Heliyon 9 (2023) e21268

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

C-kit^{pos} cells in the human left atrial appendage

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ARTICLE INFO

Keywords:
c-kit^{pos} cells
Cardiac progenitor cells
Human left atrial appendage

ABSTRACT

Background: Subpopulations of myocardial c-kit^{pos} cells have the ability to stimulate regeneration in ischemic heart disease by paracrine effects. The left atrial appendage (LAA), which is easy accessible during cardiac surgery, may represent a perfect source for c-kit^{pos} cell extraction for autologous cell therapies in the living human. So far, frequency and distribution of c-kit^{pos} cells in LAA are unknown.

Methods: LAAs of patients who underwent cardiac surgery due to coronary artery disease (coronary artery bypass graft, CABG), valvular heart disease or both and of two body donors were examined. Tissue was fixed in 4 % paraformaldehyde, embedded in paraffin, dissected in consecutive sections and stained for c-kit^{pos} cells. In parallel, grade of fibrosis, amount of fat per section and cells positive for mast cell tryptase were examined.

Results: We collected 27 LAAs (37.0 % female, mean left ventricular ejection fraction 50.4 %, 63.0 % persistent atrial fibrillation (AF)). Most of the patients underwent combined CABG and valve surgery (51.9 %). C-kit^{pos} cells were detected in 3 different regions: A) Attached to the epicardial fat layer, B) close to vascular structures and C) between cardiomyocytes. C-kit^{pos} cells ranged from 0.05 c-kit^{pos} cells per mm^2 to 67.5 c-kit^{pos} cells per mm^2. We found no association between number of c-kit^{pos} cells and type of AF, amount of fibrosis or amount of fat. Up to 72 % of c-kit^{pos} cells also showed a positive staining for mast cell tryptase.

Conclusion: C-kit^{pos} cells are frequent in LAAs of cardiovascular patients with a rather homogenous distribution throughout the LAA. The LAA can therefore be considered as a source for extraction of a reasonable quantity of autologous cardiac progenitor cells in the living human patient.

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https://doi.org/10.1016/j.heliyon.2023.e21268
Received 14 July 2023; Received in revised form 9 October 2023; Accepted 18 October 2023
Available online 26 October 2023

1. Introduction

Myocardial c-kit^{pos} cells represent a heterogenous cell population and their role in ischemia, inflammation and ageing has not yet been fully elucidated [1]. Isolated and cultured cardiac c-kit^{pos} cells can be differentiated into cells with characteristics of cardiomyocytes, endothelial cells and smooth muscle cells [2,3]. Further, these cells were identified to mediate paracrine effects that stimulate heart regeneration in ischemic heart disease in preclinical studies [4].

Only few studies report frequency and distribution of endogenous c-kit^{pos} cells in human cardiac tissue. Numbers of c-kit^{pos} cells have been described to vary according to cardiac tissue region, presence and type of cardiovascular disease and range between 0.5 and 35 cells/mm^2 or from 0.1 to 6.0 % among all cardiac cell types [5–9]. The reason for these varying numbers is not known, but it is assumed that e.g., fibrotic or inflammatory processes, are involved [9].

Murine left atrial appendages (LAAs) have been shown to constitute a reservoir for c-kit^{pos} cells with substantially higher numbers compared to left ventricular tissue [10]. Accordingly, the human LAA may represent a suitable tissue source for isolation of c-kit^{pos} cells, due to its easy accessibility during standard cardiac surgery, large tissue size, as well as acceptable intraoperative bleeding risk in case of excision. The LAA is accessible during coronary artery bypass graft (CABG) or valve surgery. Compared to implantation of left ventricular assist devices, the only relevant alternative to obtain comparable myocardial tissue amounts, these interventions are usually performed significantly earlier in the cardiovascular disease process. Therefore, autologous cell therapies from c-kit^{pos} cells from LAA tissue would also be useable in earlier cardiovascular disease stages. In addition, surgical occlusion or exclusion of the LAA during cardiac surgery in patients with atrial fibrillation is currently a class IIb indication in the European guidelines [11].

Although presumed as a reservoir for c-kit^{pos} cells, there is only insufficient data available on human LAA tissue to date. This study examined frequency and distribution of c-kit^{pos} cells in the LAA of patients with atrial fibrillation in consideration of clinical patient characteristics, clinical disease progress and fibrosis.

2. Methods

2.1. Patients and tissue samples

LAAs of patients who underwent cardiac surgery due to coronary artery disease, valvular heart disease or both and of two body donors were examined. All patients gave their written informed consent. Age, sex, left ventricular ejection fraction (LVEF) and AF type (paroxysmal or persistent) were obtained from the patient record. Information about the body donors was restricted to age and sex. The study was approved by the local ethics committee (127/17-ek, University of Leipzig). Tissue was fixed in 4 % paraformaldehyde, embedded in paraffin and dissected in consecutive slices from the ostium to the distal part of the LAA. Depending on tissue size and

Table 1
Baseline characteristics of the patients who underwent cardiac surgery.

Patient number	Sex	Age in years	Type of cardiac surgery	LVEF in %	AF type
1	male	70	AVR, MVR, TVR	28	Persistent
2	female	59	MVR, CABG, cryo	52	Persistent
3	female	77	MVR, AVR, cryo	62	Persistent
4	male	64	AVR, MVR, CABG, aorta, cryo	40	Persistent
5	female	75	TVR, CABG	56	Persistent
6	male	65	AVR, abl	62	Paroxysmal
7	male	79	MVR	50	Paroxysmal
8	male	77	AVR, CABG, aorta	50	Paroxysmal
9	female	71	CABG	56	Persistent
10	male	70	AVR, CABG	56	Persistent
11	male	76	AVR, CABG	45	Persistent
12	female	68	AVR, MVR	55	Paroxysmal
13	male	70	AVR, CABG, abl	35	Persistent
14	male	69	AVR, CABG, abl	60	Persistent
15	male	69	MVR, TVR, CABG	30	Persistent
16	male	67	MVR, TVR	35	Persistent
17	female	68	CABG, abl	63	Paroxysmal
18	male	56	AVR, CABG	53	Persistent
19	male	76	AVR, MVR, TVR, abl	48	Paroxysmal
20	male	74	CABG, aorta, abl	51	Persistent
21	female	74	AVR, CABG, abl	62	Paroxysmal
22	male	73	Aorta	42	Persistent
23	female	68	AVR, MVR, TVR, abl	44	Paroxysmal
24	female	77	AVR, MVR, abl	57	Paroxysmal
25	male	49	AVR, CABG, aorta	56	Persistent
26	male	62	AVR, CABG	59	Persistent
27	female	76	AVR, MVR, abl	55	Paroxysmal

AVR = aortic valve replacement or repair, MVR = mitral valve replacement or repair, TKR = tricuspid valve replacement or repair, CABG = coronary artery bypass graft, abl = left atrial ablation, aorta = surgery of the ascending aorta.

weight, a minimum of 1 and a maximum of 26 sections from basal to apical and of 2–3 μm were prepared and stained (Suppl. Fig. 1).

2.2. Immunohistochemistry

C-kit^{pos} cells were stained using a monoclonal primary antibody from rabbit (CD117/c-kit, YR145, Ref. 117R-15, Medac) and a biotinynlated secondary antibody from goat (Biotin-SP-conjugated AffiniPure Goat Anti-Rabbit IgG (H + L), Jackson ImmunoResearch) with detection by streptavidin horse radish peroxidase conjugate (Vectastain Elite ABC-HRP Kit, Peroxidase (Standard), Biozol) and subsequent diaminobenzidin-tetrahydrochloridstaining (DAB Substrate Kit, Peroxidase (With Nickel), vector laboratories) (Suppl. Material 1)).

Number of c-kit^{pos} cells was counted manually for complete sections (median section number n = 7 [1,12]). Negative controls were obtained by omitting the primary antibody and positive controls were prepared using gastrointestinal stromal tumour. To identify fibrosis, Masson Trichrome stain was applied. Analysis of all slides was performed using the Zeiss Axio Scan Z1 microscope with ZEN 2.6 (blue edition) software. All images were fully digitalized and analysed with Image J (ImageJ 1.52d, W. Rasband, USA [13]). The amount of collagen-stained area was determined half-automatically andexpress as a percentage. The size of the section, number of c-kit^{pos} cells per slide, amount of fat and fibrosis was analysed for each slide of each LAA separately. In addition, we applied a mast cell stain to characterise and mark c-kit^{pos} cells in adjacent sections and a CD45 staining with a monoclonal primary antibody from mouse (CD45, Leucocyte Common Antigen (Concentrate) Clone 2B11 + PD7/26, Dako).

2.3. Statistics

We tested for normality with the Kolmogorov-Smirnov test. The statistical significance of differences between groups was evaluated using the students t-test for continuous variables in normally distributed data and with the Mann-Whitney-U test if data were not normally distributed. For correlation of two continuous variables, a Pearson correlation was applied in normally distributed data and the Fisher's exact test in not normally distributed data. $P < 0.05$ was considered statistically significant. All analyses were performed using SPSS version 13 (IBM Corp., Armonk, NY).

3. Results

A total of 27 LAAs were collected and studied. Baseline characteristics of the patients who underwent heart surgery are depicted in Table 1. The cohort consisted of 37.0 % females, mean left ventricular ejection fraction was 50.4 % and 17 patients had persistent AF (63.0 %). Most of the patients underwent combined CABG and valve surgery (51.9 %), whereas CABG alone (7.4 %) was rare. One

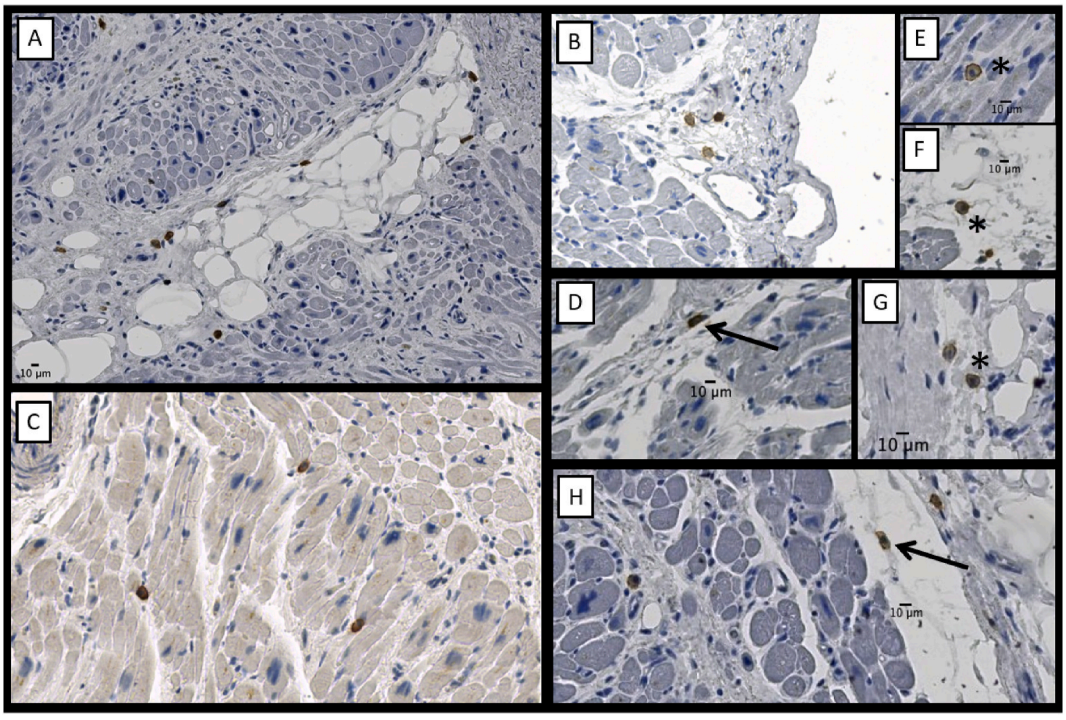

Fig. 1. Depicted are c-kit ^{pos}clusters close to epicardial fat (A), vascular structures (B) and between cardiomyocytes (C). In (D) and (H, right hand site) spindle shaped cells (arrow) and in E, F, and G (left hand site) rounded shaped cells (Asterisk) are depicted.

patient underwent aortic surgery without valve surgery or CABG. The tissue weight ranged from 0.18g to 8.57g and all recently classified shapes of LAA [14] were present (Suppl. Fig. 2).

C-kit^{pos} cells were detected in three different regions: A) Attached to the epicardial fat layer (Fig. 1, A), B) close to vascular structures (Fig. 1, B) and C) between cardiomyocytes (Fig. 1, C and Fig. 2, A-C) and formed groups of three to seven cells. We found 20.0 ± 0.9 % of cells close to epicardial fat, 2.0 ± 1.6 % to vascular structures and 58.4 ± 15.6 % between cardioymocytes. The remainig c-kit^{pos} cells (19.1 ± 16.5 %) could not be assigned to one of the aforementioned locations (e.g. fibrotic area, close to endocardial border or close to areas of fat other than the epicardial layer).

We identified two morphological distinct cell types that stained positive for c-kit (Fig. 1D–H): spindle-shaped cells and roundish cells. The rounded cell type was significantly more frequent than the spindel shaped cell type (70.7 ± 4.1 % versus 29.3 ± 4.1 %, respectively). Distribution of c-kit^{pos} cells showed no proximal - distal gradient. C-kit^{pos} cells were detected close to the orifice and very distally in the dead end. In addition, there was no relevant gradient from epicardium to sub epicardium and between cardiomyocytes (Fig. 2).

The mean LAA section size was 22.4 ± 16.6 mm^2. C-kit^{pos} cells were identified in LAAs of all patients with a wide inter-individual range of frequency (Fig. 3). The highest number of c-kit^{pos} cells per mm^{2}was detected in patient number 5 with a total of 67.5 c-kit^{pos} cells per mm^2. The lowest number was present in patient number 10 with only 0.05 c-kit^{pos} cells per mm^2. The mean number of c-kit^{pos} cells per mm^2 in all patients and sections was 5.9 ± 7.4 cells per mm^2. Both body donor LAAs showed significantly lower numbers of c-kit^{pos} cells per section compared to patients (0.3 vs 5.9 cells per mm^2, $p = 0.005$).

The amount of fibrosis was evaluated for each section in relation to total size and ranged from 0.2 % in LAA 22–36 % in LAA 13. Patients with persistent AF had a significantly higher amount of fibrosis than patients with paroxysmal AF (21.3 % versus 14.6 %, $p = 0.027$). In addition, amount of fat per slide was examined and ranged from 0.9 % in LAA 13–43 % in LAA 1. No differences in amount of fat were detected between the experimental groups.

There was no association between sex, left ventricular ejection fraction, type of AF, amount of fibrosis, amount of fat per section or LAA morphology and the number of c-kit^{pos} cells per mm^2.

Mast cell staining was applied to a subset of 10 sections that were also consecutively stained with c-kit in adjacent sections. We found 29–72 % of cells to be double positive (Fig. 4). We also used a subset of 10 sectionsthat were serial stained with CD45 to identify double positive c-kit^{pos} cells. However, frequency of c-kit^{pos} cells also positive for CD45 were scarce (Suppl. Material Fig. 2).

4. Discussion

C-kit^{pos} cells are frequent in LAA tissue of patients undergoing heart surgery with a rather homogenous distribution throughout the LAA. There are clusters close to epicardial fat, vascular structures and between cardiomyocytes.

In recent years it has been shown that c-kit^{pos} cells, although their differentiation to cardiomyocytes seems to be possible [3], mainly contribute to regeneration in ischemic heart disease models via paracrine signalling [4,15]. The detailed mechanism of the positive effect of c-kit^{pos}cells is still unclear and future studies to evaluate the interaction with other cell types in cardiac regeneration are under way [16].

C-kit is a receptor tyrosine kinase with the ligand stem cell factor and is expressed by a variety of human cardiac cells, cardiac interstitial cells, mesenchymal cells and endothelial progenitor cells [17]. In addition, c-kit is also expressed in mast cells [7]. Interestingly, in an early study examining the distribution of c-kit in human tissues, cardiovascular materials like heart, arteries, veins and capillaries were reported to not contain c-kit^{pos} cells [18]. This has been disproved by many histological studies showing varying

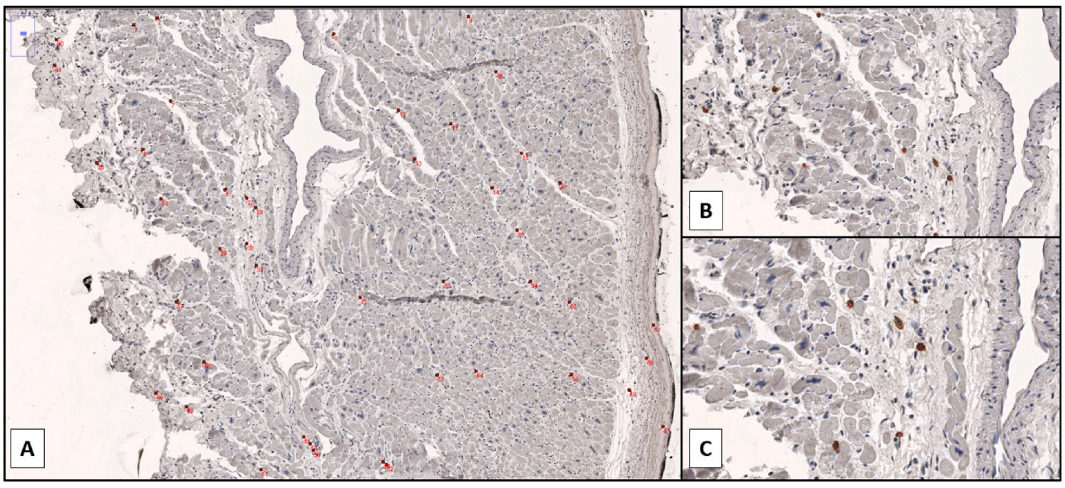

Fig. 2. Distribution of c-kit^{pos}cells was rather homogenous without a gradient from epi-to endocardial. Depicted is an exemplarily section with c-kit^{pos} cells marked in red. Left panel: overview, right panels: zoomed in.

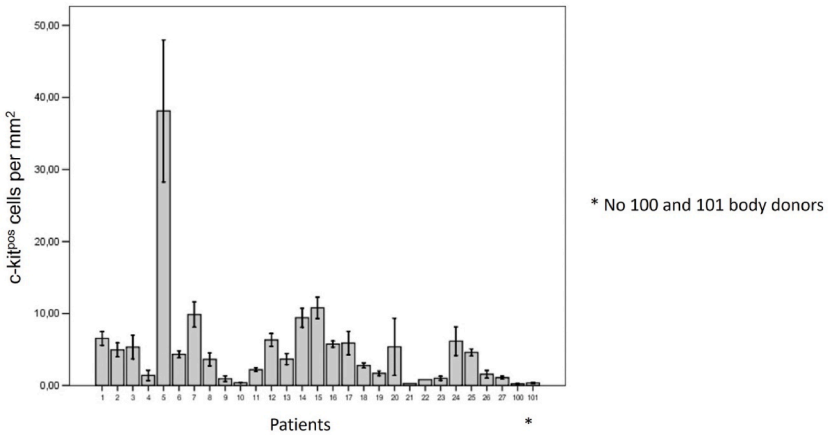

Fig. 3. Bar diagram of c-kit^{pos} cells per individual LAA. 100 and 101 are body donors.

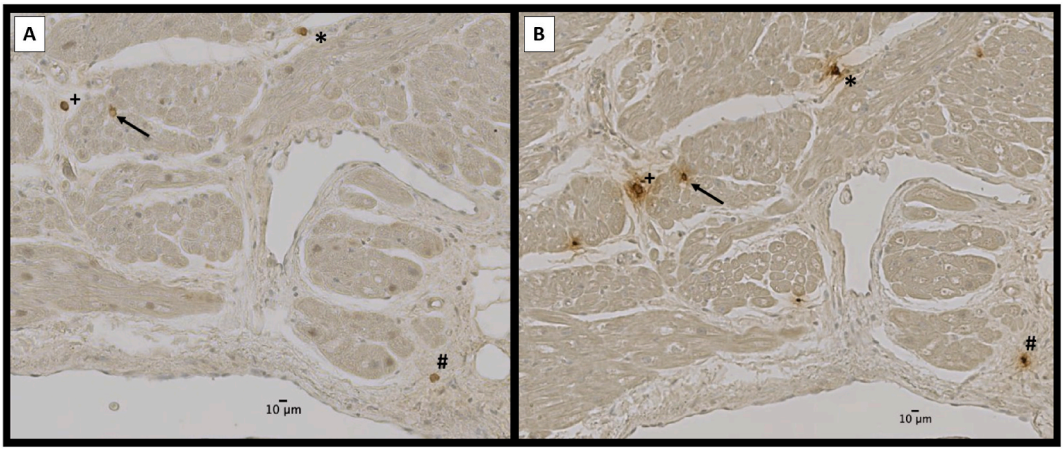

Fig. 4. Example of c-kit^{pos} cells with mast tryptase cell marker: Left panel (A): c-kit^{pos} cells, right panel (B): tryptase positive cells. Depicted are consecutive sections. The same cell is marked with the same symbol in both sections.

numbers of c-kit^{pos} cells in cardiac tissue (Table 2). Of note, the present study is the first report on distribution throughout the human LAA.

Left atrial appendages of adult mice contain a large number of c-kit^{pos} cells [10]. In addition, as the number of c-kit^{pos} cells was highest in the LAA compared to right atrial appendage and left ventricular myocardium [10] it was proposed, that the LAA may be a reservoir for progenitor cells. In human studies, cell counts for c-kit^{pos} cells varied from 0.5 to 35 cells/mm^2, representing 0.1–6.0 % of all cardiac cells, whereas no specific analysis of the LAA was performed (Table 2) [5–9,19–21]. We found a frequency of 5.8 $\pm$ 7.4 cells/mm^2 with a high inter-individual variability, but in line with previous studies. Numbers of c-kit^{pos} cells were not associated with age or sex, but whether inflammation or acute decompensation relates to c-kit^{pos} cell frequency should be analysed in further studies.

Although, c-kit^{pos} cells are frequently used in the scientific literature as synonymous for cardiac progenitor cells (CPCs), c-kit is not a sufficient marker to identify CPCs alone [1,22]. Nevertheless, c-kit is used as surface marker in a large number of studies on cardiac regeneration [23–25]. Therefore, we assume that our data indicate sufficient numbers of c-kit^{pos} cells to perform autologous CPC isolation for further characterization of these cells ex vivo [16,17,24].

Earlier reports showed that patients with ischemic end-stage heart failure have higher numbers of cardiac c-kit^{pos} cells than patients who died for non-cardiovascular reasons, what is in line with our results [6].

Contrary to our results, Castaldo and colleagues and Di Meglio found c-kit^{pos} cells with a significantly higher count in the epicardium and subepicardium compared to the myocardial layer in the left atrium and left ventricle [6,8]. However, the LAA has a relatively thin myocardial layer in relation to epicardial fat and the endocardial layer, so that distribution may appear different due to the lower diameter of atrial myocardium. Conversely, as epicardial and subepicardial borders are long due to the concertina-like structure of the LAA, the high number of c-kit^{pos} cells may be due to the specific tissue structure. Of note, there are reports by Pouly and colleagues demonstrating lower numbers of c-kit^{pos} cells in the RAA of patients undergoing CABG than in RV biopsies of patients who underwent heart transplantation (presumably healthy hearts) [5]. As they analysed presence of all c-kit^{pos} cells and in a

Table 2
Short review of all past studies that evaluated numbers of c-kit^{pos} cells in cardiac tissue.

Tissue	Patient population	c-kit^{pos} cell number	Comment	Reference
LA, RV, LV, AV junction, Apex	donorhearts and end-stage ischemic heart failure	LA donor heart 4 cells/mm^2 LA diseased heart 35 cells/mm^2	Only hematopoietic-lineage negative cells	[6]
RV biopsies and RAA	RV biopsies from heart transplant recipients (“normal hearts") RAAs from CABG operations	RV biopsies 2.7 cells/mm^2 RAA 1 cell/mm^2	Co-staining revealed all c-kit^{pos} cells as CD 45 positive	[5]
Apex, anterior and lateral LV wall	Autopsies without prior known cardiac disease	Apex 4.9 cells/mm2 Anterior LV wall 4.2 cells/mm^2 Lateral LV wall 3.9 cells/mm^2	Majority of cells are mast cells and weakly CD45 positive	[7]
Atrial appendage/left atrium, LV	Autopsies without prior known cardiac disease and ischemic heart disease	LA 4.09 cells/mm^2(no cardiac disease hearts) LV 2.48 cells/mm^2(no cardiac disease hearts) LA 35.73 cells/mm^2(ischemic hearts) LV 32.43 cells/mm^2 (ischemic hearts)	c-kit^{pos} Cells express epithelial and mesenchymal markers	[8]
RV and LV biopsies	Cardiac healthy subjects Myocarditis DCM ICM	Cardiac healthy subjects 1 cell/mm^2 Myocarditis 6.75 cells/mm^2 DCM 1.67 cells/mm^2 ICM 4 cells/mm^2	Only cells with CD 90 co-expression	[19]
Location not provided and biopsies from RV	Univentricular heart and DCM Heart transplanted children (“normal hearts")	0.15 % in heart transplanted patients 0.84 % in univentricular heart 0.22 % in DCM	Additional staining with mast cell tryptase and CD 45	[20]
LV apex	LVAD recipients with ischemic heart disease	3 to 8 cells per 50 fields examined	Co-staining with mast cell tryptase, only tryptase negative cells	[21]

LA = left atrium, LV = left ventricle, RV = right ventricle, RAA = right atrial appendage, AV junction = atrioventricular junction, CABG = coronary artery bypass grafting, CD = cluster of differentiation, DCM = dilated cardiomyopathy, ICM = ischemic cardiomyopathy, LVAD = left ventricular assist device.

second step characterized cells as lineage positive or negative, numbers of c-kit^{pos} cells should be comparable to our study. Whether the higher numbers of c-kit^{pos} cells in our studies is due to the tissue type (LAA versus RAA and RV) can only be speculated.

It has been reported that 90–100 % of all c-kit^{pos} cells are positive for tryptase markers and for CD45 as well [7]. This has consistently been confirmed by other studies, although to a varying amount of reported co-expression [20,22]. In our study, we found up to 72 % of c-kit^{pos} cells to be also positive for tryptase.

Mast cells are very frequent in cardiac tissue and have been described as enhancers, inhibitors and bystanders in cardiac fibrosis [26]. A substantial number of cardiac c-kit^{pos} cells could be assigned to mast cells with weak to moderate CD45 positivity [7], consistent with our findings that up to 72 % of ckit^{pos} cells also had positive mast cell staining. Therefore, it is currently debated whether most of the isolated ckit^{pos} cells in the myocardium are mast cells [26] and whether or not the cardiogenic potential is -limited only to the ckit^{pos} cells without mast cell markers [7,26].

The frequency of c-kit^{pos} cells also positive for CD45 was low, but the analysis was limited to serial sections.

There is still ongoing debate surrounding the involvement of hematopoietic stem cells in cardiac regeneration. The existing studies have been subject to critical discussion and have been described as “inconclusive and statistically underpowered” [12]. However, more recently there have been promising results that underline the potential role of peripheral blood - derived hematopoietic precursor cells in recovery of old myocardial infarction tissue [27]. In addition, the PERFECT trial (Intramyocardial Transplantation of Bone Marrow Stem Cells in Addition to Coronary Artery Bypass Graft Surgery) used intramyocardial transplantation of c-KIT/CD117+/CD133+, /CD34+ bone marrow derived hematopoietic stem cells in post-myocardial infarction coronary artery bypass graft patients and showed very promising results, although mainly explained by enhanced angiogenesis [28,29]. The exact mechanisms of hematopoietic bone marrow cells in cardiac regeneration thus still need to be exactly determined. Importantly, LAA represents the largest myocardial structure that can be removed without a high risk for complications during heart surgery. Left ventricular material, which is removed during implantation of left ventricular assist devices or right atrial appendage (RAA) material that is removed during intraoperative cannulation are other sources. However, the easy accessible amount of RAA tissues is significantly smaller than a completely excised LAA [8] which thus represents the most relevant cardiac structure for isolation of c-kit^{pos} cells. On the other hand, material released during heart transplantation is rare and if heart transplantation is already performed, use of autologous stem cells for further therapies is less relevant.

4.1. Limitations

We did perform additional stainings for CD45, but due to different retrieval requirement were not able to use double staining techniques. In addition, co-expression for mast cell tryptase was examined in only a subset of sections and we only used adjacent sections, no staining-restaining techniques. In addition, we had no information about presence of clinical or laboratory signs for myocarditis or acute decompensation prior to operation, both factors that may influence the numbers of c-kit^{pos} cells.

5. Conclusion

C-kit^{pos} cells are frequent in LAAs of patients undergoing heart surgery with a rather homogenous distribution. Due to the relatively easy access during heart surgery and the substantial amount of extractable heart tissue, the LAA may be qualified as a source of choice for extraction of autologous cardiac progenitor cells in the living human undergoing heart surgery.

Data availability statement

Data associated with this study has not been deposited into a publicly available repository but will be made available on request.

CRediT authorship contribution statement

Lea Schwarzkopf: Data curation, Formal analysis, Validation. **Petra Büttner:** Conceptualization, Formal analysis, Methodology, Project administration. **Karl Scholtyssek:** Data curation, Formal analysis. **Thomas Schröter:** Resources, Validation. **Ruth Hiller:** Methodology, Validation. **Gerhard Hindricks:** Project administration, Resources, Supervision, Writing – review & editing. **Andreas Bollmann:** Project administration, Resources, Supervision, Writing – review & editing. **Ulrich Laufs:** Project administration, Resources, Supervision, Writing – review & editing. **Laura Ueberham:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Funding statement

This study was supported through a grant provided by ProCordis – Verein zur Förderung der Forschung in der Herzchirurgie e. V. and the ESC First Contact Initiative Grant.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Gerhard Hindricks has received grants through the Leipzig Heart Institute from Boston Scientific (Boston Scientific Corporation, Marlborough, Massachusetts, USA), and Abbott/St. Jude Medical (Abbott Laboratories, Chicago, Illinois, USA), no personal payments are to declare. All other authors state that there is nothing to declare.

Acknowledgements

We thank Tina Fischer-Scheapmann, Sarah Werner, Angela Kricke and Martina Fügenschuh for their excellent technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21268.

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