Cardiac cell proliferation assessed by EdU, a novel analysis of cardiac regeneration

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Abstract Emerging evidence suggests that mammalian hearts maintain the capacity for cardiac regeneration. Rapid and sensitive identification of cardiac cellular proliferation is prerequisite for understanding the underlying mechanisms and strategies of cardiac regeneration. The following immunologically related markers of cardiac cells were analyzed: cardiac transcription factors Nkx2.5 and Gata 4; specific marker of cardiomyocytes TnT; endothelial cell marker CD31; vascular smooth muscle marker smooth muscle myosin IgG; cardiac resident stem cells markers IsL1, Tbx18, and Wt1. Markers were co-localized in cardiac tissues of embryonic, neonatal,

adult, and pathological samples by 5-ethynyl-2'-deoxyuridine (EdU) staining. EdU was also used to label isolated neonatal cardiomyocytes in vitro. EdU robustly labeled proliferating cells in vitro and in vivo, co-immunostaining with different cardiac cells markers. EdU can rapidly and sensitively label proliferating cardiac cells in developmental and pathological states. Cardiac cell proliferation assessed by EdU is a novel analytical tool for investigating the mechanism and strategies of cardiac regeneration in response to injury.

Keywords Cardiac cells · Proliferation · EdU · Regeneration · Injury

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Introduction

The heart was long considered as a static organ, incapable of self-renewal. In humans, myocardial infarction (MI) is characterized by the irreversible loss of heart tissue that is ultimately replaced by fibrous scar. In contrast to the prevailing viewpoints for mammals, some fish and amphibians retain a robust capacity for cardiac regeneration by cardiomyocyte proliferation (Kikuchi et al. 2010; Jopling et al. 2010) or stem cell-mediated regeneration (Lepilina et al. 2006). Growing evidence suggests that mammalian hearts maintain some capacity for regeneration. A specific study of ^{14}C dating in human heart tissue showed a continuous production of cardiomyocytes

throughout the entire lifespan (Bergmann et al. 2009). The ability of human cardiomyocytes to divide after MI was also demonstrated (Beltrami et al. 2001). Most recently, regeneration capacity was reported in the neonatal mouse heart for a short period after birth, with the majority of newly formed cardiomyocytes derived from preexisting cardiomyocytes (Porrello et al. 2011). Stem cells or precursor cells were capable of regenerating adult mammalian cardiomyocytes after injury (Hsieh et al. 2007).

The lack of a robust cellular proliferative response prevents efficient cardiac recovery upon injury. Investigation of cardiac cell proliferation may provide further insight into the requirements for cardiac regeneration. Compared with [^3H]-thymidine, 5-bromo-2-deoxyuridine (BrdU) has been extensively used, but BrdU-based immunochemistry can be problematic because of strong DNA denaturing conditions, such as strong acids and heating, which can distort the cell and tissue structure and result in the loss of antigenicity of cellular proteins, preventing immunochemical detection (co-localization) by their specific antibodies (Chehrehasa et al. 2009; Salic and Mitchison 2008; Qu et al. 2011). An alternative thymidine analogue, 5-ethynyl-2'-deoxyuridine (EdU), is a fast and specific agent that does not require DNA denaturation. EdU stains proliferating cells using a fluorescent azide that covalently binds to alkyne groups in a reaction known as “click chemistry” (Hsu et al. 2007). Though labeling with EdU showed a more increased sensitivity compared to BrdU (Cappella et al. 2008; Buck et al. 2008), and EdU has been used to studying cell proliferation in human atrial tissues, zebrafish and adult nervous system (Wada et al. 2013; Guner-Ataman et al. 2013; Zeng et al. 2010a, b), the aim of the present study was to investigate the novel method based on EdU for the analysis of proliferating cardiac cells in mice at different developmental periods: embryonic, neonatal, and adult. Furthermore, we investigated whether EdU can assess cardiac cell proliferation post-MI.

Materials and methods

EdU reagents

The 50 assay Click-iTTM EdU cell proliferation Assay Kit (Cat# C10339, Invitrogen, Carlsbad, CA, USA)

contains reagents termed component A to component G. According to the supplier's description, components are as follows: component A, EdU; B, Alexa Fluor 594 azide; C, DMSO; D, Click-iTTM EdU reaction buffer; E, CuSO₄; F, Click-iTTM EdU buffer additive; G, Hoechst 33342.

Approval of animal experiments

This study was performed according to the recommendations stated in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Committee on the Ethics of Animal Experiments of the University of Minnesota approved the study (Permit Number: 27-2956). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Myocardial infarction

The mice used in this study were anesthetized with sodium pentobarbital (40 mg/kg i.p.), and mechanically ventilated. After the heart was exposed through a lateral thoracotomy, a 6–0 polypropylene thread was passed around the left coronary artery and the artery was occluded. The ECG was recorded to confirm the presence of infarction (Yang et al. 2013; Zeng et al. 2010a, b).

EdU labeling in vivo

EdU was given at a dose of 50 mg/kg body weight in a solution of 10 mg/ml PBS (pH 7.35) (Hsu et al. 2007). Control mice received an intraperitoneal injection of 0.1 M PBS alone. Pregnant mice at embryonic day 10.5 and 17.5, neonatal mice at day 7, week 3 and adult mice at day 3, week 3 post-MI were used in the experiment. Group 1 consisted of pregnant mice and neonatal mice treated with a single intraperitoneal injection of EdU and sacrificed at 1 h after injection. Group 2 consisted of adult mice at week 3 and day 3, week 3 post-MI receiving a single intraperitoneal injection of EdU and sacrificed after 2 h. Six mice hearts of every group were harvested and cryopreserved in OCT medium (Sigma, St. Louis, MO, USA). The frozen mouse hearts were sectioned at 6 μm with a cryostat, and 12 sections of one heart were randomly chosen for EdU or BrdU staining.

EdU labeling in vitro

Primary cultures of mouse ventricular cardiomyocytes were prepared from hearts of 1–2-day old newborn mice and performed as previously described (Formigli et al. 2009). To remove fibroblasts, isolated cells were filtered through a 70 μ m cell strainer (Millipore, Billerica, MA, USA) and then pre-plated twice on uncoated dishes for 40 min. Cardiomyocytes in the supernatant were then centrifuged and plated on collagen-coated coverslips in DMEM culture medium containing 10 % horse serum, 5 % fetal calf serum. Then, cells were labeled by 2 μ M EdU with or without 2 μ M BrdU for 12 h.

Double staining of EdU and BrdU

Six pregnant mice at embryonic day 17.5 received a single intraperitoneal injection of EdU (50 mg/kg) and BrdU (50 mg/kg) (Sigma, MO). One hour after injection the hearts were processed as described above, and double staining of EdU and BrdU was performed. EdU staining was conducted using Click-iT™ EdU imaging kit (Invitrogen, CA), according to the manufacturer's protocol. Briefly, heart sections or cells were fixed with 4 % paraformaldehyde in phosphate buffered saline (PBS) for 15 min and permeabilized with 0.5 % Triton-X-100 for 20 min. The sections were then incubated with a Click-iT™ reaction cocktail for 30 min for EdU staining. The sections or cells were then incubated in 2 N HCl for 60 min at 37 °C to denature DNA and neutralized with 0.1 M boric acid (pH 8.5) for 30 min. The sections were incubated in blocking solution (3 % natural donkey serum in PBS + 1:500 Triton-X) for 1 h, and incubated with mouse anti-BrdU antibody (Sigma, 1:200) (Sigma, MO) overnight at 4 °C. After washing, sections were incubated with donkey anti-mouse IgG (1:100) (Invitrogen, CA) secondary antibody. Finally, sections or cells were examined using a fluorescence microscope.

Double staining of EdU and various cardiac cell molecular markers

Following the EdU steps described above, sections or cells were double stained using the following antibodies: goat anti-Nkx2.5 (SC-8697, 1:150) (Santa Cruz Biotechnology, CA) (Zeng et al. 2011), goat anti-Gata 4 (SC-1237, 1:200) (Santa Cruz Biotechnology, CA)

(Saravanakumar and Devaraj 2012), mouse anti-TnT (Thermo scientific Ab-1, 1:1,000) (Thermo Scientific, IL) (Porrello et al. 2011), mouse anti-CD31 (BD, 1:500) (BD Pharmingen, CA) (Shu et al. 2010), rabbit anti-SMM IgG (BTI-562, 1:300) (Biomedical Technologies, MA), goat anti-Tbx18 (SC-17869, 1:100) (Santa Cruz Biotechnology, CA) (Zeng et al. 2011), rabbit anti-Wt1 (SC-192, 1:300) (Santa Cruz Biotechnology, CA) (Zeng et al. 2011), and rabbit anti-isI1 (ab26122, 1:150) (Abcam, MA) antibodies. The sections were then incubated with the appropriate secondary antibody. The incubation for each primary antibody was performed at 4 °C overnight or at 37 °C for 1 h followed by three washes with PBS. Finally, the stained sections were examined using a fluorescence microscope (Laguerre et al. 2010; Nassiri et al. 2007).

Results

Edu and BrdU co-localized in the heart tissues and cardiomyocytes

To know how EdU compares with the conventional BrdU-labeling method, tissues from hearts at E17.5 and cardiomyocytes from 1 to 2-day old newborn mice were labeled with both EdU and BrdU. Specimen processing for EdU was simple and rapid. In addition, as shown in Fig. 1 all cells stained with BrdU also show EdU staining. Yet, the EdU staining was significantly more intense than the BrdU staining (Fig. 1).

EdU-labeled cardiac proliferating cells in embryonic, neonatal, and adult hearts

Alexa-594 staining of heart tissues from pregnant mice at E10.5 and E17.5, neonatal mice at 7 days, and adult mice at 3 weeks that received intraperitoneal injection of EdU resulted in the highest red fluorescence in the atrial and ventricular walls and ventricular septum (VS) in the fetus; staining was noticeably reduced after birth (Supplementary Fig. 1). No fluorescence signal was observed in mice that received injections of PBS (data not shown). To detect proliferating cardiac cells in mouse heart tissue, co-immunostaining was performed with EdU, cardiac transcription factors Nkx2.5 and Gata 4, specific marker of cardiomyocytes TnT, endothelial cell marker CD31, and vascular smooth muscle marker

Fig. 1 Edu and BrdU co-localized in the heart tissues and cardiomyocytes. **a** Sections from hearts at E17.5 were stained for EdU and BrdU ($\times 200$). **b** Cardiomyocytes were stained for EdU

and BrdU ($\times 200$). Note that all cells stained with BrdU also show EdU staining. But, the EdU staining is significantly more intense than the BrdU staining (Scale bars, 25 μ m)

Fig. 2 EdU-labeled cardiac proliferating cells in developing hearts. Tissue sections were co-immunostained with EdU and cardiac transcription factors (1) Nkx2.5 ($\times 200$) and (2) Gata 4 ($\times 200$), specific cardiomyocyte marker (Scale bars, 25 μ m) (3) TnT ($\times 400$), endothelial cell marker (4) CD31 ($\times 400$), and

vascular smooth muscle marker (5) smooth muscle myosin IgG. ($\times 400$). Red EdU. Staining shows that EdU can robustly label dividing cardiac cells in vivo (Scale bars, 50 μ m). (Color figure online)

SMM IgG. Figure 2 shows representative double labeling with EdU and other markers. The percentage of proliferating cardiac cell decreased from E10.5 to neonatal day 7, and no EdU incorporation was detected after 3 weeks (data not shown).

EdU-labeled cardiac proliferating cells in adult hearts after MI

To assess wound healing, proliferating cardiac cells were localized using the following markers: Nkx2.5, Gata 4, TnT, CD31, and SMM. Results showed that a subpopulation of proliferating cells were Nkx2.5, Gata4, and TnT positive, suggesting that these cardiomyocytes were proliferating at day 3 post-MI. Endothelial cells and vascular smooth muscle cells

were also proliferating at day 3 post-MI (Fig. 3a). Most proliferating cells were located at the interface of the infarct and viable tissue. Cellular proliferation ceased by week 3 (Fig. 3b).

EdU-labeled cardiac resident stem cells in embryonic hearts

To determine whether Edu labels cardiac resident stem cells in the heart, we examined the expression of cardiac resident stem cell markers IsL1, Tbx18, and Wt1. IsL1 was previously reported to identify cardiac-lineage progenitor cells within the myocardium. We found IsL1 staining to be clustered in the right atrial wall, in areas that also stained with EdU (Fig. 4a). Tbx18 and Wt1 have recently been recognized as

Fig. 3 EdU-labeled cardiac proliferating cells in adult hearts at day 3 and week 3 after MI. Cardiac cells began the renewal process including regeneration of cardiomyocytes at day 7 post-MI; cellular proliferation ceased by week 3. (1) Nkx2.5 ($\times 200$) and (2) Gata 4 ($\times 200$), specific cardiomyocyte marker (3) TnT

($\times 200$), endothelial cell marker (4) CD31 ($\times 200$), and vascular smooth muscle marker (5) smooth muscle myosin IgG ($\times 200$) (Scale bars, 25 μ m). 6, 7, 8 are higher-magnification views ($\times 400$) (Scale bars, 50 μ m) of the areas within the white squares in 3,4,5 ($\times 200$). Red EdU. (Color figure online)

Fig. 4 EdU-labeled cardiac resident stem cells and neonatal cardiomyocytes in vitro. EdU sensitively labels resident stem cells with IsL1 (a), Tbx18 (b), and WT1 (c) ($\times 200$) (Scale bars, 25 μm), and neonatal cardiomyocytes in vitro ($\times 400$) (Scale bars, 50 μm) (d)

epicardial progenitors; these markers were expressed in the epicardium, which co-localized with EdU (Fig. 4b, c).

EdU-labeled cardiomyocytes in vitro

Assessment of the labeling ability of EdU to the cells in vitro showed that incubation of neonatal cardiomyocytes in EdU-containing media resulted in intense red fluorescence in the nuclei when stained with Alexa-Fluor 594 azide (Fig. 4d).

Discussion

Accumulating evidence suggests that proliferation is involved in cardiac regeneration after injury. The proliferating cells not only include endothelial cells and vascular smooth muscle cells, but also cardiomyocytes. Understanding the mechanisms regulating proliferation may be helpful in identifying novel approaches to enhance their regenerative capacity. One main concern among the currently used approaches to label proliferating cells is the need for an improved nucleic acid labeling technique. BrdU staining has been used extensively, and share several common characteristics with EdU staining. However, studies show that EdU staining has several advantages over BrdU staining. (1) EdU staining does not require DNA denaturation and

therefore preserves the integrity of tissue; (2) EdU staining may conserve antigenicities for molecular markers; (3) EdU staining is much faster than BrdU staining (Guner-Ataman et al. 2013). Our current findings show that EdU robustly labeled dividing cardiac cells in vitro and in vivo. Specimen processing for EdU was simple, rapid, and sensitive and does not require strong DNA denaturing condition. The EdU protocol allowed structural and antigenic integrity to ensure high-resolution multiple-fluorescence microscopy using different antibodies.

The field of cardiomyocyte proliferation during developmental and pathological states has been a major issue in terms of the presence or absence of mitosis (Anversa and Kajstura 1998). Cardiomyocytes comprise only 20–30 % of the cellular heart tissue. At the same time, non-muscle cells play important roles in the heart. During embryonic cardiac development, endothelial cells share the same cardiac mesodermal precursors with cardiomyocytes (Linask and Lash 1993). Interaction between endothelial cells and cardiomyocytes in the heart regulates normal heart functions (Narmoneva et al. 2004). Furthermore, endothelial cells play an essential role in facilitating cardiomyocyte differentiation from pluripotent stem cells (Chen et al. 2010). Endothelial cell and vascular smooth muscle cell proliferation after MI may result in neovascularization, facilitating cardiac cell regeneration. Our study finds that proliferation of

cardiomyocytes, endothelial cells, and vascular smooth muscle cells can be rapidly and sensitively labeled by EdU. Pluripotent cardiac progenitor cells resident in the myocardium have received considerable attention because of their potential roles in cardiac regeneration after injury. Our study indicates that EdU can easily and sensitively identify the proliferation of different resident cardiac cells. There is an inverse relationship between proliferation and differentiation among stem cells, suggesting that signals repressing proliferation may promote differentiation of stem cells into cardiac cells (Studzinski and Harrison 1999). After MI, we observed endothelial cells and vascular smooth muscle cells re-entering the cell cycle. Most interestingly, Nkx2.5, Gata 4, and TnT positive cells also re-entered the cell cycle. Nkx2.5 and Gata 4 are well-characterized markers of early cardiomyocyte lineage (Harvey 1996; Dodou et al. 2004), and Gata4 positive cells have recently been reported to contribute to heart regeneration after injury (Kikuchi et al. 2010). Our data indicate that cardiac cells have proliferative potential and that the mouse heart maintains some capacity to renew cardiomyocyte after MI. However, the proliferation ability is limited and extremely rare after 3 weeks. Most studies attempting to induce cardiomyocyte proliferation use growth factors, cell cycle genes, and oncogenes. Although promising results have been achieved in vitro, the proliferating cardiomyocytes in hearts after MI may be “killed” due to severe pathological conditions (Zeng et al. 2008). The isolated induction of cardiomyocytes to reenter the cell cycle is inadequate. Non-muscle cell proliferation combined with modification of the environment created by pathological damage might be required for efficient cardiac regeneration.

Taken together, our results show that EdU can rapidly and sensitively label cardiac cells in the developmental and pathological heart, and cardiac cells including cardiomyocytes can be observed to re-enter the cell cycle following the stimulus of MI. In the future, we will investigate the effects of cardiac cell proliferation combined with modification of the pathological environment on cardiac regeneration by EdU-based cardiac cell staining.

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Conflict of interest The authors do not have any possible conflicts of interest.

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