In Vitro and *In Vivo* Differentiation of Mesenchymal Stem Cells in the Cardiomyocyte Direction

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The possibility of mesenchymal stem cell differentiation in the cardiomyocyte direction was studied on Wistar—Kyoto rats with myocardial infarction induced by ligation of the left coronary artery. *In vitro* treatment of mesenchymal stem cells with 5-azacitidine led to spontaneous contractions of about 15% cells in culture. Analysis of the expression of matrix RNA showed expression of fetal and functional markers of the myocardium in this cell culture. *In vivo* on day 21 after myocardial infarction and intravenous transplantation of mesenchymal stem cells into the periinfarction area, myocardial cells carrying donor label were detected. Immunohistochemical analysis showed that these cells were cardiomyocytes integrated into the myocardium. These cells can be a result of differentiation of transplanted mesenchymal stem cells or fusion of endogenous cardiomyocytes with exogenous mesenchymal stem cells.

Key Words: *mesenchymal stem cells; differentiation; myocardial infarction; cardiomyocytes*

The therapeutic effects of mesenchymal stem cells (MSC) on repair processes in the heart after myocardial infarction are now actively studied [6,9,10]. Experiments on rats showed that intravenously injected MSC migrated to the site of myocardial injury. This phenomenon was observed in all animals irrespective of the time of MSC transplantation: 14, 7, 2 days before myocardial infarction, on the day of the intervention, or 2, 7, 14 days after it. Transplantation of MSC reduced the time of tissue inflammation and cicatrization of the infarction area, appearance (or retention) of subepicardial layer of cardiomyocytes, activation of angiogenesis in the adjacent zone, formation of more elastic cicatrix, and a lesser dilatation of ventricular cavities [1,2]. Morphologically intact cardiomyocytes of unknown origin were present in the infarcted zone. It was hypothesized that stem cells could differentiate into cardiomyocytes directly in the focus of injury, in accordance with the organ niche theory [5].

We verified the possibility of MSC differentiation in the cardiomyocyte direction *in vitro* and *in vivo*.

MATERIALS AND METHODS

Experiments were carried out on male Wistar—Kyoto rats (90-130 g).

Bone marrow suspension was isolated from the femoral bones of rats directly after decapitation: epiphyses were removed under sterile conditions, while diaphyses were washed with αMEM (HyClone) supplemented with 20% FCS (Gibco) and 100 μg/ml penicillin or streptomycin (Gibco). The resultant suspension was inoculated in plastic Petri dishes (Sarstedt). Forty-eight hours after bone marrow explantation, MSC were washed twice from blood cells with PBS (20 mM PBS, pH 7.4; 0.1 M NaCl). The cells were cultured in a monolayer at

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37°C and 5% CO_2 for 6-7 days after explantation. The culture was re-inoculated every 7 days at the initial density of 1.27×10^3 cell/cm^2. Trypsin and EDTA (HyClone) were used for reinoculation of rat MSC. Nutrient medium was replaced every 3 days.

The rat MSC were phenotyped on a FACSscan cytofluorometer (Becton Dickinson). MSC were stained with antibodies to CD45 negative marker (Becton Dickinson) and antibodies to CD90 positive marker (Becton Dickinson). For staining the cells with antibodies to surface markers, the cells were removed from dishes with trypsin and EDTA (HyClone), washed twice in PBS, and placed for 1 h into solution (1:20) of fluorochrome-conjugated monoclonal antibodies. The cells were then washed twice in PBS and the fluorescence intensity was evaluated. Phenotyping was carried out after the first, second, and third reinoculation of the culture.

In order to induce MSC differentiation towards cardiomyocytes, the cells were cultured until the formation of a complete monolayer, after which 5-azacitidine (3 μmol/liter) was added to the standard culture medium. Exposure with 5-azacitidine lasted for 3 weeks, half culture medium being replaced every 3 days. Morphological changes after differentiation were evaluated visually under a microscope (Leica). Phenotyping of the resultant derivatives was carried out by analysis of the marker gene expression by reverse transcription and subsequent polymerase chain reaction.

Myocardial infarction was induced in experimental animals by ligation of the descending branch of the left coronary artery at the level of the lower edge of the left auricle. The rats were narcotized with sodium pentobarbital (40 mg/kg intraperitoneally). A longitudinal incision was made on the skin of the left side of the rat chest, thoracic muscles were separated, and ribs V and VI were drawn apart with retractors. The pericardium was cut and the heart was removed. After ligation of the left coronary artery the heart was placed back into the thoracic cavity; thoracic muscle and skin were sutured. Electrocardiograms were recorded (KFS-01, Kardiometr-MT) before the operation, several days after myocardial infarction, and before decapitation. Myocardial infarction was documented electrocardiographically. Two days after myocardial infarction the animals were intravenously (into the caudal vein) transplanted undifferentiated MSC (5 million in 100 μl αMEM) labeled with PKH26 fluorescent stain. The animals were decapitated 21 days after infarction.

Histological preparations were made directly after decapitation. A segment with the infarcted zone was resected from the heart for cryofixation. The specimen was cooled in liquid nitrogen vapor for 10 sec, plunged in liquid nitrogen for 1 h, and then put into a freezer (-70°C). The sections (7 μ) were prepared using a Leica CM 1900 Cryostat microtome (Leica), mounted on glasses, spread by warming, and dried at room temperature for 2 min. The preparations were embedded into propylgallate (5% in glycerol) and the distribution of labeled MSC was analyzed using a Leica DM LB HC fluorescent microscope (Leica). For immunohistochemical staining, the cryosections were mounted on glasses, spread by warming, dried for 2 min at room temperature, washed twice in PBS, and placed into solution of first antibodies to connexin 43 (Chemicon). FITC-conjugated asinine antibodies to mouse immunoglobulins (Chemicon) served as second antibodies. For staining with antibodies to GATA4 transcription factor and troponin I, the cryosections were washed in PBS, incubated (25 min) in Tris-HCl buffer (pH 2) in a boiling water bath, washed twice in PBS, incubated in 10% fetal serum containing 0.03% sodium azide, 0.01% Triton X-100, and put into solution of first polyclonal antibodies to GATA4 (Santa Cruz) or monoclonal antibodies to troponin I (Chemicon). The sections were then washed in PBS and placed into solution of second antibodies. Goat antibodies to rabbit immunoglobulins or asinine antibodies to mouse immunoglobulins, conjugated with Cy3, served as the second antibodies. All preparations for immunohistochemical analysis were embedded in propylgallate (5% in glycerol) and examined in a Leica DM LB HC fluorescent microscope (Leica).

RESULTS

Cardiomyocyte banks in the culture served as morphological criterion of MSC differentiation *in vitro* (Fig. 1). We detected spontaneously contracting cell conglomerations. About 15% MSC differentiated in the cardiomyocyte direction, which is in line with previous data [4].

Analysis of the expression of cardiomyocyte marker genes revealed expression of proteins GATA4 and Nkx2.5 genes (myocardial precursor determination factors in early vertebrate embryogenesis) in the population of differentiated MSC. Expression of connexin 43 (the main protein of gap junctions essential for the transmission of the contraction signal) was detected in the population of differentiated MSC, which is characteristic of differentiating myocardium [12]. Increased expression of troponins T, I, and cardiotrophin-1 (cardiomyocyte differentiation markers) was detected (Fig. 2). These results suggest that MSC differentiation under the

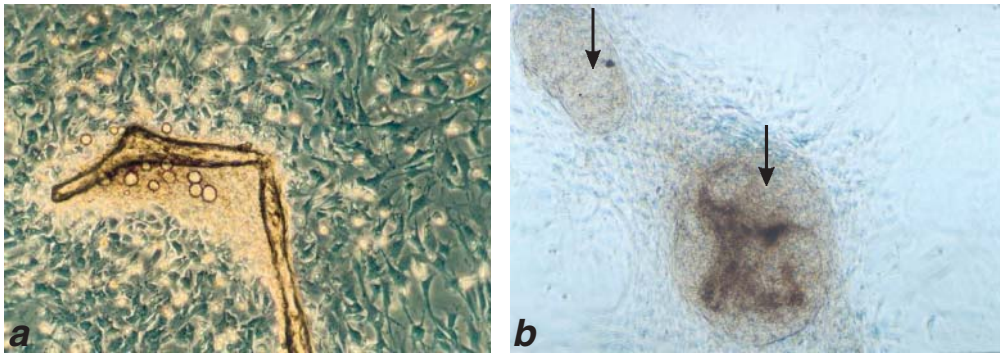

Fig. 1. Changes in the morphology of rat MSC differentiating in the cardiomyocyte direction (×60). *a*) undifferentiated MSC; *b*) MSC (shown by arrows) after treatment with 5-azacitidine.

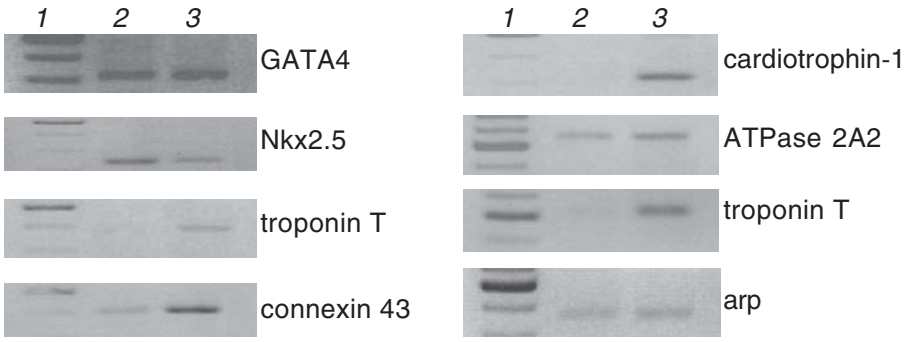

Fig. 2. Cardiomyocyte differentiation markers. *1*) undifferentiated MSC; *2*) cardiomyocyte differentiation; *3*) H_2O.

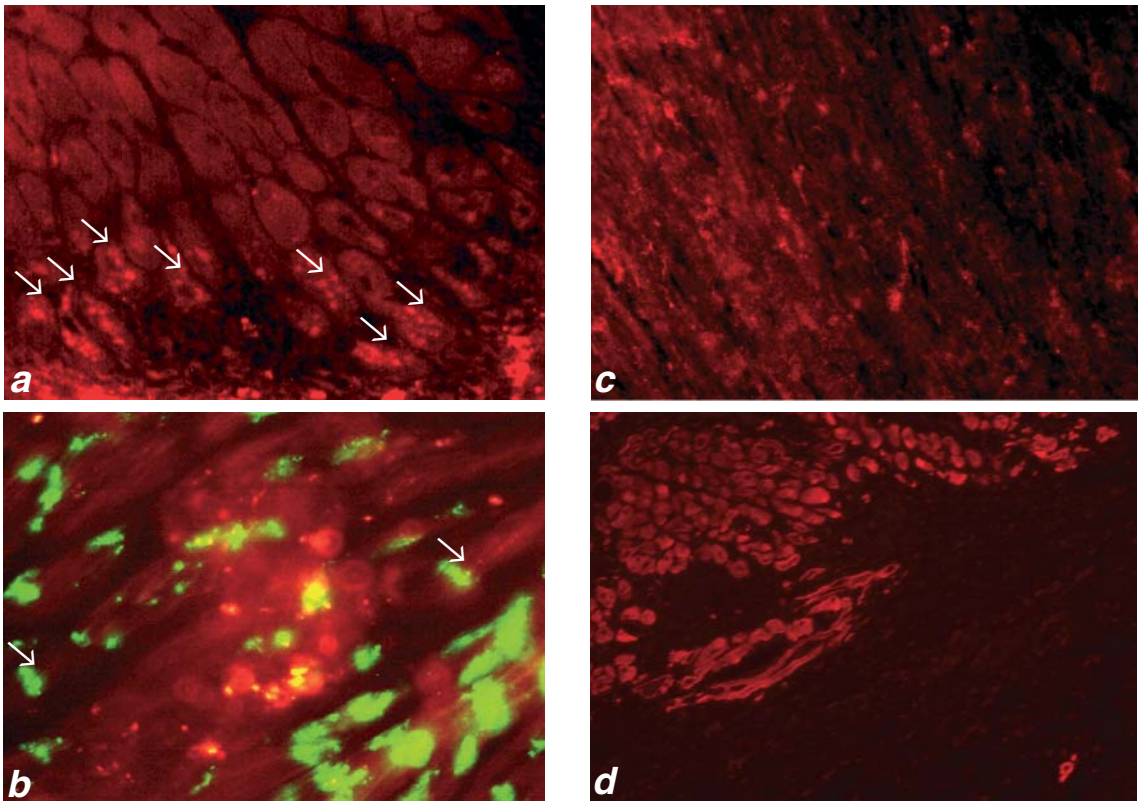

Fig. 3. Histochemical staining of the zone adjacent to myocardial infarction in rats (×45). *a*) distribution of cells with donor MSC label in myocardial cryosections from animals with myocardial infarction. Arrows show stained cardiomyocytes; *b*) staining of periinfarction zone with antibodies to connexin 43 (green); red staining: PKH26. Arrows show contact of stained and unstained cardiomyocytes; *c*) staining of periinfarction zone with antibodies to GATA4 (red); *d*) staining of periinfarction zone with antibodies to troponin T (red).

effect of 5-azacitidine led to the formation of functionally active cardiomyocytes. Expression of cardiomyocyte differentiation transcription factors was also observed in undifferentiated MSC, which indirectly attests to differentiation potential of MSC.

Cells morphologically resembling cardiomyocytes and carrying donor MSC label were detected 21 days after infarction in the myocardial periinfarction zone of animals with transplanted MSC. The layer of these cells was directly adjacent to the infarcted zone (Fig. 3, *a*).

Staining of heart cryosections with antibodies to connexin 43 showed that labeled cells contained connexin 43 and formed gap junctions with stained cells and with recipient cardiomyocytes (Fig. 3, *b*). Immunocytochemical analysis of heart sections using antibodies to other cardiomyocyte markers (troponin I and GATA4) showed that all cells adjacent to the zone of injury were stained for GATA4 and troponin I (Fig. 3, *c*, *d*). Hence, myocardial cells adjacent to the zone of injury and carrying donor label were cardiomyocytes integrated into the myocardium. However, from these data we cannot definitely conclude that donor MSC differentiated into cardiomyocytes. The phenomenon of cell fusion is actively discussed in recent years. Spontaneous fusions of cells were detected for fetal stem cells, hemopoietic stem cells, and monocytes [3,7, 8,11]. Fusion of monocytes and cardiomyocytes in the periinfarction zone is a sort of defense reaction. We cannot rule out this mechanism of emergence of cardiomyocytes with donor label.

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