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ORIGINAL ARTICLE

Absence of Myostatin Improves Cardiac Function Following Myocardial Infarction

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Background Myostatin inhibits the development of skeletal muscle and regulates the proliferation of skeletal muscle fibroblasts. However, the role of myostatin in regulating cardiac muscle or myofibroblasts, specifically in acute myocardial infarction (MI), is less clear. This study sought to determine whether absence of myostatin altered left ventricular function post-MI.

Methods Myostatin-null mice ($Mstn^{-/-}$) and wild-type (WT) mice underwent ligation of the left anterior descending artery to induce MI. Left ventricular function was measured at baseline, days 1 and 28 post-MI. Immunohistochemistry and immunofluorescence were obtained at day 28 for cellular proliferation, collagen deposition, and myofibroblastic activity.

Results Whilst left ventricular function at baseline and size of infarct were similar, significant differences in favour of $Mstn^{-/-}$ compared to WT mice post-MI include a greater recovery of ejection fraction ($61.8 \pm 1.1\%$ vs $57.1 \pm 2.3\%$, $p < 0.01$), less collagen deposition ($41.9 \pm 2.8\%$ vs $54.7 \pm 3.4\%$, $p < 0.05$), and lower mortality (0 vs. 20%, $p < 0.05$). There was no difference in the number of BrdU positive cells, percentage of apoptotic cardiomyocytes, or size of cardiomyocytes post-MI between WT and $Mstn^{-/-}$ mice.

Conclusions Absence of myostatin potentially protects the function of the heart post-MI with improved survival, possibly by limiting extent of fibrosis.

Keywords Myostatin • Myocardial infarction • Ejection fraction • Fibrosis

Introduction

Myostatin is known to be a negative regulator of skeletal muscle mass [1]. Although myostatin was discovered nearly two decades ago, its role in the heart remains controversial. The expression of myostatin in the heart (protein, mRNA or both) is up-regulated both in rat models of volume-overload, and in patients with congestive heart failure [2–5]. Myostatin also regulates the proliferation of normal and dystrophic skeletal muscle fibroblasts [6,7] and may be an important mediator between cardiomyocytes and fibroblasts, an effect consistent with the activities of other members of the TGF-β superfamily [8]. Therefore, myostatin may play a role in regulating both the viable myocardium and the extent of connective tissue deposition following myocardial infarction (MI).

The aim of this study was to assess if there was a difference in left ventricular function post-MI in the absence of myostatin when compared to controls.

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Materials and Methods

Heterozygote $Mstn^{+/-}$ mice (C57BL/6 background) were kindly gifted by Se-Jin Lee, John Hopkins University [1]. Mice were genotyped and bred to homozygosity at the Ruakura Small Animal Colony Unit. Adult male (12-week-old) $Mstn^{-/-}$ and WT (C57BL/6) mice were randomised to induction of MI or sham-control group (n = 12 per genotype and procedure). All animals and surgical procedures were performed with local ethical approval and conformed with the NIH Guidelines for the care and use of laboratory animals.

Induction of MI

General anaesthesia was achieved using a combination of Ketamine, Xylazine and Acepromazine. Mice were intubated and ventilated in a supine position on a 37 °C heated pad. A 10 mm left thoracotomy was performed at the fourth intercostal space and ligation of the LAD artery was made midway between the left atrium and apex of the left ventricle [9]. Successful ligation was attained when blanching of the distal myocardium was observed. Assisted ventilation was maintained until spontaneous breathing was restored. For sham-control, a left thoracotomy was performed without coronary artery ligation. Mice were observed daily in a small animal colony unit with provision of standard chow and water *ad libitum*.

Echocardiogram and Clinical Parameters

Total body weight (BWT) was recorded at baseline and weekly, thereafter. Heart rate (HR) and blood pressure were measured on conscious mice during daylight at baseline, and at day 28 post-surgery using a computerised blood pressure tail-cuff analysis system (Visitech Systems, Apex, NZ, USA). Each measurement was obtained from an average of 25 readings.

Transthoracic echocardiography was performed at baseline, days 1 and 28 post-surgery, using a Philips HDI 5000 Sono CT ultrasound, with a 10 MHz broadband compact linear array transducer (Phillips NZ Ltd, Auckland, New Zealand). Mice were lightly anaesthetised with the aforementioned anaesthetic combination. Imaging was obtained in the parasternal short axis view at the level of the papillary muscle using two-dimensional (2D) and M-Mode analysis [10]. Left ventricular end diastolic and systolic diameters (LVEDD and LVESD respectively) were measured and fractional shortening (FS) and ejection fraction (EF) calculated. Three readings were obtained for each measurement and then averaged. The operator was blinded to the surgical procedure.

Histological Examination

On day 28, the mice were weighed and sacrificed with carbon dioxide asphyxiation, coupled with cervical dislocation. Hearts were rapidly excised, weighed, formalin-fixed and embedded in paraffin wax. Seven micrometre sections were cut longitudinally in the centre of the infarct, and stained with haematoxylin and eosin (H&E). Each section was photographed (Leica DMI6000 B inverted microscope, Leica Microsystems, Germany). The size of the infarct was calculated as previously described [11] using Image J Software (NIH).

Collagen deposition was assessed with Van Gieson staining. A modified grid-counting system was used to estimate the amount of collagen deposition in the peri-infarct region, where viable cardiomyocytes were still present [12]. A grid of equal spaces was created over the region of interest (ie. infarcted and peri-infarcted areas) on each section of the myocardium. Grids that had over 50% of infarcted scar tissue (stained red) were graded as 1, while grids with over 50% of viable tissue (stained yellow/light brown) interspersed with collagen were graded as 2. Grids with only viable cardiomyocytes were not counted as this represents the distant non-infarcted myocardium. The amount of collagen deposition was calculated as a percentage of the infarcted scar tissue to the total region of interest (infarcted + viable).

Immunohistochemistry (IHC)

Immunohistochemistry was performed to: assess DNA synthesis [anti-5-Bromo-2′-deoxyuridine (BrdU)], quantify the size of cardiomyocytes (laminin); and assess apoptosis, programmed necrosis and survival [anti-cleaved caspase-3 (1:300) (#9664, Cell Signalling Technology, MA, USA), anti-PARP-1 (1:10,000) (#1835238, Roche Diagnostics NZ Ltd, Auckland, NZ), anti-$pAkt^{s473}$ (1:100) (#4058, Cell Signalling Technology, MA, USA), anti-$pAkt1/2/3^{s473}$ (1:2000) (SC-7985, Santa Cruz Biotechnology, CA, USA) and anti-$pAkt1/2/3^{t308}$ (1:1000) (SC-16646, Santa Cruz Biotechnology, CA, USA). All were performed according to the manufacturer's protocol. For negative controls, a mouse IgG1 antibody (Dako, # x0931, VWR, Auckland, New Zealand) was used at the same dilution as the primary antibody of interest.

BrdU (0.1 ml/10 g BWT) was injected intra-peritoneally two hours before euthanasia (Cell Proliferation kit, # RPN20, GE Healthcare Life Sciences, Auckland, New Zealand). The total labelling fraction was calculated as a ratio of the BrdU positive cells to the total number of cells counted on each field with six fields per section in the border region of the infarcted ventricle randomly selected [13]. For sham animals, the six fields were randomly taken throughout the left ventricle.

Cells were stained with laminin (Dako, #Z0097, VWR, Auckland, New Zealand) to highlight the cell membrane and counterstained with haematoxylin to visualise the nuclei. A point-counting system was used where a grid of equal spacing was overlaid onto the section. Only cells with well-defined cell membrane and visible nuclei that sit on the 'crosses' of the grid were selected. The minimal Feret diameter and the cross-sectional area were measured from at least 300 consecutive cells in each section using image J (NIH) software.

The immunointensity of the other cellular markers was obtained semi-quantitatively using a multiplicative quick

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