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Original article

Reduction of c-kit positive cardiac stem cells in patients with atrial fibrillation

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ABSTRACT

Background: We aimed to determine expression patterns of cardiac stem cells in the left atrium (LA) tissue from patients with atrial fibrillation.
Methods: LA appendages were obtained during open-heart surgery and processed for explant cell culture and tissue analysis ($n = 319$). The total number of grown cells and c-kit positive cells were analyzed by flow cytometry after 4 weeks of culture. The remaining tissue was used for Masson's trichrome staining to determine the area of the fibrosis.
Results: The diameter of the LA, as measured by echocardiography, was significantly larger in the AF group than in the sinus rhythm group. Reverse transcription polymerase chain reaction analysis revealed higher expression of collagen in the AF group and an increase in the expression of basic fibrosis growth factor and transforming growth factor-2 and -3. Masson's trichrome staining showed progression of fibrosis in the AF tissue. In addition, the expression of apoptosis-related genes were significantly higher in AF group. There was no difference in the expression of connexin-40 between groups, while the expression of connexin-43 was decreased and that of connexin-45 was increased in the AF group. The total numbers of grown cells as well as c-kit positive cells after 4 weeks of cardiac tissue culture were significantly lower in the AF group.
Conclusion: Progression of remodeling in LA tissue was observed in AF patients. The number of c-kit positive cells cultured from LA appendages was reduced in AF patients, suggesting impairments in self-renewal.

Introduction

Atrial fibrillation (AF) is the most common arrhythmia that occurs due to multiple factors such as aging, gender, obesity, hypertension, or heart disease [1–5]. Prolonged AF causes fibrotic changes or dilatation of the left atrium (LA) [6,7] known as remodeling. Causation of AF is associated with mechanical stretching of the LA [8], changes in ion channel expression [9], or autonomic nerve activity [10]. In addition, connexin family, main components of gap junction proteins, play a central role in the electrical remodeling associated with AF, and an alteration in the expression or distribution of connexin(s) has been observed in the heart of patients with AF [11–13]. These structural or electrical remodeling processes enhance intrinsic consequences of arrhythmia, resulting in persistent AF. Many studies suggest that inflammation is responsible for the development of remodeling. In addition, a recent study demonstrated that local expression of microRNA may contribute to atrial remodeling in patients with chronic AF [14]; however, the underlying mechanism is still unclear.

The heart is no longer considered to be a terminally differentiated organ and it is now known that heart cells are renewed at a certain rate annually [15,16]. Resident cardiac stem cells (CSCs) have been identified [17] and these are considered to be involved in cardiac regeneration or maintenance [18,19]. c-kit positivity is a general marker for CSC identification, and this protein plays a central role in self-renewal and the ability to differentiate into cardiac cells [20]. The expression of c-kit

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positive cells is associated with age, gender, and serum level of B-type natriuretic peptide (BNP) [21,22]. Importantly, c-kit expression is increased when the cardiac tissue is damaged, such as in the case of myocardial infarction or end-stage heart failure in animal or human hearts [22–24]. Therefore, if LA tissue in patients with AF is damaged, expression of c-kit positive cells may be affected. To test this, we investigated whether there were any changes in functional activity of c-kit positive cells in patients with AF, using human atrial tissue obtained during open-heart surgery.

Methods

Patients

Signed written consent was obtained from patients according to a protocol approved by the Internal Review Committee on Ethics of Human Investigation of the Juntendo University Hospital prior to the collection of tissue samples. Patients who underwent open-heart surgery between September 2011 and February 2014 were enrolled for this study. The patients were divided into two groups: persistent atrial fibrillation (AF) and sinus rhythm (SR). Patients diagnosed with paroxysmal AF, patients with pacemaker implants, patients on hemodialysis due to chronic renal failure, patients with history of previous open-heart surgery, and patients who had received chest irradiation for cancer therapy were excluded from this study.

Tissue collection and culture of cardiac outgrowth cells

The LA appendage was obtained during cardiac surgery. The samples were immersed in cardioplegic solution (Miotecter®, Mochida Pharma, Tokyo, Japan) immediately after tissue dissection and stored until further use. The sample was cut into small pieces and trimmed to a final weight of 20–30 mg for primary cell culture. Any remaining piece of sample was immersed in RNAlater solution (Life Technologies, Tokyo, Japan) for RNA isolation or 4% paraformaldehyde for histochemical analysis. The sample for culture was minced into small pieces (80–100 pieces) and digested for 5 min with 0.05% trypsin-EDTA (Sigma–Aldrich, Tokyo, Japan). After washing with phosphate-buffered saline (PBS, Wako, Tokyo, Japan) the explants were placed onto two fibronectin-coated dishes (BD Biosciences, Tokyo, Japan) in Iscove's modified Eagle's medium (Life Technologies) containing 10% fetal bovine serum (Thermo Scientific, Yokohama, Japan), 1% penicillin-streptomycin (Life Technologies), and 2 mM L-glutamine (Life Technologies). The media were changed every 3–4 days. A primary outgrowth of cells growing out radially in a monolayer from the cardiac tissue was seen after 4 weeks of culture. These cells were harvested with 0.05% trypsin-EDTA and counted. A portion of the cells was used for analysis by flow cytometry and the remaining cells were stored at −80 °C.

Flow cytometry

A portion of the harvested outgrowing cells was fixed in 4% paraformaldehyde in PBS (Sigma) for 15 min. The cells were then washed twice with PBS, followed by blocking with 1% bovine serum albumin (Iwai Chemicals Company, Tokyo, Japan) for 30 min. Cells were incubated with phycoerythrin-conjugated anti-human c-kit antibody (BD Bioscience, Franklin Lakes, NJ, USA) for 2 h at 4 °C. Cells were then washed with 0.1% bovine serum albumin twice followed by one PBS wash. The cells were counted using a FACSCalibur flow cytometer and CellQuest software (BD Bioscience).

Reverse transcription polymerase chain reaction

mRNA was extracted from the heart tissue by using an RNA extraction kit (Qiagen, Tokyo Japan). Extracted RNA was then reverse transcribed into cDNA (iScript cDNA Synthesis Kit, Bio-Rad, Tokyo, Japan). Polymerase chain reaction and subsequent analysis were carried out using a SYBR-green kit (Life Technologies) and the HT-7700 thermal cycler system (Life Technologies). Primers were designed to amplify and quantify GAPDH and c-kit. The relative abundance of the target gene was obtained by calculating gene-specific mRNA expression normalized to the internal control GAPDH.

Masson's trichrome staining

Masson's trichrome staining was performed to quantify the area of fibrotic change. After fixation in 4% paraformaldehyde, the tissue was dehydrated by immersing in 10%, 15%, and 20% sucrose solutions in succession prior to embedding in optical coherence tomography compound (Sakura Finetek, Tokyo, Japan), and then stored at −80 °C. Subsequently, each sample was cut into 5-μm sections using a cryostat. Sections were mounted on a glass slide and the slides were then left immersed into Bouin solution (Muto Pure Chemicals, Tokyo, Japan) overnight at room temperature. The slides were processed the next day using a staining kit (Sigma–Aldrich) according to the manufacturer's instructions. Quantification analysis of the fibrotic area was performed using ImageJ software developed by NIH.

Statistical analyses

The Kolmogorov–Smirnov test was used to assess the normality of data distribution. Area analysis of Masson's staining was evaluated by the Welch's *t*-test using Statcel 3 (O.M.S. publisher, Saitama, Japan). The difference in the mRNA expression between groups was analyzed by the Mann–Whitney *U*-test using SPSS 22.0 (IBM-Japan, Tokyo, Japan). All data are represented as the mean ± standard deviation (SD). Statistical significance was set at a level of $p < 0.05$.

Results

Patient background

A total of 319 cardiac specimens obtained from patients, including those with chronic AF ($n = 104$), were analyzed. The details of the clinical background of the patients are shown in Table 1. The AF group had older patients ($p < 0.01$), more female patients ($p < 0.01$), lower triglyceride values ($p < 0.05$), higher serum creatinine values ($p < 0.05$), higher values of serum BNP ($p < 0.01$), and higher preoperative New York Health Association (NYHA) classification ($p < 0.01$). Notably, overall there were more male patients in both the SR (71.0%) and the AF groups (53.3%). In addition, the SR group included a significantly higher number of patients who had undergone coronary artery bypass surgery (CABG) due to ischemic heart disease ($p < 0.01$), while the AF group showed a higher rate of mitral valve surgery ($p < 0.01$). Further, 89.5% of AF patients had a history of maze procedure.

Structural and electrical remodeling of the left atrium

The preoperative echocardiogram data are shown in Table 2. Patients with AF had significantly larger left atrial diameter (LAD). In addition, there was no significant difference in the left ventricular ejection fraction between groups (SR vs. AF: 59.0 ± 15.3% vs. 59.3 ± 12.1%, $p = 0.99$), or in the left ventricular

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