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Cardiac Myocytes are Recruited by Bone Marrow-Derived Cells in Intact Murine Heart

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It is generally believed that the cardiac myocytes withdraw from the cell cycle shortly after birth and thereafter any loss of myocardial tissue cannot be repaired. However, recent reports indicate that cardiac myocytes can be regenerated by stem cells derived from bone marrow in the damaged hearts. In this study, we investigated whether bone marrow-derived cells can differentiate into cardiac myocytes in the intact hearts. We performed bone marrow transplantation from syngenic male mice to female c57/B6 mice. In female mice's hearts, the presence of cells from male mice was examined by FISH method that detects Y chromosome. Using the same samples, we also performed immunohistochemical staining with muscle specific antibodies. In the heart sections of female mice, there were some cells that were considered as differentiated myocytes derived from male bone marrow (0.01~0.09% of total myocytes) and the proportion of the cells increased as the period after bone marrow transplantation became longer (3 months after vs. 8 months after). These results suggest that, not only in the damaged heart but also in the intact heart, a portion of cardiac myocytes is recruited by bone marrow-derived cells.

It has been believed that tissues, such as brain or cardiac muscle that does not contain stem cells, are not normally renewed, nor regenerate after damage. Particularly cardiac myocytes were thought to become mitotically inactive through development (3). It is generally accepted that the cardiac myocytes withdraw from the cell cycle and are established shortly after birth, so thereafter any loss of myocardial tissue cannot be repaired. However, the evidence for proliferation of cardiomyocytes in humans has been reported recently (1,6). Furthermore, it is revealed that bone-marrow-derived cells have a capacity to proliferate and transdifferentiate into cell lineages of the host organ (2,7). Bone marrow is the origin of some myocytes in murine ischemic-injured hearts or in hearts of mdx mice (a model of Duchenne's dystrophy) and those cells are thought to transfer to the damaged myocardium for rescue.

For these reasons, we hypothesized that replacement of cardiac myocytes by circulating bone-marrow-derived myogenic progenitor cells may take place even after adolescence. To address this issue, we transplanted bone marrow cells from male donor mice to female

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recipient mice and assessed the presence of donor-derived myocytes in the recipient heart using Y chromosome as a cellular marker. Here, we report that cardiac myocytes are recruited by bone marrow-derived cells in the intact murine heart.

MATERIALS AND METHODS

Bone marrow transplantation

Eight-week-old female c57/BL/6N mice (Charles River Japan Inc) were irradiated (9Gy) and subsequently injected via the tail vein with a total of $2x10^6$ bone marrow cells isolated from femurs of the age- and genetic back ground-matched male mice. The bone marrow transplantation (BMT) was performed at Fuji Gotemba Research Labs, Chugai Pharmaceutical Co. In total, 6 female mice underwent BMT, and they were maintained for either 3 months (3 mice) or 8 months (3 mice) under the specific pathogen-free (SPF) condition in the facilities of Laboratory Animal Science, Kobe University Graduate School of Medicine. All animal care was performed in accordance with institutional guidelines.

Detection of cardiac specific proteins and fluorescence in-situ hybridization (FISH) of Y chromosomes

Three or eight months after BMT, hearts were removed and frozen in isopentane at -80 ºC. Cryostat sections were fixed with acetone for 10 minutes at -20 ºC and probed for the expression of myocyte-specific proteins to distinguish cardiac myocytes. The murine monoclonal antibody against caveolin 3, which is only expressed in smooth, skeletal and cardiac muscle, was obtained from Transduction Labs. Polyclonal goat antiserum to cardiac troponin I-C (C-19), which is exclusively expressed in cardiac myocytes, was purchased from Santa Cruz Biotechnology Inc. As the secondary antibodies, anti-mouse IgG Texas Red linked antibody from Amersham Pharmacia Biotech was used for caveolin 3 and anti-goat IgG Texas Red from EY Labs was for troponin I-C, respectively. The sections were subsequently processed for fluorescence in situ hybridization (FISH) to detect Y chromosome. We used the Star FISH Chromosome Painting System (Whole Mouse Chromosome Y-specific Paint and FITC Amplification Kit) purchased from Cambio Ltd and followed the protocol recommended. The hybridization efficiency was >80% on whole nuclei. For analysis of each mouse, five sections obtained from the middle level of the left ventricle were used. The total number of all myocytes and Y chromosome-positive cells in the five sections were counted by two independent researchers. The interobserver difference in the counted cell numbers was <10% and the average values were used for analysis. All microscopic investigations were performed on a confocal laser scanning microscope.

RESULTS

In heart tissue specimens from the recipient female mice, Y chromosome-specific signals were detected occasionally after BMT. Y chromosome-positive (Y-positive) cells were detected in various areas such as cardiac myocytes or interstitial tissue in specimens. We tried to distinguish cardiac myocytes from other types of cells using immunohistochemistry with antibodies against cardiac specific proteins, caveolin 3 and troponin I. The former exists on the inner surface of the plasma membrane, so its signals partition each myocyte. The latter is the protein that is binding to actin and facilitates the interactions between actin and myosin, so the signals from it exist in the cytoplasm of myocytes. Using imuunostaining with these antibodies, we classified the Y-positive cells into two groups; "Y-positive-center cells" (Fig. A, C) and "Y-positive-other cells" (Fig. B, D). The Y-positive-center cells had Y-positive nuclei in the center of myocytes that are clearly partitioned by signals of caveolin 3 (Fig. A).

We regarded only these Y-positive-center cells as the differentiated cardiac myocytes derived from bone marrow cells of male mice. The other Y-positive cells were classified as Y-positive-other cells. When the nuclei were located in the margin of myocytes or in the interstitial tissue, we could not distinguish whether they belong to myocytes, because the nuclei and the cell boundary margined by caveolin 3 sometimes overlapped (Fig. B). This is a limitation of our methods using immunohistochemistry to detect the location of nuclei and in such a case we regarded those cells as Y-positive-other cells. Same results were obtained using troponin I antibody (Fig. C, D).

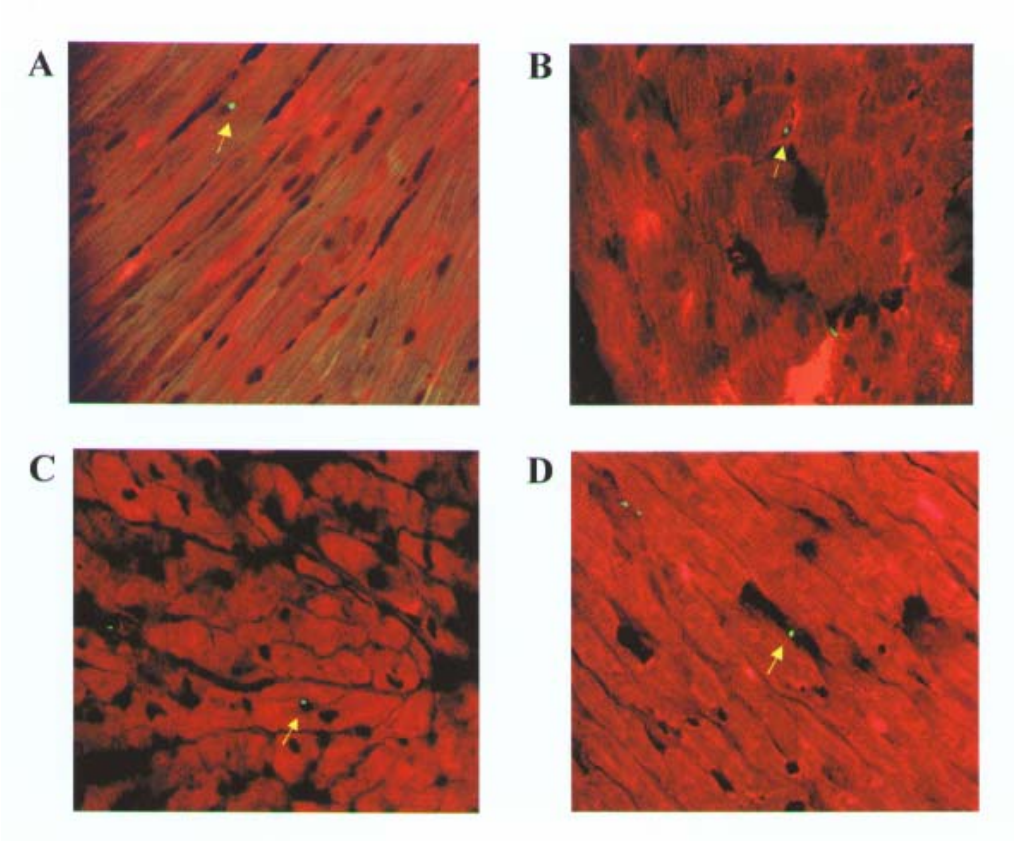


Fig. Identification of bone marrow-derived nuclei in cardiac muscles from BMT female mice by Y chromosome-specific FISH (FITC) on cryosections simultaneously immunostained for caveolin 3 (Fig. A, B) and troponin I (Fig. C, D) (Texas Red). The sections of cardiac muscle were from the female mice that were received BMT 8 months before.

A, C: Y chromosome-specific signal in the myonuclear lacuna (arrows) are located in the center of cardiac myocytes (Y-positive center).

B, D: Y-positive signals are found in peripheral positions of cardiac myocytes or in non-muscle cells (Y-positive other).

The percentage of cardiac myocyte with a donor-derived nucleus appeared to increase as time passed after BMT (Table I). At 3 months after BMT, the Y-positive center was detected in 0.04, 0.01 and 0.01% of the total cardiac myocytes. On the other hand, at 8 months after BMT, the Y-positive center was found in 0.05, 0.09 and 0.07%.

Table I. Detection of donor-derived Y chromosome after bone marrow transplantion.

Mouse	Months after BMT	Y+ center cells *(%)	Y+ other cells # (%)
No.1	3	30 (0.04)	948 (1.16)
No.2	3	14 (0.01)	468 (0.36)
No.3	3	16 (0.01)	526 (0.49)
No.4	8	61 (0.05)	2715 (2.40)
No.5	8	100 (0.09)	1572 (1.34)
No.6	8	58 (0.07)	1273 (1.57)

* Number and percentage of Y chromosome positive nuclei found in the center of cardiac myocytes in five sections analyzed. We regarded these cells as the differentiated cardiac myocytes derived from bone marrow cells.

Number and Percentage of Y chromosome positive nuclei found peripherally in the cardiac myocytes or other cells.

DISCUSSION

In this study, we showed for the first time that bone marrow cells are recruited to cardiac myocytes and undergo muscle specific differentiation in the murine intact heart without myocardial damage. Recently, it was reported that cardiac myocytes is capable of reentering mitotic cell cycles (6). Several reports have demonstrated that after myocardial damage, particularly after acute myocardial infarction, bone marrow-derived cells are mobilized to the damaged region, replicate, differentiate to cardiac myocytes, and ultimately promote myocardial repair (5,8,9). However, in the present study, bone-marrow-derived cells were found to be located within normal cardiac muscle fibers. The presence of Y-positive center cells are not explained by adhesive inflammatory cells such as macrophages, since the present study was performed in the intact heart in the absence of cardiac damage. It seemed that bone marrow-derived cells migrated to the heart and incorporated into cardiac muscle fibers. Although we found Y-positive bone-marrow-derived cells in cardiac muscle fibers, the percentage of such cells was low and most of Y-positive cells were located outside of muscle fibers, in the interstitial tissue, in the heart. We did not identify the cell types of those cells, but some of them might be vascular cells. It is revealed that there are potential bone-marrow-derived progenitor endothelial or smooth muscle cells in hearts, which are involved in angiogenesis (12).

There might be a possibility that the Y-positive center cells have another origin. Recently, some investigators demonstrated the presence of cell fusion. Transgenic female-derived neural (14) or bone marrow cells (13) fused with ES cells from male mice in vitro and hybrid cells displayed a dual phenotype. Cell fusion occurred at a frequency of 10^{-4} or 10^{-5} cells. The ratio is lower than that of our experiment, but it is possible that the Y-positive center cells are partly attributed to cell fusion. If cell fusion occurred, the cells may have a tetrapoid number of chromosomes or 4 sex chromosomes (XXXY). In this study we couldn't examine the DNA content of Y-positive center cells. More detailed studies will be necessary for molecular characteristics of cells associated with donor-derived nuclei.

It was intriguing that the percentage of the bone marrow cell-derived myocytes increased with time after bone marrow transplantation. It seems that the donor cells gradually incorporated with recipient cells in the heart, particularly cardiac myocytes. However, as cited, the percentage of bone marrow-derived myocytes was very low. Therefore it is unlikely that such cells play a major role in cardiac mechanical functions.

The role of bone marrow-derived myocytes in the heart remains to be determined. Our

finding is in accordance with a recent clinical report of Quaini et al, in which using transplanted hearts they showed that a part of myocytes of the donor heart was derived from recipient cells (11). Therefore it seems that cardiac muscle is capable of undergoing regeneration by the recruitment of circulating bone-marrow-derived cells, which might be continuously circulating with constant trafficking through all tissues. In the injured heart model, several ligands and their receptors are found to be important for mobilization, such as stem cell factor and c-kit (4), CXCR4 and SDF-1 (10). So far, it has not been clear how the bone marrow derived cells are mobilized to intact heart tissue.

In conclusion, the bone marrow-derived cells from male donors incorporated with female recipient cardiac myocytes in the intact heart tissue. Furthermore, the number of differentiated cardiac muscle increased in the longer period after BMT. The bone-marrow-derived cells appeared to restore cardiac muscle. The present findings widen the concept of myocardial regeneration and would open new perspectives for cell therapy in heart diseases.

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