Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 20 (2009) 621 – 628
Journal of Nutritional Biochemistry

Regression of copper-deficient heart hypertrophy: reduction in the size of hypertrophic cardiomyocytes

Zhanxiang Zhou[a], W. Thomas Johnson[b], Y. James Kang[a,c,*]

[a]Department of Medicine, University of Louisville School of Medicine, Louisville, KY 40202, USA
[b]Agricultural Research Service, U.S. Department of Agriculture, Grand Forks Human Nutrition Research Center, Grand Forks, ND 58202, USA
[c]Department of Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, KY 40202, USA

Received 22 April 2008; received in revised form 14 May 2008; accepted 6 June 2008

Abstract

Dietary copper (Cu) deficiency causes cardiac hypertrophy and its transition to heart failure in a mouse model. Cu repletion results in rapid regression of cardiac hypertrophy and prevention of heart failure. The present study was undertaken to understand dynamic changes of cardiomyocytes in the hypertrophic heart during the regression. Dams of FVB mice were fed a Cu-deficient (CuD) diet (0.3 mg Cu/kg) starting on Day 3 post-delivery, and weanling pups were fed the same diet until Cu repletion (6.0 mg Cu/kg) in the diet at 31 days of age. Heart samples were obtained at the end of CuD feeding or at 3, 7, 14 or 28 days after Cu repletion. Cu deficiency resulted in increases in the size and reduction in the number of cardiomyocytes in the heart. Cu repletion led to regression in the size of hypertrophic cardiomyocytes and normalization of the total number of cardiomyocytes. Although a direct reduction in the cell size would be significantly responsible for the regression of heart hypertrophy, some hypertrophic cardiomyocytes upon Cu repletion reentered the cell cycle as determined by Ki-67 staining in the cardiomyocyte-specific α-sarcomeric actin-stained cells and underwent division as determined by a mitosis-specific marker, phospho-histone 3. Quantitative analysis indicated that the replication of hypertrophic cardiomyocytes made a contribution of about one-third to the total mitosis of the regenerated myocardium. This study suggests that a direct reduction in the size of some hypertrophic cardiomyocytes and a replication of other hypertrophic cardiomyocytes with reduced size make a significant contribution to the regression of CuD heart hypertrophy, leading to normalization of the size and the number of cardiomyocytes in the heart.

Keywords: Cell cycle; Copper; Hypertrophy; Mitosis; Myocardial regeneration; Regression

1. Introduction

Copper (Cu) is an essential trace element, and dietary Cu restriction leads to cardiac hypertrophy [1]. Cu-deficient (CuD) heart hypertrophy in mouse and rat models has characteristic structural, biochemical and signaling alterations resembling those induced by pressure overload [1] and transits to heart failure [2]. We recently observed that dietary Cu repletion results in rapid regression of the hypertrophy and complete functional recovery [3]. These regression of heart hypertrophy and functional recovery indicate a cytokinetic change in the hypertrophic cardiomyocytes. Possible mechanisms underlying the change include apoptosis, leading to a cleanup of the hypertrophic cardiomyocytes; a reduction in cellular volume, making the hypertrophic myocytes smaller by an unknown mechanism; and mitosis of the hypertrophic cardiomyocytes with reduced cell size, leading to myocardial regeneration.

Apoptosis of cardiomyocytes in the failing heart is a major cellular event involved in the pathogenesis [4]. Our early studies showed that dietary Cu deficiency causes myocardial apoptosis [5], which would be highly responsible for the transition from cardiac hypertrophy to heart failure. Defective myocardial function is associated with the loss of cardiomyocytes due to apoptosis. In the regression induced by Cu repletion in Cu deficiency-induced hypertrophic hearts, the depressed function was completely recovered [3]

* Corresponding author. Department of Medicine, University of Louisville School of Medicine, Louisville, KY 40202, USA. Tel.: +1 502 852 8677; fax: +1 502 852 6904.
E-mail address: yjkang01@louisville.edu (Y.J. Kang).

0955-2863/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2008.06.007

and myocardial structures were normalized as examined by both light and electron microscopes [3]. Therefore, it is unlikely that apoptosis can be responsible for the regression of heart hypertrophy induced by Cu repletion.

Heart hypertrophy developed under physiological stimulations, such as exercise-induced myocardial growth, is reversible [6], although molecular mechanisms involved in the regression of physiological heart hypertrophy have not been fully understood. At the cellular level, the size of hypertrophic cardiomyocytes has to become smaller or normalized in order for the regression of heart hypertrophy to occur. This process would be accomplished by a direct reduction in the size of the hypertrophic cardiomyocytes. Under pathological conditions, heart hypertrophy can also be reversed under different conditions in mice [7,8]. However, this regression would be more complicated than a simple reduction in the size of hypertrophic cardiomyocytes under physiological conditions. The functional recovery of the failing heart would demand myocardial regeneration, which requires repletion of the lost cardiomyocytes during the transition from cardiac hypertrophy to heart failure.

Myocardial regeneration through mitosis of the hypertrophic cardiomyocytes with reduced cell size potentially is an attractive mechanism for regression of heart hypertrophy and recovery of myocardial function observed in the CuD hearts. Although the view of the adult mammalian heart as a terminally differentiated organ without regenerative capacity remains dominant [9], recently documented evidence challenges this belief. Several studies have shown the presence of cycling ventricular myocytes in the adult mammalian heart under both physiological and pathological conditions [10–12]. It is thus important to know whether or not the Cu repletion-induced regression of heart hypertrophy and recovery of cardiac function are associated with cell cycle reentry and mitosis of the cardiomyocytes.

The present study was undertaken to determine (1) cytokinetic changes in Cu-induced regression of heart hypertrophy in a mouse model and (2) potential contributions of mitosis of hypertrophic cardiomyocytes to myocardial regeneration. We found that besides a direct reduction in the size of hypertrophic cardiomyocytes, a replication of hypertrophic cardiomyocytes would make a significant contribution to the regression of heart hypertrophy, although differentiation of other types of cells to cardiomyocytes may also play an important role in the regeneration process.

2. Materials and methods

2.1. Experimental animals and procedure

FVB mice obtained from the Jackson Laboratory (Bar Harbor, ME, USA) were maintained in University of Louisville animal facilities and housed in plastic cages at 22°C on a 12-h light/dark cycle. Dams of the pups were fed a CuD AIN-93 diet containing 0.3 mg Cu/kg or a Cu-adequate (CuA) diet containing 6.0 mg Cu/kg starting on the third day after delivery. The AIN-93 powder diet was prepared according to a report published previously [13]. After the pups were weaned at 21 days of age, they were fed the same diet as their respective dams for seven additional days (31 days of age), at which point some of the CuD mice were sacrificed and others were fed the same diet or switched to the CuA diet for 1 or 2 weeks more (6–12 mice in each group). Mice had free access to doubly distilled water. Cages, feeding jars and water bottles were regularly rinsed first with water containing EDTA and then with distilled water. The parallel CuA mice were sacrificed at the same time as the feeding and age controls. At the conclusion of each feeding experiment, mice were anesthetized with avertin (0.4 mg/g) and blood was drawn from the dorsal vena cava. Plasma was collected by centrifugation at 10,000 rpm for 10 min and stored at −80°C until used. Hearts were removed, perfused with phosphate-buffered saline (PBS) and processed for the analyses described below. All procedures were approved by the University of Louisville Institutional Animal Care and Use Committee, which is certified by the Association for Assessment and Accreditation of Laboratory Animal Care.

2.2. Cardiac hypertrophy

At the organ level, whole hearts were fixed with 10% neutral formalin and embedded in paraplast. Longitudinal sections were cut at 5 μm and stained with hematoxylin and eosin. Whole heart images were captured using a Nikon stereomicroscope. At the cellular level, cardiomyocytes on histological sections were stained by immunofluorescence with the cytoplasmic marker α-sarcomeric actin. Cross-sections of myocardium were rehydrated and incubated with 5% normal donkey serum for 30 min to block non-specific binding sites. Sections were stained with a monoclonal mouse anti-α-sarcomeric actin antibody and a Cy3-labeled donkey anti-mouse antibody to detect cardiomyocytes. Digital images with cross-sectioned cardiomyocytes were captured with 40× objective, and for each histological section of the myocardium from six to eight mice, the diameters of 300 cardiomyocytes were measured and the total number of cardiomyocytes in a 25-mm^2 field was counted.

2.3. Immunofluorescence detection of Ki-67, phospho-histone 3 and myocyte enhancing factor-2

Double staining of Ki-67/α-sarcomeric actin and that of phospho-histone 3/α-sarcomeric actin were performed for detection of proliferating and mitotic cardiomyocytes. For staining of Ki-67/α-sarcomeric actin, Ki-67 was first stained by incubation with rabbit anti-Ki-67 antibody (Vector Laboratories, Burlingame, CA, USA) and fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit immunoglobulin G (IgG; Jackson ImmunoResearch Laboratories,

West Grove, PA, USA), and then α-sarcomeric actin was stained by incubation with a monoclonal mouse anti-α-sarcomeric actin antibody and an FITC-labeled donkey anti-mouse antibody. For staining of phospho-histone 3/α-sarcomeric actin, phospho-histone 3 was first stained with rabbit anti-phospho-histone 3 antibody (Vector Laboratories) and FITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories), and then α-sarcomeric actin was stained by incubation with a monoclonal mouse anti-α-sarcomeric actin antibody and an FITC-labeled donkey anti-mouse antibody. For triple staining of phospho-histone 3, myocyte enhancing factor-2 (MEF-2) and α-sarcomeric actin, the histological sections were first stained by incubation with rabbit anti-phospho-histone 3 antibody (Vector Laboratories) and FITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and then by incubation of goat anti-MEF-2 antibody and AMC-labeled donkey anti-goat antibody, followed by staining with a monoclonal mouse anti-α-sarcomeric actin antibody and a Cy3-labeled donkey anti-mouse antibody.

2.4. Electron microscopy

Heart tissues used for electron microscopy (EM) study were obtained through an in situ perfusion procedure described previously [14]. The free wall of the left ventricle was cut into about 1 mm^3. For conventional EM, samples were fixed in 3% glutaraldehyde in 0.1 mM sodium cacodylate buffer, pH 7.4, for 2 h at 4°C and post-fixed in 1% osmium tetroxide. The samples were embedded in resin, and ultrathin sections were cut and stained with uranyl acetate and lead citrate. For immunogold EM, samples were fixed in 2% freshly depolymerized paraformaldehyde and 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, at 4°C for 2 h. After they were rinsed in sodium cacodylate buffer, the samples were partially dehydrated with 50% and 70% ethanol and embedded in LR White resin. Ultrathin sections were incubated with the rabbit anti-Ki-67 antibody overnight at 4°C. After they were rinsed in 0.01 M PBS, pH 8.2, the ultrathin sections were incubated in 10-nm gold-conjugated goat anti-rabbit antibody (British BioCell, Cardiff, UK) diluted in 0.01 M PBS, pH 8.2, for 2 h. The ultrathin sections were then rinsed in distilled water and counterstained with uranyl acetate and lead citrate.

2.5. TUNEL assay

An ApopTag in situ detection kit was used according to the manufacturer's instruction and following a procedure described previously [5]. Briefly, the histological sections were pretreated with H_2O_2 and incubated with the reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and digoxigenin-conjugated deoxy-UTP for 1 h at 37°C. Labeled DNA was visualized with peroxidase-conjugated anti-digoxigenin antibody with DAB as the chromagen. Rat mammary gland tissue provided in the kit was used as positive control. For negative control, TdT was routinely omitted from the reaction mixture.

2.6. Statistical analysis

Data were analyzed by one-way analysis of variance followed by Duncan's multiple-range test for further determination of the significance of differences. Differences among groups were considered significant at $P<.05$.

3. Results

3.1. Reduction in the size of hypertrophic cardiomyocytes by Cu repletion in hypertrophic hearts

After mice were fed CuD diet for 4 weeks, their heart size increased as measured by heart weight-to-body weight ratio (9.7 ±0.5 vs. 5.2±0.2 mg/g). Histological sections of the whole heart showed increased wall thickness (Fig. 1). Cu repletion resulted in regression of the heart size (6.0±0.2 mg/g) and normalized wall thickness (Fig. 1). To explore the cellular events of Cu

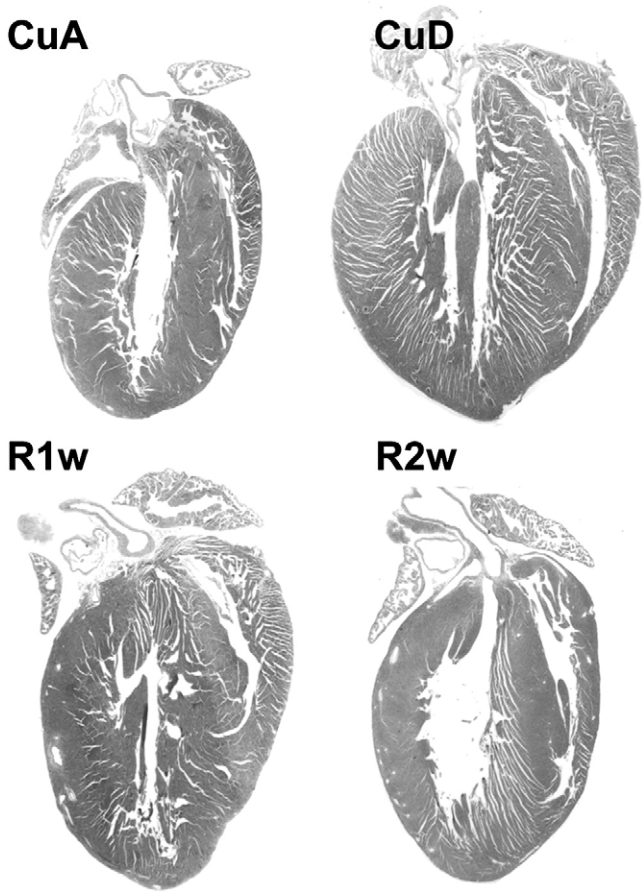

Fig. 1. Regression of CuD heart hypertrophy by Cu repletion. Mice were fed a CuD diet along with CuA controls for 4 weeks and then switched to a CuA diet for 2 weeks. The regression of heart hypertrophy was examined 1 week (R1w) and 2 weeks (R2w) after Cu repletion. Hypertrophy was evidenced by increased wall thickness and decreased chamber lumen; regression, by reversal of the changes.

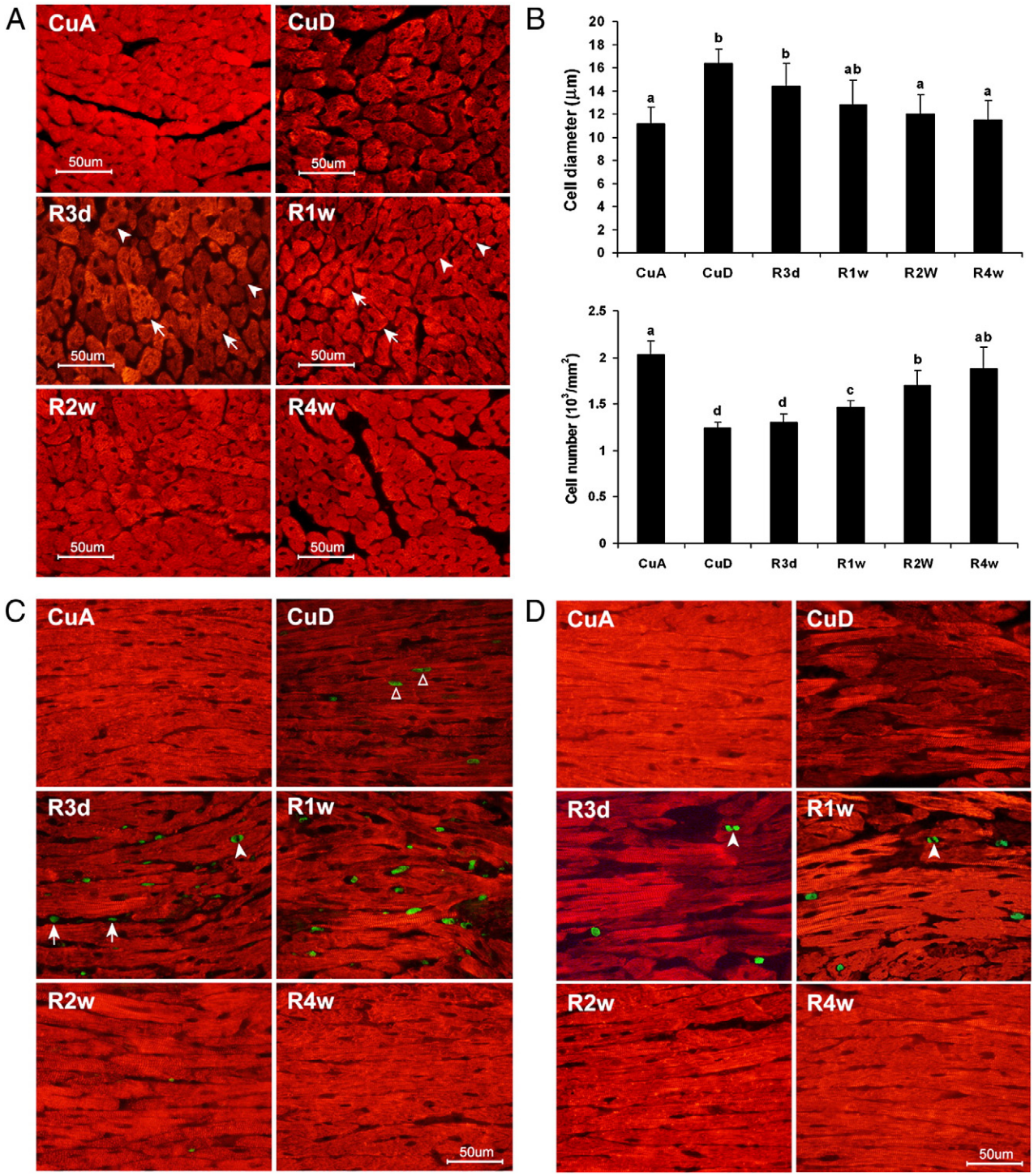

Fig. 2. Regression of Cu deficiency-induced cardiomyocyte hypertrophy by Cu repletion. (A) α-Sarcomeric actin staining of cardiomyocytes (red) shows that Cu deficiency increases the cell size compared with Cu adequacy. Cu repletion for 3 days (R3d), 1 week (R1w), 2 weeks (R2w) or 4 weeks (R4w) gradually decreases the cell size and increases the number of cells. (B) Quantitative analysis of the diameter and number of cardiac myocytes. Digital images were captured with 40× objective; for each histological section, the diameter of 300 myocytes was measured and the total number in a 25-mm^2 field was counted. Values (mean±S.E.M., n=6–8) not sharing the same letter are significantly different (P<.05). (C) Double staining of α-sarcomeric actin (red) and Ki-67 (green) to visualize co-localization of Ki-67-positive nuclei and cardiomyocyte cytoplasm. All the Ki-67-positive nuclei localized in the cardiomyocytes in the CuD heart. The Ki-67-positive nuclei were also found in cells that were not stained by α-sarcomeric actin obtained from Cu-replete mice (R3d and R1w). (D) Double staining of α-sarcomeric actin (red) and phospho-histone 3 (green) to identify mitotic nuclei and their co-localization with myocyte cytoplasm. Mitotic nuclei were not found in the CuD heart but were found extensively in the R3d and R1w hearts. Reduced intensity of α-sarcomeric actin staining in the CuD myocardium and gradually regained intensity of α-sarcomeric actin staining in the R3d and R1w hearts and normalization 2 weeks after Cu repletion (R2w) were observed in (A), (C) and (D).

repletion-induced regression of heart hypertrophy, we measured the size of cardiomyocytes at different stages of the regression. Histological sections of the myocardium were stained with α-sarcomeric actin antibody to label cardiomyocytes specifically. In the hypertrophic myocardium, the average size of cardiomyocytes was significantly increased (Fig. 2). During the regression, a progressive decrease in the number of hypertrophic myocytes and an increase in the number of normal-sized cardiomyocytes were observed 3 and 7 days after dietary Cu repletion. Normalization in the size of the cardiomyocytes within 2 weeks and that in the number within 4 weeks were observed (Fig. 2). By examination through EM, we found mitochondrial swelling and disorganization, myofibrillar damage in cardiomyocytes from the hypertrophic myocardium and transitional improvement in the morphological changes after 3 and 7 days of Cu repletion and a normalization within 2 weeks of Cu repletion (Fig. 3).

3.2. Cu repletion-induced cell cycle reentry of hypertrophic cardiomyocytes

We performed a TUNEL assay to detect apoptotic cardiomyocytes and found that, although there were a few TUNEL-positive cardiomyocytes in the CuD hearts, no TUNEL-positive cardiomyocyte was detected in any myocardial tissue section obtained from the Cu-replete mice (data not shown), excluding the possibility that apoptosis may be responsible for the clearance of the hypertrophic cardiomyocytes. Therefore, a direct reduction in the size would be ascribed to the normalization of the hypertrophic cardiomyocytes. The normalization of the number of cardiomyocytes in the heart, which had a reduction in the hypertrophic myocardium, would require repletion of the lost cardiomyocytes.

Hypertrophic cardiomyocytes may go through mitosis with reduced cell size, leading to normalization in both cellular volume and total number. To test this, we first used Ki-67, a nuclear protein present only in proliferating cells [15], to identify the cells in the cell cycle. This protein is expressed during G1, S, G2 and metaphase but is neither present in quiescent cells nor involved in DNA repair [15]. We found a few Ki-67-positive cells in the CuD hypertrophic hearts, but the number of the Ki-67-positive cells was significantly increased in the myocardium obtained from mice with Cu repletion for 3 or 7 days (Figs. 2C and 4C). There were very few Ki-67-positive cells after 2 weeks of Cu repletion (Figs. 2C and 4C). We also found that the Ki-67-positive cells in CuD myocardium were cardiomyocytes, as they were dually labeled with α-sarcomeric actin, whereas Ki-67-positive cells in Cu-replete hearts likely included other cell types (Fig. 2C).

3.3. Mitosis of hypertrophic cardiomyocytes induced by Cu repletion

To determine if all or any of these Ki-67-positive cells undergo mitosis, we used mitosis-specific phospho-histone 3 antibody [16]. We found non-mitotic cells in most cases or

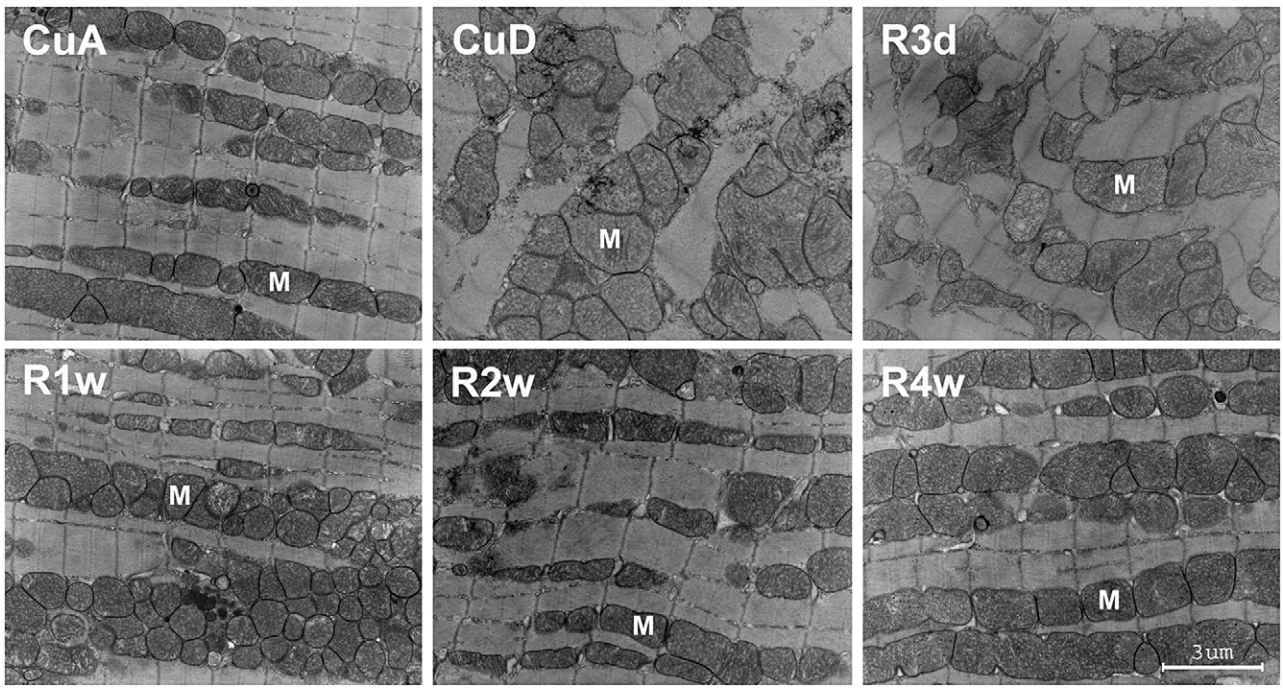

Fig. 3. Electron micrograph of cardiac myocytes obtained from CuD mice in comparison with those obtained from CuA mice. Cu repletion for 3 days (R3d), 1 week (R1w), 2 weeks (R2w) or 4 weeks (R4w) caused progressive recovery of the degenerated changes in the myocytes. Mitochondrial swelling with vacuolar disorganization and disarray of cristae was observed in the CuD myocardium and was completely recovered 2 weeks after Cu repletion. Myofibrillar damage was also observed. M=mitochondria. Bar represents 3 μm.

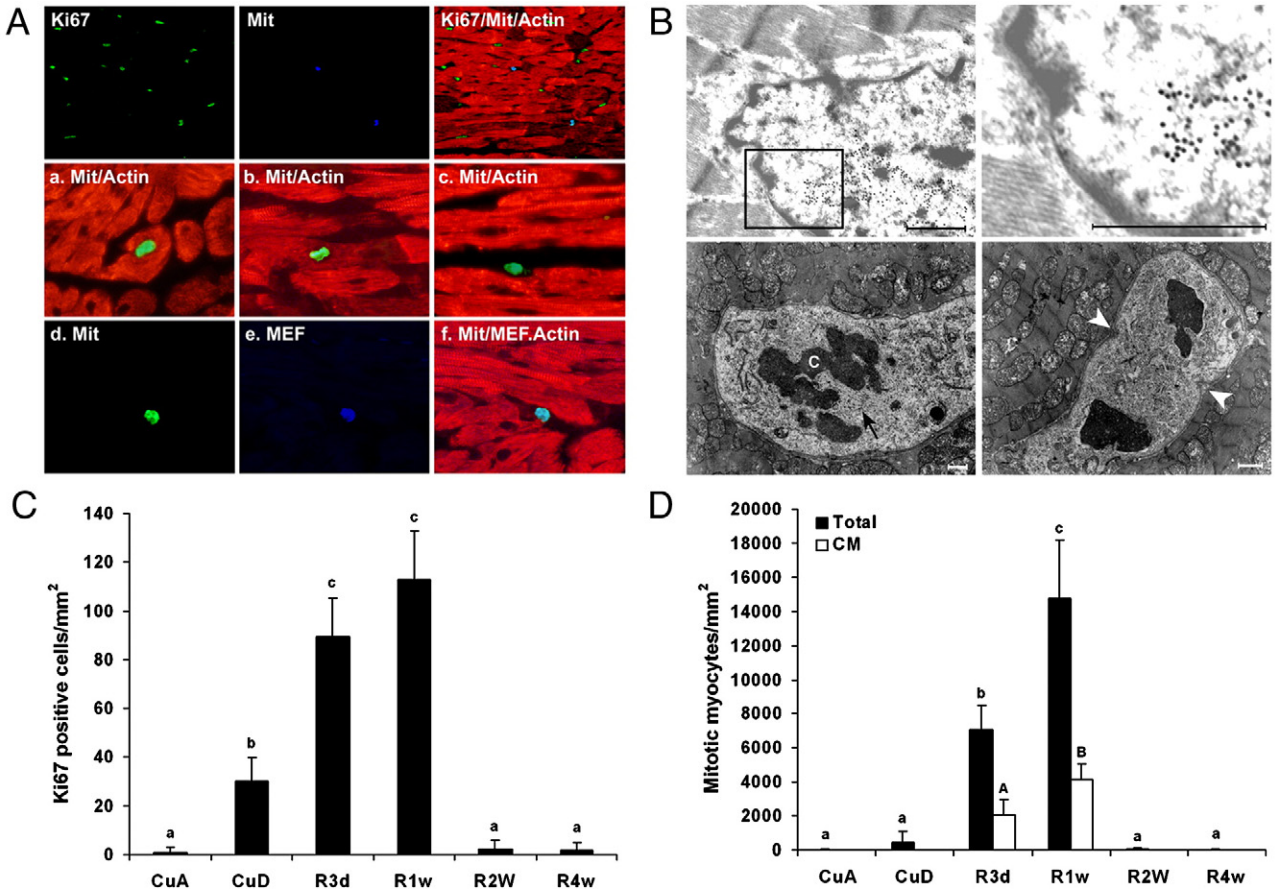

Fig. 4. Identification of the origin of mitotic cells in hypertrophic myocardium undergoing regeneration (Cu repletion for 1 week). (A) Dual staining of Ki-67 (blue) and phospho-histone 3 (green) for visualizing co-localization of the mitotic nuclei with the Ki-67 cell cycle-positive marker (top row). All the mitotic positive nuclei are Ki-67 positive (arrowheads), but not vice versa. (a) Phospho-histone 3-positive nucleus localized in hypertrophic cardiomyocyte. (b) Phospho-histone 3 nucleus localized in hypertrophic myocyte with degenerated cytoplasm (indicated by decreased intensity of α-sarcomeric actin staining). (c) Nucleus stained by phospho-histone 3 but not localized in myocyte. The micrographs of (d), (e) and (f) show that a mitotic positive nucleus (d) is also MEF-2 positive and a cardiomyocyte-specific marker (e), although the nucleus is not localized in myocyte (f). (B) Identification of cell cycle-active nuclei in the myocardium by EM. (a) Immunogold (20 nm of gold) electron micrograph of Ki-67 in cardiomyocyte demonstrating the cell cycle activity. (b) Higher magnification of the framed area of (a). (c) Electron micrograph of anaphase nucleus. (d) Early telophase nucleus. The disorganized mitochondria are also shown in (c) and (d), demonstrating the mitotic nuclei localized in the degenerated cardiomyocyte. (C) Quantitative analysis of Ki-67-positive cells under different conditions as shown in Fig. 2C. (D) Mitotic nuclei in hypertrophic cardiomyocytes (CM) were differentiated as described in the text, and the total mitotic nuclei include all phospho-histone 3-positive cells. The number of the Ki-67- or phospho-histone 3-positive nuclei among 10,000 nuclei in each histological section was counted. Each data point was obtained from six to eight heart samples and expressed as mean± S.E.M., and the mean values not sharing the same letter are significantly different ($P<05$).

only one mitotic cell for the most in any histological section from the CuD hypertrophic myocardium but a large number of mitotic cells from the myocardium of mice fed Cu-replete diet for 3 or 7 days (Figs. 2C and 4C). Considerably fewer mitotic cells than Ki-67-positive cells were present at any given time under all the conditions (Figs. 2 and 4), but all mitotic cells were Ki-67 positive (Fig. 4A). Mitosis of hypertrophic cardiomyocytes was identified by dual immunohistochemical staining with α-sarcomeric actin and phospho-histone 3 in hypertrophic cardiomyocytes (Fig. 4A). To further define that duplication of the existing cardiomyocytes plays a role in myocardial regeneration, we used EM and observed that Ki-67 immunogold staining was localized in the nuclei of cardiomyocytes (Fig. 4B). We also found cardiomyocytes with disorganized organelles in the process of mitosis (Fig. 4B).

We determined quantitatively the contribution of the duplication of hypertrophic cardiomyocytes to the regenerated myocardium. Only those mitotic myocytes clearly showing hypertrophy were counted as duplicating cardiomyocytes. Other mitotic nuclei that were not in the hypertrophic cardiomyocytes were counted only for the total number of the mitotic nuclei. However, these latter nuclei were found to contain the myocyte-specific transcription factor MEF-2, demonstrating that these cells were either of myocyte origin or readily differentiated to myocyte (Fig. 4A). With the exclusion of this type of cells, we found that about one-third of the mitotic cells were hypertrophic cardiomyocytes in the regenerating myocardium, but none of the mitotic myocytes was present in CuD hypertrophic myocardium (Fig. 4C).

4. Discussion

The results here demonstrate that Cu repletion-induced regression of heart hypertrophy is associated with normalization of the size of the hypertrophic cardiomyocytes as well as the total number of cardiomyocytes in the heart. This process is likely accomplished by a combination of several cellular dynamic changes. It appeared that a direct reduction in the size of the hypertrophic cardiomyocytes was involved in this process. The division of the hypertrophic cardiomyocytes with reduced cell size was also detected. In addition, it is possible that cardiac progenitor cells were involved in the regeneration process.

A direct reduction in the size by Cu supplementation of hypertrophic cardiomyocytes has been observed in vitro in our recent studies [17]. Primary cultures of neonatal rat cardiomyocytes were treated with phenylephrine (PE) at a final concentration of 100 μM in cultures, and the cells became hypertrophic after the treatment with PE for 48 h. In that study, we found that the purity of cardiomyocytes in the cultures was more than 95%, determined by flow cytometry cell sorting of α-sarcomeric actin-stained cardiomyocytes. The hypertrophic cardiomyocytes had increased expression of β-myosin heavy chain protein, α-skeletal actin and atrial natriuretic peptide, along with increased cell volume and protein content. The hypertrophic cardiomyocytes were exposed to Cu sulfate at a final concentration of 5 μM in cultures. This Cu treatment reduced the size of the hypertrophic cardiomyocytes, as measured by flow cytometry, protein content in cells and cell volume. Cell cycle analysis and cell sorting of phospho-histone 3-labeled cardiomyocytes indicated that cell division was not involved in the Cu-induced regression of cardiomyocyte hypertrophy. This in vitro study thus demonstrates that Cu reduces the size of hypertrophic cardiomyocytes, leading to regression of cardiac hypertrophy in the primary cultures of neonatal rat cardiomyocytes.

Reentry into cell cycle and mitosis of the hypertrophic cardiomyocytes would make a critical contribution to the regression of heart hypertrophy in vivo. This contribution to the regression of heart hypertrophy was not observed in our in vitro studies using primary cultures of neonatal rat cardiomyocytes. In the hypertrophic heart, the hypertrophic cardiomyocytes were Ki-67 positive. In the regenerating myocardium, the mitotic cardiomyocytes were those having increased volume. The time course of the regression of the hypertrophic cardiomyocytes was coincident with the mitotic active period and closely correlated with the time course of the myocardial regeneration. At the end of the 2-week regression, the hypertrophic cardiomyocytes disappeared and mitosis ceased, further indicating the contribution of duplication of hypertrophic cardiomyocytes to myocardial regeneration.

The rapid regression of heart hypertrophy following Cu repletion was initially observed in the Cu deficiency-induced heart hypertrophy in a mouse model. Our recent studies have shown that dietary supplementation with physiologically relevant levels of Cu also made a rapid regression of heart hypertrophy induced by pressure overload in a mouse model [18]. These observations suggest that pathological heart hypertrophy would be like physiological heart hypertrophy in which the regression of the hypertrophy occurs. This notion is also supported by recent human studies that the implantation of left ventricular assist devices in cardiac failure patients can result in an improved geometry of the hypertrophic heart at both organ and cellular levels, thus leading to improved left ventricular function as a result of a reduction in wall stress and improved mechanical performance [19–21]. We show here that differentiated cardiomyocytes have the capacity for self-regeneration. The potential for cell replication is likely retained in the cell but for some reasons is inhibited in the differentiated cardiomyocytes. In the CuD heart, the hypertrophic cardiomyocytes were stimulated to reenter the cell cycle but prevented from undergoing mitosis. On Cu repletion, some critical factors would be available to trigger, or to remove the inhibition of, the transition to mitosis. While Cu repletion initiated this mitotic transition in the present study, mechanical intervention using left ventricular assist devices has apparently caused analogous changes in patients with failing hearts [19–21].

A clinically relevant question is whether or not Cu supplementation to patients with heart disease would be beneficial. Previous studies have documented that the hearts of people who died from ischemic heart disease have low Cu levels [22–25], although the mechanism for Cu loss in the ischemic heart has not been explored. Furthermore, Cu deficiency leads to cardiac ischemic injury and hypertrophic cardiomyopathy [1,2,26]. Multiple signaling pathways and cross-interactions between the pathways are involved in the regulation of cardiac hypertrophy [27]. Therefore, pressure overload and dietary Cu deficiency would activate the same cascade; once the cascade of signaling transduction that leads to hypertrophy is activated, cardiac hypertrophy would be a common endpoint. An intervention targeting the etiology to prevent cardiac hypertrophy would be effective; however, an etiology-targeted approach to reverse the cardiac hypertrophy that has already developed would not be effective. Therefore, the Cu repletion-induced regression of heart hypertrophy observed in this study would not be simply explained as a counteraction of Cu repletion to Cu deficiency. This was further elucidated in the study of Cu supplementation reversing pressure overload-induced heart hypertrophy [20]. These results suggest that a shift from a myocardial remodeling program to a reverse remodeling program may be activated by Cu repletion.

In summary, Cu repletion results in rapid regression of heart hypertrophy and prevents heart failure induced by dietary Cu restriction. Although a direct reduction in the size of the hypertrophic cardiomyocytes is importantly involved in the regression of heart hypertrophy, the hypertrophic cardiomyocytes have the potential to replicate, making an

important contribution to the repletion of the lost cardiomyocytes. The hypertrophic cardiomyocytes have the potential to reenter the cell cycle but undergo mitosis only during Cu repletion-induced regression. In addition, non-myocyte proliferation and differentiation to cardiomyocytes may be involved in the regeneration of myocardium. The combination of these processes in response to Cu repletion contributes to the regression of heart hypertrophy, including the reduction in the size of the hypertrophic cardiomyocytes and the normalization of the total number of cardiomyocytes.

Acknowledgments

This study was supported in part by the National Institutes of Health through grant HL63760. Y.J.K. is a Distinguished University Scholar of the University of Louisville. We thank Xinggu Sun for providing technical assistance.

References

[1] Medeiros DM, Wildman RE. Newer findings on a unified perspective of copper restriction and cardiomyopathy. Proc Soc Exp Biol Med 1997;215:299–313.
[2] Elsherif L, Ortines RV, Saari JT, Kang YJ. Congestive heart failure in copper-deficient mice. Exp Biol Med 2003;228:811–7.
[3] Elsherif L, Wang L, Saari JT, Kang YJ. Regression of dietary copper restriction-induced cardiomyopathy by copper repletion in mice. J Nutr 2004;134:855–60.
[4] Sabbah HN, Sharov VG. Apoptosis in heart failure. Prog Cardiovasc Dis 1998;40:549–62.
[5] Kang YJ, Zhou ZX, Wu H, Wang GW, Saari JT, Klein JB. Metallothionein inhibits myocardial apoptosis in copper-deficient mice: role of atrial natriuretic peptide. Lab Invest 2000;80:745–57.
[6] Richey PA, Brown SP. Pathological versus physiological left ventricular hypertrophy: a review. J Sports Sci 1998;16:129–41.
[7] Kishimoto I, Rossi K, Garbers DL. A genetic model provides evidence that the receptor for atrial natriuretic peptide (guanylyl cyclase-A) inhibits cardiac ventricular myocyte hypertrophy. Proc Natl Acad Sci U S A 2001;98:2703–6.
[8] Zahabi A, Picard S, Fortin N, Reudelhuber TL, Deschepper CF. Expression of constitutively active guanylate cyclase in cardiomyocytes inhibits the hypertrophic effects of isoproterenol and aortic constriction on mouse hearts. J Biol Chem 2003;278:47694–9.
[9] Boluyt MO, O'Neill L, Meredith AL, Bing OH, Brooks WW, Conrad CH, et al. Alterations in cardiac gene expression during the transition from stable hypertrophy to heart failure. Marked upregulation of genes encoding extracellular matrix components. Circ Res 1994;75:23–32.
[10] Flesch M, Erdmann E, Bohm M. Changes in beta-adrenoceptors and G-proteins during the transition from cardiac hypertrophy to heart failure. J Card Fail 1996;2:S35–43.
[11] Grossman W. Diastolic dysfunction in congestive heart failure. N Engl J Med 1991;325:1557–64.
[12] Chemla D, Coirault C, Hebert JL, Lecarpentier Y. Mechanics of relaxation of the human heart. News Physiol Sci 2000;15:78–83.
[13] Reeves PG, Nielsen FH, Fahey Jr GC. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition Ad Hoc Writing Committee on the Reformulation of the AIN-76A Rodent Diet. J Nutr 1993;123:1939–51.
[14] Kang YJ, Chen Y, Yu A, Voss-McCowan M, Epstein PN. Overexpression of metallothionein in the heart of transgenic mice suppresses doxorubicin cardiotoxicity. J Clin Invest 1997;100:1501–6.
[15] Scholzen TGerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol 2000;182:311–22.
[16] Wei Y, Yu L, Bowen J, Gorovsky MA, Allis CD. Phosphorylation of histone H3 is required for proper chromosome condensation and segregation. Cell 1999;97:99–109.
[17] Zhou Y, Jiang Y, Kang YJ. Copper reverses cardiomyocyte hypertrophy through vascular endothelial growth factor-mediated reduction in the cell size. J Mol Cell Cardiol 2008;45:106–17.
[18] Jiang Y, Reynolds C, Xiao C, Feng W, Zhou Z, Rodriguez W, et al. Dietary copper supplementation reverses hypertrophic cardiomyopathy induced by chronic pressure overload in mice. J Exp Med 2007;204:657–66.
[19] Zafeiridis A, Jeevanandam V, Houser SR, Margulies KB. Regression of cellular hypertrophy after left ventricular assist device support. Circulation 1998;98:656–62.
[20] Mancini DM, Beniaminovitz A, Levin H, Catanese K, Flannery M, DiTullio M, et al. Low incidence of myocardial recovery after left ventricular assist device implantation in patients with chronic heart failure. Circulation 1998;98:2383–9.
[21] Muller J, Wallukat G, Weng YG, Dandel M, Spiegelsberger S, Semrau S, et al. Weaning from mechanical cardiac support in patients with idiopathic dilated cardiomyopathy. Circulation 1997;96:542–9.
[22] Zama N, Towns RLR. Cardiac copper, magnesium, and zinc in recent and old myocardial infarction. Biol Trace Elem Res 1986;10:201–8.
[23] Wester PO. Trace elements in human myocardial infarction determined by neutron activation analysis. Acta Med Scand 1965;178:765–88.
[24] Chipperfield B, Chipperfield JR. Differences in metal content of the heart muscle in death from ischemic heart disease. Am Heart J 1978;95:732–7.
[25] Anderson TW, Neri LC, Schreiber GB, Talbot FD, Zdrojewski A. Letter: ischemic heart disease, water hardness and myocardial magnesium. Can Med Assoc J 1975;113:199–203.
[26] Klevay LM, Saari JT. Comparative responses of rats to different copper intakes and modes of supplementation. Proc Soc Exp Biol Med 1993;203:214–20.
[27] Schaub MC, Hefti MA, Harder BA, Eppenberger HM. Various hypertrophic stimuli induce distinct phenotypes in cardiomyocytes. J Mol Med 1997;75:901–20.