Cardiac Cells in Regenerative Processes in Patients with Heart Failure Due to Ischaemic Heart Disease

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Authors' contributions

This work was carried out in collaboration between all authors. The design of the study was carried out by authors OVS and TGK. The subject recruitment and left atrial appendages sampling procedures were carried out by author AAS. Authors TGK and MPV carried out the immunofluorescence staining of the resident cardiomyocyte progenitors. The electron microscopy was done by author MPV. The isolation and cultivation of fetal cardiomyocytes was carried out by author RAP. The cultivating of these cells and their immunofluorescence staining were carried out by author OVS. Author VPM provided supervisory support during the study. Authors GTS and VPC provided assistance with clinical interpretation. Authors OVS and TGK drafted the manuscript. All authors read and approved the final manuscript.

Article Information

Original Research Article

ABSTRACT

Aims: This study aimed to find the cardiac cells which can participate in the processes of regeneration at patients with heart failure due to ischaemic heart disease. To investigate the participation of myosin activating protein kinases in sarcomerogenesis, because sarcomerogenesis

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is the crucial part of cardiomyocyte differentiation process.

Study Design: Resident cardiomyocyte progenitors and dedifferentiated cardiomyocytes were found in left atrial appendages from patients with heart failure due to ischaemic heart disease. We used a cell model of fetal cardiomyocytes with the disassembly contractile apparatus to study the forming of new myofibrils (or sarcomerogenesis) regulated by myosin activating protein kinases.

Place and Duration of Study: Cardiology Research and Production Center, Research Center for Obstetrics, Gynecology and Perinatology, Department of Fundamental and Applied Neurobiology of V. Serbsky Federal Medical Research Centre of Psychiatry and Narcology between June 2014 and October 2015.

Methodology: We included 10 patients with heart failure due to ischaemic heart disease. Resident cardiomyocyte progenitors and dedifferentiated cardiomyocytes were found by the immunofluorescence approach and the electron microscopy. To determine the myosin activating protein kinases localization in human fetal cardiomyocytes at the 8-9 week heart gestation stage immunofluorescence approach was used.

Results: We detected the cardiomyocyte progenitor cells which express c-Kit and Nkx-2.5, other cells express Mdr-1 and GATA-4. Dedifferentiated cardiomyocytes were found. It has been established that smooth muscle, nonmuscle and skeletal myosin light chain kinases are colocalized with nonmuscle myosin in premyofibrils in fetal human cardiomyocytes.

Conclusion: We demonstrated that the heart of patients with heart failure due to ischaemic heart disease contains the progenitor resident cardiomyocytes and dedifferentiated cardiomyocytes. These cardiac cells possibly can proliferate and differentiate to mature cardiomyocytes and recover heart function and structure after injury. Myosin activating protein kinases may contribute in myofibril formation during the cardiomyocyte differentiation.

ABBREVIATIONS

IHD	*: Ischaemic Heart Disease*
HF	*: Heart Failure*
Mdr-1	*: Multiple drug resistance-1*
MLCK	*: 108- smooth muscle Myosin Light Chain Kinase*
MLCK	*: 210- nonmuscle Myosin Light Chain Kinase*
sk MLCK	*: skeletal Myosin Light Chain Kinase*
NMIIB	*: Nonmuscle Myosin II type B*
PBS	*: Phosphate Buffered Saline*
FBS	*: Fetal Bovine Serum*
BSA	*: Bovine Serum Albumin*
DMEM	*: Dulbecco's modified Eagle medium*
SP	*: Side Population*
ABC	*: Adenosine Tri-Phosphate-Binding Cassette*

1. INTRODUCTION

Many years it was thought that the heart is a terminally differentiated organ, and the adult mammalian heart is not capable of regeneration. The loss of cardiomyocytes due to cardiovascular disease is a major mechanism resulting to ventricular dysfunction and heart failure (HF). Cardiovascular disease is the leading cause of death in the world today [1], and patients with heart failure due to ischaemic heart disease (IHD) too represent a large part [2-6]. Half of patients with HF will die within 5 years of diagnosis [7,8]. These statistics support the need to find complementary treatment for HF. The cardiomyocyte loss associated with the disease triggers pathological remodeling characterized by progressive changes in ventricular size, shape and function which leads to further loss of cardiomyocytes [9-11]. Standard drug therapy, ventricular assist devices, cardiac resynchronization therapy and cardiac transplantation have been used for disease treatment. But the conventional treatment cannot resolve the problem of the cardiomyocyte loss. Cardiac transplantation improves outcomes in

end-stage HF, but this procedure is connected with many difficulties. Remarkable works by Bergmann et al have established that human cardiomyocytes are renewed throughought life [12,13]. Recently it has been found that the adult mammalian heart contains progenitor (or stem) cells that can generate functional cells [14-21].

Formation of new functional cardiomyocytes may come both of dedifferentiation and proliferation of pre-existing cardiomyocytes without complete reversion to a cardiac progenitor state [22], or by cardiac differentiation of stem cells (embryological or dedifferentiated origin) [23-25]. The main features of dedifferentiated cardiomyocytes are changes of the sarcomeric structure of cardiomyocytes and fetal gene expression, the phenotype of these cells is similar to fetal cell phenotype.

Changes of the regulated sarcomeric structure of cardiomyocytes under pathological conditions are characterized by the disappearance of highly organized patterns of myosins, titin and desmin and the phenotype of these cardiomyocytes resembles the phenotype of fetal cardiomyocytes [26]. Such dedifferentiated cardiomyocytes were found in infarction border zones [27,28] and fibrillating atria [29]. Cardiomyocytes found in hibernating myocardium show a dedifferentiated phenotype with structural hallmarks of fetal cardiomyocytes [27]. All changes are accompanied by important alterations in the expression and organization of contractile and cytoskeletal proteins [30]. The dedifferentiation state might enable cardiomyocytes to survive pathological conditions [31].

Cardiomyocyte differentiation includes sarcomerogenesis. Sarcomerogenesis in cardiomyocytes is regulated by some myosin activating protein kinases. Smooth muscle myosin light chain kinase (MLCK-108) functional activity decreasing results to sarcomerogenesis inhibition, but increased skeletal myosin light chain kinase (sk MLCK) level accelerates sarcomere formation in rat cultured cardiomyocytes [32]. However their participating in sarcomerogenesis of human cardiomyocytes has not been investigated yet. Studying the molecular mechanisms of sarcomerogenesis regulated by protein kinases is important for understanding cardiomyocyte differentiation and redifferentiation mechanisms and understanding of the myocardial regeneration processes.

In our work dedifferentiated cardiomyocytes and resident cardiomyocyte progenitors have been found in left atrial appendages from patients with HF. Human cardiomyocyte sarcomerogenesis has been studied at a cell model.

2. MATERIALS AND METHODS

2.1 Tissue Samples

Ethical approval was obtained from local research ethics committee (Re: N208).

Left atrial appendages of patients with IHD and HF were obtained using standard techniques. A total 10 atrial appendages specimens were collected from 10 patients during the routine procedure of open heart surgery from Cardiology Research and Production Centre. The specimens were from left atrial appendages and the size of samples varied from 50 to 500 mg. Samples were placed in the medium for freezing tissue Tissue-Tek (Sacura Finetechnical Co., Ltd.) and frozen in liquid nitrogen for subsequent preparation of cryosections.

No information was able to be collected in terms of patient's age, sex and properties of noncardiac diseases, and other medical history of patients. A written consent agreement was obtained from all patients.

2.2 Reagents and Antibodies

General reagents of analytical grade were purchased from Sigma and Life Technologies (USA). Cell culture reagents and plastic were obtained from Hyclone (USA) and Corning (Netherlands). Electron microscopy reagents were obtained from Agar Scientific (UK) and LADD Research Industries (USA).

Following primary antibodies were used: NMIIB (Covance, USA), MLCK-108 and MLCK-210 (clone K-36, Sigma, USA), skMLCK (Santa-Cruz, USA), GATA-4 (Santa-Cruz, USA), Nkx-2.5 (Santa-Cruz, USA), Mdr-1 (Santa-Cruz, USA), c-Kit (Santa-Cruz, USA). Secondary Alexa- labeled antibodies were obtained from Molecular Probes (USA).

2.3 Cell Culture

Ethical approval was obtained from local research ethics committee (Re: N13). Fetal cardiomyocytes were isolated from 8-9 week fetuses resulting from medical abortions carried out for medical reasons. The hearts were minced and washed with phosphate buffered saline

(PBS). Cell isolation was performed with 0.2% trypsin and 1 mg/ml type II collagenase in 0.02% glucose/PBS, pH7.4 solution at 37°C. After dissection, cells were incubated on plastic culture dishes for 2 hours at 37°C to separate cells for adherent and non-adherent cells, with Dulbecco's modified Eagle medium (DMEM) containing penicillin and streptomycin and supplemented with 10% fetal bovine serum (FBS). After incubation, the supernatant with non-adherent cells was transferred to new culture dishes and maintained in a 5% CO_2 incubator at 37°C with the culture medium DMEM, 10% FBS replaced every 3 days.

2.4 Electron Microscopy

For transmission electron microscopy myocardial tissue was fixed in 2.5% glutaraldehyde buffered with 90 mM KH_2PO_4, pH 7.4, After rinsing in the same buffer for 24 hrs, the samples were postfixed for 1 hr in 2% OsO_4 buffered to pH 7.4 with 0.1 M veronal acetate. Additional impregnation of myocardium tissue was performed in 1% uranyl acetate in 0.1 M veronal acetate, pH 5.2. The myocardium samples were dehydrated in graded series of ethanol and routinely embedded in Epon (LADD Research Industries).

Ultrathin sections were counterstained with uranyl acetate and lead citrate, prior to examination in a microscope Libra-120 (Zeiss).

2.5 Immunofluorescence

Cardiomyocytes grown on glass coverslips were fixed in 4% formaldehyde/PBS for 5 min at room temperature and treatment with 1% Triton-X100 in PBS for 5 min. Cells were washed in PBS at room temperature and treatment with blocking solution 10% FBS in PBS for 30 min at room temperature. After that cardiomyocytes were incubated with appropriate primary and secondary antibodies diluted in 1% BSA (bovine serum albumin) in PBS during 1 h.

Sections (6 µm) of left atrial appendages of patients with IHD and HF were fixed in acetone for 10 min. After washes with PBS sections were treatment with blocking solution and subsequently incubated 1 h with primary and secondary antibodies diluted in 1% BSA in PBS.

Cells on coverslips and tissue sections were mounted in Aquapolymount medium (Polysciences, Warrington, PA) and

immunofluorescent images were obtained using Zeiss Observer.Z1 microscope (Zeiss, Germany) equipped with Axiocam cooled CCD camera and Axiovision v.4.8 software.

3. RESULTS

3.1 Resident Cardiomyocyte Progenitors in Left Atrial Appendages of Patients with IHD and HF

Cells expressing stem cell markers c-Kit, Mdr-1 and early cardiac transcription factors GATA-4 and Nkx-2.5 were identified in left atrial appendages from all patients with ischaemic heart disease and heart failure by immunofluorescence approach. Detected cells are small, and they have the rounded shape. Cardiomyocyte progenitor cells express c-Kit and Nkx-2.5 (Fig. 1). Other cells express Mdr-1 and GATA-4 (Fig. 2). Cell markers are located as follows: c-Kit and Mdr-1 on the cell membrane (Figs. 1A, 2A), Nkx- 2.5 and GATA-4 in the nucleus (Figs. 1B, 2B). Localization of nucleus was identified by DAPI staining (Figs. 1C, 2C).

3.2 Dedifferentiated Cardiomyocytes in Left Atrial Appendages of Patients with HF

Cardiomyocytes with hallmarks of dedifferentiation as evaluated by electron microscopy have been found in this study in left atrial appendages from patients with IHD and HF. Revealed cardiomyocytes have main features of dedifferentiated cardiomyocytes such as sarcomere disorganization, enhanced glycogen content, mitochondria disposition, changes in size and shape of cardiomyocytes (Fig. 3).

3.3 Identification of Myosin Activating Protein Kinases in Human Fetal Cardiomyocytes

The myosin activating protein kinases localization was detected in human fetal cardiomyocytes by immunofluorescence approach. It was established that skMLCK colocalized with nonmuscle myosin IIB (NMIIB) in premyofibrils (Fig. 4). MLCK-108 and its high molecular weight isoform nonmuscle myosin light chain kinase (MLCK- 210) detected in our study immunoblotting approach (unpublished data) localized along NMIIB positive premyofibrils (Fig. 5).

4. DISCUSSION

Regeneration processes are different in the body organs and are connected with development during embryogenesis. Mammalian cardiomyocytes have two ways for growth and generation. During embryogenesis the proliferation of cardiomyocytes takes place, but after the birth only the cell size increased. Many years it was believed that the postnatal human heart had no capacity to regeneration; recently this opinion has changed [12], but the rate of regeneration and the source of it are still unclear. Some studies suggest that there is a high level of differentiation of progenitors to cardiomyocytes [33] and their turnover is high [34]. Other studies demonstrate that new cardiomyocytes are made at a very low level [12, 35]. Kikuchi et al. [36] propose that the source of the new cells is the division of existing cardiomyocytes. Other researchers propose that the new cells come from progenitors residing in the heart [15] or from exogenous niches, such as the bone marrow [37].

Resident cardiomyocyte progenitors have been found in our study in left atrial appendages from patients with IHD and HF. Cardiomyocyte progenitor cells express simultaneously stem cell markers c-Kit, Mdr-1 and early cardiac transcription factors Nkx-2,5 and GATA-4 respectively. We detected that the cells express c-Kit and Nkx-2.5, other cells express Mdr-1 and GATA-4. These cells represent different populations of adult cardiac progenitor cells (c-Kit and SP).

The existence of c-Kit positive resident progenitor population in the heart was first reported in 2003, when Beltrami and colleagues reported isolating clonogenic, self renewing cells that are capable of differentiating into cardiomyocytes, vascular smooth muscle cells, and endothelial cells [15]. These cells are negative for many blood lineage markers (Lin-), and positive for c-Kit, the receptor for stem cell factor. In the adult rat myocardium, c-Kit+ cells are rare (1 per 10,000 myocytes), and heterogeneous, with a minority (7%-10%) expressing early cardiac transcription factors such as GATA-4, Mef2, and Nkx-2.5 [15].

Population of cells with stem cell-like properties has been identified in bone marrow, muscle, and skin by their ability to exclude Hoechst dye and certain anticancer drugs, named as "side population" or SP [38]. Some groups have identified SP cells in adult mouse hearts marked
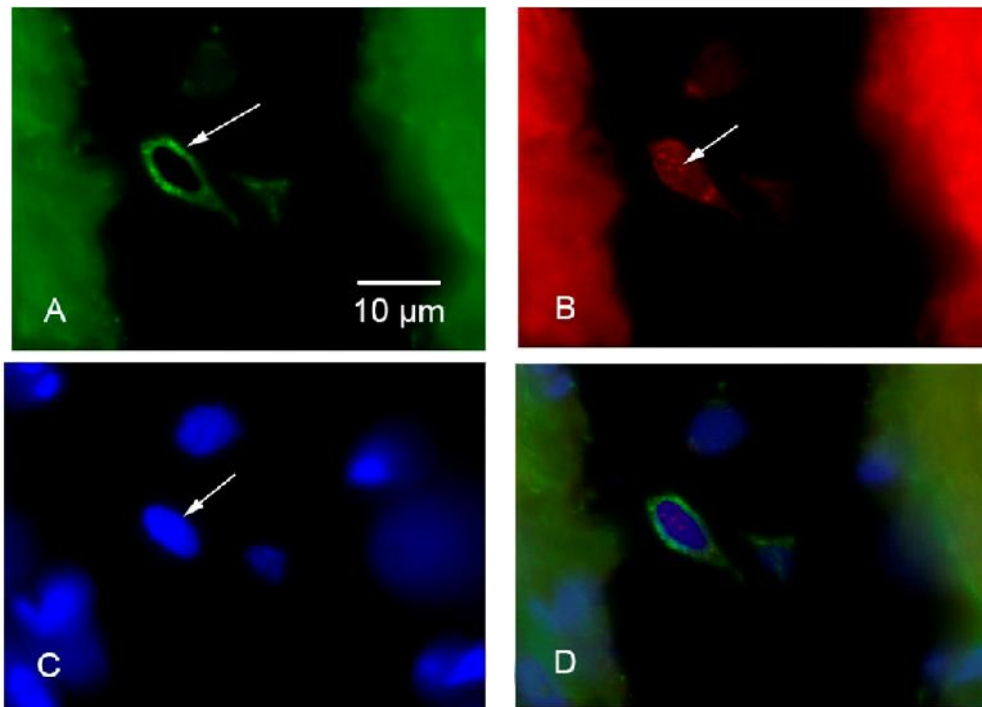
Fig. 1. Resident cardiomyocyte progenitors in left atrial appendages from patients with HF
A - c-Kit - antibodies (localization on the membrane), B – Nkx-2.5- antibodies (nuclear localization), C- DAPI, D- Merged images
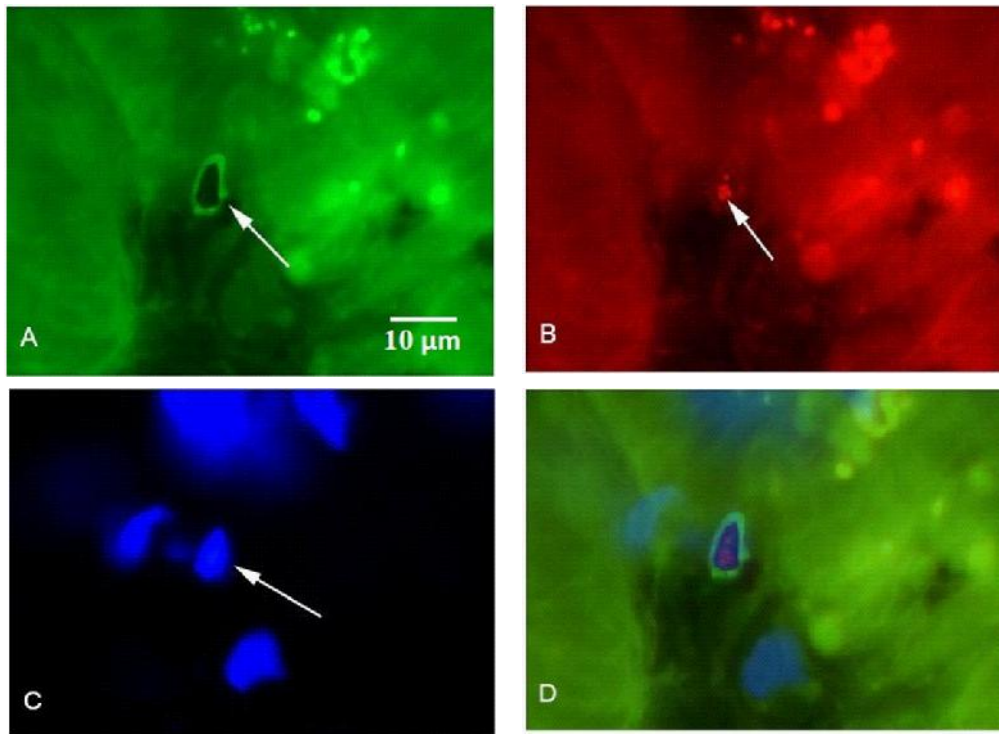
Fig. 2. Resident cardiomyocyte progenitors in left atrial appendages from patients with HF
*A- Mdr-1- antibodies (localization on the membrane), B- GATA-4- antibodies (nuclear localization), C- DAPI,
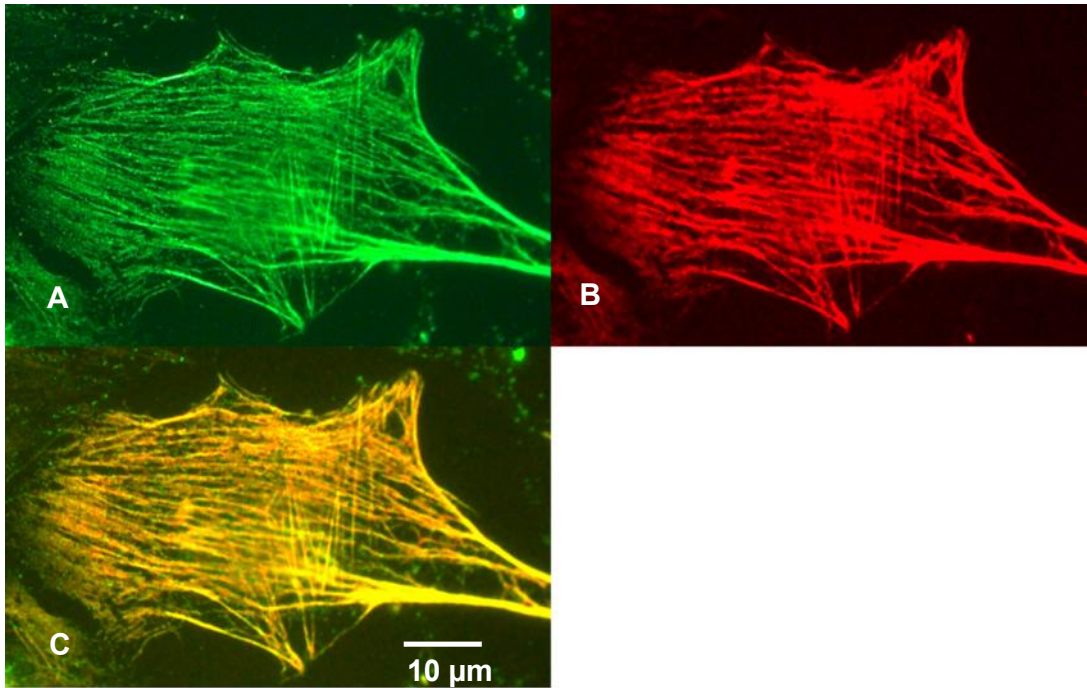D- Merged images*

by the expression of Abcg2 and Mdr-1, two genes belonging to the ATP-binding cassette (ABC) transporter superfamily that constitute the molecular basis for the dye efflux [18,39-41].While their clonogenic potential, capacity for self-renewal, and developmental origin remain to be determined, upon coculture with adult rat ventricular cardiomyocytes, these cells demonstrate not only biochemical differentiation, as evidenced by the expression of cardiac transcription factors GATA-4 and Nkx-2.5 and contractile proteins, but also functional cardiomyogenic differentiation, as determined by sarcomeric organization, intracellular calcium transients, and cellular contraction [40].

Three populations of adult cardiac progenitor cells (c-Kit+, Sca-1+, SP) represent 0.005-2% of the total cellular content in the heart, enter the cell cycle when growth of the heart is attenuated, proliferate in culture, and form cells expressing cardiomyogenic markers [42]. They appear phenotypically distinct from one another and show differential expression of surface markers [43-45]. Other studies suggest that the side population of adult cardiac progenitor cells express also the stem cell antigen-1 [46]. Recently the researchers defined four subpopulations of cardiac progenitor/stem cells in adult mouse myocardium all sharing stem cell antigen-1 (Sca1), based on side population (SP) phenotype [47]. To date, the exact lineage relationships between the adult cardiac progenitor cell populations and embryonic cardiac progenitor cells remain unknown.
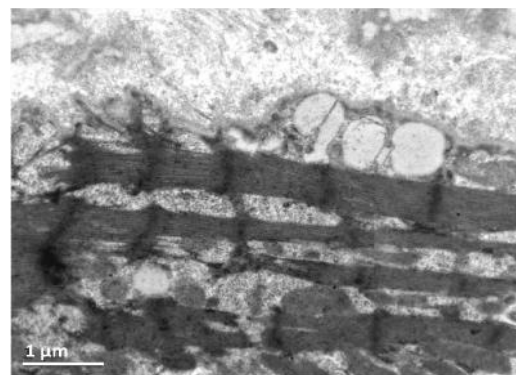
Fig. 3. Electron microscopy of left atrial appendages from patients with ischaemic heart disease and heart failure show a dedifferentiating cardiomyocyte with obvious disruption of the sarcomeric apparatus and glycogen accumulation

Fig. 4. Sk MLCK localization in human fetal cardiomyocytes
A - sk MLCK antibodies, B - Nonmuscle myosin IIB antibodies, C - Merged images
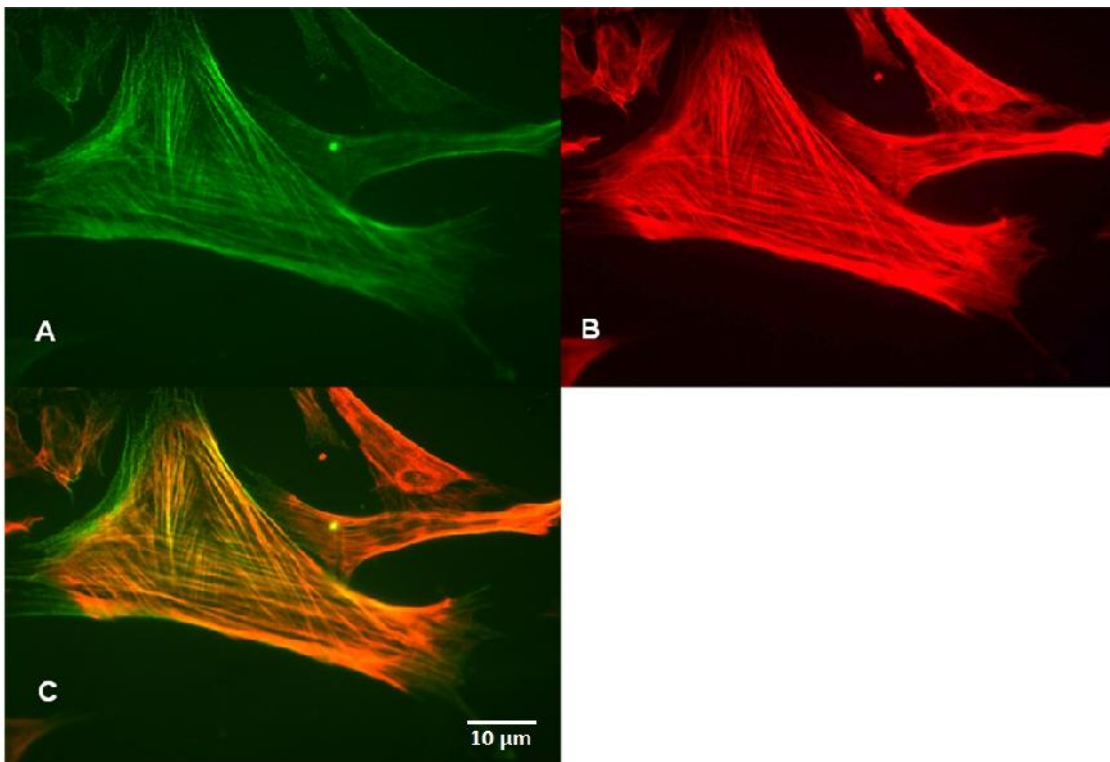
Fig. 5. MLCK-108 and MLCK-210 localization in human fetal cardiomyocytes
A – MLCK-108 and MLCK-210 antibodies, B - Nonmuscle myosin IIB antibodies, C - Merged images

The presence of endogenous cardiac progenitors in the rodent adult heart has prompted studies into whether similar populations exist in the human adult heart. As was demonstrated in rodents, a heterogeneous population of cardiac cells defined by the expression of the primitive stem cell markers c-Kit, Mdr-1, or a Sca-1-like epitope could also be found in human cardiac specimens from patients with aortic stenosis [48] myocardial infarction [49] and in the post-mortem hearts of patients who had undergone cardiac transplantation [50]. The human cardiac c-Kit+ subset was reported to give rise to cardiomyocytes, vascular smooth muscle cells, and endothelial cells *in vitro*, and following transplantation into immunodefficient mice [19].

The researchers from the laboratory Richard T. Lee showed, by combining two different pulse-chase approaches - genetic fate-mapping with stable isotope labelling, and multi –isotope imaging mass spectroscopy- that the genesis of cardiomyocytes occurs at a low rate by the division of pre-existing cardiomyocytes during normal ageing, a process that increases adjacent to areas of myocardial injury [51]. They found that cell activity during normal ageing and after injury led to polyploidy and multinucleation, but also to new diploid, mononucleate cardiomyocytes [51]. The data of this work at a mouse model revealed pre-existing cardiomyocytes as a dominant source of cardiomyocyte replacement in normal mammalian myocardial homeostasis as well as after myocardial injury [51].

Dedifferentiated cardiomyocytes have been found in our study in left atrial appendages from patients with IHD and HF. Revealed cardiomyocytes have main features of dedifferentiated cardiomyocytes such as sarcomere disorganization, enhanced glycogen content, mitochondria disposition, changes in size and shape of cardiomyocytes. The transient dedifferentiation of the sarcomeric structure during cytokinesis supports the notion of normal cell division resulting in two functional cardiomyocytes. This phenomenon was previously demonstrated in fetal cardiomyocytes [52,53]. Investigators showed that neonatal cardiomyocytes transiently dedifferentiate, stop beating, undergo cytokinesis, redifferentiate and begin beating again [54]. We propose that obvious disruption of the sarcomeric apparatus is characteristic of dedifferentiated cardiomyocyte.

Formation of functional cardiomyocytes may come both of dedifferentiation and proliferation of pre-existing cardiomyocytes without complete reversion to a cardiac progenitor state [22] and their subsequent differentiation (so called redifferentiation), or by cardiac differentiation of stem cells (embryological or dedifferentiated origin) [23-25]. Sarcomerogenesis is an important part of the formation of functional cardiomyocytes. We used a cell model of fetal cardiomyocytes with the disassembly contractile apparatus to study this process. The phenotype of dedifferentiated cardiomyocytes resembles the phenotype of fetal cardiomyocytes [26].

The cardiomyocyte differentiation includes myofibrils formation process – sarcomerogenesis. The premyofibril transition to myofibril, containing sarcomeres and replacement of premyofibril nonmuscle proteins to sarcomeric proteins is a crucial sarcomerogenesis stage. Premyofibril stability is an important part in sarcomerogenesis. The main premyofibril protein is nonmuscle myosin II type B, and its phosphorylation is an obligatory condition for stable filament formation. NMIIB phosphorylation is necessary for nonmuscle myosine filament assembly from monomers and myosine activation [55]. It was shown *in vitro* that NMIIB can be phosphorylated by smooth muscle myosin light chain kinase and skeletal myosin light chain kinase [56]. Nonmuscle myosin IIB is likely to be a natural substrate for MLCK-108 since this kinase is collocated with nonmuscle myosin both in premyofibrils and in the Z- disks of mature sarcomeres [57]. MLCK-108 functional activity decreasing results to sarcomerogenesis inhibition, but increased sk MLCK level accelerates sarcomere formation in rat cultured cardiomyocytes [32]. All these data testify to MLCK-108 is a natural nonmuscle stabilizer. Controversy reigns in the sarcomerogenesis regulation mechanisms area: which myosin activating protein kinases take part in this process for premyofibril stabilization. In this study the myosin activating protein kinases localization was studied in fetal cardiomyocytes.

In our research myosin light chain kinase isoform MLCK-210 was found in studied fetal cardiomyocytes by quantitive immunoblotting approach (unpublished data). To localize MLCK-108 and MLCK-210 isoforms by immunofluorescence approach we used monoclonal antibodies (clone K-36 Sigma) which can detect both isoforms. So the detected localization of both isoforms coincide (Fig. 1A). It was established that MLCK-108 and its high molecular weight isoform MLCK- 210, sk MLCK

colocalized with non-muscle myosin in premyofibril composition. Non-muscle myosin may be the substrate for above mentioned myosin-activating protein kinases, its phosphorylation by these protein kinases may contribute to premyofibril stabilization during cardiomyocyte differentiation.

5. CONCLUSION

This study aimed to find the cardiac cells which can participate in the processes of regeneration at patients with heart failure due to ischaemic heart disease. Our study demonstrated that the heart of these patients contains the progenitor resident cardiomyocytes and dedifferentiated cardiomyocytes. Such cardiomyocytes possibly can recover heart function and structure after injury. Myosin activating protein kinases may contribute in myofibril formation during the cardiomyocyte differentiation and these protein kinases may be pharmacological tools in regenerative medicine.

CONSENT

All authors declare that written informed consent was obtained from all participants for publication of this data.

ETHICAL APPROVAL

Ethical approval (Re: N208) for this study was permitted by the local research ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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