Myocardial Infarction

Chronic Akt1 Deficiency Attenuates Adverse Remodeling and Enhances Angiogenesis After Myocardial Infarction

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Background—Akt1 is a key signaling molecule in multiple cell types, including endothelial cells. Accordingly, Akt1 was proposed as a therapeutic target for ischemic injury in the context of myocardial infarction (MI). The aim of this study was to use multimodal in vivo imaging to investigate the impact of systemic Akt1 deficiency on cardiac function and angiogenesis before and after MI.

Methods and Results—In vivo cardiac MRI was performed before and at days 1, 8, 15, and 29 to 30 after MI induction for wild-type, heterozygous, and Akt1-deficient mice. Noninfarcted hearts were imaged using ex vivo stereomicroscopy and microcomputed tomography. Histological examination was performed for noninfarcted hearts and for hearts at days 8 and 29 to 30 after MI. MRI revealed mildly decreased baseline cardiac function in Akt1 null mice, whereas ex vivo stereomicroscopy and microcomputed tomography revealed substantially reduced coronary macrovasculature. After MI, Akt1−/− mice demonstrated significantly attenuated ventricular remodeling and a smaller decrease in ejection fraction. At 8 days after MI, a larger functional capillary network at the remote and border zone, accompanied by reduced scar extension, preserved cardiac function, and enhanced border zone wall thickening, was observed in Akt1−/− mice when compared with littermate controls.

Conclusions—Using multimodal imaging to probe the role of Akt1 in cardiac function and remodeling after MI, this study revealed reduced adverse remodeling in Akt1-deficient mice after MI. Augmented myocardial angiogenesis coupled with a more functional myocardial capillary network may facilitate revascularization and therefore be responsible for preservation of infarcted myocardium. (Circ Cardiovasc Imaging. 2013;6:992-1000.)

Key Words: Akt1 protein, mouse ■ angiogenesis, pathologic ■ heart ■ myocardial infarction

Cardiovascular disease remains a leading cause of mortality in Western nations.1 Thus, molecular pathways affecting cardiac disease are of interest as potential targets for therapy and companion diagnostics. Akt kinases are serine threonine kinases controlling essential cellular functions including survival, proliferation, metabolism, and patterned gene expression.2 Akt1 is the predominant isoform in vascular endothelial cells and has been shown to play a crucial role in physiological and pathological angiogenesis.3−5 Many of the angiogenic functions attributed to vascular endothelial growth factor A (VEGF-A)6 are mediated by intracellular activation of the phosphoinositide 3-kinase–Akt signaling pathway.3 Previous studies, including those from our laboratory, have reported reduced angiogenesis in Akt1-deficient placenta4,6 and bone.5,7 These findings, as well as the complex interplay between VEGF-A and Akt,3 led us to investigate myocardial angiogenesis before and after myocardial infarction (MI) in mice that systemically lacked Akt1 (Akt1−/−).

Clinical Perspective on p 1000

After MI, neovascularization in the border zone adjacent to the ischemic region helps to preserve cardiac function and thereby attenuate adverse left ventricular (LV) remodeling.8 Neovascularization is orchestrated by interactions between proangiogenic and antiangiogenic factors, the extracellular matrix, and endothelial cells and pericytes at the border zone.9 Given the role of Akt signaling in the heart in modulating cardiac growth, contractile function, and coronary angiogenesis,8 Akt has emerged as a potential target for gene therapy approaches to improve neovascularization and thus cardiomyocyte survival within the healing heart.10 For example, acute Akt overexpression demonstrated cardioprotective

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behavior via enhanced angiogenesis and reduced dysfunction in rats after MI. Recently, Akt has been described as playing a key role in stimulation of mesenchymal stem cell–mediated cardiac repair. However, the role of Akt1 in cardioprotection is multifaceted because chronic Akt1 overexpression was shown to impair angiogenesis in mice, resulting in increased contractile dysfunction at 6 weeks after activation of the transgene. Moreover, human samples with advanced heart failure demonstrated enhanced Akt phosphorylation, suggesting that Akt activation may not be sufficient for long-term cardioprotection.

In vivo cardiac MRI has emerged as a reference standard modality for sequential and minimally invasive 3-dimensional characterization of ventricular structure and function in patients and preclinical mouse models. In the current study, we aimed to investigate the role of Akt1 deficiency first in myocardial angiogenesis in the healthy heart and then in structural and functional aspects of infarct healing, applying multiple in vivo and ex vivo imaging techniques.

### Methods

#### Animals

Animal experiments were approved by the Weizmann Institutional Animal Care and Use Committee following US National Institutes of Health, European Commission, and Israeli guidelines. Overall, 33 wild-type (Akt1+/+), 32 heterozygous (Akt1+/–), and 32 Akt1–/– mice were studied before and after MI. Male Akt1+/+, Akt1+/–, and Akt1–/– mice were generated through heterozygous crossbreeding pairs, and age-matched littermates were used. Mice were housed at 21°C±1°C, 40% to 50% humidity, on a 12-hour light–dark cycle, with ad libitum access to water and standard rodent food. At 13±0.5 weeks of age, normal male adult mice (Akt1+/+: n=15; Akt1+/–: n=15; Akt1–/–: n=16) were imaged using cardiac MRI and underwent MI surgery a few days later (see below). In addition, normal adult mice were euthanized for analysis of macrovasculature by ex vivo microcomputed tomography (n=1 for each genotype) for functional analysis of blood vessels and histology (Akt1+/+: n=5; Akt1+/–: n=5; Akt1–/–: n=5) or for quantitative real-time polymerase chain reaction (qRT-PCR) (Akt1+/+: n=5; Akt1+/–: n=4; Akt1–/–: n=4). One day after successful MI surgery, infarct size was determined using late gadolinium-enhanced (LGE) MRI. Mice were randomly assigned to either an MRI follow-up study terminated at day 29 after infarction for histological examination (Akt1+/+: n=7; Akt1+/–: n=8; Akt1–/–: n=8) or were euthanized at day 8 after MI for functional analysis of blood vessels and histology (Akt1+/+: n=4; Akt1+/–: n=6; Akt1–/–: n=3) or for RT-PCR (Akt1+/+: n=4; Akt1+/–: n=4; Akt1–/–: n=3).

#### Genotyping

See Methods in the online-only Data Supplement.

#### Surgically Induced MI

MI was induced as previously described by ligating the left coronary artery. Briefly, mice were anesthetized by intraperitoneal administration of ketamine (75 mg/kg; Fort Dodge, IA) and xylazine (3 mg/kg; VMD, Arendonk, Belgium). Analgesia included buprenorphine (0.05 mg/kg IP) and lidocaine (1 mg/kg) at the incision site. The trachea was intubated for positive pressure ventilation (12 strokes/min, 200 mL stroke volume; Harvard Ventilator 687, Harvard apparatus, Holliston, MA), and the chest was clipped and aseptically prepared. A left thoracotomy was performed in the fourth intercostal space, and the left coronary artery was permanently ligated (8-0 prolene suture; Johnson & Johnson Medical Ltd, Wokingham, United Kingdom) at its emergence from under the left atrium. Survival after experimental MI was similar (Akt1+/+: 85.7%; Akt1+/–: 84.6%; Akt1–/–: 83.3%).

#### Cardiac MRI

MI was performed at age 13±0.5 weeks and 1 day after MI in all mice. In mice assigned to an MRI follow-up study, MI was performed on days 8, 15, and 29 to after MI. MI was performed on a 9.4T horizontal scanner using a linear resonator for excitation and an actively decoupled 2-cm surface coil for detection (Bruker, Ettlingen, Germany). Mice were anesthetized with isoflurane (3.5% induction; 1.5% maintenance), and body temperature was maintained at 37°C±0.4°C using a circulating water system and monitored with rectal thermometry. Heart rate was monitored using ECG electrodes placed on the front paws, and isoflurane levels were adjusted to maintain heart rate at 485±25 bpm throughout imaging. Respiration rate was monitored and maintained at 110±11 bpm with a respiratory sensor balloon placed on the abdomen (SA Instruments Model 1025, Stony Brook, NY).

#### Cine Imaging

Acquisition of cine images at both long-axis and multiple short-axis orientations was performed using a retrospectively gated fast low angle shot (FLASH) sequence. Specific parameters included 10 frames per cardiac cycle for both short-axis and long-axis images. Imaging parameters for short-axis acquisition were: 6 to 8 slices; slice thickness=0.8 mm; interslice distance=1 mm; flip angle=10°; repetition time=6,2716 ms; echo time=2.9106 ms; number of repetitions=80; matrix=256×128 with zero-filling to 256×256; field of view=25.6×25.6 mm2; and acquisition time=7 minutes. Imaging parameters for long-axis acquisition: slice thickness=1 mm; flip angle=10°; repetition time=5.97 ms; echo time=2.878 ms; number of repetitions=300; matrix=256×128 zero-filling to 256×256; field of view=40×40 mm; acquisition time=4 minutes.

#### Late Gadolinium-Enhanced MRI

Infarct size was assessed using LGE MRI at 1 day after MI after acquisition of all precontrast images as previously described. One hundred fifty microliters of gadolinium-diethylenetriamine-pentaacetic acid (Gd-DTPA, 0.1 mg/kg; Magnevist, Bayer Healthcare, Germany) was injected via an indwelling intraperitoneal line, and T1-weighted 3-dimensional images were acquired with a flip angle of 50°.

#### Basal Slice Definition

The basal slice of the LV was defined as the last slice that the myocardium was present on the LV cavity on short-axis images assigned on 2-chamber and 4-chamber views at end diastole (ED).

#### Image Analysis

End-diastolic volume, end-systolic volume, LV mass, wall thickness, LV inner radius, stroke volume and ejection fraction, cardiac output (CO), and infarct size were measured using freely available semiautomatic Segment software v1.9R1852 (http://segment.heiberg.se). CO was calculated as stroke volume×heart rate, and then indexed to the body mass (cardiac output index). Mass-to-volume ratio was calculated as the ratio of LV-mass-to-end-diastolic volume. Wall thickness and LV inner radius were measured at the midventricular short-axis image. Endocardial and epicardial borders were manually drawn on short-axis images. Infarct size was calculated via threshold-level analysis, with pixels demonstrating signal intensity >2.4 SD above the mean signal intensity of nonenhanced remote myocardium on LGE magnetic resonance images defined as infarcted myocardium.

#### Scar Size

Scarc size was measured on days 8, 15, and 29 after MI as the percent myocardial area with wall thickness <0.7 mm at ED in short-axis images. The border zone was defined as a 1-mm area circumferentially extending from the scar area (or the gadolinium-enhanced infarct for day 1) where the wall thickness varied from 0.7 mm to normal (0.8±0.3 mm). Wall thickness was calculated as a percentage of wall thickness (Th) during end systole (ES) relative to Th during ED, namely [Th (ES)−Th (ED)]×100/Th (ED).

#### Ex Vivo Microcomputed Tomography Imaging of Myocardial Macrovasculature

Noninfarcted mice were euthanized using an overdose of pentobarbital, and immediately afterward, the cardiac arteries were filled via the apex of the heart with radio-opaque silicone rubber microfil MV120 (FlowTech, Carver, MA). Whole hearts were rapidly excised, fixed...
with 4% paraformaldehyde, and imaged with 12.9-µm resolution using a Micro-XCT 400 system (Xradia, Pleasanton, CA). Image analyses, including volume rendering, were performed using Avizo (VSG International, Burlington, MA).

Functional Assay of Myocardial Vasculature

Mice were anesthetized by intraperitoneal administration of ketamine (75 mg/kg; Fort Dodge, IA) and xylazine (3 mg/kg; VMD, Arendonk, Belgium). Before euthanasia, bovine serum albumin (BSA) labeled with red-fluorescent 6-carboxy-X-rhodamine (BSA-ROX, 10 mg/ml in PBS, 10 µL/g) was intravenously injected via an indwelling tail vein catheter. When perfusion of red-fluorescent BSA-ROX was complete at 5 minutes after administration, animals were euthanized via pentobarbital overdose, hearts were excised, and the left coronary vein (LCV) was visualized using stereomicroscopy (SZX-RFL-2, Olympus, Tokyo, Japan) equipped with a fluorescence illuminator and a Pixelfly QE-CCD camera (PCO, Kelheim, Germany; excitation: 550 nm; emission: 605 nm; fluorescence exposure: 25 ms). Aterward, hearts were fixed in Carnoy’s solution for histological examination of functional myocardial capillaries. For analysis of stereomicroscopic images, ImageJ was used to threshold images, and AngioTool was used to evaluate the LCV2 (National Institutes of Health, Bethesda, MD).

Histology and Fluorescence Microscopy

Hearts from each group of mice were isolated and fixed before MI, and at 8 to 29 to 30 days after MI; hearts were embedded in paraffin and sectioned serially at a thickness of 4 µm. Baseline tissue sections that contained BSA-ROX were stained with either hematoxylin and eosin for morphology or Bandeiraea simplicifolia isolecitin B4 (lectin-fluorescent isothiocyanate [FITC]) staining for endothelial cells and endothelial precursor (1:100; Sigma-Aldrich L2895, Saint Louis, MO). Tissue sections acquired 8 days after MI from hearts containing BSA-ROX were stained with lectin for endothelial cells, and Mac-2 staining for activated macrophages (1:200; Cedarlane). The border zone was defined as a 1-mm area circumferentially extending from the scar area (see above). In long-axis sections of the LV, the border zone was analyzed on the anterior wall. The number of blood vessels was quantified on sections containing BSA-ROX day 8 after MI. Sections of hearts 29 to 30 days after MI were stained with Trichrome Masson for collagen and alpha smooth muscle actin (αSMA) for (myo)fibroblasts. Light microscopic images were taken with a Nikon E800 camera. Fluorescent images were acquired on an Axio observer microscope (Zeiss, Jena, Germany) equipped with a fluorescence illuminator and an Olympus DP72 camera using the Cell^A camera-controlling software (Olympus, Center Valley, PA).

Quantitative Histological Analysis

See Methods in the online-only Data Supplement.

RNA Extraction and qRT-PCR

qRT-PCR was performed on hearts excised from mice at 13±0.5 weeks of age and at 8 days after MI. After euthanasia, hearts were rapidly excised, frozen, and RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel, Duren, Germany). Total RNA (200 ng) was used for reverse transcription using SuperScript II RNase H-reverse (Invitrogen, Carlsbad, CA). qRT-PCR was performed (StepOnePlus QRT-PCR system, Applied Biosystems, Carlsbad, CA) with the following primers: mouse VEGF-A (forward: 5′-cattcggcccctgcatca and reverse: 5′-gggtagaaccgctatta). The transcription level was normalized to the expression of hypoxanthine phosphoribosyltransferase (forward: 5′-GGTCTTITITTACCAACGCAA and reverse: 5′-GCAGTACGCCCCAAATGTGG). Each sample was run at least in duplicates.

Statistical Analysis

All data are presented as mean±SEM unless otherwise noted. Statistical analysis was performed with Statistix 8 (Analytical Software, Tallahassee, FL). Data were analyzed for normal distribution by Shapiro–Wilk test and for equal variances between groups by Bartlett test. Baseline MRI data, histological, and immunohistochemical data were analyzed using 1-way ANOVA followed by Tukey (honestly significant difference) HSD All-Pairwise Comparisons test. After MI, cardiac MRI parameters were analyzed using repeated measures ANOVA, followed by (least significant difference) LSD All-Pairwise Comparisons test. Pearson χ² test analysis was used to compare proportional data. Differences were considered significant at P<0.05.

Results

Reduced Cardiac Function and Altered Morphology in Adult Akt1-Deficient Mice

Representative long-axis and midventricular short-axis end-diastolic magnetic resonance images of each genotype are shown in Figure 1A and 1B. MRI revealed changes in cardiac structure and function in Akt1−/− mice. LV cavity size, indicated by end-diastolic volume and end-systolic volume, and LV mass were significantly lower in adult Akt1−/− mice compared with Akt1+/− mice (Table). When cardiac parameters were normalized to size relative to age-matched Akt1+/+ and Akt1−/− mice, these alterations were normalized because of a significantly reduced body weight in Akt1−/− compared with Akt1+/− mice. Consequently, the ratio of LV-mass-to-body-mass ratio showed no statistically significant differences between Akt1+/+ and Akt1−/− mice. LV inner radius was not statistically different for all groups. Both mass-to-volume ratio and wall thickness were significantly increased in Akt1−/− mice, suggesting modest hypertrophy, whereas stroke volume and CO were lower when compared with control mice (Table). Heart rates were similar for all 3 groups. Reduced ejection fraction, stroke volume, and CO were observed in baseline Akt1−/− mice. However, when CO was normalized to the body mass, cardiac output index showed the same trend, but was not significantly different between the groups (Table). Although myocyte cross-sectional area, a histological measure for hypertrophy, was not increased in Akt1−/− hearts, it was significantly reduced in Akt1−/− hearts compared with Akt1+/+ hearts (Figure 1A and 1B in the online-only Data Supplement).

Increased Functional Microcirculation Accompanies Reduced Coronary Macrovasculature in Adult Akt1-Deficient Mice

Volume-rendered microcomputed tomography images of the coronary vasculature filled with radio-opaque silicone rubber revealed smaller and less branched left coronary artery in Akt1−/− heart (Figure 1C). Stereomicroscopic images of the LCV after intravenous injection of red-fluorescent BSA-ROX revealed reduced branching of the LCV in Akt1−/− hearts (Figure 1D). The number of junctions and total vessel length/LV mass of the LCV were significantly reduced in Akt1−/− hearts (19.0±1.9, 1.2±0.04 mm/g, respectively; P<0.0310, P=0.0179, respectively; Figure 1E and 1F). Akt1−/− hearts showed intermediate phenotype between that of Akt1+/+ and Akt1+/− hearts (Figure 1C–1F), consistent with previous reports. Despite the large difference in the macrocirculation, the endothelial cell density in Akt1+/− hearts appeared similar to Akt1+/+ as assessed by percent area of lectin-FITC stained endothelial cells. However, the functional volume of the same vessels, assessed as the area demonstrating red-fluorescent
signal from vascular BSA-ROX, was increased in Akt1−/− hearts (3.78±0.29%) compared with Akt1+/+ hearts (2.31±0.63%; P=0.0157). Interstitial BSA-ROX was negligible. Vascular BSA-ROX was mainly increased because of enhanced capillary dilatation in Akt1−/− hearts as a larger capillary vessel diameter was measured (14.0±0.3 μm) than in Akt1+/+ hearts (10.9±0.4 μm; P=0.0006; Figure 2A–2E). qRT-PCR of baseline hearts revealed mildly augmented VEGF-A expression in Akt1−/− mice (1.24±0.05) compared with Akt1+/+ (1.00±0.2; P=0.0335) and Akt1+/− mice (1.08±0.2; Figure 2F).

Improved LV Function and Remodeling in Akt1−/− Mice After MI
Sequential cardiac MRI was applied to image the hearts of adult mice before and on days 1, 8, 15, and 29 to 30 after MI (Figure 3A). After permanently occluding the left coronary artery, permanent MI was induced in Akt1−/−, Akt1+/−, and Akt1+/+ mice. Table 1 shows that after MI, Akt1−/− mice had improved LV function and remodeling compared with Akt1+/+ mice. LV end-diastolic volume was reduced in Akt1−/− mice (46.5±1.6 μL) compared with Akt1+/+ mice (56.8±2.6 μL; P=0.0115). Similarly, LV end-systolic volume was reduced in Akt1−/− mice (23.4±1.5 μL) compared with Akt1+/+ mice (24.2±1.7 μL; P=0.0748). LV mass was reduced in Akt1−/− mice (76.5±3.1 mg) compared with Akt1+/+ mice (88.8±2.5 mg; P=0.0001). Body mass was also reduced in Akt1−/− mice (22.7±0.7 g) compared with Akt1+/+ mice (26.9±0.6 g; P=0.0008). LVM/body mass ratio was reduced in Akt1−/− mice (3.31±0.15 mg/g) compared with Akt1+/+ mice (3.80±0.13 mg/g; P=0.0046). LV wall thickness was reduced in Akt1−/− mice (0.90±0.02 mm) compared with Akt1+/+ mice (1.00±0.03 mm; P=0.0604). LV inner radius (LVri) was similar across genotypes (1.84±0.05 mm vs. 1.76±0.04 mm; P=0.3380). Wall thickness/LVri ratio was also similar across genotypes (0.49±0.018 vs. 0.59±0.024; P=0.1106). Heart rate was similar across genotypes (475±14 bpm vs. 469±18 bpm; P=0.5177). Ejection fraction was increased in Akt1−/− mice (51.7±1.4%) compared with Akt1+/+ mice (55.1±1.2%; P=0.0023). Stroke volume was increased in Akt1−/− mice (26.6±1.1 μL) compared with Akt1+/+ mice (22.6±1.0 μL; P=0.0008). Cardiac output was also increased in Akt1−/− mice (11.2±0.6 μL/min) compared with Akt1+/+ mice (12.2±0.7 μL/min; P=0.0007). Cardiac output index was increased in Akt1−/− mice (498±25 μL/min/g) compared with Akt1+/+ mice (449±32 μL/min/g; P=0.0542).

Table 1. Baseline Left Ventricular Morphology and Function

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Akt1+/+ (n=15)</th>
<th>Akt1+/− (n=15)</th>
<th>Akt1−/− (n=16)</th>
<th>P Value</th>
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<tr>
<td>End-diastolic volume, μL</td>
<td>56.8±2.6</td>
<td>48.5±2.1</td>
<td>46.5±1.6*</td>
<td>0.0115</td>
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<td>End-systolic volume, μL</td>
<td>24.2±1.7</td>
<td>22.0±1.3</td>
<td>23.4±1.5</td>
<td>0.0748</td>
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<td>LVM, mg</td>
<td>88.8±2.5</td>
<td>95.0±2.5</td>
<td>76.5±3.1*</td>
<td>0.0001</td>
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<td>Body mass, g</td>
<td>26.9±0.6</td>
<td>25.3±0.8</td>
<td>22.7±0.7*</td>
<td>0.0008</td>
</tr>
<tr>
<td>LVM/body mass, mg/g</td>
<td>3.31±0.07</td>
<td>3.80±0.13*</td>
<td>3.40±0.15</td>
<td>0.0046</td>
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<td>MVR, mg/μL</td>
<td>1.60±0.07</td>
<td>2.01±0.10*</td>
<td>1.68±0.10</td>
<td>0.0073</td>
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<td>Wall thickness, mm</td>
<td>0.90±0.02</td>
<td>1.00±0.03*</td>
<td>0.95±0.04</td>
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<td>LVr, mm</td>
<td>1.84±0.05</td>
<td>1.76±0.04</td>
<td>1.72±0.06</td>
<td>0.3380</td>
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<tr>
<td>Wall thickness/LVr</td>
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<td>0.59±0.024</td>
<td>0.49±0.024</td>
<td>0.1106</td>
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<td>Heart rate, bpm</td>
<td>475±14</td>
<td>469±18</td>
<td>459±16</td>
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<td>Ejection fraction, %</td>
<td>58.6±1.2</td>
<td>55.1±1.2</td>
<td>51.7±1.4*</td>
<td>0.0023</td>
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<tr>
<td>Stroke volume, μL</td>
<td>31.9±1.5</td>
<td>26.6±1.1*</td>
<td>24.5±0.9*</td>
<td>0.0008</td>
</tr>
<tr>
<td>Cardiac output, μL/min</td>
<td>15.1±0.8</td>
<td>12.2±0.7*</td>
<td>11.2±0.6*</td>
<td>0.0007</td>
</tr>
<tr>
<td>Cardiac output index, μL/min/g</td>
<td>568±33</td>
<td>499±32</td>
<td>498±25</td>
<td>0.0542</td>
</tr>
</tbody>
</table>

LVM indicates left ventricular mass; LVr, LV inner radius; and MVR, mass-to-volume ratio.

*P<0.05 different from Akt1+/+.
Infarct size measured by LGE MRI (Figure 3B) was similar for all groups at 1 day after MI (Akt1<sup>+/+</sup>: 29.9±0.9%; Akt1<sup>+/−</sup>: 29.7±0.2%; Akt1<sup>−/−</sup>: 29.8±0.7%), as was the surgical survival rate (P=0.9936). Representative end-diastolic long-axis images (Movies I–III in the online-only Data Supplement) and corresponding Trichrome-stained long-axis sections acquired 29 days after MI are shown in Figure 3C–3E. Akt1<sup>−/−</sup> hearts demonstrated reduced loss of function, as indicated by significantly higher ejection fraction compared with Akt1<sup>+/+</sup> and Akt1<sup>+/−</sup> at days 8, 15, and 29 after MI (Figure 4A). Akt1<sup>−/−</sup> mice demonstrated significantly reduced LV remodeling as assessed by end-diastolic volume/body weight and wall thickness/LV inner radius. The time course of structural LV remodeling appeared abrogated in Akt1<sup>−/−</sup> mice from day 8 after MI, as evidenced by the absence of progressive LV dilation in Akt1<sup>−/−</sup> mice (Figure 4B and 4C). Although infarct size was similar among genotypes at day 1 after MI, the extent of scar expansion at 29 days after MI was reduced in Akt1<sup>−/−</sup> mice (Figure 4D). Border zone wall thickening of Akt1<sup>−/−</sup> infarcted hearts was significantly enhanced at 15 and 29 days after MI (Figure 4E).

**Enhanced Angiogenesis and Altered Scar Tissue Composition in Akt1 Null Mice After MI**

The remote myocardium in Akt1<sup>−/−</sup> mice exhibited an increased blood volume in microcirculation compared with Akt1<sup>+/−</sup> and Akt1<sup>+/+</sup> mice as measured by a larger percent area of intravenously administered red-fluorescent BSA-ROX. Interstitial BSA-ROX was increased in Akt1<sup>−/−</sup> myocardium (3.24±0.14%) compared with Akt1<sup>+/−</sup> myocardium (0.18±0.12%; P=0.0010), probably because of enhanced leakage. Vascular BSA-ROX also showed the same trend as in baseline mice, with elevated capillary dilatation in Akt1<sup>−/−</sup> mice (7.40±0.58%) compared with Akt1<sup>+/−</sup> mice (5.49±0.50%; P=0.0095). However, endothelial cell density (measured by percent area stained with lectin-FITC) was similar among groups (Figure 5A–5C, 5H, and 5J).

The border zone of Akt1<sup>−/−</sup> myocardium at day 8 after MI revealed less blue Trichrome staining and less densely packed collagen compared with Akt1<sup>+/−</sup> and Akt1<sup>+/+</sup> infarcted myocardium (Figure 5D). Infarct border zone blood vessels from all genotypes presented similar Mac-2 staining for activated macrophages (Figure 5E). Akt1<sup>−/−</sup> mice exhibited an increased amount of small- (110±17) and medium-size (35.6±4.4) blood vessels compared with Akt1<sup>+/−</sup> border zone blood vessels (44±15, 13.5±2.6, respectively; P=0.0022, P=0.0001, respectively; Figure 5E and 5I). qRT-PCR of the infarcted hearts 8 days after MI revealed increased expression of VEGF-A in Akt1<sup>−/−</sup> mice (2.58±0.03) compared with Akt1<sup>+/−</sup> (1.00±0.03; P=0.0023) and Akt1<sup>+/+</sup> mice (1.24±0.10; Figure 5K).

The scar tissue in infarcted hearts of Akt1<sup>−/−</sup> and Akt1<sup>+/−</sup> mice at 29 to 30 days after MI demonstrated collagen fibers...
that were well organized, dense, and tightly packed. However, the collagen matrix at 29 to 30 days after MI was less densely packed in Akt1^{−/−} hearts (44.7±4.3%) compared with collagen in Akt1^{+/−} hearts (71.7±4.3%; P=0.0027) as assessed by reduced Trichrome staining (Figure 5F and 5L). At 29 to 30 days after MI, αSMA staining, a marker for activated myofibroblasts, was increased in Akt1^{−/−} hearts (12.9±0.7%) compared with Akt1^{+/−} (7.2±1.3%; P=0.0031) and Akt1^{+/+} hearts (7.2±0.8%; Figure 5G and 5M).

**Discussion**

Systemic Akt1 deficiency is reported here to result in the following: (1) smaller adult hearts with reduced function, (2) shorter and less branched coronary macrovasculature, (3) greater functional capillary network, and (4) attenuated loss of global cardiac function after MI concomitant with an increased microcirculation and enhanced cardiac VEGF-A expression. Cardiac morphology and function were assessed using ex vivo imaging techniques, including microcomputed tomography, stereomicroscopy, and histological analysis.

The magnetic resonance images of baseline Akt1^{+/+} hearts, showing only slightly different Akt1^{+/+} cardiac function and morphology compared with Akt1^{+/−} mice, illustrated the ability of Akt1^{+/−} mice to compensate for reduced myocardial macrovasculatization. Cardiac MRI revealed a smaller but significant reduction of Akt1^{+/−} ejection fraction, which has not been detected before. Structural and functional measurements in Akt1^{+/−} mice were comparable with previously published data. The shorter and less branched coronary macrovasculature found in Akt1^{+/−} hearts is consistent with reduced vasculogenesism during Akt1^{+/−} cardiac development. We found that Akt1^{+/−} mice exhibited similar endothelial cell density but elevated volume inside functional myocardial capillaries with enhanced capillary dilatation, with enhanced VEGF-A expression compared with Akt1^{+/−} mice, possibly signaling a mechanism by which Akt1^{+/−} mice compensate for reduced macrovascular density, which resulted in preserved ventricular function in the noninfarcted heart. Such a change can be explained by microvascular dilatation.

Regulation of angiogenic responses by the Akt pathway has been proposed to rely on several compensatory mechanisms. The enlarged functional microcirculation in chronically Akt1^{+/−} mice seemed to compensate for reduced coronary macrovasculatization. These findings are consistent with previous findings of chronic Akt1 overexpression yielding impaired angiogenesis and contractile function and further illustrates the complex and differential short-term and long-term roles of Akt1 in myocardial and endothelial cells. This consideration applies also to the slight hypertrophic features seen in baseline Akt1^{+/−} hearts.

Serial imaging of ventricular structure and function after MI revealed that systemic ablation of Akt1 decreased adverse remodeling and improved global cardiac function. Abrogation of LV remodeling with reduced scar extension in Akt1^{+/−} mice was first observed at 8 days after MI. At day 8, we reported increased angiogenesis in Akt1^{+/−} hearts, with a larger functional capillary network, elevated leakage, augmented VEGF-A levels in the infarcted heart, reduced scar expansion, and enhanced border zone thickening. Although the overall effects of angiogenesis on the myocardium after MI remain unclear, enhanced angiogenesis may have a positive effect on adverse remodeling. For example, increased VEGF-A expression in the infarct border zone has been shown to inhibit apoptosis of cardiomyocytes. Enhanced angiogenesis within the healing heart enables greater delivery of nutrients, bioactive molecules, and oxygen and thereby may contribute to reduced scar expansion.

Histological analysis showed less densely packed collagen fibers within scar tissue at 8 and 29 days after MI in
Akt1−/− hearts, with increased myofibroblast density at 29 days after MI. Impaired collagen deposition may result from reduced fibronectin assembly by Akt1−/− fibroblasts as described previously. Abnormalities in extracellular matrix composition, associated with enhanced angiogenesis in Akt1−/−, were reported as a long-term effect of deficient Akt1 activation. Expansion of extracellular matrix has been linked to conductive abnormalities and life-threatening arrhythmias. Therefore, reduced collagen deposition in Akt1−/− might be beneficial for global cardiac function after MI. Persistence of myofibroblasts in the infarct area is vital because myofibroblasts counteract LV dilatation and hence restrict the development of chronic heart failure. Moreover, αSMA-immunoreactive myofibroblasts at Akt1−/− infarcts may reduce LV remodeling. No difference in staining of activated macrophages was observed between the genotypes 8 days after MI, suggesting no change in immune response.

This study made use of mice in which Akt1 was systemically ablated. Scar formation and adverse remodeling involve the interaction of multiple cell types, including macrophages, myofibroblasts, endothelial cells, as well as numerous other inflammatory and reparative cell types. The vascular phenotype reported here for the Akt1−/− hearts could reflect cell autonomous effects on processes mediated by endothelial cell expression of Akt1. However, it is clear that the observed vascular phenotype can also be mediated by expression of Akt1 in other cells including cardiomyocytes and stroma. Ongoing investigation of the effects of cell type–specific conditional deletion of Akt1 on the heart should focus not only on cardiac myocytes, but also on macrophages, stromal fibroblasts, vascular smooth muscle cells, and endothelial cells.

In conclusion, this noninvasive longitudinal study provides clear evidence that mice with chronic loss of Akt1 exhibit improved heart function and reduced LV remodeling after experimental MI. We postulate that reduced Akt1−/− LV remodeling after MI resulted from the presence of a larger functional collateral network and enhanced VEGF expression within the

**Figure 4.** Analysis of cardiac function using cardiac MRI follow-up after myocardial infarction (MI) showing reduced LV remodeling in Akt1−/− mice. **A**, Ejection fraction dropped immediately after MI in all groups, but did not deteriorate further in Akt1−/− mice as compared with other groups. **B**, End-diastolic volume/body weight (μL/g) was reduced in Akt1−/− hearts starting from day 8 after MI. **C**, Wall thickness/left ventricular (LV) inner radius measured at a midventricular short-axis image revealed decreased adverse remodeling in Akt1−/− mice. **D**, Although infarct size at day 1 was similar, time-dependent extension of the scar was reduced in Akt1−/− hearts at 29 days after MI. **E**, Border zone wall thickening in Akt1−/− hearts was enhanced at days 15 and 29 after MI (*P<0.05).
Akt\(^{1+/–}\) heart after MI with preserved function of the Akt\(^{1+/–}\) border zone. Because patients with major remodeling demonstrate progressive worsening of cardiac function, slowing adverse remodeling will be a goal of heart failure therapy. This study underlines that care should be taken when upregulating Akt in cardiac stem cells as a stimulator for cardiac repair. Long-term inhibition of Akt1 might offer an alternative therapeutic strategy aiming to reduce secondary damage caused by cardiac remodeling.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Akt1, the main isoform of Akt in the heart and the endothelium, regulates essential cellular functions within the cardiovascu-
lar system. In preclinical models, gene therapy approaches that upregulate Akt target improved neovascularization, and thus,
cardiomyocyte survival within the healing heart. Acute overactivation of Akt has shown cardioprotective features; however, human samples with advanced heart failure displayed enhanced Akt phosphorylation, suggesting that Akt activation alone may be insufficient for long-term cardioprotection. Because of the paradoxical functions of Akt in cardiovascular disease, the mechanism of cardioprotection with Akt1 deficiency remains unclear. This study uses multiple in vivo and ex vivo imaging techniques to demonstrate reduced adverse remodeling in mice systemically lacking Akt1 after experimental myocardial infarction. Preservation of infarcted myocardium with augmented myocardial angiogenesis and a greater functional myocardial capil-
lary network were observed in Akt1 knockout mice. The potential role of Akt1 in enhancing neovascularization within infarcted tissue is an attractive clinical target for reduced adverse remodeling and preserved function in heart failure therapy. Long-term pharmaceutical or genetic blockade of Akt1 may offer a novel therapeutic approach for reducing structural and vascular remodel-
ing. Moreover, our results highlight that care should be taken in upregulating Akt as a stimulator for cardiac repair.
Chronic Akt1 Deficiency Attenuates Adverse Remodeling and Enhances Angiogenesis After Myocardial Infarction

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Genotyping

Tail samples were lysed overnight at 55°C in the presence of proteinase-K and DNA lysis buffer (Sigma-Aldrich, Saint-Louis, US), and then denatured by heating at 85°C for 45 min. The resultant supernatant was used in a polymerase chain reaction (PCR) reaction to identify the genotype of weaned mice. The following primers were used in the PCR reaction: (a) \textit{Akt1} forward- 5’-TTGTTCCTACGCTTTCTCG-3’, (b) \textit{Akt1} reverse – 5’-5’-CCTGCTGGGTAGCTAGAAGA-3’, (c) LacZ-cassette – 5’-GCGGATTGACCAGTAATGG-3’; The amplified PCR product was subjected to agarose-gel electrophoresis and visualized using an infra-red detection system.

Quantitative histological analysis

To analyze positively stained cells or tissue on separate sections a computer-based image analysis with ImageJ was used. For the analysis of light microscopic images, namely Trichrome Masson’s and αSMA, a color threshold mask for immunostaining was defined to detect respectively red or blue color by sampling, and the same threshold was applied to all sections (3-5 sections/animal). The percentage of the total area with positive color for each section was calculated. Collagen volume fraction equals the sum of stained tissue divided by the total muscle area and connective tissue in the field of view (FOV) of the section. For fluorescent images, namely total BSA-ROX, lectin and Mac2, a threshold was defined for a certain red or green fluorescence intensity, dependent on the fluorescent marker and the same threshold was applied to all sections (3-5 sections/animal). For calculation of percent area interstitial BSA-ROX, the holes in the capillaries were filled in the thresholded lectin images and
subsequently subtracted from the thresholded BSA-ROX images (3-5 sections/animal). Vascular BSA-ROX was calculated by subtracting values for interstitial BSA-ROX area from values for total BSA-ROX area. For calculation of myocardial capillary diameter, 30-50 capillaries per section were measured from BSA-ROX images (3-5 sections/animal). For calculation of the number of vessels per FOV of the border zone 8 days after MI, 1mm² of the anterior wall myocardium adjacent to the scar was taken to count the amount of blood vessels of different sizes (0-100µm², 100-500µm² and >500µm²; 3-5 sections/animal). All measurements were done using imageJ software (NIH, Bethesda, MD, USA, http://imagej.nih.gov/ij/).

**Supplementary Figure and Figure legend**

**Supplementary Figure 1.**

**Histological analysis of adult Akt1⁺/⁺, Akt1⁺/⁻ and Akt1⁻/⁻ cardiomyocytes.** (a) Histological cardiac tissue sections of baseline hearts, stained with hematoxilin and eosin. (b) Myocyte cross-sectional areas from randomly selected high power fields were calculated on short axis H&E slices (40–60myocytes/heart). Quantification of myocyte cross-sectional area revealed smaller Akt1⁻/⁻ cardiomyocyte size.
Video Legends

Supplemental Movie 1. Four-chamber long axis cine image of Akt1+/+ 29 days after myocardial infarction.

Supplemental Movie 2. Four-chamber long axis cine image of Akt1+/− 29 days after myocardial infarction.

Supplemental Movie 3. Four-chamber long axis cine image of Akt1−/− 29 days after myocardial infarction, showing reduced LV remodeling.