20]. Therefore, we used c-kit and CD45 co-staining to rule out the cardiac mast cells and to specifically identify the quantity of c-kit⁺ CSCs in the Ad-HGF-treated infarcted hearts. The c-kit⁺/CD45⁻ cells, namely the c-kit⁺ CSCs, amounted to $7.3\pm0.5\%$ of the population in Ad-HGF-treated infarcted hearts (Fig. 2D).

Fig. 4. Ad-HGF induced necroptosis in the hearts of aged rats following MI. (A) Representative Western blots showing RIP1 and RIP3 expression in the indicated groups. The bar graph shows the quantification of these proteins. (B) Immunofluorescence analysis of PI (red), cTnI (green) and DAPI (blue) co-staining in the indicated groups. The bar graph shows the quantitative analysis of the PI-positive cardiomyocytes among the indicated groups. Data are expressed as the means $\pm$ SD, n=4, *P<0.05.

Ad-HGF promoted c-kit⁺ CSC proliferation and differentiation and enhanced cardiomyocyte regeneration and angiogenesis in aged rats following MI

When we used a Ki67-specific antibody to evaluate the number of proliferating c-kit⁺ CSCs, significantly more Ki67⁺/c-kit⁺ CSCs were detected in Ad-HGF-treated cardiac tissue than in tissue from Ad-null-treated hearts ($6.15 \pm 0.8\%$ vs. $1.5 \pm 0.3\%$) and the sham controls ($6.15 \pm 0.8\%$ vs. $0.3 \pm 0.05\%$) (Fig. 3A). Thus, Ad-HGF overexpression induced active c-kit⁺ CSC proliferation in the ischaemic myocardial of aged rats.

Many c-kit⁺ CSCs express the transcription factors Nkx-2.5, Ets-1 or Gata6, suggesting that these cells are committed to myocyte, endothelial and smooth muscle lineages, respectively [8]. Compared to the abundance levels in the Ad-null-treated and sham hearts, respectively, the abundance levels of the committed myogenic (c-kit⁺/Nkx2.5⁺ cells) ($4.7 \pm 0.5\%$ vs. $1.38 \pm 0.1\%$ vs. $0.13 \pm 0.06\%$), endothelial (c-kit⁺/Ets-1⁺ cells) ($3.7 \pm 0.24\%$ vs. $1.36 \pm 0.17\%$ vs. $0.18 \pm 0.06\%$) and smooth muscle (c-kit⁺/Gata6⁺ cells) ($3.8 \pm 0.23\%$ vs. $1.12 \pm 0.2\%$ vs. $0.31 \pm 0.12\%$) progenitors significantly increased in Ad-HGF-treated hearts (Fig. 3B-3E). To further assess the Ad-HGF-induced myocardial regeneration, we determined the quantity of proliferating cardiomyocytes in the heart by Ki67 and cTnI double-staining on day 28 after MI. The number of Ki67⁺ cardiomyocytes was lowest in the sham group, and the numbers increased 4.7-fold following Ad-HGF treatment relative to the number in the Ad-null group (Fig. 3F). To analyse the angiogenesis mediated by Ad-HGF, small blood vessels in the CD31-stained sections were counted by two investigators blinded to the experimental groups. The Ad-HGF treatment group showed significantly more capillaries in the myocardium than the Ad-null (64 ± 7.6 vs. 36 ± 5.9 , P<0.05) and sham (64 ± 7.6 vs. 18 ± 4.7 , P<0.05) groups (Fig. 3G).

Taken together, these results suggested that Ad-HGF overexpression promoted c-kit⁺ CSC proliferation and their differentiation into myocyte, endothelial and smooth muscle lineages, likely further differentiating into cardiomyocytes and capillaries in the ischaemic myocardial tissue of aged rats.

Fig. 5. Inhibition of necroptosis attenuated endogenous c-kit⁺ CSC activation and decreased cardiomyocyte proliferation and angiogenesis induced by Ad-HGF. (A) Representative immunofluorescence images of c-kit (green) and DAPI (blue) co-staining in the indicated groups. Bar=40 μ m. (B) Representative immunofluorescence images of c-kit (green), ki67 (red) and DAPI (blue) co-staining in the indicated groups. Bar=40 μ m. (C) Bar graph showing the quantification of the c-kit/Nkx2.5, c-kit/Ets-1 and c-kit/Gata6 co-stained cells in the indicated groups. (D) Immunofluorescence image of Ki67 (red) and cTnI (green) co-staining. The bar graph shows the statistical analysis of the Ki67-positive cardiomyocytes. (E) Representative images showing the CD31⁺ microvessels in the indicated groups. Bar=50 μ m. Data are expressed as the means $\pm$ SD, n=4, *P<0.05.

Ad-HGF induced necroptosis in the hearts of aged rats following MI

Necroptosis, a novel pathway of regulated cell death, occurs in various diseases, including MI [10]. However its impact on the c-kit⁺ CSCs remained unknown. Therefore, we first evaluated the effect of Ad-HGF on necroptosis in aged rat hearts following MI. RIP1 and RIP3 levels have been reported to positively correlate with the extent of necroptosis [21]. Therefore, we examined RIP1 and RIP3 expression in the heart following MI. Western blot analysis results revealed that the levels of these proteins increased in the cardiac tissues following MI. Notably, the Ad-HGF treatment markedly enhanced RIP1 and RIP3 levels (Fig. 4A). These observations were further supported by the results of PI staining, widely used as a marker of necroptosis [22]. The Ad-HGF treatment significantly increased necroptosis in the infarct and peri-infarct zones of aged rat hearts following MI. The necrotic apoptosis

Fig. 6. Necroptosis induced by Ad-HGF increased the HMGB1 levels and enhanced the c-kit⁺ cell number in the bone marrow. (A), (B) Western blot analysis of HMGB1 levels in the indicated groups. The bar chart shows the statistical analysis of the HMGB1 protein. (C) ELISA assay showing the serum levels of HMGB1 in the indicated groups before and 7 days after surgery. (D) Immunofluorescence images of bone marrow smears showing c-kit (green) and DAPI (blue) co-staining in the indicated groups. Bar=40 μ m. The bar graph shows the statistical analysis of the relative levels of the c-kit⁺ cells. Data are expressed as the means $\pm$ SD, n=4, *P<0.05.

levels in the non-infarcted areas in all groups were similar. Necrostatin-1 (Nec-1), a potent inhibitor of necroptosis, dramatically blocked the Ad-HGF-induced necroptosis (Fig. 4B). Collectively, these results clearly suggested that Ad-HGF induced necroptosis in aged rat hearts following MI.

Inhibition of necroptosis attenuated endogenous c-kit⁺ CSC activation and suppressed cardiomyocyte proliferation and angiogenesis induced by Ad-HGF

We further assessed the impact of Ad-HGF-induced necroptosis on c-kit⁺ CSCs. The inhibition of necroptosis by Nec-1 markedly decreased the numbers of total and proliferating c-kit⁺ CSCs induced by Ad-HGF in infarcted aged hearts (Fig. 5A, 5B). The numbers of committed myogenic (c-kit⁺/Nkx2.5⁺ cells), endothelial (c-kit⁺/Ets-1⁺ cells) and smooth muscle (c-kit⁺/Gata6⁺ cells) progenitors all significantly decreased after the inhibition of necroptosis (Fig. 5C). Additionally, the numbers of proliferating cardiomyocytes and capillary vessels in the myocardium also markedly declined following the inhibition of necroptosis (Fig. 5D, 5E).

Taken together, the results demonstrated that the inhibition of necroptosis suppressed c-kit⁺ CSC proliferation and their differentiation into myocyte, endothelial and smooth muscle lineages and further reduced myocardial regeneration and angiogenesis in infarcted aged rat hearts. Additionally, the data suggested that Ad-HGF induced c-kit⁺ CSC proliferation and differentiation potentially by enhancing necroptosis in aged rats following MI.

Necroptosis induced by Ad-HGF increased the HMGB1 levels and enhanced the c-kit⁺ cell number in the bone marrow

HMGB1 is a DNA-binding protein secreted into the extracellular space from the inflammatory and necrotic cells and acts as a cytokine. It has been reported that exogenous HMGB1 treatment induces myocardial regeneration after infarction by enhancing cardiac

Fig. 7. Ad-HGF treatment improved cardiac remodelling 28 days after MI. (A) Representative images of TUNEL (green)-, cTnI (red)- and DAPI (blue)-stained sections. Bar=60 μ m. The bar graph shows the quantification of the apoptotic cardiomyocytes. (B) Representative Western blots showing the expression levels of pro-survival and pro-apoptotic proteins. The bar graph shows the quantification of the proteins. CCP3, cleaved caspase 3; CP3, caspase 3; I, infarct zone; P, peri-infarct zone; N, normal zone. (C) Representative M-mode echocardiograms of aged rats before and 4 weeks after MI. The line graph shows the analysis of LVEF and LVFS in the indicated groups. (D) Representative images showing Masson's staining of the left ventricular tissue sections four weeks after MI. Bar=100 μ m. The bar graph shows the quantification of myocardial fibrotic areas. Data are expressed as the means $\pm$ SD, n=4, *P<0.05.

c-kit⁺ cell proliferation and differentiation [23]. In the present study, we evaluated the HMGB1 protein levels after Ad-HGF treatment. Compared with the Ad-null-treated group, the Ad-HGF-treated group showed significantly increased HMGB1 expression, an increase that was effectively inhibited by Nec-1 (Fig. 6A, 6B). The serum HMGB1 levels were similar in the groups before surgery but were significantly elevated in the Ad-HGF treatment group, a change that was effectively reversed by the inhibition of necroptosis (Fig. 6C). Homing of the stem cells mobilized from the bone marrow has been reported to play an important role in the regulation of neo-vascularization during acute ischaemia and tumour growth [24]. Immunofluorescence staining of the bone marrow smear revealed that the inhibition of necroptosis significantly decreased the quantity of c-kit⁺ cells in this tissue induced by the Ad-HGF treatment (Fig. 6D).

Collectively, these data suggested that the protective effect of Ad-HGF-induced necroptosis might be mediated by increased HMGB1 levels and the elevated c-kit⁺ cell numbers in the bone marrow.

Ad-HGF treatment improved cardiac remodelling on the 28th day after MI

Compared with the Ad-null hearts, the Ad-HGF-treated hearts showed significant decreases in the TUNEL-positive nuclei in the infarct and peri-infarct zones (Fig. 7A). Consistent with these results, cleaved caspase-3 and Bax expression levels in the infarct and peri-infarct zones of the Ad-HGF group were significantly lower than those in the Ad-null group, and the Bcl-2 and Bcl-xL levels were markedly elevated in the Ad-HGF group (Fig. 7B). These results suggested that the Ad-HGF treatment activated prosurvival pathways, resulting in the suppression of cardiomyocyte apoptosis.

Echocardiography was performed prior to and post-MI. The basal LVEFs prior to LAD coronary artery ligation were similar in all groups. The LVEFs were approximately 55% in the Ad-HGF-treated group and 45% in the Ad-null-treated group after four weeks of MI. There was a 9% improvement in the LVFS in the Ad-HGF-treated group relative to that in the Ad-null-treated group (Fig. 7C).

To determine whether Ad-HGF treatment attenuated cardiac injury, we assessed the size of the fibrotic area using Masson trichrome staining 28 days after MI. The fibrotic areas in the infarct and peri-infarct zones of the Ad-HGF-treated hearts (35–42%) were significantly smaller than those of the Ad-null-treated hearts (68–77%) (Fig. 7D). Together, our findings suggested that the Ad-HGF treatment improved cardiac remodelling as observed on the 28th day following MI.

Discussion

The present results revealed that the HGF/p-Met expression levels and the numbers of c-kit⁺ CSCs gradually decreased with age. Our results demonstrated for the first time that necroptosis induced by the Ad-HGF treatment activated endogenous c-kit⁺ CSCs and promoted cardiomyocyte proliferation and angiogenesis and further improved cardiac remodelling in aged rats following MI.

Acute MI-related morbidity is much higher in the elderly than in younger adults [9]. Therefore, there is an urgent need for the development of methods that facilitate myocardial regeneration in the elderly. The discovery of c-kit⁺ CSCs provides new avenues for cardiac repair. However, the mechanisms regulating c-kit⁺ CSC proliferation and differentiation in aged hearts remain largely uncharacterized. Moreover, the isolation and expansion of endogenous CSCs for autologous cell transplantation is a slow and expensive process that produces cells of uneven quality [7, 8]. As a result, promoting endogenous c-kit⁺ CSC proliferation and differentiation in aged hearts following MI is fast becoming a new research hotspot.

The results presented here showed that the numbers of total c-kit⁺ CSCs and the proliferating c-kit⁺ CSCs and cardiomyocytes were all significantly higher in neonatal rat hearts than in adult hearts. In addition, the c-kit expression levels gradually declined in the heart with age, from early perinatal (2–3 days) to later stages (6–10 months and 18–22 months). We also found that the c-kit protein level positively correlated with HGF/p-Met expression at three different stages in the rat heart. These results, which strongly suggested that the excellent regenerative capacity of the neonatal heart might be related to the higher abundance of c-kit⁺ CSCs, prompted us to investigate whether HGF promoted c-kit⁺ CSC proliferation and differentiation in aged rats.

To assess the effect of HGF on c-kit⁺ CSC proliferation and differentiation in aged hearts, we injected Ad-HGF into the cardiac infarct border zone after MI. Our previous study had demonstrated that Ad-HGF expression from the CMV promoter insures high expression in the myocardial tissue on the first day post-injection and that the expression is maintained for at least four weeks [18]. In the present study, we tentatively chose two time points, days 7 and 28, to assess the effect of Ad-HGF on c-kit⁺ CSCs. The numbers of c-kit⁺ cells were significantly higher in the peri-infarct and infarct areas of Ad-HGF-treated hearts on 7th day following MI. Moreover, nearly 90% of the c-kit⁺ cells expressed the p-Met

receptor. To specifically identify the quantity of c-kit⁺ CSCs in the Ad-HGF-treated infarcted heart, we ruled out cardiac mast cells using c-kit/CD45 co-staining. The c-kit⁺/CD45⁻ cells, representing the c-kit⁺ CSCs, amounted to 7.3% in Ad-HGF-treated infarcted hearts. Like proliferating cell nuclear antigen (PCNA) and bromodeoxyuridine (BrdU), Ki67 has been widely used as a marker of proliferating cells [25, 26]. Therefore, we used Ki67/c-kit co-staining to investigate the effect of Ad-HGF on c-kit⁺ CSC proliferation. We found that there was significantly higher abundance of proliferating c-kit⁺ CSCs in the Ad-HGF-treated group than in the Ad-null and sham groups. These results demonstrated that Ad-HGF promoted the proliferation of endogenous c-kit⁺ CSCs in aged rat hearts.

Recent studies have demonstrated that necroptosis, a novel type of regulated cell death, occurs in various diseases, including MI [10]. Here, we found that Ad-HGF administration significantly augmented necroptosis in infarcted aged rat hearts. Necrostatin-1, a specific necroptosis inhibitor, efficiently blocked the Ad-HGF-induced necroptosis after MI and markedly decreased the quantities of total and proliferating c-kit⁺ CSCs. These findings clearly suggested that Ad-HGF-induced necroptosis played a key role in promoting endogenous c-kit⁺ CSC proliferation.

Next, we assessed the impact of Ad-HGF-induced necroptosis on c-kit⁺ CSC differentiation. Cardiomyocytes, endothelial cells and smooth muscle cells constitute the basic units of the heart and play important roles in the maintenance of normal heart function [27]. Nkx2.5, Ets-1 and Gata6 expression profiles in precursor cells indicate their commitment to cardiomyocyte, vascular endothelial cell and smooth muscle cell lineages, respectively [8, 28]. Co-staining of these specific markers and c-kit revealed that Ad-HGF administration significantly increased c-kit⁺ CSC differentiation into these cardiac precursor cells in the peri-infarct and infarct areas. Twenty-eight days after Ad-HGF administration, we found that the treatment caused sharp increases in the numbers of proliferating cardiomyocytes and CD31⁺ microvessels. In contrast, the inhibition of necroptosis reversed these effects, suppressing c-kit⁺ CSC proliferation and differentiation into myocyte, endothelial, and smooth muscle cell lineages and further reducing myocardial regeneration and angiogenesis in infarcted aged rat hearts. For the first time, these data demonstrate that Ad-HGF treatment efficiently activates endogenous c-kit⁺ CSCs in aged infarcted hearts and further promotes cardiomyocyte proliferation and angiogenesis. Our results suggest that Ad-HGF induces c-kit⁺ CSC proliferation and differentiation, likely by enhancing necroptosis after MI.

HMGB1, a highly conserved, ubiquitously expressed nuclear protein released into the extracellular space by necrotic and inflammatory cells, has been identified to be involved in inflammatory responses and tissue repair [29]. Exogenous HMGB1 induces myocardial regeneration after infarction by enhancing cardiac c-kit⁺ cell proliferation and differentiation [23]. Notably, we found that Ad-HGF treatment significantly increased HMGB1 levels in the myocardial tissue and peripheral blood of aged rats following MI. Further, necroptosis inhibition produced an opposite effect. These data suggested that HMGB1 might be partly responsible for the beneficial effect of necroptosis on c-kit⁺ CSC proliferation and differentiation in aged rat hearts. We also found that the number of c-kit⁺ cells in the bone marrow was markedly enhanced by the Ad-HGF-induced necroptosis. Necroptosis is characterized by disrupted plasma membranes and is believed to contribute to innate immunity in humans and mice by alerting the status of the immune system through the release of danger signals [13]. It is likely that unknown substances released from the cells undergoing necroptosis may mobilize and recruit the c-kit⁺ cells from the bone marrow and further contribute to the myocardial regeneration. Further investigation is warranted to examine this possibility. The present results showed that the Ad-HGF treatment decreased apoptosis and myocardial fibrosis and improved the heart function in rats four weeks after MI, suggesting that the Ad-HGF therapy was effective in the treatment of MI in the aged rats.

The present study had several limitations. First, our study mainly investigated the roles of Ad-HGF and necroptosis on the proliferation and differentiation of c-kit⁺ CSCs in aged rats. Its effects *in vitro* were not explored and warrant further research. Second, the therapy was administered immediately after coronary artery ligation. This study was targeted to acute MI

in an attempt to reduce mortality by avoiding a second surgery. Future studies are needed to address these issues and to study the therapeutic effects of this approach in chronic MI.

Taken together, the results of our study demonstrated that the injection of Ad-HGF into the peri-infarct zones of aged rat hearts efficiently activated endogenous c-kit⁺ CSCs and promoted cardiomyocyte proliferation and angiogenesis potentially through the induction of necroptosis. This study opens new avenues for the treatment of MI in the elderly. The usefulness of Ad-HGF administration in the treatment of patients with cardiac injury warrants further investigation.

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Disclosure Statement

The authors declare that no conflicts of interest exist.

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