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Absence of Regeneration in the MRL/MpJ Mouse Heart Following Infarction or Cryoinjury

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Summary

The MRL/MpJ mouse has been reported to exhibit minimal scarring and subsequent cardiac regeneration after cryoinjury of the right ventricle. We studied the temporal evolution of infarcts and cryoinjuries in MRL/MpJ and C57BL/6 control mice. Both types of injury in MRL/MpJ mice heal similarly to controls by typical scar formation rather than muscle regeneration, and no differences in cell proliferation, angiogenesis or scar contraction between the two mouse strains were observed.

Background—Myocardial infarcts in mammals heal by scar formation rather than formation of new muscle tissue. The MRL/MpJ mouse, however, has been reported to exhibit minimal scarring and subsequent cardiac regeneration after cryoinjury of the right ventricle. Other groups have reported that permanent and temporary ligation of the coronary artery resulted in scarring without regeneration.

Methods—To clarify these contradictory results, we studied the temporal evolution of infarcts in MRL/MpJ and C57BL/6 control mice from 1 to 90 days post-injury, and the effects of intrathoracic cryoinjury to 28 days.

Results—After infarction, the conversion from necrotic myocardium to granulation tissue and then to scar proceeded identically in the two groups. Infarct DNA synthesis, measured by incorporation of a BrdU pulse, peaked at 4 days in both strains and did not differ between strains at any time point. Endothelial cell and total vascular density in the both the infarcted and non-infarcted cardiac tissue did not differ between groups at any time. Histological analysis of directly cryoinjured right and left ventricular myocardium showed indistinguishable wound healing in both strains, and final scar size was identical in each group.

Conclusions—These studies demonstrate that both myocardial infarcts and cryoinjuries in MRL/MpJ mice heal by typical scar formation rather than muscle regeneration, in a manner very similar to C57BL/6 controls. We conclude that the MRL mouse is not a model for myocardial regeneration.

Keywords

MRL; Cardiac Regeneration; Myocardial Infarction; Cryoinjury; Scar

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1. Introduction

The MRL/MpJ (Murphy Roths Large derived by the Murphy (Mp) group of the Jackson Laboratory) mouse has gained considerable attention recently as a mammalian model for regenerative wound healing. The MRL/MpJ mouse completely regenerates punched ear tissue by one month [1], including regrowth of cartilage and hair follicles in the wounded region with minimal fibrotic scarring. This phenomenon had been correlated to increased matrix metalloproteinase (MMP) expression in the MRL/MpJ mouse compared to the C57BL/6 control [2]. Incisional and excisional skin wounds, however, do heal with scar [3]. In addition to these findings, the MRL mouse's regenerative phenotype has been reported in the heart in response to trans-diaphragmatic cryoinjury of the right ventricle: the C57BL/6 showed extensive scarring on the basal surface of the right ventricle while the MRL/MpJ showed little to none [4]. Heber-Katz and colleagues explained this as both the result of diminished scarring and a myoregenerative phenotype [4–7].

The observed regenerative capabilities of the MRL/MpJ mouse put it in consideration for a model of cardiac regeneration and suggested an alternative healing response to injury in the heart. We hypothesized that the distinct regenerative phenotype observed after cryoinjury to the heart would also be observed in response to cardiac ischemic injury from coronary artery ligation. The observed cardiac regeneration could be the result of increased populations of cardiac stem cell or the ability of surviving cardiomyocytes to re-enter the cell cycle. An alternative explanation for the observed differences between strains could be attributed to differences of the inflammatory fibrotic response to injury. The potential implications of a differential regenerative capacity or altered remodeling properties in a mammalian model of cardiac injury demanded additional investigation.

Concurrent with our investigation, two other reports concerning MRL/MpJ heart injury have cast doubt on the extent that this strain regenerates cardiac muscle without scar [8,9]. Oh and colleagues used magnetic resonance imaging at one and four months after permanent occlusion of the coronary artery to show no difference of infarct size over time. Histological data at four month indicated extensive scar in the injured heart [8]. Abdullah *et al* showed that MRL/MpJ and C57BL/6 strains did not differ in myocardial infarct size 10 weeks after transient ischemia. The MRL/MpJ mice were neither resistant to injury and scarring, nor able to regenerate cardiac muscle [9]. One potential explanation for the discrepancy would be a different regenerative response of the MRL/MpJ heart to cryoinjury versus infarction. We therefore compared the cardiac responses to infarction and cryoinjury in MRL/MpJ and C57BL/6 mice.

2. Methods

MRL/MpJ mice were obtained from the Jackson Laboratories (Bar Harbor, ME) and C57BL/6 mice were obtained from Harlan (Indianapolis, IN). Animals were housed under specific pathogen-free conditions and used in accordance with NIH guidelines. The Institutional Animal Care and Use Committee at the University of Washington approved this study.

2.1 Murine Myocardial Injury

Murine myocardial infarction was performed as previously described [10,11]. Thirty-eight male MRL/MpJ mice and 35 male C57BL/6 mice aged 6–10 weeks were used in this study. We created permanent ischemic injury in 32 MRL/MpJ and 28 C57BL/6 mice by ligation of the left anterior descending (LAD) coronary artery. Under Avertin (Sigma, St. Louis, MO) anesthetic, 25–35 g mice were orotracheally intubated, ventilated and their hearts exposed by left sided thoracotomy. The pericardium was reflected and the LAD was visualized with a surgical microscope and ligated using 8–0 polypropylene suture. The chest was closed, animals

were rehydrated and allowed to recover in a heated chamber. One C57BL/6 animal was excluded from the study after no evidence of infarction was observed by histology, and survival was equivalent (C57BL/6 = 92%, MRL/MpJ = 95%). Two MRL/MpJ and three C57BL/6 mice died of respiratory failure during the open chest procedure. Four animals from each strain received thoracotomies and pericardial reflection but no intervention and were sacrificed at 90 days as sham controls. Survivors used for this study are listed in Figure 1.

Cryoinjury was generated in 8 MRL/MpJ and 9 C57 BL/6 mice ventilated as above. Hearts were collected for analysis at 1 and 4 weeks. A liquid nitrogen-cooled 3 mm diameter aluminum probe was applied directly on the anterior left ventricle for 10 seconds. The frozen myocardium thawed completely 10–15 seconds following probe removal, and the injured area exhibited a deep red coloration of approximately 4 mm diameter. The incisions were surgically closed and animals allowed to recover as above. From this set of animals, one C57BL/6 mouse died as the result of lung injury.

2.2. Tissue Collection, Histology and Analysis

Animals from the infarcted group received a 2 mg bolus of 5-bromo-2-deoxyuridine (BrdU) in saline by intraperitoneal injection one hour prior to sacrifice. Under deep anesthesia and laparotomy, hearts were arrested in diastole by intravenous KCl injection into the abdominal inferior vena cava at 1, 4, 7, 14, 28 and 90 days. The distribution of animals in each group and time point is indicated in Table 1. Extracted hearts were sectioned into 2 mm thick slices and fixed in zinc fixative [11,12]. Blinded histological assessment for infarct size, cell proliferation and vascular density was performed. Infarct histomorphometry was calculated from hematoxylin and eosin (H&E) stained slides and total infarct size was calculated as a fraction of the left ventricular circumference [13]. Micrographs of 2 mm spaced sections H&E-stained slides were taken at 20× magnification using a SPOT RT digital camera and the SPOT imaging software (Diagnostic Instruments, Sterling Heights, MI). The images were randomized and the investigator was blinded to the strain and time point. Arc lengths were traced in ImageJ software (NIH, Bethesda, MD). The sum of arc lengths of the infarct's inner and outer walls was divided by the sum of the inner and outer circumferences of the entire left ventricle. Values from each 2 mm spaced slice were summed over the entire left ventricle.

Immunostaining for sarcomeric actin, CD31 and BrdU followed previously described protocols [11], and used tissue sections approximately 2 mm from the apex. For histological analysis, the investigator was blinded to time point and experimental condition. Briefly, endothelial cells were stained using anti-CD31 (biotinylated rat anti-mouse monoclonal; PharMingen, #553371) at a 1:2000 dilution. BrdU incorporation was detected using peroxidase-conjugated anti-BrdU (Roche, #1585860) at a 1:40 dilution. Cardiomyocytes were stained for the muscle specific sarcomeric α -actin (monoclonal clone 5C5; Sigma #A2172) at a 1:5000 dilution. Three fields representing endocardial, epicardial and mid-myocardial regions of the infarct were counted at the microscope using a 10 × 10 grid reticle eyepiece. One additional field was selected from the infarct's border zone for a total of ten fields per slide. Cells were counted at 400× magnification. Numbers were expressed as the percentage of labeled cells (both BrdU and CD31 phenotype marker-positive) in more than 500 total cells. Vessel density was measured using the 10×10 reticle at 200× magnification, representing an area of 0.25 mm².

2.3 Statistics

Correlation and regression analyses were done to describe relationships between the experimental conditions. Statistical significance between groups was determined by analysis of variance followed by Bonferroni post-hoc analyses. Single data points were compared using a two-tailed T-test with unequal variance. All statistics were performed using Instat (GraphPad,

San Diego, CA) and significance levels were $P < 0.05$. Values presented in text and figures are mean $\pm$ standard error of the mean (SEM).

3. Results

3.1 Infarct Morphometry

Hematoxylin and eosin-stained sections 2 mm from the apex are shown at each time point for C57BL/6 and MRL/MpJ mice in Figure 1A. These images demonstrate that infarct repair progressed identically for both strains. Myocyte karyolysis (1C) at 1 day was followed by typical granulation tissue formation at 4 days (1D) in both strains. The progression from provisional extracellular matrix at 4 days to mature scar was complete at 14 days, with little residual necrotic material in the infarct. By 90 days, wall thinning, scar contraction and ventricular aneurysm in conjunction with extreme compensatory septal wall hypertrophy were seen in both strains. The MRL/MpJ mouse heart was slightly larger than the C57BL/6 owing to the differences in growth in the two strains. Subendocardial sparing occurred similarly between the strains. The extent of scar formation was verified by sirius red staining for collagen as shown in Figure 1B. Morphometric analysis of these data using sirius red stained sections is shown in Figure 1E. Initial infarct size induced by permanent coronary ligation was comparable in the two groups (38% of the left ventricular circumference in MRL/MpJ vs. 36% in C57BL/6; $p = \text{NS}$). Infarct size generally followed a decrease from the maximum of 47% at 4 days to 40% at 90 days, and scar shrinkage was comparable between groups.

3.2 Cell Proliferation and Endothelial Density

Figure 2 demonstrates that DNA synthesis rates during infarct repair were identical between the MRL/MpJ and C57BL/6 strains (2C). There was an initial wave of cell proliferation at day 4 associated with granulation tissue formation as previously described [11]. This peak of DNA synthesis occurred in both strains (MRL/MpJ = $6.5 \pm 1.2\%$ BrdU+ nuclei, C57BL/6 = $8.5 \pm 2.2\%$ of total cells; $p = \text{NS}$) and did not differ between strains at any time point. Endothelial cell density in both the infarcted ($22 \pm 0.3\%$ of nucleated cells in MRL/MpJ and $22 \pm 0.5\%$ in C57BL/6 at 7 days; $p = \text{NS}$) and non-infarcted ($39 \pm 3\%$ in MRL/MpJ and $35 \pm 5\%$ in C57BL/6 at 7 days; $p = \text{NS}$) cardiac tissue did not differ between groups at any time. Representative images of MRL/MpJ and C57BL/6 infarcts at 4 days are shown in Figure 2A and B. Extensive histological analysis found only one candidate BrdU+ cardiomyocyte nucleus in an MRL/MpJ heart section (Figure 2A, upper inset). This nucleus was not fully surrounded by the sarcomeric actin staining, however, and therefore may represent a proliferating interstitial cell.

Both groups showed a significant decline in endothelial density (expressed as a percentage of total cells) post infarction, as well as declines in total vascular density, with no differences between strains. No differences in endothelial density in the non-infarcted region were detected. Figure 3 shows representative histology of endothelial cells in the uninjured border region (A and B) and in the middle regions of the infarct (D and E) at 7 days as well as the progression of endothelial cell density to 90 days (C and E) and the vessel density per mm^2 in the infarct. No discernable differences in any of these metrics was seen.

3.3 Cardiac Response to Cryoinjury

Although our studies clearly demonstrated that the MRL heart does not regenerate after myocardial infarction, the original reports of cardiac regeneration in this strain used cryoinjury to induce myocardial necrosis [4]. To test whether this distinct form of injury would heal by regeneration, we performed cryoinjuries in both MRL and C57BL/6 mice and examined their hearts histologically after injury. MRL/MpJ and C57BL/6 hearts respond identically to direct cryoinjury of the left and right ventricles after 7 and 28 days. H&E and picrosirius red stained sections are shown in Figure 4. In each strain, a thick, amuscular scar formed, often with

endocardial sparing. The total scar volume per heart was equivalent at 1 week ($8.4 \pm 1.0 \text{ mm}^3$ for MRL/MpJ and $7.7 \pm 1.3 \text{ mm}^3$ for C57BL/6; $p = 0.66$) and 4 weeks ($11 \pm 1.9 \text{ mm}^3$ for MRL/MpJ and $12 \pm 1.2 \text{ mm}^3$ for C57BL/6; $p = 0.57$) by morphometric analysis (Figure 4C). As originally observed by Vracko *et al*, myocytes at the border zone often extended irregular processes into the scar [14]. This normal response to necrotizing injury occurred in both groups of mice (Figure 4D). No colocalized staining for BrdU and sarcomeric actin was found at 7 days following cryoinjury (Figure 4E), indicating no significant DNA synthesis occurred in cardiomyocytes in either group. This shows that neither cryoinjury nor myocardial infarction heal by regeneration in the MRL mouse.

4. Discussion

4.1 Myocardial Infarction in the MRL/MpJ mouse

Strategies to achieve myocardial regeneration are currently under intense study [15,16] and early clinical trials of cell-based cardiac repair are underway [17–21]. Model organisms capable of heart regeneration include the zebrafish [22] and newt [23], but until recently no mammalian model of heart regeneration existed. The MRL/MpJ mouse achieved significant attention at the first report of its ability to respond without scar to cardiac injury [4]. A mammalian system of cardiac regeneration could point to alternative mechanisms for cardiac repair, such as resident or circulating stem cell populations, re-entry of cardiomyocytes into the cell cycle or differences in the inflammatory or fibrotic phases of infarct healing. In contrast to the original report, however, we found that the histological indices of infarct repair were identical between the MRL/MpJ and C57BL/6 strains.

Regarding changes in fibrosis, previous reports have shown increased MMP2 and MMP9 expression and enzyme activity in the MRL mouse ear punch model [2], which could be a causative factor in any change in cardiac remodeling. MMPs have been shown to be an important contributors to the state of cardiac fibrosis [24] and can be used as markers for heart failure [25]. In addition, elevated MMP activity in the heart accelerates cardiac remodeling and prevents proper healing after myocardial infarction [26,27]. The similarity of injury repair between hearts from the C57BL/6 and MRL/MpJ mice demonstrates that previously identified differences in MMP activity do not correlate to a different cardiac phenotype between the two strains. In the infarct, cell proliferation rates in the MRL/MpJ infarct followed a pattern identical to C57BL/6 controls, and the microvascular response to injury was equivalent between the two. This data suggests no particular difference in regenerative capacity by MRL/MpJ cardiomyocytes.

4.2 Cryoinjured Myocardium

The original report of regeneration in the MRL/MpJ mouse used cryoinjury rather than infarction [4]. Cryoinjury differs from infarction in that necrosis proceeds from epicardium to endocardium and that the necrosis is much more confluent in cryoinjured tissue [28]. We therefore examined the response to direct cryoinjury in MRL/MpJ and C57BL/6 hearts and found no difference between strains. We used an open thoracotomy approach to induce a cryoinjury on the heart, because this allowed direct visualization of the freezing process and immediate validation of the resulting freeze-thaw injury. In contrast, Leferovich *et al* used a trans-diaphragmatic approach to cryoinjury in which the heart's location is only approximated and in which successful injury cannot be documented [4]. The resulting injury (and the differences reported by Leferovich *et al*) of the right ventricle could vary with the heart's position in the chest and unknown anatomical differences between the strains. The authors of that study also suggest that myocardial regeneration progresses by extension of myocyte fingers into the injury. Vracko *et al* were the first to describe these finger-like extensions at the border zone of cryoinjuries or infarcts in rats [14]. They performed a detailed time course by electron

microscopy and showed that these extensions arose from the blunt stumps of intercalated disks in the surviving cardiomyocytes. This phenomenon occurs ubiquitously in both strains and is not a sign of cryoinjury. We should note that our approach caused injury on both the right and left ventricles (Figures 4A–B), indicating that the right ventricle possesses no special regenerative properties in MRL/MpJ mice. That no colocalization of BrdU and sarcomeric actin was found in 7 day old infarcts suggests that cardiomyocytes are not dividing in response to cryoinjury.

4.3 Comparison with Previous Reports

Our findings agree with Oh *et al* and Abdullah *et al* that myocardial infarct repair in the MRL/MpJ mouse is not scarless [8,9], and with Abdullah *et al* that no difference exists between C57BL/6 and MRL/MpJ in the response to ischemic injury [9]. Our data confirm and expand these findings by showing that scar size progresses equivalently between both strains, that original vessel density and endothelial repopulation of the infarcted heart is equivalent, and that no differences in DNA synthesis occur in the infarcted or the cryoinjured heart, including in the border regions. In addition, this study indicates that cryoinjury does not progress with scarless healing, and that the scar formed in C57BL/6 and MRL/MpJ mice is the same size. We attribute the discrepancy between our findings and the previous report of regeneration following cryoinjury to the increased reproducibility of applying a cryoprobe directly to the heart versus injury across the diaphragm.

5. Summary

Cardiac regeneration in the MRL/MpJ mouse strain does not occur following myocardial infarction or cryoinjury. The reaction to ischemic injury follows the well-described progression of necrosis, granulation tissue, collagenous scar formation, scar contraction and compensatory ventricular hypertrophy. No difference between the MRL/MpJ and C57BL/6 control mice was observed in a blinded analysis of cell proliferation or vascular content. Cryoinjured hearts at 7 and 28 days were identical in morphology and morphometry between both strains. These results suggest that the MRL/MpJ mouse does not serve as a mammalian model of cardiac regeneration.

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Figure 1.

Infarct repair in MRL/MpJ and C57BL/6 mouse hearts. Midlevel cross-sections of C57BL/6 and MRL/MpJ mouse hearts from sham operated mice and 1, 4, 14 and 90 days after myocardial infarction stained by hematoxylin and eosin (A) and sirius red and fast green (B) show a progression from necrosis at day 1 (C) to granulation tissue at day 4 (D) to scar and compensatory septal hypertrophy (days 14 and 90). Each feature is comparable between strains. (Bars = 1 mm and 50 μ m) (E) Infarct size as a percent of the left ventricular circumference is similar in both strains at all times.

Figure 2.

DNA synthesis determined by BrdU incorporation (brown nuclear staining) in the infarcts of (A) MRL/MpJ and (B) C57BL/6 at 4 days do not co-localize with sarcomeric actin immunostaining (red). Arrows indicate BrdU positive nuclei (bar = 50 μ m). (C) Total BrdU incorporation rates between 1 and 14 days is equivalent in both strains.

Figure 3.

Endothelial density determined by CD31 staining (brown cytoplasmic staining) 7 days after myocardial infarction in the injury border region of the (A) C57BL/6 and (B) MRL/MpJ mouse strain (Bar = 50 μ m). The insets show vessel structure (Inset Bar = 10 μ m). Example endothelial cells are indicated by (+) and CD31-negative cells are marked with (-). CD31 staining in the infarcted region of C57BL/6 and MRL/MpJ mouse strains indicates similar vessel density between the strains. No significant differences exist in endothelial cell density between the two strains in (C) the uninjured myocardium and (F) the infarct scar to 90 days. Vessel density measurements (G) are also equivalent between the two strains.

Figure 4.

Cardiac scar morphology by hematoxylin and eosin and picosirius red/fast green staining in response to a 10 second direct cryoinjury is equivalent in the MRL/MpJ and C57BL/6 mouse 28 days (A) and 7 days (B) after injury. (Bar = 1mm) Extent of cryoinjury includes the right ventricle in each intervention, indicating lack of regeneration in that area. (C) Scar volume measured from serial sirius red stained sections is equivalent between each strain at both 1 and 4 weeks. (D) Each strain showed both sharp and jagged transitions at the infarct border zones owing to the muscle fiber orientation at that area of the tissue section. (Bar = 50 μm.) (E) No proliferating cardiomyocytes were found 7 days after cryoinjury as measured by immunostaining for BrdU (brown nuclei) and sarcomeric actin (red cytoplasm). (Bars = 50 and 20 μm.)

Table 1

Group sizes and average age in weeks at surgery for each data point.

C57BL/6			MRL/MpJ	
Day	Group Size	Age (weeks)	Group Size	Age (weeks)
<i>Sham Surgery</i>	4	8.2	4	7.9
<i>Infarction</i>				
1	5	8.5	5	7.0
4	5	9.0	7	9.7
7	5	9.2	4	8.5
14	5	6.4	7	7.8
28	4	7.5	4	8.0
90	4	7.1	5	8.6
<i>Cryoinjury</i>				
7	5	6.5	4	6.5
28	3	7.2	4	8.0