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Pim-1 Mediated Signaling during the Process of Cardiac Remodeling Following Myocardial Infarction in Ovine Hearts

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Abstract

Objectives—The serine/threonine kinase Pim-1 was recently identified as a cardiomyocyte survival regulator downstream of Akt. The present study aims to examine Pim-1 activity and its association with the post MI remodeling myocardium in a clinically relevant large animal model.

Methods—Apical myocardial infarction of approximately 25% left ventricular mass was created in an ovine model. Regional post-infarction deformation of the left ventricle was monitored by sonomicrometry and quantified using areal remodeling strain (i.e., areal expansion). Myocardial tissues were harvested at 12 weeks from the adjacent and remote regions of the infarct for analysis of Pim-1 mediated survival signaling proteins as well as apoptotic activity.

Results—The cDNA coding sequences of two ovine Pim-1 kinase isoforms, 44 and 33 kDa, were identified. Both isoforms were detected in heart tissue and the overall Pim-1 expression was found to be tightly controlled at multiple molecular levels. Pim-1 as well as the Pim-1 mediated survival signaling proteins Bcl-2, Bcl-xL, and phospho-Bad (Ser112), were upregulated in the adjacent region at 12 weeks post-infarction and their expression correlated positively with the degree of the remodeling, which was accompanied by significant upregulations of the PP2A/BAD mediated apoptotic signaling proteins. However these upregulations were imbalanced, such that p-BAD (Ser112)/BAD decreased in the adjacent region of the infarcted hearts. Apoptotic activity also increased with remodeling strain.

Conclusions—Despite an observed intrinsic upregulation of survival proteins, the imbalanced activation of apoptotic pathways resulted in evident apoptosis in the adjacent region.

Keywords

Pim-1; myocardial infarction; cardioprotection; apoptosis; remodeling

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Disclosures

None declared.

1. Introduction

Myocardial infarction (MI) is a common cause of mortality and morbidity. After an MI, the heart undergoes progressive left ventricular (LV) dilatation and systolic dysfunction with increased risk of heart failure, known as cardiac remodeling [1, 2]. Such changes in LV geometry and function are the results of both acute and chronic hemodynamic and neurohumoral responses, and are brought about by perturbations and subsequent responses of individual cardiac cells and the molecules contained therein.

Recently, the serine/threonine kinase Pim-1 was identified as a survival regulator downstream of Akt in cardiomyocytes [3], having been implicated in multiple cellular functions such as proliferation, differentiation, and anti-apoptosis by phosphorylating a number of intracellular signaling molecules (reviewed in Ref. [4]). Humans and mice express two isoforms of Pim-1 – 33 kDa and 44kDa – which are produced from the same gene by utilizing a normal (AUG) and an alternative (CUG) translation initiation codon [5, 6]. Both isoforms can physically interact with Bad by phosphorylating it at Ser112 under *in vivo* and *in vitro* conditions [7], promoting the release of Bcl-xL and increases the availability of Bcl-xL for its anti-apoptotic activity [8]. Phosphorylation of two additional substrates – p21^{Waf} and NFATc1 – has been reported to modulate cardiac development and failure [9, 10]. Pim-1 also supports the expression of the anti-apoptotic protein Bcl-2 to inhibit cell death through a Bcl-2-dependent mechanism [11]. Overexpression of Pim-1 in transgenic mice can enhance cardiomyocyte survival by preserving mitochondrial integrity [12]. Gain and loss of function studies have shown that Pim-1 can improve contractility and mitigate hypertrophy *in vitro* and *in vivo* [13]. Taken together, Pim-1 can exert cardioprotective effects by promoting survival of cardiomyocytes, while preventing their hypertrophy. Genetic alteration of cardiac Pim-1 may become a promising approach of myocardial repair. Recent studies on cardiac progenitor cells [14] and bone marrow cells [15] enhanced with Pim-1, demonstrated that Pim-1 may also serve as an ideal cellular therapeutic target in preventing cardiac remodeling. The regenerative capacity of cardiac progenitor cells was boosted by Pim-1 to repair damaged myocardium. Additionally, recruitment of endogenous stem cells, improved cardiomyocyte hypertrophy and promoted recovery of myocardial structure with Pim-1 modified bone marrow cell treatment. Although several mechanisms were involved, all of treatments means blunted pathological remodeling after MI.

These early investigations have mostly been conducted at the cellular level or using small animal models. In the present work, we explored the cardioprotective effects of Pim-1 on cardiomyocytes and the regulations of cardiac Pim-1 in an ovine model. We identified the *PIM-1* cDNA sequence and investigated the association of Pim-1 mediated survival signaling with the degree of myocardial remodeling. We hypothesized that expression of Pim-1 and its related proteins would increase with progressively increased myocardial strain and attempted to examine whether these intrinsic survival signals could rescue cardiomyocytes and prevent remodeling.

2. Materials and methods

2.1. Surgical Protocol

Twelve Dorsett hybrid sheep (male, average weight: 55 kg, Thomas Morris, Reisterstown, MD) were used. Eight animals underwent a left thoracotomy and were instrumented with 16 sonomicrometry transducers for monitoring myocardial structural deformation of the infarcted hearts (MI group). The surgical procedure and implantation of the transducers were performed as previously described [16]. Apical MI of about 25% LV free wall mass was created by ligating the coronary arterial branches supplying blood to the apex. Four sheep

that underwent the same left thoracotomy and were instrumented with sonomicrometry transducers without MI, were used as the sham control (Sham group). The sheep were allowed to recover after the MI or sham surgery and survived for 12 weeks. All the animals received treatment in compliance with the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health (National Institutes of Health publication 85–23, revised 1996). The surgical procedures and postoperative care were carried out according to the protocol approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore.

2.2. LV function data collection and analysis

Echocardiograms were collected with a Sonos 5500 machine (Philips Medical, Andover, MA) 30 minutes post-MI, and at termination. Sonomicrometry data were collected pre-MI, 30 minutes post-MI, and twice per week until termination. The global LV function of each sheep at each time point was evaluated by analyzing end-diastolic volume (LVEDV), end-systolic volume (LVESV), ejection fraction (LVEF), and wall motion abnormality (WMA) as measured by echocardiography. The remodeling strain was defined as the post-infarct to pre-infarct ratio of the diastolic area of a given segment of LV free wall [17–19] and can be expressed as:

$$S = \frac{A(t) - A_0}{A_0}$$

where S is the remodeling strain, $A(t)$ is the area of a segment at a time t post MI, and A_0 is the baseline, pre-infarct area of the same segment. This was the formula used to quantify regional myocardial expansion during the course of cardiac remodeling [20].

2.3. Isolation of RNA and cDNA synthesis

Myocardial tissues from the adjacent (approx. 2 cm wide) and remote regions of the infarct were harvested, frozen in liquid nitrogen, and stored at -80°C . Total RNA was isolated using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instruction. First-strand cDNA was synthesized from 1 μg of total RNA using SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen, Carlsbad, CA).

2.4. Sequencing analysis of ovine *PIM-1* mRNA

The sequence of ovine *PIM-1* corresponding to the 33 kDa protein was amplified by Pfu DNA polymerase (Stratagene, La Jolla, CA). The 5'-end sequence was amplified by Phusion DNA polymerase (Finnzymes Oy, Espoo, Finland). The GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) was used in the amplification, and the primers are listed in Table 2. The PCR products were separated on agarose gel. The 942 bp band corresponding to 33 kDa Pim-1 coding sequence and the 479 bp band corresponding to N-terminal sequence of 44 kDa Pim-1 were excised. The PCR products were extracted, purified through QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA), and sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA) on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA).

2.5. Quantitative real-time PCR

Expression of *PIM-1*, *Bcl-2*, *Bcl-xL* and *GAPDH* mRNA was analyzed using Applied Biosystems 7900HT Fast Real-Time PCR System. Fifty nanograms of RNA products were added to a 25 μl reaction mixture with SYBR® Green PCR Master Mix (Applied

Biosystems, Foster City, CA). Primer sequences were designed using the program AlleleID (PREMIER Biosoft International, Palo Alto, CA) and are listed in Table 3. All samples were measured in duplicate. The quantification of gene expression levels was performed using the relative quantification 2^{-CT} method. Reactions without reverse transcription were run as negative controls.

2.6. Preparation of whole cell, nuclear and cytosolic extracts for immunoblot analysis

To measure protein expression, whole cell extracts were prepared in RIPA lysis buffer (Upstate, Temecula, CA). Nuclear and cytosolic extracts were fractionated using the NE-PER Nuclear and Cytosolic Extraction Reagents (Pierce Biotechnology, Rockford, IL) according to manufacturer's recommendation. Forty micrograms of protein from each sample were separated on 4–15% SDS-PAGE gel and immunoblotted with antibodies against Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated Akt (p-Akt), Akt, Pim-1, Bcl-xL, phosphorylated BAD (Ser-112) (p-BAD), protein phosphatase type 2A (PP2A) A subunit and BAD (Cell Signaling Technology, Danvers, MA). Immunoreactivities were detected using enhanced chemiluminescence (ECL) (Amersham Biosciences, UK). For loading control, the same blots were then probed with the antibody against GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) for whole cell and cytoplasmic extracts, and the antibody against Histone H3 (Cell Signaling Technology, Danvers, MA) for nuclear extracts.

2.7. Immunoprecipitation analysis of Pim-1 related heat shock proteins

Whole cell extracts from the heart tissues of the sham group as well as the remote and adjacent regions of infarcted hearts were immunoprecipitated with Pim-1 antibody overnight at 4°C, followed by incubation with protein A-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitates were then separated by SDS-PAGE and immunoblotted with antibodies against heart shock proteins (Hsp): Hsp70 and Hsp90 (Assay Designs, Inc., Ann Arbor, MI). The immunoreactive signals were measured by densitometry.

2.8. Immunohistochemistry analysis by confocal microscopy

The heart tissues were fixed in formalin overnight, embedded in paraffin, and cut into 5 μ m sections. After deparaffinization and rehydration, the sections were treated for antigen retrieval and permeabilization, and were blocked in donkey serum PBS. Rabbit anti-Pim-1 (Cell Signaling Technology, Danvers, MA) and mouse anti-tropomyosin (Santa Cruz Biotechnology, Santa Cruz, CA) were incubated overnight at 4°C, followed by labeling with FITC-conjugated donkey anti-rabbit IgG and Texas Red-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA). After DAPI staining (Invitrogen molecular probes, Eugene, OR), images were acquired using a Zeiss LSM410 confocal microscope (Carl Zeiss, Thornwood, NY).

2.9. Evaluation of apoptotic cell death by TUNEL assay

Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) with MEBSTAIN Apoptosis Kit II (MBL international corporation, Woburn, MA). The positive control was prepared by pre-treating the sections with 3 μ g/ml DNase I (Roche Diagnostics, Indianapolis, IN) before labeling, while the negative control was obtained by omitting TdT from the reaction mixture. Histological sections were prepared and stained according to the manufacturer's instruction. The stained sections were examined using a Zeiss Axioskop microscope (Carl Zeiss, Thornwood, NY) and the number of TUNEL positive myocytes was visually counted as previously described [21].

2.10. Statistical analysis

Data are given as means $\pm$ SEM. One-way repeated measures analysis of variance (ANOVA) was used to compare temporal changes in the echocardiographic and strain data. One-way ANOVA was used to compare the regional changes in the strain data at a particular time point, as well as the levels of protein and mRNA expression at termination. All ANOVAs were followed by multiple comparisons with the least significant difference correction when applicable. Pearson's correlation analysis was conducted between protein expression and regional remodeling strain. The significance level was set at 0.05.

3. Results

3.1. Cardiac remodeling after myocardial infarction

The indicators of the LV performance, including LVEDV, LVESV, LVEF, and WMA, are summarized in Table 1. Both the LVEDV and the LVESV increased significantly, while the LVEF decreased over the 12 weeks post-MI. Based on analysis of the short-axis echocardiogram at the level of the papillary muscles, infarct expansion was present with an increase in the length of WMA. Likewise, the ratio of WMA to endocardial circumference increased significantly, indicating the expansion of infarct tissue. These data indicate that the LV underwent progressive cardiac remodeling, as evidenced by chamber dilation and decreased LVEF.

The percent expansions of the remote, adjacent, and infarct regions are presented in Figure 1. The remodeling strains of the infarct and the adjacent regions increased significantly after MI, showing a progressive expansion, while the remote region exhibited a much lower remodeling strain. These data show that the cardiac remodeling post-MI is an inhomogeneous process over the LV free wall.

3.2. DNA sequence of ovine *PIM-1*

The cDNA sequence of ovine *PIM-1* is shown in Figure 2a. Like human and mouse *PIM-1*, the ovine *PIM-1* has the normal translational initiation codon AUG corresponding to 33 kDa Pim-1 translation. The sequence of this isoform is 942 bp in length and shares 95%, 98%, 95%, 89%, and 89% similarities in nucleotides with the *PIM-1* of human, bovine, cat, rat and mouse, respectively (data not shown). The deduced amino acid sequence of ovine Pim-1 is exactly the same as that of bovine Pim-1, but has 98%, 98%, 97%, and 93% similarity to the Pim-1 of human, cat, rat and mouse (data not shown). In addition, ovine *PIM-1* contains alternative upstream open reading frames, in which an alternative translational initiation codon, CUG is employed as the putative translation initiation site (Figure 2a). The N-terminal sequence of 44 kDa ovine Pim-1 is highly homologous to that in human and mouse, and shares 57% and 70% similarities of the deduced amino acid sequence, respectively (Figure 2b). Moreover, three PXXP motifs are also identified in the N-terminal of 44 kDa ovine Pim-1, suggesting that the ovine Pim-1 could share the biological functions observed in 44 kDa Pim-1 of human and mouse [22].

3.3. Pim-1 expression in remodeling myocardium

No significant increase in *PIM-1* mRNA levels was observed in the heart tissue from the remote region of the MI group compared with the same region of the sham group. However, the *PIM-1* mRNA level in the tissue from the adjacent region of the MI group was 1.6-fold higher than that in the tissue from the sham group (Figure 3a). Furthermore, we confirmed that both the 44 kDa and the 33 kDa protein isoforms existed in the ovine myocardium. Consistent with the previous study [3], the 33 kDa isoform of Pim-1 appeared in immunoblots as double bands, corresponding to the phosphorylated and unphosphorylated forms, respectively (Figures 3b and 3d). Quantification of the immunoblots revealed that the

expression of 44 kDa Pim-1 in the tissue (whole cell extracts) from the adjacent region of the MI group increased significantly by 1.4-fold compared to that from the sham group while the 33 kDa Pim-1 increased 2-fold. No significant difference was observed between the tissue from the remote region of the MI group and the sham group (Figure 3c).

Subcellular fractionation analysis shows that the increase in Pim-1 expression in the adjacent region of the MI group occurred mainly in the cytoplasm, whereas the nuclear fraction remained unchanged. There was 1.7-fold increase in Pim-1 expression for the 44 kDa isoform and 1.9-fold increase for the 33 kDa isoform in the cytoplasm from the adjacent region of the MI group compared to those from the sham group (Figure 3e). The increase of Pim-1 protein in the cytoplasm is also confirmed by immunofluorescence staining of the tissue sections (Figure 3f).

3.4. Elevational Pim-1 levels

The tissue from the adjacent region of the MI group exhibited 1.5-fold and 1.3-fold increase in p-Akt and Akt expression compared to that from the sham group (Figures 3b and 3c). Given that Pim-1 has been shown to be downstream of Akt, these results suggest that the elevated expression of Pim-1 may be associated with the elevations in p-Akt and Akt.

Increased Pim-1 may also be a result of an alternative turnover rate. In order to examine this possibility, we performed immunoprecipitation using Pim-1 antibody with whole cell extract from the heart tissues of both the MI and sham groups. Hsp70 and Hsp90 were precipitated with the Pim-1 antibody to examine how Pim-1 physically interacted with Hsp70 and Hsp90 in the heart tissues. Hsp70 and Hsp90 are two important chaperones regulating protein stabilization and degradation. The ratio of Hsp90 and Hsp70 bound to Pim-1 significantly increased (1.7-fold) in the tissue from the adjacent region of the MI group compared to that in the tissue from the sham group (Figure 4), suggesting that Pim-1 had more association with Hsp90 than with Hsp70 in the adjacent region of the infarcted hearts. This effect could consequently contribute to an increased stability of Pim-1.

3.5. The promotion of anti-apoptotic protein expression by elevated Pim-1

To evaluate the anti-apoptotic effect of Pim-1, we analyzed the expression of Bcl-2 and Bcl-xL as well as the phosphorylation status of Bad. They have all been reported to be critical anti-apoptotic factors downstream of Pim-1 [7, 11]. The mRNA and protein levels of Bcl-2 in the tissue from the adjacent region of the MI group increased significantly by 1.7 and 2.5-fold, respectively, compared to those in the sham group. Although the mRNA of *Bcl-xL* had no significant change in the tissue from the adjacent region of the MI group, its protein level elevated 2.9-fold compared to that in the sham group (Figures 3a, 3b, and 3c). Moreover, the p-Bad (Ser112) was significantly increased (1.6-fold) in the tissue from the adjacent region of the MI group compared to that of the sham group. These results suggest that survival proteins were upregulated in the remodeling myocardium of the infarcted hearts.

3.6. Upregulated survival proteins were not sufficient to protect heart cells from apoptosis

To examine whether elevated Pim-1 and its downstream proteins decreased apoptosis in the remodeling myocardium, the expression of PP2A and BAD, the ratio of p-BAD (Ser112) to BAD was analyzed (Figure 5a, 5b). Protein levels of PP2A and BAD in the tissue from the adjacent region increased significantly by 1.4 and 1.8-fold, respectively, compared to those in the sham group. Although p-BAD (Ser112) in the tissue from the adjacent region of the MI group (Figure 3b, 3c) was elevated, the ratio of p-BAD (Ser112) to BAD (p-BAD (Ser112)/BAD) decreased 1.3-fold compared to the remote zone, which indicated the dephosphorylation of BAD in the adjacent zone. Apoptotic DNA fragmentation was detected by TUNEL assay (Figure 5c). The number of TUNEL-positive cells in the tissue

from the adjacent region of the MI group was significantly greater (7.1 ± 0.9 per field) than those from the remote region of the MI group (1.8 ± 0.2 per field, $p < 0.05$) and in tissues of the sham group (0.8 ± 0.1 per field, $p < 0.05$). The results suggest that the elevated expression of anti-apoptotic factors was not sufficient to completely inhibit apoptosis – an observation that correlates well with functional observations.

3.7. The correlation of the regional remodeling strain and protein expression

The functional and molecular alterations of the myocardium of the infarcted hearts were region-specific. Figure 6 shows the correlations between the regional remodeling strain and the expressions of the Akt/Pim-1 mediated survival signaling proteins and the PP2A/BAD mediated apoptotic signaling proteins. Positive correlations were observed between the myocardial remodeling strains and the expressions of Pim-1 44 kDa Pim-1 33 kDa, Bcl-2, Bcl-xL, and PP2A, BAD. Negative correlation was observed in p-BAD (Ser112)/BAD. No positive correlation was observed in p-Akt/Akt, the upstream protein of Pim-1. The adjacent region experienced larger remodeling strain, which was accompanied by significant upregulations of the Akt/Pim-1 mediated survival signaling proteins and PP2A/BAD mediated apoptotic signaling proteins. But these kinds of upregulations were imbalanced, so that p-BAD (Ser112)/BAD decreased in the adjacent region of the infarcted hearts.

4. Discussion

Apoptosis is the major type of cell death during chronic and progressive cardiac remodeling, although necrosis has been reported to be the predominant process in the infarct region during the first 24 to 48 hours post-MI [23, 24]. Previous studies in our group have demonstrated a positive relationship between strain and regional expression of apoptotic proteins and regional imbalanced activation of apoptotic pathway and survival pathway during cardiac remodeling [16, 21]. In the present study, we have provided evidence that Akt/Pim-1 anti-apoptotic signals were upregulated in response to myocardial expansion at 12 weeks post-MI.

Both the 44 and 33 kDa isoforms of Pim-1 were present in ovine myocardium (Figures 3b and 3d). The sequence analysis shows that ovine Pim-1 is highly homologous to that of the human and the mouse in primary structure [5, 25] suggesting that Pim-1 function may be similarly conserved across species. Both Pim-1 protein and mRNA were elevated in the adjacent region of the infarcted hearts compared with the remote region of the infarcted and the non-infarcted sham hearts. This could be a consequence of increased p-Akt and Akt expression in the myocardium, activating translational machinery and subsequent increasing of Pim-1 expression [3, 26]. Additionally, Pim-1 is shown to physically interact with Hsp90 and Hsp70. An earlier study has reported that Hsp90 specifically associates with nonubiquitinated Pim-1 and acts to maintain and protect Pim-1 from degradation under cellular stress. In contrast, Hsp70 mainly binds with ubiquitin-conjugated Pim-1 and promotes Pim-1 protein degradation through ubiquitin-proteasome pathway [27]. The increased association between Pim-1 and Hsp90 observed in the myocardium from the adjacent region of the infarcted hearts (Figure 4) suggests that the overall elevation of Pim-1 might also be attributable to a heat shock protein dependent mechanism.

Pim-1 has been reported to be present in cytoplasm, nucleus, and cell surface, depending on cell type and cell physiology [28–31]. The different localizations of Pim-1 may regulate distinct substrates. As an oncogene, Pim-1 may regulate the activity of cell's transcriptional machinery in the nucleus, and modulate levels of critical second messenger molecules in the cytoplasm [32]. Our results demonstrated that Pim-1, both 44 and 33 kDa isoforms, were elevated significantly in the cytoplasm of cardiomyocytes from the adjacent region of the infarcted hearts (Figure 3e and 3f) and acted as a cytoplasmic kinase, which suggests that it

may play important roles in intracellular signal transduction during the chronic phase of cardiac remodeling.

We observed that the levels of anti-apoptotic proteins Bcl-2, Bcl-xL and p-BAD (Ser112) significantly increased in the myocardium from the adjacent region of the infarcted hearts at 12 weeks after MI. It has been reported that increased expression of the Bcl-2 and Bcl-xL proteins was associated with elevated Pim-1 levels [11], whereas no difference in Bcl-xL mRNA levels was observed. These findings suggest that elevated Pim-1 is a response to exert a protective effect against apoptosis.

Our prior investigations in the ovine MI model have demonstrated that regional imbalanced activation of the calcineurin/BAD apoptotic pathway and the PI3K/Akt survival pathway after MI was that calcineurin could increase the expression of BAD and dephosphorylate BAD through serine 136. Dephosphorylated BAD is targeted to the mitochondria, where it causes cell apoptosis by binding Bcl-2 and releasing cytochrome c [21]. It was reported that pro-apoptotic activity of BAD is regulated by 14-3-3 and PP2A in vitro. Phosphorylated BAD Ser136 recruits the 14-3-3 dimer to BAD. The p-BAD (Ser112) stabilizes 14-3-3 binding. PP2A dephosphorylation of p-BAD (Ser112) functions as the gatekeeper and the key initiating event for BAD-mediated apoptosis [33]. In the sheep MI model, we found PP2A increased and the ratio of p-BAD (Ser112) to BAD decreased in the adjacent region of the infarcted hearts. Our results showed that similar to our previous reports the molecular events involved in the activation of PP2A/BAD apoptotic pathway had also a strong correlation with the regional strain, which was followed by apoptosis.

Collectively, although these anti-apoptotic signals, together with their upstream regulator Pim-1, exhibited a region-specific response and were positively correlated with myocardial remodeling strain (myocardial expansion) during cardiac remodeling, they were overwhelmed by the activation of apoptotic pathway. Gene expressions are known to be regulated by mechanical forces [34]. Shear stress has been shown to induce Pim-1 expression in rat MSC [35]. It could be postulated that the upregulation of Pim-1 mediated signaling might be induced by myocardial remodeling strain through a negative-feedback mechanism. The significant increases of these survival signals in the chronic phase of cardiac remodeling suggest that these signals played key roles in the intrinsic response of the cardiomyocytes to the MI insult. However, these endogenous and passive responses seemed inadequate to provide complete cardioprotection during progressive cardiac remodeling. Pim-1 upregulation has been found soon after MI and overexpression of cardiac Pim-1 mitigated infarction injury in mice [3]. Pim-1 mediated survival signaling might be involved in both initiation and development of cardiac remodeling. Therefore, the long-term manipulation of cardiac Pim-1 during the course of cardiac remodeling is likely a novel, effective therapeutic strategy. Because of the complexity and diversity of cellular signal transduction pathways involving PP2A and Hsp70/90, the exogenous enhancement of Pim-1 expression by overexpression may be the most direct approach. Future studies will focus on the effects of long-term Pim-1 overexpression on cardiac remodeling. Pim-1 is ubiquitous and exerts many effects in different organs. New delivery systems need to be developed for the efficient, organ- and time-specific overexpression of Pim-1 in large animals. Recently, the systemic administration AAV9-Pim-1 was found to attenuate cardiomyocyte apoptosis [36], illustrating a convenient and feasible approach of intravenous gene therapy with Pim-1. Pim-1 may also enhance stem-cell-based myocardial repair. Pim-1 modified cardiac progenitor cells [14] and bone marrow cells [15] were capable of imparting protective effects to the infarcted heart. These studies on genetic engineering of progenitor cells with Pim-1 [14, 15] provided a promising therapeutic option for cardiac remodeling.

5. Conclusions

In the present study, the molecular mechanisms of Pim-1 mediated cardioprotection during progressive cardiac remodeling were investigated in an ovine MI model. At 12 weeks post-MI, the adjacent region of the infarcted hearts experienced higher remodeling strain and exhibited concomitantly elevated levels of Pim-1 and the related survival proteins p-Akt, Akt, Pim-1, Bcl-2, Bcl-xL, and p-Bad (Ser112). Despite this observed abundance of survival factors, the imbalanced activation of PP2A/BAD apoptotic pathway resulted in evident apoptosis in the adjacent region. The results suggest that remodeling cardiomyocytes were in a chronic and constant survival mode; however, these endogenously elevated signals were not sufficient to protect cardiomyocytes from apoptosis. Although we have demonstrated that there was the association between the up-regulation of Pim-1 and the degree of remodeling, the causal relationship to intrinsic reparative processes could not be established because of the limitation of the large animal models by nature.

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Highlights

- Pim-1 activity was involved in a large animal model of post-ischemic remodeling.
- Expression of Pim-1 was upregulated in region adjacent to infarct.
- PP2A/BAD apoptotic pathway was favored despite increased Pim-1 activity.

Figure 1. The change in average strain over time in the infarct, adjacent, and remote regions
For healthy sham controls, strain is taken to be zero at all time points. * $P < 0.05$ compared to normal control at the same time point, # $P < 0.05$ compared to the remote region at the same time point.

(a)

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ctcttggtgcggggcgagccggcgctctgtgcagcgccgcggtggcgaggaggccg 60
gagaggacttggcgggcgcggtggcgggaccggcaacagacagacagacgtacagcta 120
cacactcctgtgcccagagcgtgccacaccagccctcgagcgccacccctgcgcgc 180
M P H Q F L E F P T L F P
ggcgcgctgctgaacctgcggcggtgtccatcagcgccagcgcccccagtgctctcc 240
A A L F D P A G V S I S R Q R P Q S S S
gactcgccctcggtctccgcgcagcgccagcgccgcaacgcacccgcagcctc 300
D S P S A F R A S R S R S R N A T R S L
agccccgccttaggcacagcgccgcgcagccctagccctactgcagtagctctcc 360
S P G L R H S R R R S P S P Y C S S S S
agacacagcctcagcgccgacacccctggaggtgggatgctcttgtccaaatcaactcg 420
R H S L S A D T L E V G M L L S K I N S
cttgcccactgcgcgcgcgcctgcagcgatctgcagcgccacagctggcgccgggc 480
L A H L R A A P C S D L H A T K L A P G
aaggagagagagccctggagtcgacgtaccaggtggcgccgctcctggcgagcgccgc 540
K E K E P L E S Q Y Q V G P L L G S G G
ttcggtcggtgactcaggcattcgtgtgcgcgacacgtggcggtggccatcaagcac 600
F G S V Y S G I R V A D N L P V A I K H
gtggagagagagcggatttcgactgggagagctgcctaatggcaccgcagtgcccatg 660
V E K D R I S D W G E L F N G T R V P M
gaagtgttctgctgaagaagtgagctcgggtcttcggcgctcattaggtcctctggac 720
E V V L L K K V S S G F S G V I R L L D
tcgttcgagagcccgacagtttcgtcctgactcctggagagcgccgagcagtgcaagac 780
W F E R P D S F V L I L E B P E P V Q D
ctcttcgactttatcacggaagagggccctgcaggagagctgcggcgagcttcttc 840
L F D F I T E R G A L Q E E L A R S F F
tggcaggtgctggagcggtgcgacactgccagcactgcgggtgcttaccgcgacatc 900
W Q V L E A V R H C H D C G V L H R D I
aaggagagagacatccttatcgacactcaatcgccgagcagctcaagctcatcgactcgg 960
K D E N I L I D L N R G E L K L I D F G
tcggggcgctgctcaaggacacgctctacacggacttcgatggagccagtgatgtagt 1020
S G A L L K D T V Y T D F D G T R V Y S
cctccagagtgatccgctacatcgctacatggcagtgccgagcgtctggtctctg 1080
P P E W I R Y H R Y H G R S A A V W S L
gggatcctgctgtacatggtgtgcgagatattccctttgagcacgatgaggagatc 1140
G I L L Y D M V C G D I P F E H D E E I
gtcagggcccaagttttcttcagcgacgggtctcctcagagtgtaacatctcattaga 1200
V R G Q V F F R Q R V S S E C Q H L I R
tgggtccttgctgagaccatcagatcgcccaacttcgaagaatccagaaccatcca 1260
W C L A L R P S D R P T F E E I Q N H P
tggatgcaagagctcctcctgcggcaggaactgctgagatccatctccacagcctgtcg 1320
W N Q D V L L P Q E T A E I H L H S L S
ccaggcgccagcaaatagctt 1341
P G P S K -

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(b)

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Human MHEPHEPLTPPFSAIPDAGAPSRQSPQRLSSDSPSAFRASRSHSRNATRSRSH 60
Ovine MHEPHEPLTPPFSAIPDAGVSI---ISRQSPQSSDSPSAFRASRSHSRNATRSR 60
Mouse M---GFAAFLALFPFALPDAGEFARGQPRQSPQSSDSPSALRASRSQSRNATRSR 60
      * * : * :***** . ,***** *****;*****;***** *

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Human SFRHSLRHSFGSGSGSGHRCADILEVMLSKINSIAHLRAAPCNDLHATKLAPGK 120
Ovine GLHRSRRRSP-SPYCSRRHSLADTLEVMLSKINSIAHLRAAPCNDLHATKLAPGK 120
Mouse GRKLS--PSSLRRRCCSS-RHRRRTDTLEVMLSKINSIAHLRAAPCNDLHATKLAPGK 120
      . * * . , * * * * : * ****

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Figure 2. The cDNA sequence of *PIM-1*

(Genebank accession No. FJ155993, FJ155994). (a) The nucleotides and the deduced amino acid sequences of ovine *PIM-1* cDNA. The putative alternative translation initiation site CUG and the start codon AUG are in bold. (b) Comparison of N-terminal sequences of 44 kDa *PIM-1* from ovine, human and mouse. The proline-rich sequences are underlined and in bold. Two translational initiation sites are in bold. Asterisks indicate identical amino acid residues. The multi-alignments were performed using Clustal X2 (Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. 2007).

Figure 3. The survival signals in the adjacent region of the infarcted hearts at 12 weeks post-MI
(a) Quantification of *PIM-1*, *Bcl-2*, and *Bcl-xL* mRNA by qRT-PCR. **(b)** Immunoblots of p-Akt, Akt, Pim-1(44 kDa), Pim-1(33 kDa), Bcl-2, Bcl-xL, and p-Bad (Ser112). GAPDH is used as loading control. **(c)** Quantification of protein expression levels from immunoblots. **(d)** Immunoblots of 44 kDa and 33 kDa of Pim-1 proteins from nuclear and cytosolic fractions. GAPDH is used as cytosolic loading control and histone H3 as nuclear loading control. **(e)** Quantification of Pim-1 expression level from immunoblots. **(f)** Confocal micrographs of paraffin-embedded sections stained for tropomyosin (red), Pim-1 (green) and nucleus (blue). Scale bar, 25 μ m. Arrows indicate cardiomyocytes expressing Pim-1.
^{*}P<0.05 compared to sham control, [#]P<0.05 compared to the remote region.

Figure 4. The ratio of Pim-1-bound Hsp90 to Hsp70 in the adjacent region of the infarcted hearts
(a) Immunoblots of Hsp90 and Hsp70. **(b)** Quantification of the changes in Hsp90/Hsp70 ratio. * $P < 0.05$ compared to normal control, # $P < 0.05$ compared to the remote region.

Figure 5. The apoptotic analysis in the adjacent region of the infarcted hearts at 12 weeks post-MI

Evidence of apoptosis are increased expression of PP2A and BAD and decreased ratio of p-BAD (Ser 112)/BAD in the adjacent zone after MI (a,b). Nuclei of apoptotic cells were labeled by FITC and are green in color in positive control and adjacent zone. There were more TUNEL-positive cardiomyocytes in the adjacent zone (c). All slides shown are in x40 magnification. P: positive control; S: sham control animal; R: remote zone; A: adjacent zone.

Figure 6. Normalized levels of survival and apoptotic proteins with respect to remodeling strain
 Each data point represents the remodeling strain of a specific region (remote or adjacent region) in one sheep and the corresponding protein expression level. : remote region, : adjacent region.

Table 1

Summary of echocardiographic data

Study point	LVESV(ml)	LVEDV(ml)	LVEF(%)	WMA length (cm)	WMA/Circ
Pre-infarct	36 ± 4	70 ± 4	50 ± 4	N/A	N/A
Post-infarct (30 min)	46 ± 5	84 ± 5	46 ± 3	3.04 ± 0.14	0.33 ± 0.02
Terminal	68 ± 7 ^{*#}	105 ± 10 ^{*#}	36 ± 3 [*]	5.12 ± 0.72 [#]	0.50 ± 0.06 [#]

LVESV: left ventricular end-systolic volume, LVEDV: left ventricular end-diastolic volume, LVEF: left ventricular ejection fraction, WMA: wall motion abnormality, Circ: circumference. All values are represented as mean ± SEM.

^{*} P<0.05 compared to pre-infarct.

[#] P<0.05 compared to post-infarct.

Table 2

Primer sequences used for sequence analysis

Gene	Accession No.		Sequence	Product Size
<i>PIM-1</i> (33 kDa)	FJ155993	Sense	GGGATGCTCTTGTCCTCAAATCAAC	942bp
		Antisense	AAGCTATTTGCTGGGCCCTGGCGA	
<i>PIM-1</i> (5' end)	FJ155993	Sense	CTCCTGGCTGCGGGGCGAGCCGGGCGTCT	479bp
		Antisense	CCCGGCGCCAGCTTGGTGGCGTGCAGATC	

Table 3

Primer sequences used for quantitative real-time PCR

Gene	Accession No.		Sequence	Product Size
<i>PIM-1</i>	FJ155993	Sense	TCCTGCTGTATGACATGGTGTG	88bp
		Antisense	ACCCGCTGCCTGAAGAAA	
<i>Bcl-xL</i>	NM_001009226	Sense	GTGCGTGGAAGCGTAGAC	83bp
		Antisense	GGTGGTCATTGAGGTAAGTAGC	
<i>GAPDH</i> *	U94889	Sense	GAGAAACCTGCCAAGTATGATG	95bp
		Antisense	ACCTGGTCCTCAGTGTAGC	
<i>Bcl-2</i>	AY_423861	Sense	CACCTGACGCCCTTCACC	131bp
		Antisense	TTGACGCTCTCCACACAC	
<i>GAPDH</i> **	U94889	Sense	GAGAAACCTGCCAAGTATGATG	123bp
		Antisense	AGTGTCGCTGTTGAAGTCG	

*
Used with *PIM-1* and *Bcl-xL*,

**
Used with *Bcl-2*.