Regional remodeling strain and its association with myocardial apoptosis after myocardial infarction in an ovine model

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Objective: Progressive left ventricular remodeling after myocardial infarction has been viewed as an important contributor to progressive heart failure. The objective of this study was to investigate the relationship between myocardial apoptosis and strain during progressive cardiac remodeling.

Methods: Before creation of an anterolateral left ventricular infarction by ligation of diagonal arteries, 16 sonomicrometry transducers were placed in the left ventricular free wall of 8 sheep to assess regional deformation in the infarct, adjacent, and normally perfused remote myocardial regions over 8 weeks' duration. Hemodynamic, echocardiographic and sonomicrometric data were collected before infarction and then 30 minutes and 2, 6, and 8 weeks after infarction. At the end of the study, regional myocardial tissues were collected for apoptotic signaling proteins.

Results: At terminal study, an increase in left ventricular end-diastolic pressure of 8.1 ± 0.1 mm Hg, a decrease in ejection fraction from $54.19\% \pm 5.68\%$ to $30.55\% \pm 2.72\%$, and an end-diastolic volume increase of 46.08 ± 5.02 mL as compared with the preinfarct values were observed. The fractional contraction at terminal study correlated with the relative abundance of apoptotic protein expressions: cytochrome c ($r^2 = 0.02$, $P < .05$), mitochondrial Bax ($r^2 = 0.27$, $P < .05$), caspase-3 ($r^2 = 0.31$, $P < .05$), and poly (adenosine diphosphate–ribose) polymerase ($r^2 = 0.30$, $P < .05$). These myocardial apoptotic activities also correlated with remodeling strain: cytochrome c ($r^2 = 0.02$, $P < .05$), mitochondrial Bax ($r^2 = 0.28$, $P < .05$), caspase-3 ($r^2 = 0.43$, $P < .05$), and poly (adenosine diphosphate–ribose) polymerase ($r^2 = 0.37$, $P < .05$).

Conclusion: Increase in regional remodeling strain led to an increase in myocardial apoptosis and regional contractile dysfunction in heart failure.

Cardiac remodeling after myocardial infarction (MI) is characterized by progressive global left ventricular (LV) dilatation and systolic dysfunction associated with an increased risk of congestive heart failure.^{1,2} Post-MI remodeling has been described as a change in the structural properties between the ischemic zone and the nonischemic zone that leads to increases in regional wall stress, myocyte cell loss, side-to-side myocyte slippage, chronic lengthening, and hypertrophy of myocytes.^{3,4} Increased mechanical stresses in the myocardium during cardiac

Abbreviations and Acronyms

ANOVA	= analysis of variance
GAPDH	= glyceraldehyde-3-phosphate dehydrogenase
LAD	= left anterior descending coronary artery
LV	= left ventricular
LVEDP	= left ventricular end-diastolic pressure
MI	= myocardial infarction
PARP	= poly (adenosine diphosphate-ribose) polymerase
TUNEL	= terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
VDAC	= mitochondrial porin

remodeling have been shown to activate stretch-activated signaling pathways and apoptotic molecular events.^{5,6}

Studies have shown that myocyte apoptosis occurs in the infarct, peri-infarct (adjacent), and remote zone of myocardium after MI.⁷ Apoptosis is programmed cell death characterized by cell shrinkage, membrane blebbing, and DNA fragmentation.^{8,9} Both intrinsic and extrinsic pathways of apoptosis leading to cell death have been described. The intrinsic pathway relies on the release of mitochondrial cytochrome c into the cytosol whereas the extrinsic pathway relies on ligation of membrane-bound death receptors. Induction of either pathway leads to the activation of the aspartate-specific cysteine proteases such as caspase-3 and cleavage of poly (adenodiphosphate-ribose) polymerase (PARP).^{10,11}

The objective of this study was to correlate changes in remodeling strain (end-diastolic dimensional stretch) and fractional contraction with molecular changes observed in the infarct, adjacent, and remote regions of the myocardium in a post-MI model of cardiac remodeling.

Methods**Study Design**

Eight Dorsett hybrid sheep (male; weight, 60–70 kg) bred for laboratory use (Thomas Morris, Reisterstown, Md) were instrumented with subsequent creation of an anterolateral MI by coronary ligation. Four noninstrumented animals served as healthy tissue controls. LV geometry and function were measured with an echocardiogram. Sonomicrometry transducers were placed in the free wall of the LV to quantify the regional myocardial contractile function. At the time of terminal study, LV myocardial tissue samples from the excised heart from each animal were harvested in ice-cold solution and separated into the infarct region, adjacent region (which was defined as less than 2 cm from the edge of the infarct), and remote region for analysis of protein expression. 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 approved protocol by the Institutional Animal Care and Use Committee of the University of Maryland at Baltimore.

Surgical Protocol

In each animal, anesthesia was induced with thiopental sodium (10 mg/kg) and maintained by mechanical ventilation with 1% to 2% isoflurane mixed with oxygen (Draeger anesthesia monitor; North American Draeger, Telford, Pa). Surface electrocardiogram, arterial blood pressure, pulse oximeter, and esophageal temperature were continuously monitored during the surgical operation. The instrumented group underwent a left anterolateral thoracotomy with polypropylene snares placed around the first and second diagonal coronary arteries of the left anterior descending artery (LAD) and tunneled subcutaneously. The snares were momentarily tightened (<30 seconds) to demarcate the border of the future infarct. Sixteen sonomicrometry transducers (2 mm; Sonometrics Corporation, London, Ontario, Canada) were placed in the free wall of the LV. The transducers were placed in 3 groups, each having 5 transducers placed circumferentially from anterior wall to posterior LV wall. One transducer was placed at the LV apex. An ultrasound flow probe (20 mm; Transonic Systems, Inc, Ithaca, NY) was placed around the main pulmonary artery for cardiac output monitoring.

Infarction

Ten days after the initial operation, the sheep were reanesthetized. The subcutaneous snares were permanently tightened to cause an anterolateral MI and the animal was supported with epinephrine infusion according to MI protocol described previously.¹² The pre-MI and post-MI data (sonomicrometry, echocardiogram, and hemodynamics) were collected.

Data Collection and Analysis

Hemodynamic, echocardiographic, and sonomicrometry data were collected in the pre-MI and post-MI periods and 2, 6, and 8 weeks after MI. The hemodynamic parameters were measured with a Millar pressure transducer (Millar Instruments, Inc, Houston, Tex) placed in the LV cavity through the aortic valve via the femoral artery with the aid of a fluoroscope. Parameters measured included heart rate, systolic and diastolic arterial pressure, mean arterial pressure, and LV end-diastolic pressure (LVEDP). Cardiac output was measured with the transonic flow probe placed around the main pulmonary artery with the flowmeter. Transdiaphragmatic echocardiographic data were collected with a Sonos 5500 machine with a sterile covered transducer (Philips Medical, Andover, Mass). LV end-systolic and end-diastolic volumes, wall motion abnormalities, and ejection fractions were measured as previously described.¹³ Sonomicrometry data were collected with a commercially available digital sonomicrometry system (Sonometrics Corporation) to determine 3-dimensional motion and deformation of the LV free wall. Sonomicrometry data were used to calculate the fractional contraction (end-systolic strain) from end-diastole to end-systole over a cardiac cycle to quantify the regional contractile function of the myocardium. The end-diastolic and end-systolic time points were determined from the pressure and flow waveforms. Negative fractional contraction values as percent change over diastolic reference indicate functional contraction and positive values indicate dysfunctional dilatation during systole. The remodeling strain calculated between the geometries at end-diastole at one data collection time point and at end-diastole at the pre-MI measurement was used to quantify the progression of LV remodeling. We described the calculation of these strains previously.¹² In brief, an area strain measure

was used and calculated by comparing the area change of the paired triangles between different time points. Strain measurements were then calculated from the collected sonomicrometry transducer coordinate data to compile (1) fractional strain during cardiac contraction and (2) remodeling strain over the progression of myocardial remodeling as described above.

Immunohistochemistry Staining

The tissues sampled from the 3 regions (remote, adjacent, and infarct) were fixed in formalin for 24 hours, embedded in paraffin, and 5- μ m sections were obtained. The sections were then deparaffinized with xylene and rehydrated with a series of graded alcohol. The immunohistochemistry of cleaved caspase-3 was performed as previously described.¹⁴

Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-end Labeling Analysis

Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) with a commercially available apoptosis detection kit (Roche Diagnostics, Indianapolis, Ind). Histologic sections were prepared and stained according to the manufacturer's instructions. Positive control was prepared with DNase I (3 μ g/ml) (Roche Diagnostics) to induce DNA strand fragments. Negative control was obtained by omitting terminal transferase during the labeling procedure. For cell death, tissue sections were semiquantitatively evaluated by light microscopy (Carl Zeiss, Inc, Oberkochen, Germany).

Western Blot Analysis

Protein extraction and Western blotting were performed as described previously.¹² Primary antibodies used were anti-caspase-3, anti-cleaved caspase-3 (Asp175), anti-PARP, anti-VDAC (mitochondrial porin; Cell Signaling Technology, Danvers, Mass), anti-Bax, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies (Santa Cruz Biotechnology, Santa Cruz, Calif).

Isolation of Myocardial Cytosolic Fraction of Cytochrome c

Mitochondria were isolated from remote and adjacent regions of the LV myocardium by a mitochondria-isolation kit for tissue (Pierce, Rockford, Ill). Isolation of cytosolic fraction of cytochrome c was performed as described previously.¹⁵ The soluble cytosolic fraction was assayed for cytochrome c. Western blot was performed with specific anti-cytochrome c and GAPDH antibodies (Santa Cruz Biotechnology) under the conditions recommended by the manufacturer.

Statistical Analysis

All data are expressed as the mean $\pm$ SEM. One-way repeated-measures analysis of variance (ANOVA) was used to compare temporal changes in hemodynamic, echocardiographic, and strain data as well as the abundance of protein expression. One-way ANOVA was used to compare regional changes in strain data. All ANOVAs were performed by multiple comparisons with the least significant difference correction. The significance level was set at .05. Protein expression was correlated with the regional strain to explore the association of the strain with protein expression.

Results

Hemodynamics

The heart rate, mean arterial pressure, and cardiac output remained unchanged during the course of the study, but the LVEDP progressively increased over the 10-week period. The LVEDP at baseline, 30 minutes after MI, 2 weeks after MI, 6 weeks after MI, and at terminal study were 1.4 ± 0.4 , 1.4 ± 0.3 , 3.9 ± 0.7 ($P < .05$), 6.9 ± 0.7 ($P < .05$), and 8.1 ± 0.8 mm Hg ($P < .05$), respectively.

Echocardiographic analysis

Figure 1 summarizes the echocardiographic data at the different study time points. Results from the measurement of cardiac function showed increases in LV end-diastolic volume from 86.39 ± 11.02 mL to 132.47 ± 16.04 mL ($P < .05$) and LV end-systolic volume from 40.49 ± 8.15 mL to 93.46 ± 12.85 mL ($P < .05$) with a decrease in ejection fraction from $54.19\% \pm 5.68\%$ to $30.55\% \pm 2.72\%$ ($P < .05$) during the study period.

Strain Data

Figures E1 and E2 summarize the time-dependent regional strain calculated from sonomicrometry. The fractional contraction values in the infarct, adjacent, and remote regions of the LV myocardium decreased from $-12.85\% \pm 1.48\%$ to $-0.36\% \pm 0.79\%$, $-14.05\% \pm 0.79\%$ to $-5.35\% \pm 0.76\%$, and $-15.23\% \pm 1.20\%$ to $-14.69\% \pm 1.33\%$, respectively, at terminal study. This implies the infarct zone lost its contractile function (akinetic) immediately, the adjacent zone showed a gradual decrease in its fractional contraction (hypokinetic), and the remote zone remained almost

Figure 1. Summary of changes observed in left ventricular end-systolic volume (LVESV), left ventricular end-diastolic volume (LVEDV), and ejection fraction (EF) over time on echocardiogram. Increase in LVEDV and LVSV with decrease in EF show a global LV chamber dilatation and systolic dysfunction. All values are given as mean $\pm$ standard error of the mean. Symbols *, †, ‡, and Δ indicate $P \leq 0.05$ as compared with preinfarct, postinfarct, 2 weeks, and 6 weeks, respectively.

unchanged. Over the course of the 10-week post-MI period, the remodeling strain was measured as $51.06\% \pm 11.48\%$, $39.86\% \pm 8.47\%$, and $16.04\% \pm 5.02\%$ in the infarct, adjacent, and remote regions of the LV myocardium, respectively. Both the infarct and adjacent zones showed significant degrees of dilatation during cardiac remodeling whereas the remote region exhibited a lesser areal expansion.

TUNEL Analysis

Figure 2 shows photographs of typical TUNEL-stained sections of tissue from remote, adjacent, and infarct regions of the LV myocardium. The nucleus of apoptotic cells stains brown. The number of TUNEL-positive cells in the infarct and adjacent regions of the myocardium was higher than in the remote and normal tissues.

Immunohistochemistry Staining With Caspase-3

As expected, caspase-3 intensity was higher in the infarct region than in the adjacent and remote regions, as shown in Figure 2. The adjacent region showed mild caspase-3 activity and the remote region stained for minimal caspase-3 activity.

Mitochondrium-mediated Apoptotic Protein Expression

The infarct region showed more than a 2-fold increase in expression of mitochondrial Bax, cleaved caspase-3, and PARP as compared with the remote region (Figure 3). There was no significant regional change in the protein expression of cytochrome c. Although the infarct region showed the most alteration in the expression of cleaved caspase-3 and PARP from normal, the remote region exhibited some level of caspase-3 and PARP activity.

Correlation of Protein Expression With Strain

A positive relationship is observed for fractional contraction versus expression of mitochondrial Bax ($r^2 = 0.27$), cleaved caspase-3 ($r^2 = 0.31$), and PARP ($r^2 = 0.30$), whereas a weak relationship was demonstrated for cytochrome c ($r^2 = 0.02$). Likewise, a positive relationship was observed for remodeling strain versus expression of mitochondrial Bax ($r^2 = 0.28$), cleaved caspase-3 ($r^2 = 0.43$), and PARP ($r^2 = 0.37$), and a weak relationship was observed for cytochrome c ($r^2 = 0.02$). Figures 4 and 5 illustrate that the more contractile and less remodeled the region of the myocardium, the lower the expression of mitochondrial Bax, cytochrome c, cleaved caspase-3, and PARP.

Discussion

Programmed cell death has been reported in patients with several clinical heart diseases and experimental heart failure models. Progressive cardiac remodeling is characterized by progressive loss of myocytes in the adjacent region, thinning of infarct wall, and chamber dilatation.¹⁶⁻¹⁸ Although necrosis as a form of cell death is the predominant process in the infarct region in the first 24 to 48 hours after MI, apoptosis is the major form of cell death during progressive cardiac remodeling after MI, with apoptosis higher in the adjacent region than remote region and control myocardium.^{16,19}

There is increasing evidence that increased wall stress from myocardial stretch (strain) during cardiac remodeling induces the apoptotic pathway, and apoptotic cell loss contributes to progressive ventricular dilation and systolic dysfunction.^{5,6,20} Apoptosis can be induced by stimuli with membrane death receptor or by mitochondrium-mediated pathway via the release of cytochrome c, resulting in the downstream activation of caspase-3, an important molecule

Figure 2. The infarct region shows a greater number of TUNEL-positive cells and the highest caspase-3 intensity. The adjacent region shows a lesser number of TUNEL-stained cells with a mild caspase-3 activity and remote region shows the fewest TUNEL-positive cells with minimal caspase-3 staining. Normal tissue is not shown but stained similar to the remote region of the LV myocardium.

Figure 3. A, Representative Western blot. B, Relative abundance of protein expression: Poly (ADP-ribose) polymerase (PARP), caspase-3, and cytochrome c (Cyto c) normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and mitochondria Bax (Mito Bax) normalized to mitochondrial porin (VDAC). Symbols * and † indicate $P \leq .05$ as compared with tissue in the adjacent region and the infarction region of the LV myocardium, respectively. All values are given as mean $\pm$ SEM.

in the determination phase of apoptosis.²¹ The pathway of interest is via the mitochondrial origin, and on induction of apoptosis, the mitochondrial membrane destabilizes with translocation of cytosol Bax into the mitochondria and altering the release of cytochrome c into the cytosol.²² Cytochrome c then activates caspase-9 and caspase-3 sequentially with resultant cleavage of PARP leading to apoptosis.^{23,24}

In the present study, apoptosis was documented histologically with the TUNEL assay and protein expression of mediators by Western blot. Significantly higher levels of proapoptotic Bax, cytochrome c, caspase-3, and PARP proteins were seen in the adjacent region than remote region of the myocardium. Results from our strain measurement indicate that there are regional strain differences in the remote, adjacent, and infarct regions of the myocardium after MI

during cardiac remodeling. Although there was a general increasing trend in the remodeling strain, the largest increase was found to be in the infarct region. The fractional contraction was noted to be decreasing from remote to the infarct region. In this study, we investigated the relationship of both the fractional contraction and remodeling strain (areal dilatation) to alterations in apoptotic protein expression (mitochondrial Bax, cytochrome c, caspase-3, and PARP) during cardiac remodeling. Our results show a direct correlation between apoptosis versus fractional contraction and remodeling strain during cardiac remodeling after MI. This observation is supported by the regional distribution of apoptosis being significantly higher in the infarct region, in which strain (mechanical stress) is typically higher than that in the adjacent and remote regions of the myocardium. The remodeling changes were consistent with global changes in

Figure 4. Normalized cytochrome c, mitochondrial Bax, caspase-3 and poly (ADP-ribose) polymerase versus fractional contraction. For abbreviations, see Figure 3.

vivo in LVDEP, echocardiogram-confirmed LV end-diastolic volume, ejection fraction, and wall motion abnormalities. Significant cardiac remodeling was observed by 8 weeks after MI in our ovine model as the LV chamber enlarged, and significant increase in mechanical wall stress (strain) induced with decreased fractional contraction in the adjacent region of the myocardium and with compensatory hypercontractility in the remote region.²⁵ These changes during cardiac remodeling are associated with an increase in angiotensin II, catecholamines, and various cytokines and stretch stress, all of which are potential inducers of apoptosis leading to cardiac functional impairment.²⁶ Our study indicates that regional activation of apoptosis correlates strongly with fractional contraction and remodeling

strain during cardiac remodeling. We speculate that myocardial wall stretch (strain) through mechanotransduction is the initial driving force and impetus behind myocardial apoptotic signaling cascade and myocardial cell death after MI during progression of cardiac remodeling into heart failure. Various studies have shown reversal of cardiac remodeling and restoration of cardiac protein expression and function by reducing myocardial strain in post-MI heart failure models through LV assist devices, passive constraints, and ventricular endocardial restoration procedures.²⁷⁻²⁹ It is our hope that reducing myocardial strain by mechanically unloading the LV during cardiac remodeling will inhibit progressive apoptosis in the adjacent and remote regions of the myocardium.

Figure 5. Normalized cytochrome c, mitochondrial Bax, caspase-3, and poly (ADP-ribose) polymerase versus remodeling strain. For abbreviations, see Figure 3.

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Discussion

Dr Robert C. Gorman (Philadelphia, Pa). This is an interesting and potentially important study because you are trying to assess how mechanical events in the heart that occur after an infarct affect the biology of the remaining normally perfused myocardium.

As surgeons, we have focused primarily on influencing those geometric properties with our surgical treatments for heart failure, with the tacit assumption that the remaining myocardium is inherently normal. This line of work is very important because it may help to explain why our results for surgical heart failure are not as good as we would expect after we return the heart to normal geometry, being that the existing myocardium has some inherent changes in it that have altered it beyond surgical repair.

That being said, I have 2 questions in regard to the technical performance of your study and then one theoretical question with regard to the therapeutic implications for your study.

You presented your sonomicrometry data that demonstrated the regional contractile function, and I am a little concerned that the contractile function within the infarct itself still showed contractile properties. In our long experience with sonomicrometry in this model, typically we see a very dyskinetic effect within the infarcts demonstrating a lot of infarct expansion. You did not show that, so I would be interested in your explaining that to us.

Second, when you are measuring cytochrome c, are you trying to measure cytosolic cytochrome c?

Dr Yankey. Yes, sir.

Dr Gorman. I am a little concerned about your normal tissue, which shows a tremendous amount of cytosolic cytochrome c, which we would not expect to see. That is a typical problem with trying to measure that property. You have to degrade the myocardium, and you wind up banging up the mitochondria and getting cytochrome c all over the place. It is an important end point, but we found it difficult to measure.

Dr Yankey. With regard to the first question pertaining to the fractional contraction of the infarct region, we looked at about 8 sheep and we took an average of the various fractional contraction values. When we looked at it over an 8-week period, it was a positive value that indicates dysfunctional dilatation of the heart.

Some of the sonomicrometric data that we looked at did have a few values showing the dilatation, but we evaluated only an 8-week period. I believe that if we had looked longer than that, we would have seen much greater positive values of the fractional contraction.

Pertaining to cytosolic cytochrome c, I do understand the problem you are addressing, but it was very difficult to actually get the normal value. We just did the best that we could. That is the main reason why we chose to also look at the mitochondrial Bax and then show the values for caspase 3 and PARP as well. I do understand, but that was the best that we could do to show that.

Dr Gorman. I want to expand on my question. The issue is that you showed how stretch affects, potentially, apoptosis. Should we be trying to treat mechanical stretch early with surgical means or should we be looking for pharmacologic techniques for preventing apoptosis?

Dr Yankey. I think we should try to treat stretch in the earlier phases, so that we can at least prevent the progressive apoptotic cell loss in the adjacent regions and then the ongoing problem in the remote areas as well. It is very important that we do intervene as soon as possible so that we can prevent this apoptotic cascade.

Dr Gorman. One other thing: When you do your correlation analysis with your apoptosis and stretch, you really should not put your infarct data in that. Apoptosis in the infarct, of which you showed a tremendous amount, is not really the phenomenon we are interested in. We are interested in apoptosis in the normally perfused regions. I am not sure that apoptosis in the infarct is not a good thing. That may be associated with scar remodeling and things like that. That is one thing to consider.

Figure E1. Summary of changes observed in fractional contraction and in infarct, remote, and adjacent zones of LV myocardium at different study time points. The infarct region shows loss of contractile immediately after the MI becoming akinetic. Adjacent region shows a statistically significant loss of functional contraction by 2 weeks and becomes hypokinetic by 6 to 8 weeks. The remote region shows preserved contractile function by the end of the study. All values are given as mean $\pm$ SEM. Symbols * and † indicate $P \leq .05$ as compared with remote and adjacent regions, respectively. Symbols ‡ and Δ indicate $P \leq .05$ as compared with preinfarct and 6 weeks, respectively.

Figure E2. Summary of changes observed in remodeling strain in infarct, remote, and adjacent zones of LV myocardium at different study time points. The infarct displays the greatest remodeling strain. The difference in the degree of remodeling between the adjacent and the remote zones is statistically significant at both 6 weeks and time of termination. All values are given as mean $\pm$ SEM. Symbols * and † indicate $P \leq .05$ as compared with remote and adjacent regions, respectively. Symbols ‡, Δ, and # indicate $P \leq .05$ as compared with postinfarct, 2 weeks, and 6 weeks, respectively.