Colony-Forming Cells in Rat Myocardium after Destructive Exposure and Intramyocardial Transplantation of Bone Marrow Cells

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The content of colony-forming cells in myocardial cell culture from the perinecrotic zone of rat heart was evaluated on day 40 after cryodestruction. The mean cellularity after cryodestruction was 12-fold lower than in intact animals. Intramyocardial transplantation of bone marrow cells (mononuclears, mesenchymal stem cells, and mesenchymal stem cells treated with 5-azacitidine) into the perinecrotic zone increased the content of colony-forming cells. After transplantation of mesenchymal stem cells and mesenchymal stem cells treated with 5-azacitidine, the number of colonies reached 33±7 and 11±3, the mean cellularity being 2975±80 and 1105±42 cells/cm^2, respectively. Hence, intramyocardial transplantation of mesenchymal stem cells created an appreciable pool of colony-forming cells in the myocardial perinecrotic zone. Treatment with 5-azacitidine reduced survival of mesenchymal stem cells after intramyocardial transplantation.

Key Words: *myocardium; cell transplantation; mononuclears; mesenchymal stem cells; 5-azacitidine*

It is assumed in regeneration medicine that damaged tissues stimulate proliferation and differentiation of regional stem cells [14]. The possibility of target homing of stem cells from the peripheral blood in the damaged area is hypothesized [7,10]. The intensity of these effects is essential for the development of approaches to the use of regeneratory medicine potentialities. Cell technologies are regarded as a prospective trend in the treatment of myocardial infarction and heart failure [1,2]. It is now clear that bone marrow cells are preferable for transplantation [2,13]. The bone marrow contains precursor blood cells and mesenchymal stem cells (MSC) capable of differentiating into other cells [14]. Factors promoting directed differentiation of MSC, including differentiation into cardiomyocytes, at the stage of ulturing were identified [4,6,8]. The efficiency of using nonfractionated bone marrow cells for transplantation is discussed [1,11].

We evaluated the content of colony-forming cells in the myocardial perinecrotic zone after destructive exposure of the cardiac muscle and intramyocardial transplantation of bone marrow cells.

MATERIALS AND METHODS

The study was carried out on male Wistar rats (200-250 g). The myocardium was damaged by cryodestruction [4]. The operation was carried out under light ether narcosis. The thorax and the pericardium were opened and left-ventricular wall was frozen with a 5-mm metal rod cooled to the liquid nitrogen temperature. After antibiotic treatment and removal of the air from the thoracic cavity, the wound was sutured by layers.

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The animals were divided into 4 groups, 6 per group. After 9 days, the chest was re-opened and 100 μl culture medium was injected into the myocardium. Animals of groups 2-4 were injected with medium containing $1\text{-}2\times10^6$ mononuclears (MN), MSC, or pre-differentiated MSC (pdMSC), respectively [4]. Group 1 (control) animals received medium without cells. The medium was injected in 5-6 points of the perinecrotic zone along the perimeter of the focus. After repeated surgery the animals were kept under vivarium conditions for 30 days.

Cell material was derived from 4 other animals. Mononuclears were isolated from the femoral bone marrow. The bone marrow was homogenized by passing it through a 100-μ capron mesh. The resultant mass was centrifuged (20 min, 2000 rpm). Cell suspension was layered onto Ficoll-hypaque density gradient (1.077 g/cm^3). The cell layer formed on the gradient was collected and washed with RPMI-1640 containing BSA. The viability of MN and total cell content in the resultant material were evaluated [3].

Mesenchymal stem cells were isolated from the MN fraction. The count of viable cells was brought to 5×10^6/ml, the cells were inoculated (10 ml) in plastic culture flasks (50 ml) and cultured routinely for 12 days [4]. The medium was then discarded and the feeder layer was treated with 0.1% trypsin and collagenase. The cell suspension was transferred into tubes and washed from enzymes with RPMI-1640. The content of viable cells and total cell count were evaluated. 5-Azacitidine in a concentration of 6 μmol/liter was added in the culture medium for directed differentiation of MSC into cardiomyocyte-like cells [4].

Thirty days after transplantation the experimental and 6 intact animals were sacrificed, the hearts were isolated under sterile conditions and washed from the blood in sterile saline. The left-ventricular wall was resected, the myocardium was crushed and treated with 0.25% trypsin with EDTA at 37°C for 10 min [5]. The resultant mass was then disaggregated by passing it through needles of decreasing diameters and filtering through a 100-μ capron mesh. The cell suspension was filtered twice in RPMI-1640 with BSA. The precipitate was resuspended in 4 ml culture medium. The total cell count and viability of nuclear cells were evaluated. Optimal volume of cell suspension for inoculation in 50-ml flasks was calculated. The content of the flasks was brought to 5 ml with culture medium and culturing was carried out for 16 days according to the standard protocol [4]. The medium was then removed, the flasks dried, and their content was fixed and dried. The colonies and total cell count in the preparations were estimated [3,5].

The results were processed by methods of variation statistics using Student's *t* test and Mann—Whitney nonparametric *U* test (Statistica 6.9 software, Stat Soft Inc.).

RESULTS

The results of culturing of myocardial cells depended on the cell mass (Fig. 1). Culturing of the intact myocardium resulted in the formation of a cell colony, the total cellularity being 246±20 cells/cm^2 (Table 1). No colonies were detected after culturing of group 1 animal myocardium, the mean cellularity being 12±8 cell/cm^2. Decreased content of colony-forming cells in group 4 indicated that the pool of these cells was virtually completely activated for regeneration of the myocardium. Myocardial injury did not seem to be an important factor stimulating endogenous mechanisms responsible for mobilization and homing of stem cells from other tissues, primarily from the bone marrow. This conclusion is in line with the report indicating that granulocytic CSF (G-CSF) stimulated an increase in the count of colony-forming cells in rat myocardium after experimental infarction [5], but the content of colony-forming cells in the myocardium decreased on day 14 after G-CSF application.

Cell transplantation significantly changed the results of culturing. Single transplantation of MN

TABLE 1. Results of Culturing of Cells from Rat Left-Ventricular Myocardium (*M*±*m*; *n*=6)

Parameter	Groups				
	intact	experimental			
		1 (control)	2	3	4
Number of colonies	1	No	3±1	33±7	11±3
Mean cell content, cell/cm^2	246±20	12±8*	284±32	2975±80*+	1105±42*+

Note. $p<0.05$ compared to: *intact animals, +group 2.

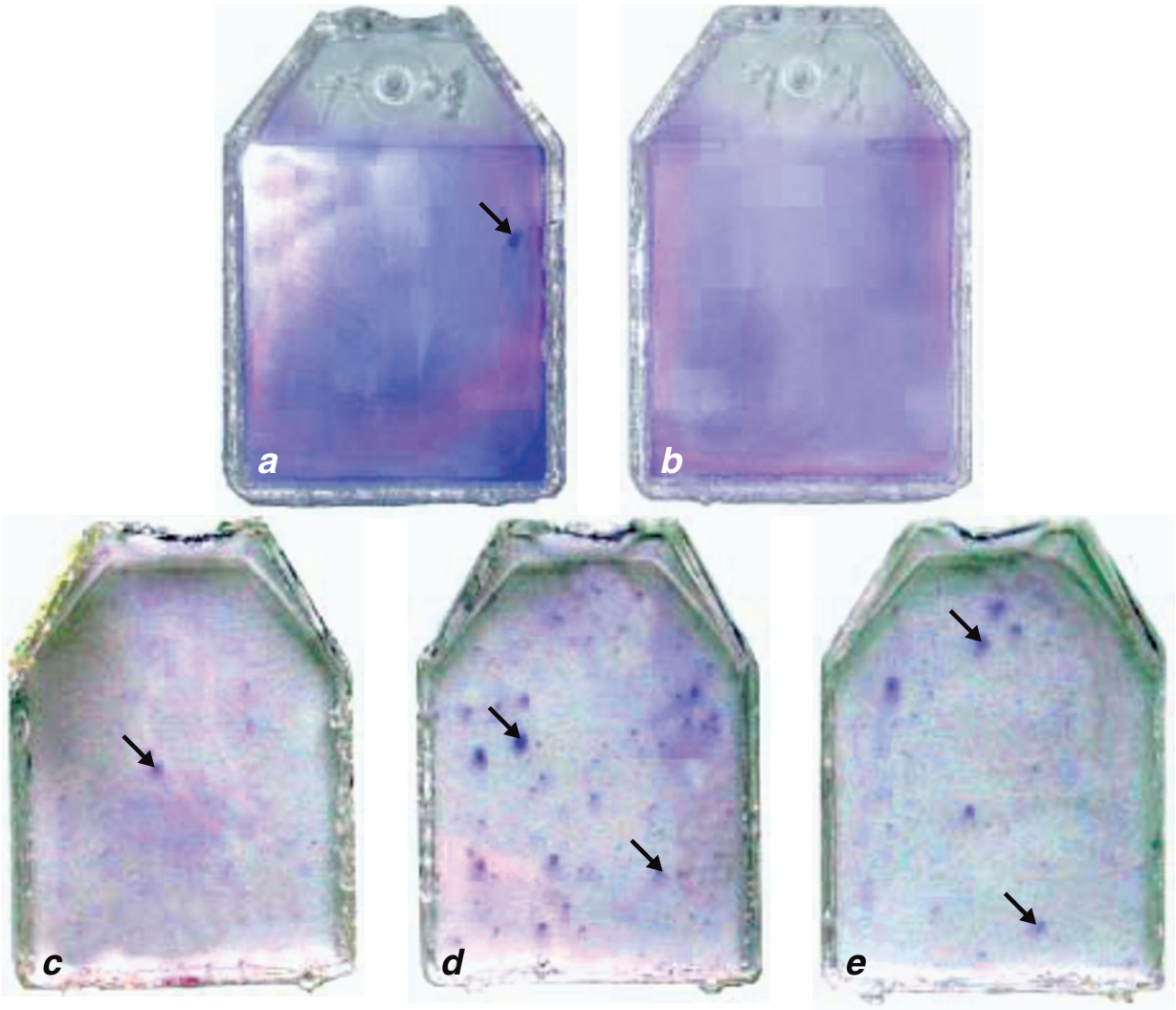

Fig. 1. Flasks after 16-day culturing of the rat myocardium cells. *a*) intact myocardium; *b*) myocardium after control cryodestruction; *c-e*) cryodestroyed myocardium with subsequent transplantation of MN, MSC, and pdMSC, respectively. Arrows show cell colonies.

(group 2) provided normalization and maintenance of colony-forming cell pool at the level of the intact myocardium even 30 days after transplantation. This could be due to common recovery of the cell pool at the expense of the transplant and to more rapid organization of the focus of lesion as a result of activation of the cytokine cascade by transplanted cells [12,15]. This result is in line with the reports indicating that transplantation of bone marrow MN is characterized by high regeneration potential [1,11]. However, low content of stem cells capable of becoming cardiomyocyte precursors in the myocardium does not permit us expect full-value morphofunctional recovery of the cardiac muscle, while MN transplantation seems to be insufficient for the realization of substitute cell transplantation.

Qualitative changes were observed after MSC and pdMSC transplantation (groups 3 and 4). In group 3, the mean cell count increased 12-fold in comparison with the intact myocardium and 33±7 colonies formed. However, the number of colonies in group 4 myocardium was 2.6 times lower. The mean cell count was also significantly lower, but higher than in intact animals and in group 2.

Virtually the same volume of cell material was used for transplantation in groups 3 and 4, and hence, the detected difference was presumably due to modification of cell characteristics under the effect of 5-azacitidine. This substance is really toxic for cultured cells [9]. We used 5-azacitidine according to the protocol adapted for stimulation of MSC differentiation towards cardiomyogenesis [4]. In addition, our data indicate, that 5-azacitidine virtually does not modify cell viability before their transplantation. This fact suggests that the pre-differentiation scheme used in our experiment did not reduce MSC viability, but had a negative impact on their capacity to survive after the transplantation

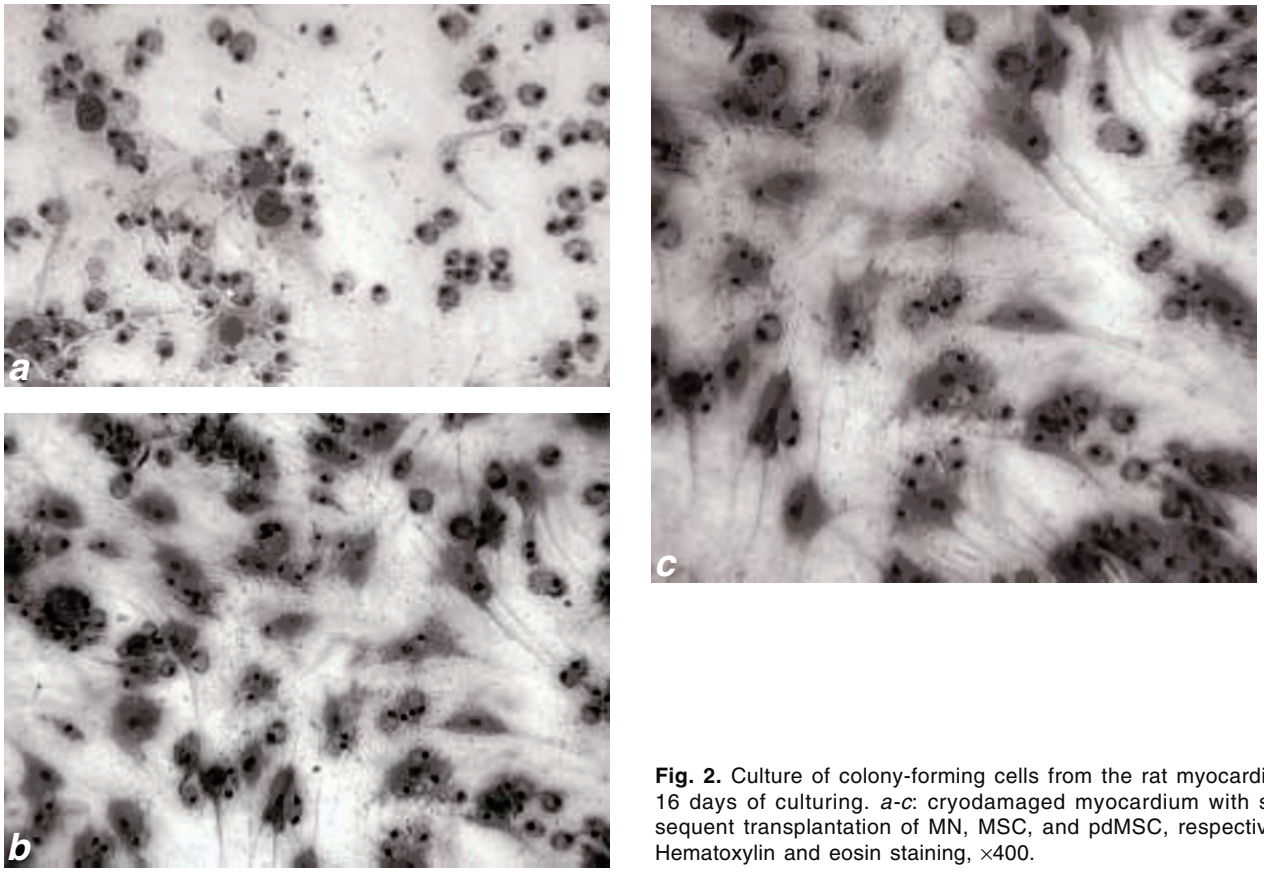

Fig. 2. Culture of colony-forming cells from the rat myocardium: 16 days of culturing. *a-c*: cryodamaged myocardium with subsequent transplantation of MN, MSC, and pdMSC, respectively. Hematoxylin and eosin staining, ×400.

procedure and to adapt to the conditions of a pathologically changed myocardium.

The composition of cells detected after myocardium culturing in groups 2-4 did not differ qualitatively (Fig. 2). Cells 15-20 μ in diameter with processes and fibroblast-like spindle cells were detected.

Hence, destruction of the myocardium virtually completely mobilizes local myocardial stem cells. Intramyocardial transplantation of MSC is preferable to transplantation of the MN fraction and leads to a several-fold increase of the pool of colony-forming cells in the pathological myocardium. Use of 5-azacitidine for pre-differentiation of MSC reduces cell survival after their transplantation into pathological myocardium.

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