CELL TECHNOLOGIES IN BIOLOGY AND MEDICINE

Resident Stem Cells in the Myocardium of Patients with Obstructive Hypertrophic Cardiomyopathy

T. V. Sukhacheva[1], Yu. A. Chudinovskikh[1], M. V. Eremeeva[1], M. V. Samsonova[2], A. L. Chernyaev[2], R. A. Serov[1], and L. A. Bockeria[1]

Translated from *Kletochnye Tekhnologii v Biologii i Meditsine*, No. 2, pp. 63-67, April, 2012
Original article submitted December 5, 2011

Interventricular septum myocardium was studied in 40 patients with obstructive hypertrophic cardiomyopathy. Immunohistochemical assay revealed c-kit-positive resident cardiac stem cells in 82.5% patients. The content of the connective tissue and myofibrillar disarray zones and the degree of cardiomyocyte hypertrophy and myolysis were determined. In 30% cases, cardiomyocytes containing atrial natriuretic peptide were detected in the interventricular septum myocardium. The data were compared with clinical and functional parameters of patients. It was found that cardiac stem cells are present in patients, whose myocardium was characterized by increased density of the connective tissue, hypertrophy of mature cardiomyocytes, medium degree of myolysis in them, and accumulation of natriuretic peptide, a cardiac failure marker, in cardiomyocytes.

Key Words: *hypertrophic cardiomyopathy; human myocardium, resident myocardium stem cells; cardiomyocyte structure*

Resident cardiac SC (RCSC) are a population of cardiomyocytes phenotypically corresponding to SC and capable of resuming the cell cycle in mature myocardium [12]. The appearance of RMSC is usually explained by activation of the regeneration potential of the heart. RMSC were detected in patients with aortic stenosis [13], dilated cardiomyopathy [9], and in the periinfarction zone in acute myocardial infarction [14] Hypertrophic obstructive cardiomyopathy (HOCMP) is a complex genetically determined disease characterized by pronounced myocardial hypertrophy with thickening of the interventricular septum (IVS) and left ventricular (LV) wall and often accompanied by LV outflow tract obstruction and a decrease in LV cavity [4]. IVS thickening is primarily caused by cardiomyocyte hypertrophy, but possible role of cardiomyocyte precursors RCSC remains unclear.

The aim of this study was to detect RCSC in IVS of patients with HOCMP and to compare these data with morphological parameters of the myocardium IVS and clinical and morphological data of patients.

MATERIALS AND METHODS

The study included 40 HOCMP patients at the age of 16-61 years (mean age 39±12 years), in whom right ventricular outflow tract obstruction was surgically removed by the method of Bockeria. According to echocardiography data, IVS thickness in patients was 12.0-35.0 mm (mean 24.1±5.1 mm), LV end-diastolic

[1]A. N. Bakulev Research Center of Cardiovascular Surgery, Russian Academy of Medical Sciences; [2]Institute of Pulmonology, Federal Biomedical Agency, Moscow, Russia. ***Address for correspondence:*** tatiana@box.ru. V. T. Sukhacheva

0007-4888/12/1534-0535

volume 39.0-139.0 ml (mean 80.3±24.1 ml), LV end-systolic volume 9.0-47.0 ml (mean 23.3±10.2 ml), LV outflow tract pressure gradient 27.0-199.0 mm Hg (mean 94.1±36.9 mm Hg.), LV ejection fraction (EF) 56-91% (mean 74.3±7.3%).

Biopsy specimens from IVS myocardium obtained during surgery were fixed in 4% paraformaldehyde (Immunofix; Bio-Optica), embedded in paraffin, and 5-μ sections were prepared.

For visualization of the connective tissue, the paraffin sections were stained after Mallory using a Picro Mallory trichrome kit (Bio-Optica). Volume density of the connective tissue in IVS myocardium was evaluated in 20 randomly selected fields of view (objective ×40) using Image-Pro software. Myofibrillar disarray was semiquantitatively evaluated in 10 randomly selected fields of view (objective ×20) as described elsewhere [10] by a 4-point scale: no disarray (0); myofibrillar disarray occupies <25% (1), ~50% (2), and >75% (3).

In semithin sections, the zones of myolysis were detected and the degree of cardiomyocyte hypertrophy was evaluated. To this end, the specimens were fixed in 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffered saline (pH 7.4), postfixed in 1.5% OsO_4, dehydrated, and embedded in araldite. The sections (1 μ) were stained by PAS method and poststained with methylene blue. The diameters of cardiomyocytes and their nuclei were measured on longitudinal sections crossing the nucleus under a light microscope (objective ×100); at least 50 cardiomyocytes for each patient were analyzed. In the same cells, the degree of myofibrillos was evaluated by a 4-point scale: no myofibril loss (0); minor (1), moderate (2), and pronounced loss (3), *i.e.* myofibril-free zones were not detected or occupied <10%, 10-50%, and >50% cardiomyocyte section, respectively.

For immunofluorescent detection of RCSC, the sections were treated with 0.01 M citrate buffer (pH 6.0) at 100°C for antigen unmasking. Then the sections were incubated with a mixture (1:1) of primary antibodies to SC marker c-kit (Abcam) and sarcomere α-actin (Abcam) and then with a mixture (1:1) of second antibodies (Alexa 488 and Alexa 546, Invitro-

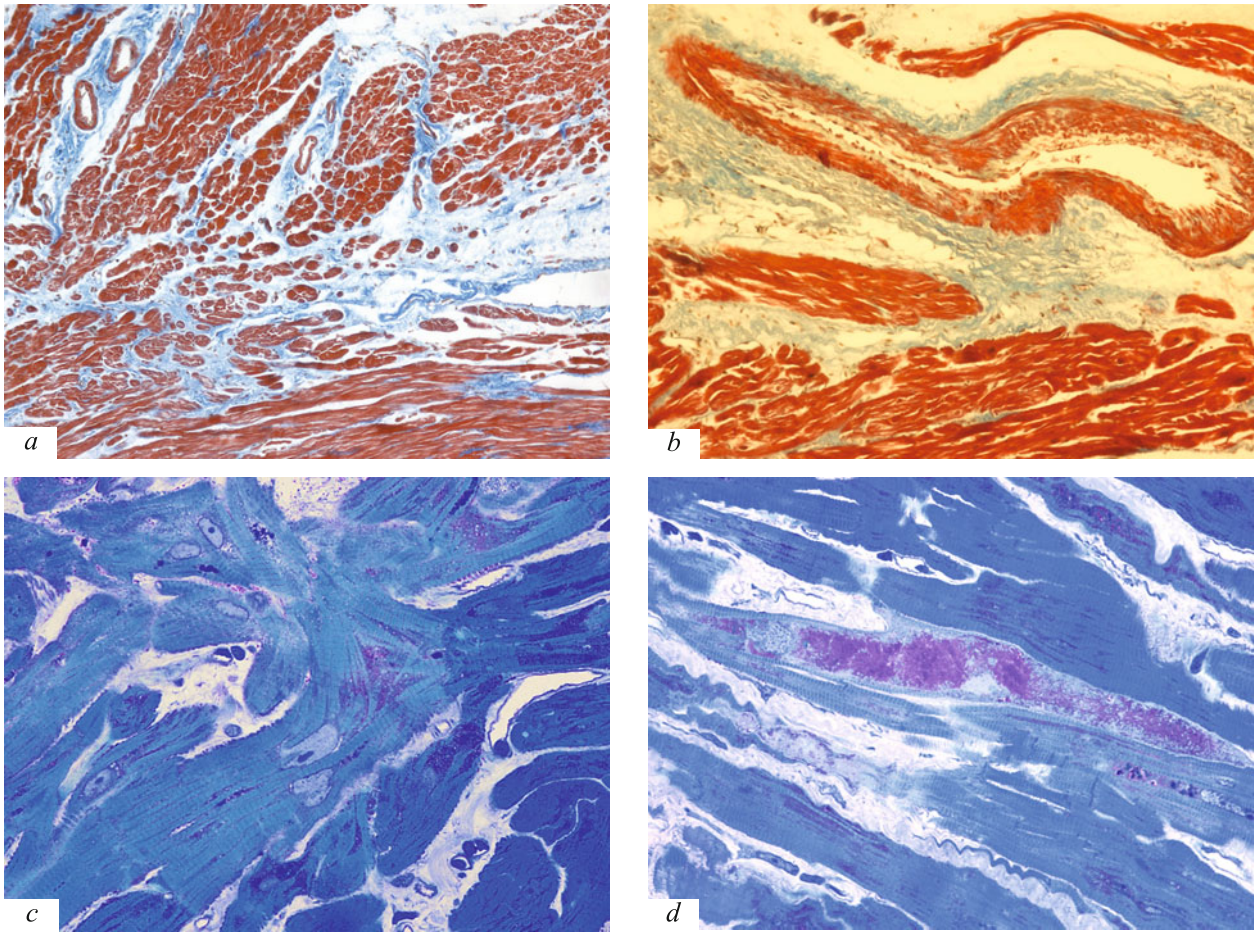

Fig. 1. IVS myocardium of HOCMP patients at the age of 24 (*a*), 25 (*b*), and 58 (*c*, *d*) years: interstitial (*a*) perivascular (*b*) fibrosis of IVS myocardium, myofibrillar disarray (*c*), cardiomyocytes with zones of partial myolysis filled with glycogen (*d*). Mallory staining, ×100 (*a, b*); semithin sections, PAS--methylene blue staining ×1000 (*c, d*).

gen). Nuclei were poststained with DAPI (Sigma). The preparations were embedded into Immu-mount (Thermo-Shandon) and examined under a Leica TCS SPE confocal microscope. In preparations, RCSC containing sarcomere α-actin and membrane SC marker c-kit were detected. In 10 fields of view (objective ×25), cardiomyocyte density per section unit area was determined. RCSC count per section and section area were determined and the relative content of RCSC in the myocardium per 10^6 cardiomyocytes was calculated. The data are presented as medians and value ranges.

For immunohistochemical detection of granules containing cardiac failure marker atrial natriuretic peptide (ANP), paraffin sections of the myocardium were treated with 3% H_2O_2, incubated with antiatrial natriuretic peptide-antibody (CBL66, Chemicon), treated with EnVision reagents (DAKO), and poststained with hematoxylin. The percentage of ANP-containing cardiomyocytes in the myocardium was evaluated.

The results were compared with morphological characteristics of IVS myocardium and clinical and functional features of the examine patients. The data were processed using Spearman and Mann–Whitney tests at $p<0.05$.

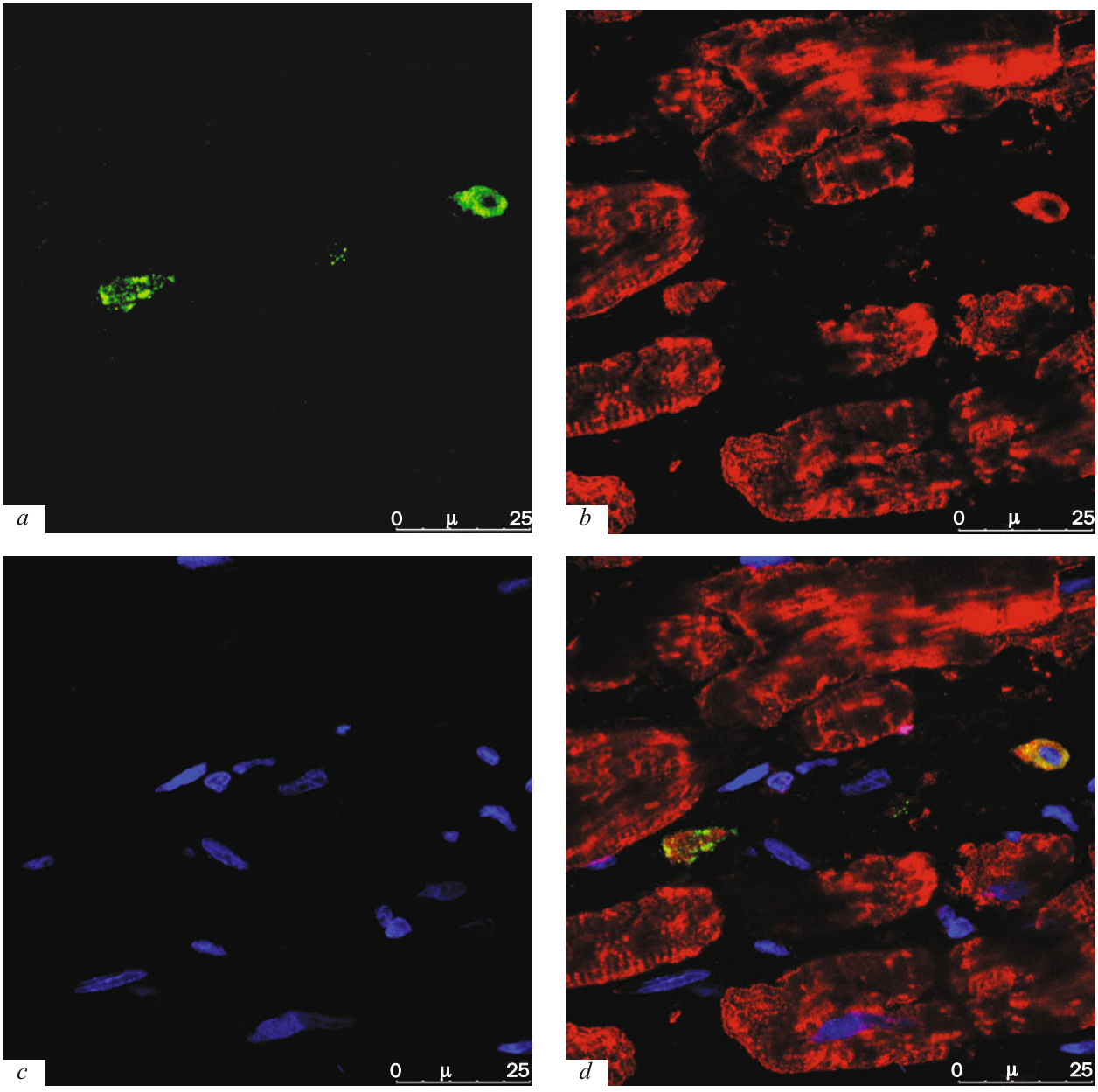

Fig. 2. A 44-old patient 44 years: RCSC in IVS. Double staining with antibodies to c-kit and sarcomere α-actin. Immunoconfocal microscopy. *a*) green fluorescence (c-kit; Alexa 488); *b*) red fluorescence (sarcomere α-actin, Alexa 546), *c*) blue fluorescence (nucleus post-staining, DAPI); *d*) superposition of images *a-c*.

RESULTS

IVS myocardium in most patients with HOCMP was characterized by pronounced interstitial and/or perivascular fibrosis (Fig. *1, a, b)*. Volume density of the connective tissue in different patients constituted from 7 to 72% (mean 42±15%) of the examined IVS myocardium area. Myofibrillar disarray, a phenomenon more typical of HOCMP than of other pathologies and described in details at both histological [5,8,10,15] and ultrastructural [5,8] levels was noted in 29 (72.5%) of 40 HOCMP patients (Fig. 1, *c*). Non-parallel myofibrils were more often seen in younger HOCMP patients (r=-0.46, p=0.004), which agreed with published data [15]. The degree of myofibrillar disarray correlated with the degree of IVS cardiomyocyte hypertrophy (r=0.52, p=0.001) and thickness of the posterior LV wall (r=-0.50, p=0.005). In previous reports, no correlations between myofibrillar disarray and LV wall and IVS thickness in HOCMP patients were noted.

Most IVS cardiomyocytes of HOCMP patients were characterized by moderate hypertrophy (cardiomyocyte diameter 21-25 μ); in some cases, pronounced hypertrophy was observed (cardiomyocyte diameter >26 μ, according to classification proposed by B.Kunkel *et al*.) [3]. The mean diameter of cardiomyocytes varied from 10.5 to 34.0 μ (mean 24.0±5.0 μ). Moderate myolysis was observed in 24 of 40 HOCMP patients in 2-71% cardiomyocytes (mean 26±20%) and pronounced myolysis was found in 7 of 40 patients in 3-38% cardiomyocytes (mean 19±15%). Light microscopy of PAS-stained preparations showed the presence of glycogen granules in myofibril-free zones (Fig. *1, d)*. Cardiomyocytes with moderate myolysis were more often observed in patients with higher LV EF (r=0.37, p=0.03) and reduced LV end-systolic volume (r=-0.45, p=0.01). According to classical studies [5], the absence of myofibrils in the myocardium of HOCMP patients is regarded as a signs of degenerative changes in cells. At the same time, the appearance of myofibril-free zones in cardiomyocyte sarcoplasm is typically observed in the myocardium under conditions of ischemia and pressure or volume overload. This stereotypic rearrangement of cardiomyocytes maintains their viability under conditions of energy deficiency [1].

c-kit-Positive RCSC were detected in the myocardium of 33 HOCMP patients (82.5%). The relative content of these small cells (diameter of 5.3-12.5 μ; mean 7.1±1.7 μ) with thin cytoplasmic rim containing sarcomeric α-actin was 47 cells per 10^6 cardiomyocytes. Their content varied from 6 to 8224 cells per 10^6 cardiomyocytes (Fig. *2, a-d)*. RCSC were primarily located in the interstitial space between mature cardiomyocytes as individual cells or small groups. Similar c-kit-positive RCSC with a diameter of 6±2 μ have been described in LV myocardium of adult patients with aortic stenosis [13], in patients with dilated cardiomyopathy [9], and in CHD patients; it should be noted that in patients with acute myocardial infarction the content of these cells increased primarily in the periinfarction zone [14].

The content of RCSC was higher in IVS myocardium with increased density of the connective tissue (r=0.46, p=0.003), more pronounced hypertrophy of mature cardiomyocytes (r=0.53, p=0.0005), and predominance of cardiomyocytes with moderate myolysis (r=0.45, p=0.02). Myofibrillar disarray in IVS did not correlate with the content of RCSC (r=0.12, p=0.45).

Cardiomyocytes containing heart failure marker ANP were detected in 12 of 40 HOCMP patients; they constitute 0.0004-0.8000% IVS cardiomyocytes. ANP granules were seen in solitary IVS cardiomyocytes, primarily in the perinuclear zone and sometimes in cardiomyocytes sarcoplasm (Fig. 3). ANP-positive cardiomyocytes were more often detected in IVS myocardium characterized by increased volume density of the connective tissue (r=0.32, p=0.04) and more pronounced myofibrillar disarray (r=0.43, p=0.007). Higher degree of fibrosis and myofibrillar disarray in LV and right ventricular myocardium containing ANP-positive cardiomyocytes in comparison with cardiomyocytes without ANP was reported previously [11]. Published data suggest that plasma content of ANP in HOCMP patients is higher than in controls [2] and in patients with dilated cardiomyopathy [7]. In HOCMP patients included in our study, ANP-positive cardiomyocytes were detected only in cases when IVS thickness surpassed 24 mm and the mean cardiomyocyte diameter in IVS was above 21.4 mm. The content of ANP-positive cardiomyocytes increased with increasing IVS thickness (r=0.4, p=0.01) and aggravation of IVS cardiomyocyte hypertrophy (r=0.4, p=0.009), as

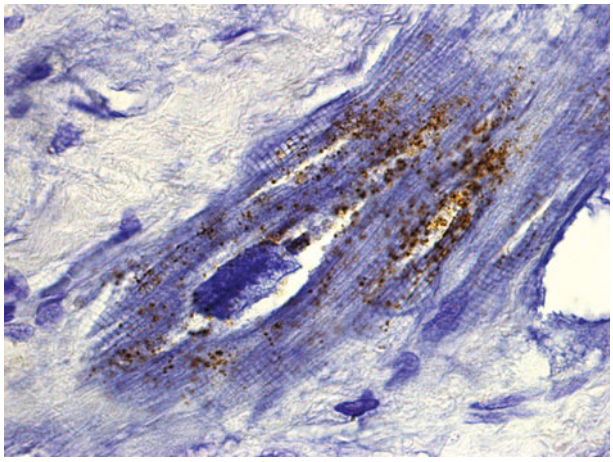

Fig. 3. HOCMP patient, 27 years: ANP-containing cardiomyocyte in IVS myocardium. Immunoperoxidase staining, ×1000.

was reported previously [11]. IVS myocardium apart from ANP-positive cardiomyocytes contained numerous RCSC (r=0.52, p=0.0006).

Thus, cardiomyocyte precursors, c-kit-positive RCSC, were detected in IVS myocardium of HOCMP patients in content 47 cells her 10^6 cardiomyocytes (their content varied from 6 to 8224 cells per 10^6 cardiomyocytes). The myocardium of these patients was characterized by increased density of the connective tissue, hypertrophy of mature cardiomyocytes, medium degree of myolysis in them, and accumulation of natriuretic peptide, a cardiac failure marker, in cardiomyocytes.

REFERENCES

1. M. Borgers, *Exp. Clin. Cardiol.,* **7,** Nos. 2-3, 69-72 (2002).
2. Y. Hirata, A. Matsumoto, T. Aoyagi, *et al., Cardiovasc. Res.,* **51,** No. 3, 585-591 (2001).
3. B. Kunkel, H. Lapp, G. Kober, and M. Kaltenbach, *Cardiomyopathy and Myocardial Biopsy*. Berlin, Heidelberg, New York (1978).
4. B. J. Maron, *JAMA*, **287,** No. 10, 1308-1320 (2002).
5. B. J. Maron, V. J. Ferrans, W. L. Henry, *et al., Circulation*, **50,** No. 3, 436-446 (1974).
6. B. J. Maron, V.J. Ferrans, and W. C. Roberts, *Am. J. Pathol.,* **79,** No. 3, 387-434 (1975).
7. Y. Mizuno, M. Yoshimura, E. Harada, *et al., Am. J. Cardiol.,* **86,** No. 9, 1036-1040 (2000).
8. W. C. Roberts and V. J. Ferrans, *Hum. Pathol.,* **3,** 287-342 (1975).
9. H. Sato, I. Shiraishi, T. Takamatsu, and K. Hamaoka, *J. Mol. Cell. Cardiol.,* **43,** 254-261 (2007).
10. M. G. Sutton, J. T. Lie, K. R. Anderson, *et al., Br. Heart. J.,* **44,** 433-443 (1980).
11. G. Takemura, H. Fujiwara, M. Mukoyama, *et al., Circulation,* **83,** No. 1, 181-190 (1991).
12. D. Torella, G. M. Ellison, S. Mendez-Ferrer, *et al., Nat. Clin. Pract. Cardiovasc. Med.,* **3,** Suppl. 1, 8-13 (2006).
13. Urbanek K., Quaini F., Tasca G. *et al., Proc. Natl. Acad. Sci. USA.,* **100,** No. 100, 10,440-10,445 (2003).
14. K. Urbanek, D. Torella, F. Sheikh, *et al., Proc. Natl. Acad. Sci. USA.,* **102,** No. 24, 8692-8697 (2005).
15. A. M. Varnava, P. M. Elliott, S. Sharma, *et al., Heart,* **84,** No. 5, 476-482 (2000).