Immunolocalization of annexins IV, V and VI in the failing and non-failing human heart

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Abstract

The failing human heart is characterized by changes in the expression and function of proteins involved in intracellular Ca\textsuperscript{2+} cycling, resulting in altered Ca\textsuperscript{2+} transients and impaired contractile properties of cardiac muscle. The role of the cardiac annexins in this process remains unclear. Annexins may play a role in the regulation of Ca\textsuperscript{2+} pumps and exchangers on the sarcolemma, and have been shown to be altered in some cardiac disease states. Objective: The goal of this study was to compare the immunolocalization and expression of annexins IV, V and VI in failing and non-failing human hearts. Methods: We used immunostaining to identify the subcellular location of annexins IV, V and VI proteins within the myocardial cell, and Western blot analysis to quantify the proteins in the same hearts. Results: Annexin IV showed a cytoplasmic distribution in both failing and non-failing human heart cells. Annexin V was localized at the z-line, around lipofuscin granules, and in the cytosol in the non-failing heart cells. Annexin VI was localized at the sarcolemma and intercalated disc. Protein levels of annexins IV and V were up-regulated in failing human hearts, while the expression of annexin VI was unchanged. Conclusions: Alterations in the intracellular localization of annexins, along with up-regulation of annexins IV and V in the failing human heart cells, suggests differential regulation of these Ca\textsuperscript{2+} regulatory proteins during heart failure. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Ca-pump; Calcium (cellular); Cardiomyopathy; Heart failure; Sarcolemma

1. Introduction

In an attempt to elucidate the intracellular mechanisms associated with contractile dysfunction in congestive heart failure, a number of laboratories have investigated the processes of excitation–contraction coupling in failing and non-failing human hearts. Studies have demonstrated altered levels of intracellular Ca\textsuperscript{2+} [1] impaired sarcoplasmic reticulum function [2,3], altered transcripts for Ca\textsuperscript{2+} regulatory proteins [4–6], a more controversial change in the corresponding proteins [7], altered protein phosphorylation [8,9] and changes in ion channels [10,11] in the failing human heart.

In spite of the number of studies which have been devoted to Ca\textsuperscript{2+} cycling in heart failure, only recently has attention focused on the annexins, a novel family of Ca\textsuperscript{2+}-binding proteins which are abundant in the heart and may play an important role in cardiac excitation–contraction coupling. Ca\textsuperscript{2+}-binding proteins, such as calmodulin, troponin C and the annexins, are intermediates for the Ca\textsuperscript{2+} signal, regulating the function of other proteins involved in excitation–contraction coupling.

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Annexins are a family of structurally related proteins with 13 members (annexins I–XIII) identified to date [12,13]. Within this family, annexins IV, V and VI have been implicated in the regulation of channels, exchangers, and intracellular signaling processes in various cell types [14–19]. Annexin IV has been found to inhibit the \(Ca^{2+}\) activated chloride conductance in epithelial cells [14] and is itself a substrate for phosphorylation by protein kinase C [14]. Annexin V has been demonstrated to form voltage-gated cation-selective channels in phospholipid bilayers [15], to mediate \(Ca^{2+}\) movement into phospholipid vesicles [16], to be involved in ANF secretion [20] and to be a high affinity inhibitor of protein kinase C [21,22]. Annexin VI has been shown to regulate the skeletal muscle SR \(Ca^{2+}\) release channel [17,23], to increase conductance through the L-type \(Ca^{2+}\) channel [24] and to modulate activity of the cardiac \(Na^{+}/Ca^{2+}\) exchanger [18]. In the heart, studies have suggested the presence of these three annexins, as well as annexin II and possibly annexins III and VIII [25–29].

The importance of annexins in the cardiovascular system has not been established. All members of the family are anti-coagulants [12]. Preliminary studies have suggested a role for the annexins in ischemia–reperfusion injury, where they may regulate phospholipid homeostasis [27] or mediate increased \(Ca^{2+}\) influx [30]. Annexins III, IV and V have been shown to increase in the plasma of patients following myocardial infarction, presumably being released from injured heart cells [31] or possibly from vascular endothelial cells [12]. Gunteski-Hamblin and colleagues have recently shown that overexpression of annexin VI in the heart results in decreased intracellular \(Ca^{2+}\) cycling and impaired contractility [19], suggesting a role for at least one of the cardiac annexins in excitation–contraction coupling.

In pathophysiological states, the relative amount of annexins found in the heart may be altered. Work by Song and co-workers, examining annexin levels in the explanted hearts of cardiac transplant recipients, has demonstrated a significant increase in the expression of annexin V and a decrease in the expression of annexin VI [29]. These data are provocative and suggest that the annexins, like other \(Ca^{2+}\) regulatory proteins, may be altered in human heart failure.

In the current study, we have sought to extend the observations of Song and colleagues to the intracellular localization of the annexins. We have examined the protein levels of annexins IV, V and VI in failing and non-failing human hearts, and have used immunolocalization to compare their subcellular distribution. Results presented here confirm their findings that the annexins are differentially regulated in human heart failure, and further demonstrate that this regulation involves not only the steady-state amounts of the proteins, but also their localization.

Table 1. Characteristics of the patients from whom failing hearts were obtained

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>LVEF (%)</th>
<th>Medications*</th>
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Table 2. Characteristics of the patients from whom non-failing hearts were obtained

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<td>CVA</td>
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* GSW=gun shot wound; CVA=cerebrovascular accident (stroke); MVA= motor vehicle accident; LVEF= left ventricular ejection fraction; NA=not available; DB=dobutamine; DP=dopamine; LB=labetolol; LV=levophed; NM=nimodipine; NS=neonaprin; NT= nitroglycerine; TH=thyrroxine; *medications administered in Emergency Room/Intensive Care Unit.
Once in the laboratory, tissue was immediately separated by heart chamber, frozen in liquid nitrogen, and stored at \(-80^\circ\text{C}\) until use.

The lung, liver, kidney and skeletal muscle samples were specimens collected by the Department of Pathology at the Cleveland Clinic Foundation at the time of routine surgical procedures. All protocols described in this manuscript were approved by the Institutional Review Board of the Cleveland Clinic Foundation and the investigation conforms with the principles outlined in the Declaration of Helsinki.

2.2. Immunocytochemistry

Endocardial trabecular muscles were dissected from the left ventricle and right atrium of each heart. Muscles were secured on small pieces of wax and frozen in liquid nitrogen. The frozen muscles were stored at \(-80^\circ\text{C}\) until preparation for sectioning.

Frozen longitudinal sections (4 \(\mu\text{m}\)) were cut from the surface of each muscle, using a cryostat (Shandon A5620, Analytical instruments, Plymouth, MN). These sections were used for all immunocytochemical reactions.

After fixation in acetone at \(-20^\circ\text{C}\) for 20 min, the sections were air dried, rinsed in phosphate buffered saline (PBS), incubated at room temperature with blocking buffer (PBS, containing 2% goat serum, 5% Triton X-100, 5% glycine) for 1 h and then incubated with the primary antibody, 1:25 mouse anti-annexin IV clone 4 (Transduction Laboratories, Lexington, KY), 1:25 goat anti-annexin V R-20 (Santa Cruz Biotechnology, Santa Cruz, CA), and 1:150 mouse anti-annexin VI clone 73 (Transduction Laboratories, Lexington, KY), diluted in PBS containing 2% goat serum, 0.5% Triton X-100 and 5% glycine, at room temperature for 1 h (annexin VI) or overnight at 4\(^\circ\text{C}\) (annexin IV and V). After rinsing with PBS, the secondary antibody, diluted 1:250 in the same buffer, was applied and incubated at room temperature for 1 h in the dark. The detection system was fluorescein isothiocyanate (FITC) labelled antimouse IgG antibody (Vector Laboratories, Burlingame, CA) or anti-goat IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). The sections were rinsed again with PBS and mounted with Vectashield\textsuperscript{\textregistered} mounting medium containing propidium iodide to label the nuclei (Vector Laboratories). Slides were examined using a Leica Confocal Laser Scanning microscope.

2.3. Western blot analysis

Human lung, liver, kidney, skeletal muscle and heart left ventricle and right atrium, devoid of fibrotic or adipose tissue, were homogenized in a threefold volume of glycercerol buffer at pH 7.4 for 3\(\times\)30 s using a Polytron (model PT 10/35, Brinkman Instruments, Westbury, NY) homogenizer at 4\(^\circ\text{C}\). Protein concentration was determined according to the method of Bradford [33] with bovine serum albumin as the standard. Homogenates were stored at \(-80^\circ\text{C}\) until use.

Samples were solubilized in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) for 5 min at 95\(^\circ\text{C}\). Equal amounts of protein from each sample were subjected to SDS–PAGE using the Laemmli buffer system [34] in a Mini-Protean II Dual Slab Cell (Bio-Rad Laboratories). All samples were run until complete elution of the dye front. Proteins were transferred to nitrocellulose in a Mini Trans-Blot Transfer Cell (Bio-Rad Laboratories), according to the procedure of Towbin [35]. The transfer was completed at 4\(^\circ\text{C}\) at a constant voltage setting of 100 V for 1 h. The blots were blocked in 5% gelatin diluted in Tris-buffered saline (TBS), composition: 20 mmol/l Tris–HCl, pH 7.4, 150 mmol/l NaCl, for 1 h at room temperature. The blots were washed three times for 5 min each and then incubated in the primary antibody solution diluted 1:500 in TBS, containing 0.1% gelatin, overnight at 4\(^\circ\text{C}\). The same primary antibodies were used for both Western blots and immunolocalization (described above). The blots were washed three times for 15 min in TBS and were incubated in secondary antibody at a dilution of 1:10 000 in the same buffer as above for 1 h at room temperature. The blots were again washed three times for 15 min, and developed using the Vistra ECF Western blotting system (Amersham, Chicago, IL). Proteins were quantified using the Molecular Dynamics Storm Imager and ImageQuant PC software (Molecular Dynamics, Sunnyvale, CA). For each band, normalization was performed by the value of a non-failing heart used as a standard on all blots. The secondary antibodies used for Western blots were: antirabbit IgG alkaline phosphatase conjugated (Vistra systems) and rabbit F(ab')\textsuperscript{2} anti-goat IgG alkaline phosphatase conjugated (Southern Biotechnology Associates, Birmingham, AL).

2.4. Statistical analysis

Protein quantitation data are expressed as mean\(\pm\)S.E.M. Comparison between non-failing and failing human tissue was performed by unpaired \(t\)-test (NF vs. ICM and NF vs. DCM) or Mann–Whitney test. A value of \(P<0.05\) was accepted as evidence that the groups were significantly different from each other.

3. Results

As a positive control for the specificity of the primary antibodies used in this study, we performed Western blot analysis using a variety of human tissues samples. Fig. 1 shows Western blots of left ventricular tissue from the heart (HR), skeletal muscle (SK), liver (LI), kidney (KD)
and lung (LN). Anti-annexin IV antibody recognized a major band at approximately 34 kD identified as annexin IV, and a minor band of approximately 68 kD, which may represent a cross reaction with the annexin VI protein. Anti-annexin V antibody recognized a specific band of approximately 32.5 kD in all tissues. The other bands observed in the blot are due to non-specific binding of the secondary antibody and were also present when the blot was incubated with secondary antibody alone (data not shown). Anti-annexin VI antibody specifically recognized a band at approximately 68 kD in all tissues studied.

3.1. Annexin IV

For the immunolocalization studies, one representative failing heart is compared to one representative non-failing heart. Among the failing hearts, there were no differences in the location of any of the annexins between DCM and ICM hearts. When the heart sections were stained for annexin IV (Fig. 2A–D), a strong staining was observed around the yellow lipofuscin granules (Fig. 2A, open arrow). Additionally a punctate longitudinal staining was also evident as shown in the non-failing left ventricle (Fig. 2A, triangles). Also, the cross striation pattern observed in the non-failing atrium (Fig. 2C) was absent in the failing atrium (Fig. 2D), which showed diminished intracellular staining. In summary, a loss of annexin IV organization was evident in both failing tissues, (Fig. 2B and D) when compared with the non-failing tissues (Fig. 2A and C).

Interestingly, although our Western blots showed a possible cross reaction of the anti-annexin IV antibody with the annexin VI protein, in these immunostaining experiments the characteristic plasma membrane staining for annexin VI was not evident when the tissues are incubated with anti-annexin IV antibody, demonstrating no important cross-reactivity in these studies.

Fig. 3 shows a representative Western blot and bar graph of annexin IV protein levels in the left ventricle and right atrium from non-failing, ICM and DCM hearts. There was a statistically significant increase in the amount of annexin IV protein in both ICM and DCM hearts as compared to non-failing hearts. In the right atrium, however, there were no differences in the amount of protein between either DCM and non-failing or ICM and non-failing hearts.

3.2. Annexin V

Longitudinal sections from left ventricle and right atrium stained with anti-annexin V antibody are shown in Fig. 2E–H. In these sections, a clear cross-striated pattern is observed in all tissues (Fig. 2E–H). In the failing ventricle (Fig. 2F) and atrium (Fig. 2H), however, a more disorganized cross-striated pattern is evident, with areas where annexin V is localized as green brilliant dots in the cytosol (brackets in Fig. 2F and H). In addition, staining for annexin V accumulated around the lipofuscin granules and at the intercalated discs (arrowhead in Fig. 2E). Staining for annexin V in the cytosol of non-myocyte cells is also evident in the majority of the sections and is clearly shown in Fig. 2G (bidirectional arrow).

Fig. 4 shows representative Western blot analyses and the corresponding bar graphs for annexin V in ventricle and atrium from failing and non-failing hearts. Results show a significant increase in the protein levels of annexin
Fig. 2. Immunolocalization of annexins IV, V and VI in the non-failing and failing human heart. In all images, the specific fluorescent stain for annexins is green, and the nuclei are red. Yellow autofluorescent granules are lipofuscin. The magnification for all the images is 63× and the scale bar (in image L) represents 20 μm. Cryostat sections of non-failing left ventricle (A, F and I), failing left ventricle (B, F and J), non-failing right atrium (C, G and K) and failing right atrium (D, H and L) were incubated overnight at 4°C with either anti-annexin IV (A–D), anti-annexin V (E–H) antibody or anti-annexin VI (I–L) antibody and then with FITC-labelled secondary antibody. Tissue sections incubated with the secondary antibody alone were negative for the green fluorescence (not shown). Specific localization of each annexin in each tissue type is discussed in the text.
V in the left ventricle of ICM and DCM as compared to non-failing. In the right atrium, there were no significant changes in the expression of annexin V between non-failing and either group of failing hearts.

3.3. Annexin VI

Annexin VI (Fig. 2I–L) was localized at the sarcolemma and intercalated disc in the ventricle and atrium from both non-failing (Fig. 2I and K) and failing (Fig. 2J and L) human hearts. However, in the non-failing left ventricle (Fig. 2), a striation pattern was also observed in the majority of the sections (closed arrow). This pattern was absent in the majority of failing left ventricle sections (Fig. 2J). The failing atrium (Fig. 2L) also showed staining in a longitudinal pattern, but this pattern was not observed in the non-failing atrium (Fig. 2K).

A representative Western blot analysis and the corresponding bar graphs for annexin VI in total homogenate of left ventricle and right atrium of failing and non-failing human hearts are shown in Fig. 5. There were no significant differences in the protein levels of annexin VI between non-failing and ICM or non-failing and DCM hearts in either left ventricle or right atrium.

4. Discussion

We chose to study left ventricular tissue because of the large contribution of left ventricular performance to overall cardiac output and its impairment during heart failure. Right atrium was studied in preference to left atrium because the human right atrium is trabeculated, facilitating the removal of muscle preparations comparable to those found in the ventricle, and thus more easily compared.

In this study, we have shown that (1) annexin IV, V and
VI are localized in distinct cellular compartments within the cardiomyocytes of the human heart and, in some cases, the characteristic intracellular location of these proteins is altered in the failing human heart; (2) annexin IV and V protein levels are up-regulated in the left ventricle but not in the right atrium of failing human hearts; and (3) annexin VI is not significantly altered in the ventricle or atrium of failing human hearts.

It has been established that human heart failure is characterized by contractile changes, which are accompanied by abnormalities in intracellular Ca$^{2+}$ cycling [36] and alterations in the steady state levels of both mRNA and proteins involved in Ca$^{2+}$ cycling, (see Ref. [37] for review). Changes in the expression of E–F hand motif Ca$^{2+}$ binding proteins, such as calmodulin [24] have also been reported. Disorganization of sarcomeres and the cytoskeleton, as well as an abnormal arrangement of contractile proteins, has been reported in human heart failure [38,39]. Differential regulation of both the amount and the location of other proteins involved in Ca$^{2+}$ cycling, such as the annexins, has also recently been reported in human heart failure [29].

The fact that annexins IV, V and VI immunolocalize in different compartments within cardiomyocytes may suggest a different role for each annexin in the heart. Moreover, the up-regulation of two of these proteins in the left ventricle, the most dysfunctional chamber in the failing heart, along with other reported abnormalities in Ca$^{2+}$ cycling, supports the hypothesis that annexins may play a role in Ca$^{2+}$ handling in the human heart.

Annexin IV is one of the most abundant annexins present in many cell types. Neither the function nor the location of the annexin IV protein has been investigated in the heart. However, in epithelial cells, annexin IV is known to be a cytoplasmic marker of the polarization of the cell, either at the basolateral domain [40] or at the
Fig. 5. Representative Western blot analysis for annexin VI and the summarized bar graphs for left ventricle and right atrium from non-failing (N), ischemic (I) and dilated cardiomyopathic (D) human hearts. Western blot analysis was performed on all 18 hearts. For conditions of the Western blot analysis, see Materials and methods. On all blots 17, 33 and 51 µg of total left ventricle (top) and right atrium (bottom) protein from the same non-failing heart (standard heart) was blotted. For normalization purposes, densitometric units obtained from each heart were divided by the average value of the standard heart from the same blot. Each value represents the mean of three independent determinations. A value of \( P \leq 0.05 \) was accepted as statistically significant.

Apical domain [41] and it has also been shown to modulate a \( \mathrm{Ca}^{2+} \) dependent chloride channel in these cells [14]. In the human heart, our results show that annexin IV is localized in the cytoplasm of cardiomyocytes and that in the failing tissues there is a loss of intracellular organization. Annexin IV protein levels are also significantly increased in the ventricle of both DCM and ICM hearts. [28]. Similarly, in the human heart, our current results show that annexin V immunolocalizes with a cross-striated localization at the z-line. In the failing heart, we show that annexin V tends to cluster in the cytosol of cardiomyocytes with a lack of organization. These results are consistent with the findings of Hein and co-workers [39] whose immunohistochemistry experiments show changes in both the amount and organization of titin, myosin and thin filament complex in human hearts.

The localization of annexin V at the z-line, and also the up-regulation of annexin V protein in the left ventricle, are
consistent with a role for annexin V in altered Ca\(^{2+}\) handling in the human heart during failure. The up-regulation of annexin V in DCM and ICM human hearts agrees with the recent results of Song and colleagues, who showed an increase in both the mRNA and protein levels for annexin V in the failing human heart [29].

The immunolocalization of annexin VI is less controversial. It has been shown to be present at the sarcolemma and the intercalated disc of rat and porcine cardiomyocytes [26,43]. In the human heart, our results also show a sarcolemmal and intercalated disc distribution of annexin VI in both atrium and ventricle. Functionally, Sobota and co-workers reported that annexin VI could reverse the inhibition of Na\(^+\)/Ca\(^{2+}\) exchange activity of cardiac sarcolemmal vesicles [18], which is consistent with its localization. This function may be particularly relevant in human heart failure, where a number of studies have demonstrated changes in the amount or function of the Na\(^+\)/Ca\(^{2+}\) exchanger [5,45].

Evidence for the physiological importance of annexin VI in the heart comes from studies of Gunterski-Hamblin and colleagues, who overexpressed annexin VI in the mouse heart and reported that the cells isolated from these hearts showed a frequency-dependent reduction in the percent shortening as well as decreased rates of contraction and relaxation. The transgenic animals had lower basal levels of intracellular free Ca\(^{2+}\) and a reduced rise in free Ca\(^{2+}\) following depolarization. After contraction, intracellular free Ca\(^{2+}\) returned to basal levels faster in transgenic cells than in those taken from control animals [19]. The relative importance of annexin VI in regulating Ca\(^{2+}\) levels and contractility may be exaggerated in the failing heart, where these processes are altered.

Song and co-workers described a decrease in the protein level, as well as the mRNA level, for annexin VI in failing human hearts compared with non-failing human hearts [29]. Our results show no significant difference in the protein levels of annexin VI between DCM or ICM hearts and non-failing human hearts, although it is worth mentioning that levels in the DCM hearts showed a trend towards a decrease, while in the ICM hearts the trend was an increase in our study. The reason for the difference in annexin VI results between our study and the study of Song and co-workers is not clear. We speculate that differences in the human population used for the studies, particularly the non-failing patient population, may partially explain this discrepancy. Clarification of this issue awaits further study.

In conclusion, the differential subcellular location of annexins and their close proximity with the proteins involved in the handling of Ca\(^{2+}\), as well as changes in their cellular organization and the up-regulation of two of these proteins in the failing heart, may suggest a role for annexins in the regulation of Ca\(^{2+}\) cycling in the heart. Further studies are needed to clarify the functional implications of these findings.

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References


