Original Article
Downregulated protein expression of transcriptional activator ELK-1 in atrial myocardium of chronic AF patients

Xiangjun Zeng, Xuejun Xiao, Yueheng Wu, Huanlei Huang

Department of Cardiovascular Surgery, Guangdong Provincial Cardiovascular Institute, Guangdong General Hospital/Guangdong Academy of Medical Sciences, 106 Zhong Shan Er Lu, Guangzhou 510080, Guangdong Province, China

Received June 23, 2015; Accepted July 27, 2015; Epub September 1, 2015; Published September 15, 2015

Abstract: Background: The structural alterations in atrial myocytes appear to be an adaptive response of dedifferentiation during chronic atrial fibrillation (AF). Transcriptional activator ELK-1, one of the members of ETS family, has been shown to play an important role in regulating cell differentiation. It is reasonable to presume that ELK-1 participate in the molecular and structural remodeling by which AF is sustained. To prove this hypothesis, the expression of ELK-1 protein in chronically fibrillating atria compared to that in normal rhythmic atria was detected.

Methods: Right atrial myocardium were obtained from twenty-four patients undergoing valve replacement surgery, twelve patients were in chronic AF (>6 months), whereas the others were in sinus rhythm (SR). The protein expression level of ELK-1 was quantified by Western blot analysis, and the cellular localization and expression pattern of ELK-1 was examined by immunohistochemical staining and indirect immunofluorescence. Results: Western blot analysis showed that the protein expression of ELK-1 was significantly reduced in the atrial tissue of chronic AF patients compared to that in the controls. Immunohistochemistry showed that ELK-1 immunostaining occurred in both cytosolic and nuclear compartments of atrial myocardium. Indirect immunofluorescence showed that the nuclei of normal rhythmic atrial cells were densely labeled, whereas the nuclei in chronically fibrillating atrial cells were very faintly labeled. Conclusions: Our results suggest that the downregulated expression of transcriptional activator ELK-1 may play an important role in the pathogenesis of AF.

Keywords: Atrial fibrillation, dedifferentiation, transcriptional activator, ELK-1

Introduction

Atrial fibrillation (AF) has the tendency to become more persistent over time [1]. Experimental and clinical studies showed that electrical and structural remodeling involved in the progression of AF [2]. However, the mechanisms of the electrical change and structural remodeling in atria during chronic AF are not well understood.

Some convincing evidence supports that chronic AF induced myocardial dedifferentiation [3, 4]. The dedifferentiation of cardiomyocyte renders itself to adapt stress conditions including higher electrical activity and stretch in atrial tissue [5]. Transcriptional activator ELK-1, one of the members of ternary complex factor (TCF) family, has been shown to play an important role in regulating cell differentiation and proliferation [6]. These clues lead to hypothesize that ELK-1 may play a role in the molecular remodeling of atrial myocytes by which AF is sustained.

In present study, the expression and distribution of ELK-1 protein in chronically fibrillating atria were compared to that in normal rhythmic atria. Our results show that the protein expression of ELK-1 is significantly reduced in the atrial tissue of chronic AF patients, implying a possible correlation between the downregulated expression of ELK-1 and the pathogenesis of chronic AF.

Materials and methods

Patients and atrial tissue collecting

Patients suffering from mitral valve disease either had sinus rhythm or chronic AF for duration of at least 6 months. The patients with...
latent or manifest hyperthyroidism, hypertension, diabetes, hyperlipidemia, and bacterial endocarditis in their history were excluded. During heart surgery, right atrial free wall tissue was obtained from 24 patients (12 in SR, 12 in chronic AF). Immediately after excision, the specimens were snap-frozen and stored in liquid nitrogen. The clinical characteristics are shown in Table 1. Hemodynamic parameters did not differ significantly between SR and AF patients but the left atria were slightly enlarged and the LVEF was slightly depressed in AF patients. The study was approved by the local ethics committee and all patients gave written informed consent.

**Cell culture**

HeLa cells were cultured with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine (Sigma, USA), 100 IU/ml penicillin G, and 100 µg/ml streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C. The medium was renewed every 2-3 days. At approximately 80% confluence, the cells were harvested.

**Protein preparation**

Frozen atrial specimens of all patients and the harvested cells were lysed in RIPA buffer containing 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mmol/L EDTA, 0.1% SDS and protease inhibitor cocktail. The extracts were centrifuged at 20000× g for 30 min at 4°C. The supernatants were used for protein concentration measurement and stored at -70°C before use. The total protein concentrations of the extracts were determined by the Bradford method.

**Polyacrylamine gel electrophoresis and immunoblotting**

Western blot analysis was carried out following standard procedure. Briefly, equal amounts of proteins were mixed with 6× SDS reducing sample buffer and boiled for 5 min before loading. Proteins were separated by 10% SDS-PAGE and electrotransferred to polyvinylidene difluoride (PVDF) membrane. Non-specific binding sites were blocked in TBST containing 5% non-fat milk. The membrane was incubated overnight at 4°C with primary antibodies and washed three times with TBST, then incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. The antigen-antibody complexes on the blot are reacted with a chromogenic substrate (either 3,3-diaminobenzidine [7]). The density of bands was scanned and quantified by LEICA550IW image analysis. Changes in protein expression of ELK-1 (Cell Signaling, USA) were studied in relation to protein levels of GAPDH (Chemicon, USA).

**Immunohistochemistry**

Frozen specimens were sectioned at 3 µm in a cryostat at -18°C and mounted on glass slides. The sections were fixed with cooled acetone for 20 min, incubated in 3% H₂O₂ for 15 min, then permeabilized with 1% Triton X-100/PBS for 30 min respectively. Normal goat serum at 5% in PBS for 30 min to prevent non-specific binding of the first antibody. Thereafter, the primary antibodies were applied at a dilution of 1:300 in PBS at 4°C overnight. Afterwards, the sections were washed three times in PBS and then incubated with rabbit anti-biotinylated immunoglobulin at 1:200 dilutions. After 1 h of incubation with the secondary antibody, the sections were incubated with a standard streptavidin-biotin-complex. The chromogen DAB was used to localize the peroxidase in tissues. Positive immunostaining was demonstrated with a red dish brown precipitate in the sections. The slides were counterstained with Mayer's hematoxylin. Control of the immunostaining included omission of the primary antibody, no signal was obtained following incubation with only the secondary antibody.
ELK-1 in atrial myocardium of chronic AF patients

Indirect immunofluorescence

Fixation, permeabilization and blocking were performed as described above. Sections were stained with antibodies against ELK-1 at a dilution of 1:200 in PBS at 4°C overnight, and detected with a TRITC-conjugated anti-rabbit IgG secondary antibody (Sigma, USA). Nuclei were hematoxylin counterstained. Indirect immunofluorescence examination was performed on a Leica microscope with excitation and emission wavelength of 568 and 585 nm, respectively. In the same situation, the location of nucleus and the silhouette of myocytes were observed in visible light.

Data analysis

All values are expressed as means ± SD. Comparisons were performed using One-way ANOVA, and P<0.05 was considered to indicate a significant difference.

Results

Downregulated protein expression of transcriptional activator ELK-1 in atrial myocardium of chronic AF patients

Western blot analysis was performed in 24 mitral valve replacement patients (12 in AF, 12 in SR). The supernatants aliquots contain 20 µg total protein per lane. The level of protein expression is estimated by the protein of interest/GAPDH ratio. Two protein bands were recognized by polyclonal antibody against ELK-1. One is at 62 KD, the other is a wide band, migrating at approximately 50 KD (Figure 1A). Hela cells were selected as positive control, in which ELK-1 was high-expressed [8], the 62 KD protein band was testified as an ELK-1 specific band (Figure 1B). The protein expression of ELK-1 at 62 KD band was significantly reduced in patients with chronic AF (0.87±0.38 vs 1.69±0.65, P<0.01) (Figure 1C).

Immunohistochemistry and indirect immunofluorescence

Immunohistochemical studies were performed to investigate the distribution of ELK-1 in atrial tissue. In light microscopy, the brown signals indicate ELK-1 or desmin, and the nuclei appear blue with hematoxylin counterstain (Figures 2, 3). The immunostaining of the muscle-specific desmin indicates the atrial myocardium (Figure 3). Darker signals were observed when brown and blue signals overlap in the nuclei (Figure 2). There was less expression of ELK-1 in the cytoplasm and nuclear of atrial myocardiums in chronic AF patients than in SR control group (Figure 2). Indirect immunofluorescence was...
ELK-1 in atrial myocardium of chronic AF patients

performed to investigate the expression of ELK-1 in the nuclei of atrial myocardium. Immunofluorescence staining with anti-ELK-1 antibody showed that the nuclei of normal rhythmic atrial myocardium were densely labeled, whereas nuclei in chronically fibrillating atrial myocardium were very faintly labeled (Figure 4).

Discussion

In the present study, we demonstrated the protein down-expression of transcription activator ELK-1 in atrial myocardium of chronic AF patients. We observed that: 1) Western blot analysis showed that the protein expression of ELK-1 was significantly reduced in the atrial myocardium of chronic AF patients compared to that in the controls. 2) Immunohistochemistry showed that ELK-1 immunostaining occurred in both cytosolic and nuclear compartments of atrial myocardium. 3) Indirect immunofluorescence against ELK-1 showed that the nuclei of normal rhythmic atrial myocardium were very densely labeled, whereas nuclei in chronically fibrillating atrial myocardium were very faintly labeled.

Severe tissue and cellular remodeling are observed in the atrial myocardium of patients with chronic AF. All the observed structural changes as well as the alterations in expression and organization of the AF remodeling-relate proteins were indicative of fetal-like phenotype of cardiomyocytes, namely dedifferentiation. For example, α-SMA and β-MHC, which belong to the fetal gene program expressed in embryonal/fetal atrial myocytes, are re-expressed during AF [3, 9].

As mentioned earlier, the ELK-1 gene belongs to the ETS family of TCF. ELK-1 functions as a nuclear transcriptional activator via its association with serum response factor (SRF) and serum response element (SRE). Recent evidence suggests that ELK-1 is a target of all three classes of MAP kinases, ERK, JNK, and p38 MAPK. Thus ELK-1 represents a key link between signal transduction and induction of gene transcription [10].

To the best of our knowledge, no data yet indicate the role of ELK-1 in atrial dedifferentiation during chronic AF. Noteworthily, over expression of ELK-1 in Smooth muscle cells down-regulates transcription of α-SMA. Knocking-down endogenous ELK-1 levels demonstrate that
ELK-1 is playing an important role in regulating muscle-specific gene expression in the partially dedifferentiated smooth muscle cells [11]. By using mutant ELK-1 alleles, Vickers confirmed a role for TCF-SRF-regulated gene activity in controlling cell proliferation and survival [12]; Wang et al. has proved that ELK-1 was involved in smooth muscle differentiation and proliferation [13], Khurana et al. has also revealed that overexpression of inactive ELK-1 enhanced skeletal muscle differentiation [14]. Based on the important role of ELK-1 in regulating the transcription of genes responsible for differentiation and development, our data suggests a possible correlation between the downregulated expression of ELK-1 and the pathogenesis of chronic AF.

Bukowska and his group have determined that the other transcription factors NFAT3 and NFAT4 in atrial myocytes were both markedly increased during chronic AF. The activity and protein level of calcineurin which activates NFAT3 were significantly upregulated in patients with chronic AF [15]. Since calcineurin has been identified as the major phosphatase targeting ELK-1, ELK-1 and Calcineurin might synergically regulate the expression of genes responsible for the molecular remodeling of atrial myocytes during chronic AF.

In conclusion, our results suggest that the downregulated expression of transcriptional activator ELK-1 might play an important role in controlling the molecular remodeling of atrial myocytes during chronic AF. In the present study, although we showed that transcription activator ELK-1 is downregulated in atrial myocardium of chronic AF patients, a direct link between the molecular remodeling of fibrillating atrial myocytes and the down-expressed ELK-1 is lacking. In the future, we need to answer how chronic AF downregulates the expression of ELK-1 in atrial myocytes and whether down-expressed ELK-1 regulates the remodeling-relative genes such as α/β-MHC, ANF, α-SMA, desmin, NCX1, and SERCA2 etc.

Disclosure of conflict of interest

None.
ELK-1 in atrial myocardium of chronic AF patients

Address correspondence to: Dr. Xuejun Xiao, Department of Cardiovascular Surgery, Guangdong Provincial Cardiovascular Institute, Guangdong General Hospital/Guangdong Academy of Medical Sciences, 106 Zhong Shan Er Lu, Guangzhou, Guangdong Province, China. Tel: +86 20 83827812; Fax: +86 20 83827812; E-mail: xuejunxiaoxj@126.com

References


