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Left-Ventricular Heart Aneurism as a New Source of Resident Cardiac Stem Cells

K. V. Dergilev[a, b], K. A. Rubina[b]*, Z. I. Tsokolaeva[a], V. Yu. Sysoeva[b], A. I. Gmyzina[b], N. I. Kalinina[b], T. M. Belyavskaya[a], R. S. Akchurin[a], E. V. Parfenova[a], and V. A. Tkachuk[a, b]

[a] *Russian Cardiology Research Center, Moscow, Russia*

[b] *Moscow State University, Faculty of Medicine, Moscow, Russia*

**e-mail: rkseniya@mail.ru*

Abstract—In the past few years, it has been established that the heart contains a reservoir of stem and progenitor cells. These cells are able to differentiate in cardiomyogenic, endothelial, and smooth muscle lineages in vitro and in vivo and, following injection into an infarcted myocardium, these cells prevent the remodeling of the left ventricle and improve cardiac function. The aim of the present study was the immunophenotypic characterization of resident stem cells in the tissue of a chronic left aneurism, an analysis of their localization, and distribution in various aneurism areas.

Fifteen samples of aneurism tissue obtained surgically were analyzed. The number of cells positive for c-kit stem-cell marker was about 3327 ± 234 cells per 1 cm^3 tissue. Single cells were found in fibrous, muscle, and adipose parts of aneurism tissue. Most of the cells were revealed in fibrous tissue near large vessels; however, the coexpression of c-kit and markers of endothelial cells was not observed. C-kit-positive cells did not express CD34 and CD45 markers and, thereby, were not of hematopoietic origin. The majority of c-kit-positive cells expressed MDR1, another stem-cell marker. These c-kit-positive cells did not proliferate, since they did not express Ki67. About 20% of these cells expressed an inhibitor of cyclin-dependent kinase $p21^{cip/Waf1}$. C-kit cells did not express markers of cardiomyocytes (Nkx 2.5, Gata 4, Mef2c, α-actinin), endothelium (Ets 1, CD105, wW), and smooth muscle cells (Gata 6, SMA). Single-cell clusters made up of 70–100 c-kit-positive cells (in cell niches) were observed; however, no markers of cardiomyogenic differentiation were expressed in these cells. Thus, aneurism tissue of the left ventricle contains c-kit-positive cells that, are most likely resident cardiac stem cells; however, their regenerative capacity should be further explored.

Keywords: cardiac stem cells, left ventricular aneurism, c-kit-positive cells.

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Ischemic heart disease and myocardial infarction are the most frequent causes of mortality and invalidization worldwide. The incidence of infracted myocardium has increased in recent years, especially in young and middle-aged patients. Despite the improved methods of medical treatment, mortality due to myocardial infarction remains very high.

Myocardial infarction pathogenesis is characterized by the acute failure of coronary circulation, which causes the ischemic necrosis of cardiac muscles and the death of cardiomyocytes. Because of the limited capacity of cardiomyocyte to proliferate, the systolic and diastolic functions of the cardiac left ventricle (LV) are disordered, which generates a complex process of remodeling that results in the development of postinfarction cardiac failure. The area of necrosis formed in the LV results in the thinning and straining of the heart wall, which may cause a cardiac aneurism. Morphologically, an aneurism appears as rigid fibrous tissue with embedded muscle fibers surrounded by adipose tissue in some cases. The enlarged size of the left ventricle and cardiac failure progression may be an indication for the surgical resection of the aneurism.

Until recently, it was considered that the regeneration of the myocardium is only possible via cardiomyocyte hypertrophy, which restores the organ mass. However, the identification of stem cells able to differentiate into cardiomyoctes and endothelial and smooth muscle cells in vivo and in vitro in human (Urbanek et al., 2005; Urbanek et al., 2005) and animal hearts (Hierlihy et al., 2002; Beltrami et al., 2003; Oh et al., 2003; Matsuura et al., 2004; Pfister et al., 2005) transformed the concept. Now, the heart is not regarded as a terminally differentiated organ. Depending on the isolation techniques or the pattern of surface or intracellular markers, different authors describe stem cells as cells expressing the c-kit marker (SCF, stem cell factor) (Anversa et al., 2006), or sca-1 antigen (in mice) (Beltrami et al., 2003), or as a side population (Pfister et al., 2005), or as cells that express the islet-1 transcription factor (Laugwitz et al., 2005),

as well as cells that form cardiospheres (Messina et al., 2004).

The most studied are resident cardiac stem cells first described by Beltrami with colleagues (Beltrami et al., 2003), which express c-kit, MDR and sca-1 surface markers and lack hematopoietic markers (CD34, CD45, CD20 and CD8). Resident stem cells are capable of renewal, proliferation and differentiation into various lineages: cardiomyocytes, smooth muscle cells, endothelial cells and fibroblasts. It is supposed that the heart has stem cell pool localized between cardiomyocytes in so-called "niches" (Fuchs et al., 2004). Niches are located in the heart apex and atriums, which are areas with the least hemodynamic pressure. In niche stem cells, division results in the generation of two cells. One cell is a stem cell that remains in the niche, while the other becomes a progenitor cell, which proliferates and differentiates into the types of cells present in myocardium tissue. Stem cells are needed to maintain the cell homeostasis in the myocardium and to regenerate damaged areas.

The development of methods for stimulating the regeneration of an myocardial infarction by activating resident stem cells in the area adjacent to the damaged myocardium or by stem cell transplantation is one of the important theoretical and practical tasks of cardiology. A number of publications have been dedicated to studying the implication of stem cells in myocardium regeneration (Beltrami et al., 2003; Dawn et al., 2005; Wang et al., 2006; Rachmat-Zade et al., 2006, 2007; Bearzi et al., 2007). C-kit-positive cells labeled with bromodeoxyuridine were grafted into peri-infarcted zone of rats with experimentally induced infarct. It was shown that transplanted cells stayed viable and facilitated a reduction of necrosis, the dilation of the left ventricle cavity, and tissue vascularization, and improved cardiac function. It was demonstrated that injected cells were able to differentiate into functionally mature cardyomyocytes, which expressed heavy chains of cardiac myosin and connexin-43, a protein of gap junctions.

Similar results were obtained by the intracoronary injection of cardiac stem cells into rats with modeled myocardium ischemia–reperfusion (Dawn et al., 2005). After cell transplantation, the infarcted zone decreased by 29%, the remodeling process of the left ventricle was attenuated, and disordered contractile heart function was improved. Later, c-kit-positive cells were shown to be present in the human myocardium and could be isolated from intramyocardial biopsy material (Bearzi et al., 2007). The cells were capable of self-renewal; proliferation; and differentiation into cardiomyocytes, smooth muscle, and endothelial cells. Upon the local transplantation of c-kit-positive cells into the postinfarct myocardium of immunodeficient animals, areas of newly formed myocardium composed of human cells structurally and functionally coupled to recipient cells were found, which indicates that c-kit-positive cells are implicated in the regeneration of postinfarct myocardium.

An obstacle for studying the function of cardiac stem cells is the limited amount of cells that can be isolated from a myocardium biopsy. Here, we attempted to utilize the tissue of cardiac aneurism obtained from surgery as a source of cardiac stem cells. The aim of the study was to identify the resident cardiac stem cells in the tissue of postinfarct myocardium aneurism of the left ventricle and characterize them immunophenotypically, as well as to study their localization and distribution in various areas of aneurism.

MATERIALS AND METHODS

Fifteen samples of chronic myocardium aneurism of the left ventricle obtained from coronary artery bypass surgery and simultaneous LV aneurism excision were studied. The mean age of patients was 58 ± 10 years. For a quantitative assay of c-kit-positive cell distribution in undamaged LV myocardium, eight samples of LV myocardium from patients who died in accidents and had no cardiovascular diseases were analyzed.

Immunohistochemical staining. The tissue of post-myocardial infarction aneurism was frozen in liquid nitrogen. Aneurism cryosections (7 μm) were fixed in 3.7% formalin in phosphate saline for 15 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 in phosphate saline for 10 min. To reduce unspecific antibody binding, sections were incubated with 1% bovine serum albumin and 10% horse serum in phosphate saline for 30 min. The blocking solution was removed and samples were incubated with mouse monoclonal antibody to human c-kit antigen in dilution 1 : 100 (Becton Dickson, United States) for 1 h. Nonspecific mouse IgG (Invitrogen, United States) was used as a control.

Antibody binding with c-kit antigen was detected using Vectors Laboratories kit (United States). After washing in phosphate, saline sections were incubated with polyclonal biotinylated goat antibody in dilatation of 1 : 2000 for 30 min. The localization of the antibody complex was visualized with DAB (Sigma, United States). Samples were dehydrated and mounted in medium compatible with xylene Cytoseal XYL (Thermo Scientific, United States).

Immunofluorescence. Cryosections of post-infarcted myocardium aneurism tissue (7 μm) were fixed in 3.7% formalin for 15 min. To reduce unspecific antibody binding, sections were incubated with 10% bovine serum albumin in phosphate saline for 30 min. The blocking solution was removed and samples were incubated for 1 h with antibodies against c-kit antigen (Dako, Biolegend, United States), sarcomeric α-actinin (Sigma, United States), CD31 (Becton Dickson, United States), MDR1 (Chemicon, United States), N-cadherin (Invitrogen, United States), CD45 (Abcam, United States), CD34 (Becton Dickson,

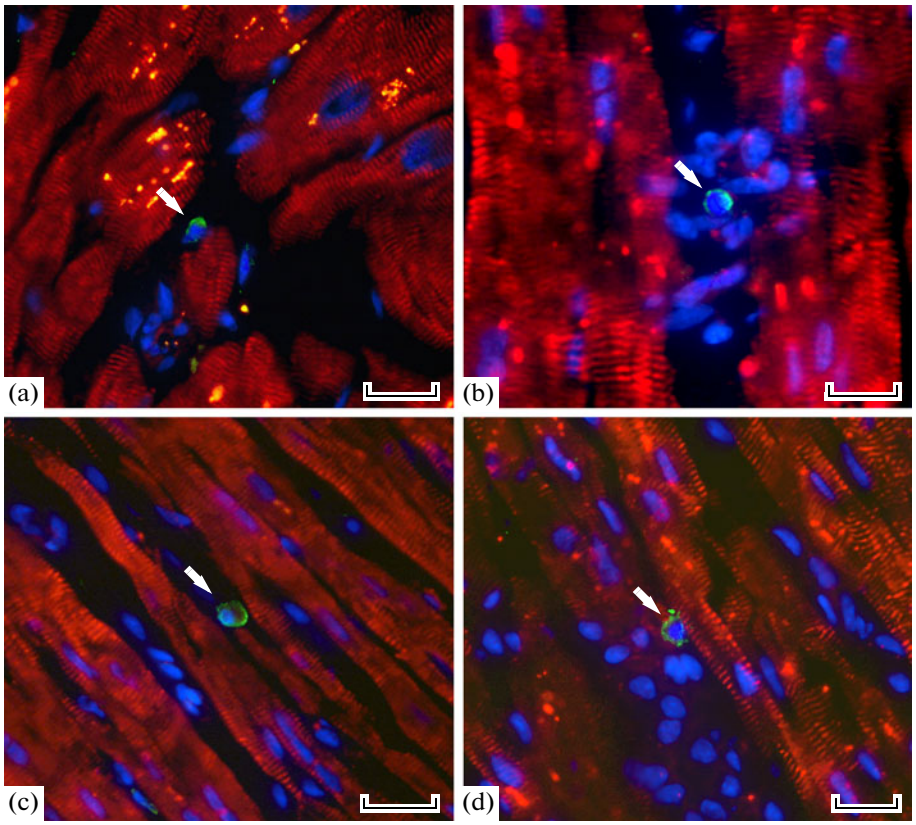

Fig. 1. Localization of c-kit-positive cells in human aneurism tissue and normal myocardium of the left ventricle. Double immunofluorescent staining with antibodies to c-kit (*green*) and sarcomeric α-actinin (*red*). Here and in Figs. 5–8, nuclei are stained with DAPI: (a) Aneurism tissue; (b) normal myocardium of 22-year patient; (c) normal myocardium of 3-year old child; (d) normal myocardium of 45-year patient. *Arrows* point to c-kit expressing cells. Scale bar is 20 μm.

United States), α-smooth muscle actin (Sigma, United States), VEGFR2 (Abcam, United States), CD105 (AbD Serotec, United States), NG2 (Chemicon, United States), Nkx 2,5 (Abcam, United States), Gata 4 (Santa Cruz, United States), Ki-67 (Becton Dickinson, United States), $p21^{Cip/Waf1}$ (Cell Signaling, United States) in dilutions recommended by manufacturers. Sections were washed in phosphate saline and incubated for 1 h with AlexaFluor labeled secondary antibody (Invitrogen, United States) (dilution 1 : 2000) in 1% bovine serum albumin in phosphate saline. Afterwards, samples were washed in phosphate saline. Cell nuclei were stained with DAPI (4', 6-diamidino-2-phenylindol), sections were washed and mounted in Aqua Poly/Mount (Polysciences Inc., United States). Preparations were visualized using an Axiovert 200M fluorescent microscope (Zeiss, Germany) equipped with an Axiocam CCD digital camera (Zeiss, Germany). Images were processed using Axiovision 3.1 (Zeiss, Germany) and Adobe PhotoShop (Adobe Systems, United States) software. For each sample, the number of c-kit-positive cells was counted in 1 cm^2 of myocardium tissue in 10 random areas and averaged. The amount of c-kit-positive cells per 1 cm^3 was calculated based on the section thickness.

Statistical treatment. The results are presented on charts as mean values with standard deviations. Statistical analysis was performed with Statistica 6.0 software (Statsoft, United States). To verify the normal distribution, the Shapiro–Wilk statistical criterion was applied. A sampling comparison was done using the Mann-Witney U criterion. The differences were considered to be significant at $P < 0.05$.

RESULTS

Immunohistochemical and immunofluorescent analysis of myocardium tissues from patients who died in accidents and postinfarction aneurism samples revealed the presence of cells that express stem-cell c-kit markers (Kondo et al., 2003) and MDR1 (multiple drug resistant protein) (Sellers et al., 2001). These cells are presumably cardiac resident stem cells.

C-kit-positive cells were identified in the autopsy tissue of an undamaged myocardium (Figs. 1b–1d) in patients of various ages who died in accidents. These cells were also found in myocardium aneurism tissue of the left ventricle (Fig. 1a). Overall the number of these cells decreased with age and was minimal in postinfarct aneurism tissue (Fig. 2). The average num-

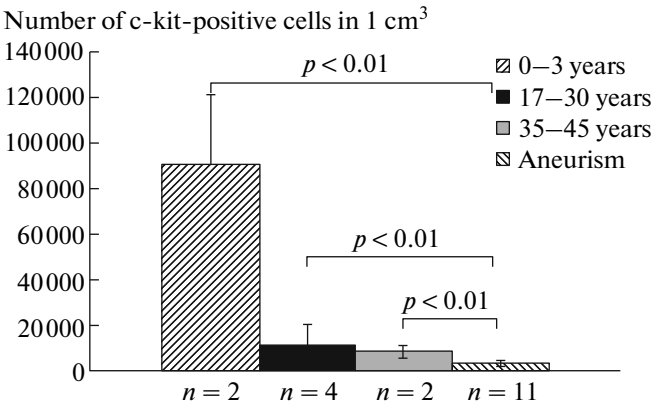

Fig. 2. Quantitative distribution of c-kit-positive cells in myocardium of the left ventricle of patients of various ages.

ber of c-kit cells in 1 cm^3 of chronic aneurism was 3327 ± 1234 (n = 11). In healthy people up to 3 years old the number of these cells was bout 30 times more (89964 ± 31224). In patients 17–30 and 35–45 years old, the number of c-kit cells was 11353 ± 9031 and 8518 ± 2643, respectively. C-kit positive cells had elongated or round shapes and were located in different parts of aneurism tissue, i.e., muscle (Fig. 3a), fibrous (Fig. 3b), adipose (Fig. 3c), or mixed (Fig. 3d). About 70% of c-kit cells were found in fibrous part of aneurism; their number in muscle and fibrous tissue was 20% and 10%, respectively (Fig. 4).

C-kit-positive cells were observed in aneurism tissue as single cells (Fig. 5b) or as clusters organized in niches (Fig. 5c). Most of these cells were identified in proximity to large vessels (Fig. 5a); However, these cells did not express markers of endothelial or vascular smooth muscle cells, such as Ets 1 (transcription factor expressed in differentiating endothelial cells) and CD31 (marker of mature endothelium) (Fig. 5a). C-kit cells also did not express markers of activated endothelium or endothelial precursor cells, such as VEGFR2 (vascular endothelial growth factor receptor type 2) (Fig. 6c) and CD105 (Fig. 6d) or Gata 6 (transcription factor expressed in the cells of smooth muscle lineage), smooth muscle α-actin (marker of mature smooth muscle cells and/or pericytes) (Fig. 6b) or NG2 (pericyte marker) (Fig. 6f). These

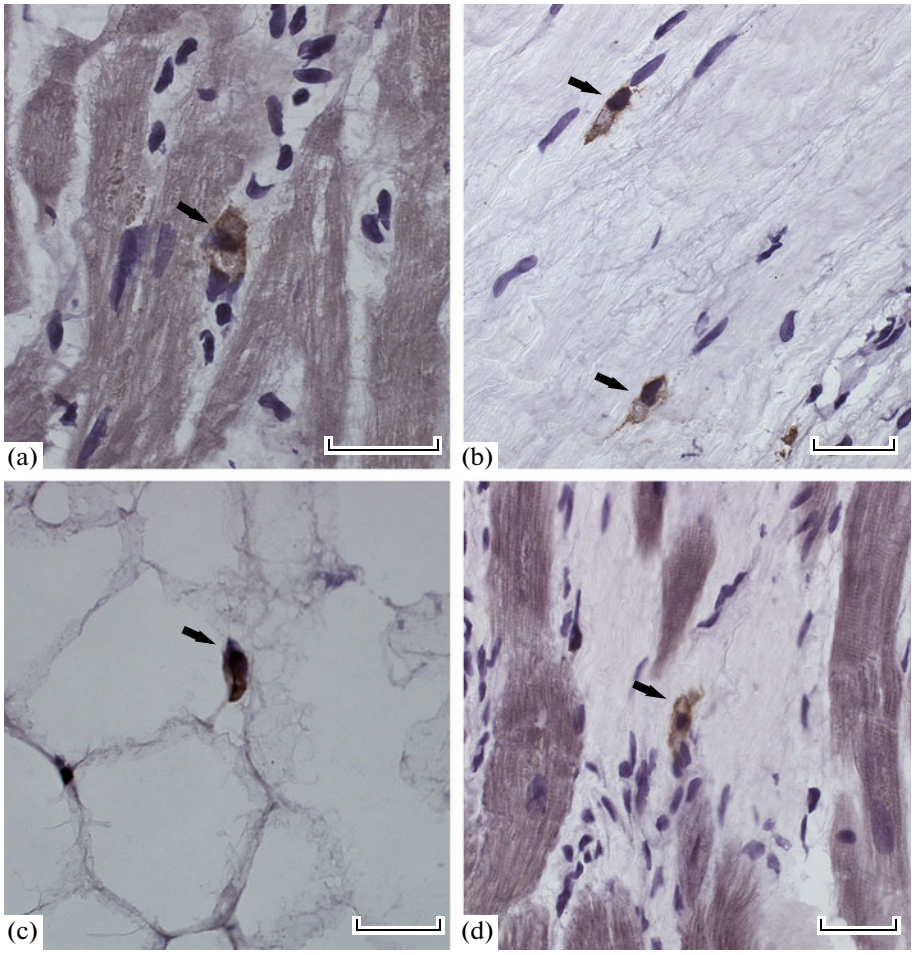

Fig. 3. Cells that express c-kit in various aneurism areas. (a) Muscle area; (b) fibrous part; (c) adipose area; (d) fibrous-muscle area. *Arrows* indicate cells expressing c-kit. Scale bar—10 μm.

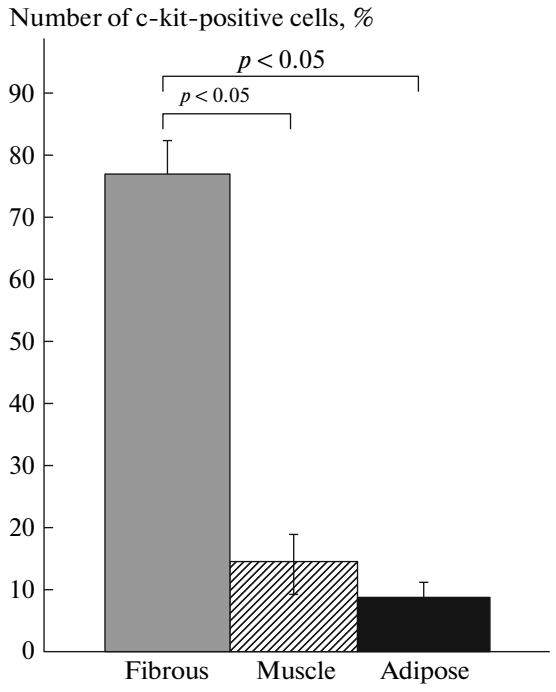

Fig. 4. Quantitative distribution of c-kit-positive cells in fibrous, muscle and adipose parts of chronic aneurism tissue of the left ventricle.

findings indicate that c-kit-positive cells that we revealed in aneurism tissue were not vascular cells. Moreover, they were not the cells that differentiated into cardiomyocytes because they lacked markers of cardiomyogenic differentiation, such as Nkx 2,5 (Fig. 6f), Gata 4 (Fig. 6g), and α-actinin (Fig. 5b).

It is known that the receptor to the c-kit stem cell factor (SCF) is also expressed by hematopoietic precursor cells, which are mobilized from bone marrow upon heart injury, migrate into the infarct zone, and participate in regeneration (Menasche, 2008; Pouly et al., 2008). To determine whether c-kit-positive cells in the aneurism tissue are hematopoietic cells, we analyzed the coexpression of c-kit and CD45 (CD34) by double immunofluorescence. It was found that c-kit-positive cells in aneurism tissue expressed neither CD45 (common leukocyte antigen, Fig. 6a) nor CD34 (marker of hematopoietic and endothelial precursor cells).

Most of the progenitor cells positive for c-kit were also positive for another stem-cell marker, i.e., MDR 1, which is the protein responsible for multiple drug resistance (Figs. 7a, 7b). Moreover, these cells expressed N-cadherin, the protein of intercellular contacts (Figs. 7d, 7e), which are expressed in cardiac stem cells (Fuchs et al., 2004; Leri et al., 2005) and are

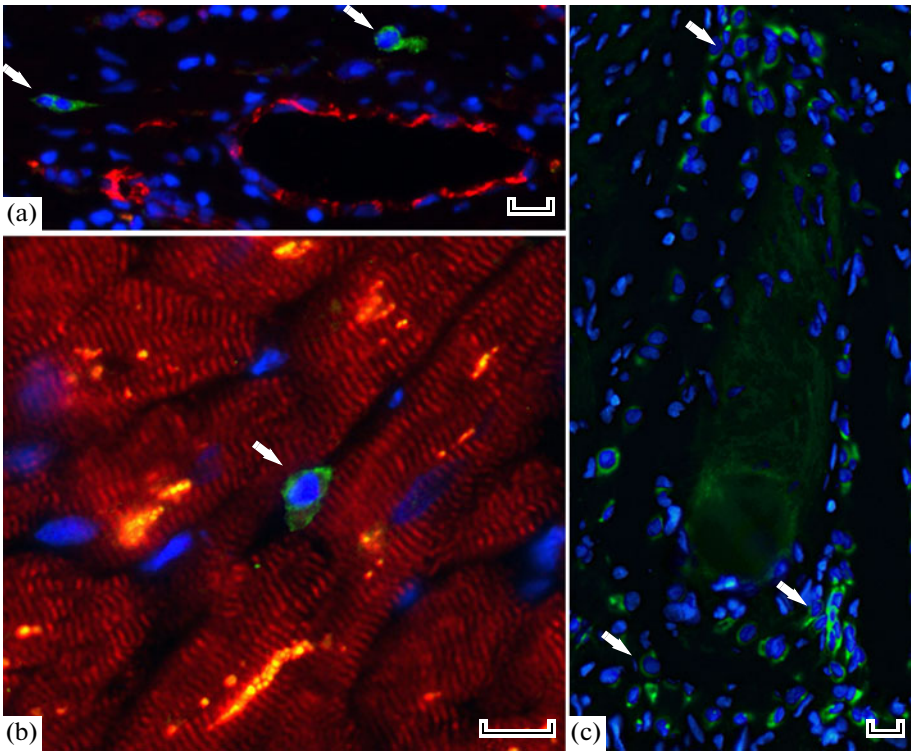

Fig. 5. Location of c-kit-positive cells in tissue of chronic post-infarct aneurism. (a, b) Single c-kit-positive cells (*green*) nearby large vessels (a) and in myocardium (b). *Red*, CD31; α-actinin (b); (c) c-kit-positive cells in clusters in *cell* niche". *Arrows* point to c-kit expressing cells. Scale bar is 10 μm.

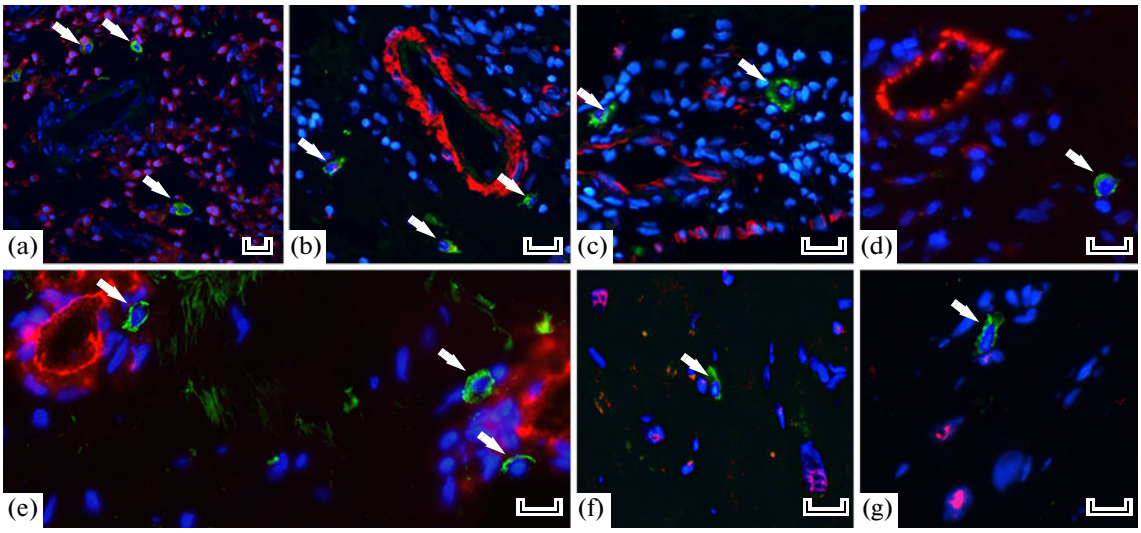

Fig. 6. Expression of c-kit and markers of hematopoietic, endothelial, smooth muscle and cardiomyogenic differentiation in aneurism. Double immunofluorescent staining of aneurism tissue for c-kit and CD45 (a), smooth muscle α-actin (b) VEGFR2 receptor (c), CD105 (d), NG2 (e), Nkx2,5 (f), Gata 4 (g). *Red*, c-kit expression; *red*, other markers. *Arrows* point to c-kit cell localization. Scale bar—10 μm.

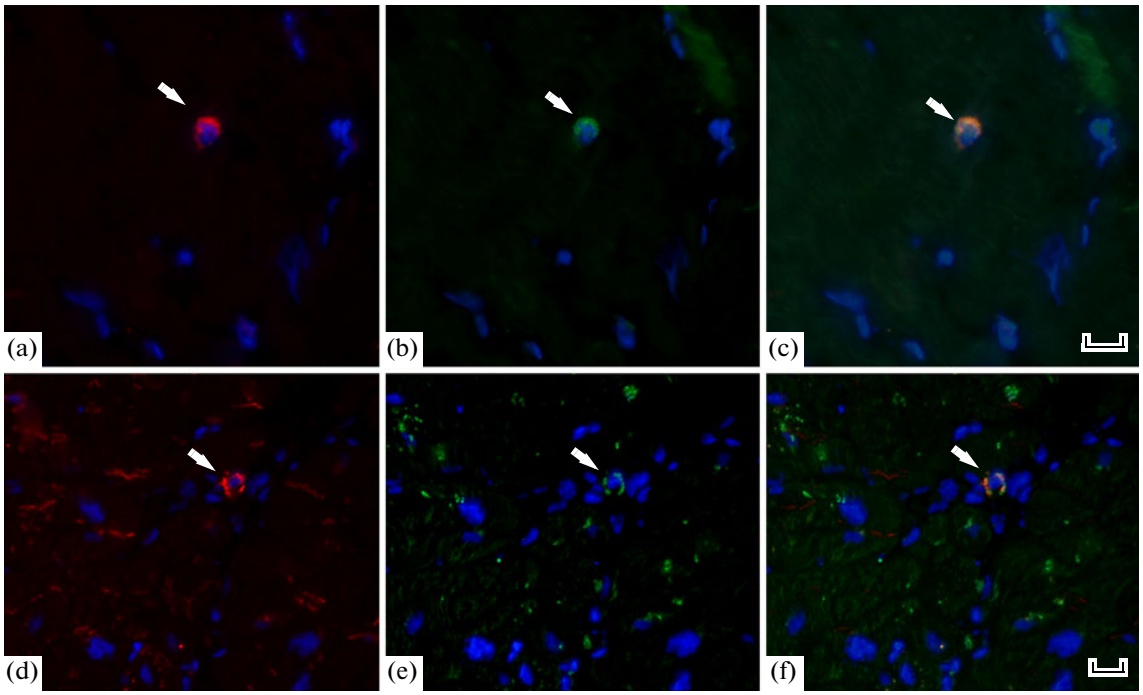

Fig. 7. Double immunofluorescent staining of aneurism tissue for stem-cell markers MDR1 (a) and c-kit (b), N-cadherin (d), and c-kit (e), (c) and (f) merged images; *arrows* point to stained cells. Scale bar—10 μm.

required for their interaction with adjacent cells in the cell niche.

To reveal proliferating c-kit-positive cells, samples were double immunostained for c-kit and Ki-67 (nuclear antigen identified at all stages of the cell cycle, except for the cells at G_0). It was found that c-kit-positive cells lacked Ki-67. These data indicate that c-kit cells in aneurism tissue remain in the G_0 phase and do not proliferate. It has been suggested that the proliferation of these cells may be suppressed by inhibitors of the cell cycle. To prove this, the cells were double stained with antibodies against c-kit and $p21^{Cip/Waf1}$ (inhibitor of cyclin-dependant kinases), which regulates the cell's transition into the G_1 phase. It was found that 20% of c-kit positive cells also expressed $p21^{Cip/Waf1}$ (Fig. 8).

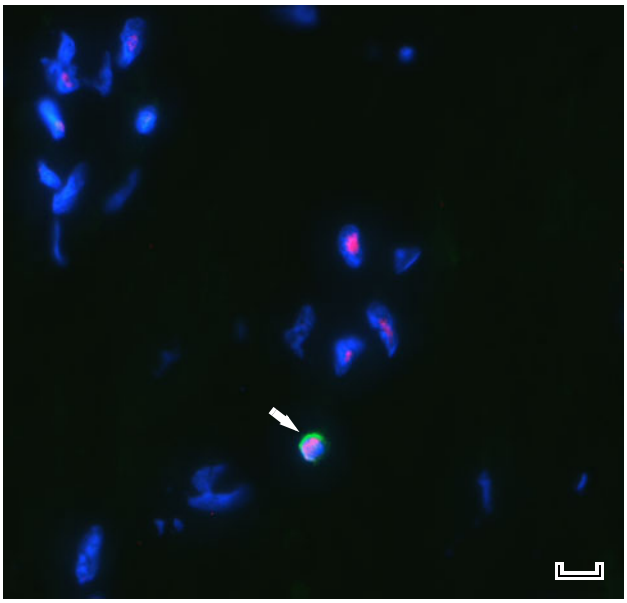

Fig. 8. Expression of cell cycle inhibitor $p21^{Cip/Waf1}$ in cðkit-positive cells in aneurism tissue. Double immunofluorescent staining of aneurism tissue for c-kit (*green*) and $p21^{Cip/Waf1}$(*red*). *Arrows* indicate cells that express both c-kit and $p21^{Cip/Waf1}$.

DISCUSSION

It has been shown that myocardial infarction is accompanied by hematopoietic and mesenchymal precursor cell migration from the bone marrow into the area of damage and ischemia (Askari et al., 2003; Kawamoto et al., 2003; Ceradini et al., 2004). Cells of bone-marrow origin have been revealed in postinfarction myocardium. It was shown in animals using the model of experimental infarction that these cells could differentiate into vascular cells, as well as exert paracrine effects that facilitate cardiomyocyte viability in peri-infarct zone and prevent postinfarct remodeling of the left ventricle (Lindbaek et al., 2007; Reinecke et al., 2008). Although it has been reported that in vitro bone marrow mesenchymal cells are able to differentiate into spontaneously beating cardiomyocytes, their regenerative capacity to restore damaged myocardium in vivo or the ability of mesenchymal cells to differentiate into functional cardiomyocytes remains unsupported (Fazel et al., 2006; Lindbaek et al., 2007; Reinecke et al., 2008).

It was shown in experimental models of myocardial infarction that bone-marrow hematopoietic cells also possess the regenerative potential, however, neither mechanisms of their action nor their capacity to transdifferente into mature cardiomyocytes and/or fuse with existing cardiomyocytes in vivo was demonstrated (Orlic et al., 2001; Murry et al., 2004; Rota et al., 2007; Scherschel et al., 2008).

Upon infarction, resident stem cells in the heart become activated in the peri-infarct zone. The cells proliferate, differentiate into cardiomyocytes, vascular cells and fibroblasts and become engaged in myocardium regeneration (Leri et al., 2005). However, upon extensive myocardial infarction endogenous reparation process turns to be insufficient for effective tissue regeneration. As a result a scar is formed at the place of infarction whereas undamaged areas become hypertrophied and left ventricular chamber is dilatated resulting in aneurism formation and heart failure (Quaini et al., 2002)

Experiments carried out in recent years using animal models of experimental infarct with subsequent application of stem (progenitor) cells of various origins demonstrated that the state of the myocardium and heart function may be improved by cell therapy (Leri et al., 2005; Reinecke et al., 2008). The improvement is accomplished mostly by the secretion of angiogeneic and antiapoptotic factors by transplanted cells, which facilitates maintaining cardiomyocyte viability in the damaged area and stimulates the proliferation and differentiation of resident stem cells, as well as angiogenesis in the peri-infarct zone (Barile et al., 2007; Lindbaek et al., 2007; Reinecke et al., 2008). These findings encouraged the search for the most advantageous cell source for cardiac cell therapy. The available data suggest that multipotent resident stem cells from an adult heart may be the type of the cells with the best regenerative potential for cell therapy of the damaged myocardium, since they not only exhibit paracrine effects upon application, but are also able to differentiate into myocardium tissue specific cells, e.g., cardiomyocytes, endothelial, smooth muscle cells, and fibroblasts (Beltrami et al., 2003; Anversa et al., 2003; Urbanek et al., 2003; Urbanek et al., 2005a; Anversa et al., 2006).

The restrictions in the studies on human resident stem cells and their possible clinical application are attributed to difficulties in obtaining these cells, which require myocardial biopsy. In our experiments, we surgically removed myocardial tissue of postinfarcted aneurism to identify and characterize human resident stem cells.

Cardiac multipotent stem cells express c-kit and MDR1 markers in humans and sca-1 in mice (Anversa et al., 2003, 2006). In our experiments, we used c-kit (receptor of stem cell factor SCF) as a marker of resident cardiac stem cells. C-kit-positive cells were found in both the tissue of undamaged myocardium and aneurism of the left ventricle. However, the number of these cells in an aneurism was several times lower than in undamaged myocardium tissue of adult patients. These cells were observed in all parts of the aneurism tissue as single cells or in clusters in cell niches. Double immunofluorescent staining showed that c-kit-positive cells express another stem-cell marker, MDR1, which is responsible for stem-cell resistance to drugs, and N-cadherin, a protein of adhesive contacts engaged in intercellular adhesion of stem cells in the niche and cells of their microenvironment (Barile

et al., 2007; Leri et al., 2005). C-kit-positive cells were frequently located perivascularly, but as a rule, were not observed in vessel lumens. This data indicates that c-kit cells in aneurism tissue are most likely not blood-circulating precursors or cells that migrate from blood into myocardial tissue as a response to damage (Oh et al., 2003) because they do not express CD31, transcription factor Ets 1, VEGFR2, and CD105 (markers of mature or activated endothelium or endothelial precursors).

Recently, it was found immunohistochemically that CD45 and CD34 (markers of mature or progenitor hematopoietic and endothelial cells) are expressed by most c-kit-positive cells of the endomyocardial biopsy of the right ventricle obtained from patients after heart transplantation or of the atrium appendages obtained during coronary artery bypass grafting from patients with ischemic heart disease. The authors concluded that cells that express c-kit in myocardium tissue were of bone-marrow origin (Menasche, 2009; Pouly et al., 2008). However, according to our data, c-kit-positive cells do not carry CD45 and CD34 markers and, therefore, are most likely resident cardiac progenitor cells.

According to the most acceptable concept, cardiac resident cells are involved in maintaining the homeostasis of the heart. Myocardial infarction or aortal stenosis activates resident stem cells, which initiate proliferation and differentiation into progenitor cells, which afterwards become committed precursors (Urbanek et al., 2003; Urbanek et al., 2005b; Linke et al., 2005). In these c-kit-positive cells, transcription factors of cardiomyogenic differentiation (Gata 4, Nkx 2,5, Mef 2C), smooth muscle (Gata 6), or endothelial (Ets 1) lineages are expressed and after structural proteins of cardiomyocytes (α-sarcomeric actin, connexin 43, troponin I), endothelium (CD31), or smooth muscle (smooth muscle α-actin) cells are synthesized (Linke et al., 2005). In the present study, it was found that c-kit-positive cells in aneurism tissue were neither proliferating cells (were not expressing Ki-67) nor differenting precursors, since they did not express transcription factors of differentiating cells or structural proteins of mature cells. It is possible to assume that the microenvironment of c-kit-positive cells that survived in the aneurism tissue with disturbed intercellular interactions does not promote c-kit proliferation and differentiation. It is also possible that ischemic damage to the myocardial tissue occurs with the apparent remodeling of the wall of the left ventricle, i.e., the depletion of the compartment of cardiac stem and progenitor cells, which results in the appearance of silent niches. It is known that myocardial infarction, especially with chronic heart failure, increases the number of senescent cardiac stem cells with reduced proliferation activity. Stem cells in these patients have shortened telomeres and express inhibitors of the cells cycle (Urbanek et al., 2005b). We assayed the expression of the cell-cycle inhibitor $p21^{Cip/Waf1}$, i.e., cyclin-dependant kinase, which regulates the cell transition into the G_1 phase of the cell cycle. It was found that about 20% of c-kit-positive cells expressed $p21^{Cip/Waf1}$. The expression of the cell-cycle inhibitor could be a cause of the reduced proliferation activity of the c-kit-positive cells.

Taking into account the presented data, it possible to assume that even small amounts of c-kit-positive cells isolated from aneurism tissue could be a source of cardiac resident stem cells, which are appropriate for the study of their biology and regenerative potential under normal and pathological conditions. Thus, the tissue of human postinfarct aneurism contains cells that express c-kit, which most likely represent a population of resident cardiac stem cells suitable for studying their biology and regenerative potential. The development of effective methods of isolating c-kit-positive cells from aneurism tissue and their expansion in vitro may be prospective as a new source of stem cells for autologous cell therapy for these patients.

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