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Myocyte apoptosis during acute myocardial infarction in rats is related to early sarcolemmal translocation of annexin A5 in border zone

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Myocyte apoptosis during acute myocardial infarction in rats is related to early sarcolemmal translocation of annexin A5 in border zone. Am J Physiol Heart Circ Physiol 291: H965–H971, 2006. First published February 24, 2006; doi:10.1152/ajpheart.01053.2005.—Annexin A5 is a Ca2+-dependent phospholipid binding protein well known for its high phosphatidylserine affinity. In vitro, translocation to sarcolemma and externalization of endogenous annexin A5 in the cardiomyocyte has recently been demonstrated to exert a proapoptotic effect. To determine whether these in vitro findings occurred in vivo, we performed myocardial infarction (MI) and studied the time course of apoptosis and annexin A5 localization (0.5 to 8 h) in the border zone around the infarcted area. This zone that was defined as Evans blue unstained and triphenyltetrazolium chloride (TTC) stained, represented 42.3 ± 5.5% of the area at risk and showed apoptotic characteristics (significant increases in caspase 3 activity 2.3-fold at 0.5 h; P < 0.05), transferase-mediated dUTP nick-end labeling-positive cardiomyocytes (15.8 ± 0.8% at 8 h), and DNA ladder. When compared with sham-operated rats, we found that in this area, annexin A5 was translocated to the sarcolemma as early as 0.5 h after MI and that translocation increased with time. Moreover, the amount of annexin A5 was unchanged in the border zone and decreased in the infarcted area after 1 h (77.1 ± 4.8%; P < 0.01 vs. perfused area), suggesting a release in the latter but not in the former. In conclusion, we demonstrated that annexin A5 translocation is an early and rapid event of the whole border zone, likely due to Ca2+ increase. Part of this translocation occurred in areas where apoptosis was later detected and suggests that in vivo as in vitro annexin A5 might be involved in the regulation of early apoptotic events during cardiac pathological situations.

ANNEXINS are a structurally related family of proteins known to bind phospholipids in a Ca2+-dependent manner (17). After a rise in intracellular Ca2+, annexins are translocated toward nuclear or plasmic membranes (3, 16, 17, 33) and might even be inserted into membranes in vitro (27). They are involved in a number of intra- and extracellular processes such as signal transduction, differentiation, membrane trafficking, and organization (17, 34). Although the physiological function of annexins is as yet poorly understood, a growing body of evidence suggests that dysregulation in their expression and/or localization can play a role in numerous diseases leading to the term of “annexinopathies” (7, 8, 21, 32).

Among other annexins, annexin A5 has a high affinity for phosphatidylserine and has been reported to form Ca2+ channel (12). However, to date, no direct role for endogenous annexin A5 has been evidenced except in apoptosis, during which a proapoptotic role has been proposed in vitro. Hawkins et al. (20) showed that DT40 cells lacking annexin A5 were resistant to various apoptotic stimuli, and Wang et al. (39) reported that Ca2+ influx mediated by annexin A5 regulates chondrocyte maturation and apoptosis. In line with these findings, Monceau et al. (28) have previously demonstrated in cardiomyocytes that endogenous annexin A5, which was externalized during apoptosis, exhibited a proapoptotic effect. Annexin A5, an abundant protein of the cardiomyocyte, has been located in both the cytosol and the sarcolemma in control hearts and is found in the interstitial tissue in the failing heart (4, 38). Apoptosis of cardiomyocytes has been largely reported in acute myocardial infarction (MI) and ischemia reperfusion and occurs preferentially in the border area around the necrotic core (10, 18, 23, 30). It is thought to play a role in cardiac remodeling and progression of myocardial dysfunction (19, 25). Although the initiating proapoptotic stimuli are probably diverse, there is strong evidence that reactive oxygen species liberated after ischemia activate the intrinsic death pathway (2, 6, 14, 26, 40). We have shown that in an in vitro model of reactive oxygen species-induced apoptosis, externalization of annexin A5 occurred at a very early stage of apoptosis preceding mitochondrial activation (28). It was thus tempting to assume that endogenous annexin A5 might be involved in in vivo apoptosis.

Therefore, to test this hypothesis, we studied both annexin A5 localization and the occurrence of apoptosis during in vivo ischemia induced by ligation of the left anterior descending coronary artery (LAD) in rats. We found that cytosolic annexin A5 was relocated to sarcolemma early after injury in the area at risk where apoptotic cells were characterized, suggesting that annexin A5 is involved in the development of apoptosis as it is in vitro and in the remodeling process after MI.

MATERIALS AND METHODS

Myocardial Ischemia

Left ventricular ischemia was induced in rats by ligating the LAD. All animal procedures were conducted in accordance with both institutional guidelines and those formulated by the European Com-

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munity for experimental animal use. Adult male Wistar rats weighing 250–300 g were anesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (50 mg/kg), and a left thoracotomy was performed under mechanical ventilation (36). The heart was then raised from the thorax, and the LAD was occluded near its origin with a 6-0 silk suture. Sham-operated rats underwent an identical procedure, except that the suture was passed through the myocardium without tying. Experimental and sham-operated animals were randomly assigned to one of the following six groups (5 animals in each group): 0.5, 1, 2, 4, and 8 h and 1 mo of LAD occlusion. After the occlusion period, the rats were anesthetized and 6 ml of 1% Evans blue dye (Sigma Aldrich Chimie, Saint Quentin Fallavier, France) was injected retrogradely into the vena cava to delineate the region of myocardial perfusion. The hearts were rapidly excised and chilled in saline at 0°C, atria were removed, and ventricles were sliced transversely into 2-mm thick slices (Fig. 1A). Slices were then fixed in an embedding medium Tissue-Tek (Miles-Diagnostics, Elkhart, IA) and immediately frozen in liquid nitrogen-cooled isopentane except from sections representing the different areas (10 × 10–15 μm) were then cut for biochemical studies (DNA ladder, caspase 3 activity, and immunoblots) (Fig. 1D).

Detection of Apoptosis

Caspase 3 activity. Tissue sections were solubilized in 100–150 µl of ice-cold lysis buffer containing 50 mM HEPES, pH 7.4, 0.1% Triton X-100, 100 mM DTT, 1 mM EDTA, and then centrifuged at 10,000 g for 10 min. Caspase 3 activity was measured in the supernatants by Biomol QuantiZyme Assay System CASPASE-3 Cellular activity Assay Kit PLUS (Le Perray-en-Yvelines, France) according to the manufacturer’s recommendations.

DNA ladder. Fragmented DNA was isolated from tissue sections (10 × 10–15 μm) of the three zones (Fig. 1) according to Herrmann et al. (22). Supernatants from 1% Nonidet P-40 (NP40) extracts were precipitated, and DNA electrophoresis was carried out in 1.5% agarose gels containing ethidium bromide. Analysis of the DNA fragments was performed under UV with a Gel Doc 1000 analyzer (Bio-Rad, Ivry s/Seine, France).

In situ end labeling. Cryosections (10 μm) were fixed and permeabilized with ice-cold acetone-methanol (50/50 vol/vol). TUNEL was performed with an in situ cell death detection kit as recommended by the manufacturer (ApopDETEK, Enzo Diagnostis). Nuclei were counterstained with hematoxylin. The percentage of TUNEL-positive myocytes was counted in the border area and determined on 1,000 total nuclei (representing approximately seven to eight fields). We verified that apoptosis occurred in the myocytes by immunohistochemical staining of α-actinin (monoclonal antibody, 1:100 dilution) (data not shown). Only nuclei that were clearly located in cardiac myocytes were scored.

The specificity of the reaction was assessed by omitting TdT (no signal) or by pretreatment with DNase I (positively stained nuclei).

Fig. 1. Experimental protocol for assessment of distribution of the perfused, border, and infarcted areas. A: heart was cut transversely into 6 slices. B: Evans blue (slice 4, S4) and triphenyltetrazolium chloride (TTC, slice 5, S5) staining seen on cryostat sections (50 μm) 8 h after myocardial infarction (MI). Combination of the two stainings allowed to delineate the perfused area (P) (Evans blue-perfused and TTC-stained area), the infarcted area (I) (Evans blue unstained and TTC unstained), and the border area adjacent to the infarcted area (B) (Evans blue unstained and TTC stained). C: transverse serial cryostat sections (7–10 μm) showed red fluorescence of Evans blue, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), and immunohistochemical labelings (annexin A5 and Na+–K+–ATPase). D: remaining frozen tissue was then divided into radial segments representing the different areas and cryostat tissue sections (10 × 10–15 μm) of each area were collected, frozen, and used for biochemical studies.
Annexin A5 Localization

To compare the localization of TUNEL-positive myocytes with those of annexin A5 and of Na⁺-K⁺-ATPase, we used serial cryosections (7–10 μm) because primary antibodies were both rabbit polyclonal antibodies (4, 9). After fixation in acetone-methanol (1:1) for 20 min at −20°C, sections were saturated with 5% bovine serum albumin in phosphate-buffered saline and incubated with either anti-annexin A5 antibodies or anti-Na⁺-K⁺-ATPase antibodies (1:20, 1:50, respectively). The sections were incubated with secondary anti-rabbit fluorescein isothiocyanate fluorochrome-conjugated antibodies (1:50), washed, and covered with FluoroGuard antifade mounting reagent.

Labelings were visualized by using a DMR P Leica microscope equipped with a JVC color video camera KY-F50 coupled to an imaging analysis system (Histolab software, Microvision, France).

Immunoblot Analysis

Radial segments of the sections (10 × 10–15 μm) representing the different areas (Fig. 1D) were homogenized in 2% SDS conventional lysis buffer and allowed the recovery of 750 to 1,500 μg protein. Protein extracts (20 μg) were resolved on 10% SDS-PAGE gels and electrotransferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech) as previously described (4). Membranes were saturated with low-fat milk and incubated with rabbit polyclonal anti-annexin A5 antibodies or mouse monoclonal anti-β-actinin antibodies (Sigma, Saint Quentin Fallavier, France) diluted 1:2,500 and 1:8,000, respectively, and then with anti-rabbit IgG coupled to peroxidase. Quantification of ECL+ spots and Coomassie blue staining of total proteins were performed using Gel Doc 1000 Molecular Analysis System (Bio-Rad). ECL spots were normalized by integrated density of Coomassie blue staining of the lane.

Statistical Analysis

Results are expressed as means ± SE. The differences between control and experimental groups of animals were evaluated using a two-tailed unpaired Student’s t-test or one-way ANOVA, followed by Scheffé’s comparison. A value of $P < 0.05$ was considered significant.

RESULTS

Detection of Infarcted, Border, and Perfused Areas

At 8 h after MI, the area at risk (Evans blue unstained) represented 35.8 ± 3.2% of the left ventricle. The infarcted (Evans blue and TTC unstained) and border area (Evans blue unstained and TTC stained) represented 56.7 ± 6.2% and 42.3 ± 5.5% of the area at risk, respectively. These values were in agreement with Takashi et al. (37).

Myocyte Apoptosis During Acute Myocardial Infarction

It is well established that apoptosis mainly occurs in the border zone around the infarcted areas (10, 23, 29, 30, 40). To characterize apoptotic areas and to assess the time course of apoptosis in our model, caspase 3 activity, DNA laddering, and TUNEL assays were performed in the different areas at various times after MI. In agreement with an early activation of caspase 3 in apoptotic cardiomyocytes, increased enzymatic activity was found at 0.5, 1, and 2 h after MI in the border zone of the infarcted area then returned to the basal level at 4 h (2.3±, 2.1±, 2.4±, and 1-fold, respectively, compared with the perfused zone) (Fig. 2A). Caspase 3 activities were similar in sham-operated animals and in perfused area, whereas there was a trend toward a decrease in the infarcted area, likely due to damaged cells in this area.

Cardiac DNA was analyzed for DNA fragmentation at various times after MI (Fig. 2B). DNA ladders were completely absent in sham-operated rats (not shown) or 2 h after ischemia. Typical DNA ladders were observed in the border and the infarcted zone 4 and 8 h after ischemia but were attenuated in the infarcted zone.

Representative sections of TUNEL staining from hearts of sham-operated animals and 0.5, 4, and 8 h after MI were shown in Fig. 3. No TUNEL-positive cardiomyocytes were detected in sham-operated animals (Fig. 3A) or 0.5 h after ischemia (Fig. 3B), whereas TUNEL-positive nuclei were prevalent in the border zone at 4 and 8 h (Fig. 3, C and D). The concentration of the TUNEL-positive cardiomyocytes in the border zone was very few at 0.5 h (1% or 2%) then significantly increased at 4 and 8 h [6.9 ± 0.5% (n = 3) and 15.8 ± 0.8% (n = 3), respectively; $P < 0.05$, $<0.01$, compared with perfused zone]. The number of TUNEL-positive nuclei has not been determined in the infarcted area.

Immunolocalization of Annexin A5

To investigate the relationship between annexin A5 localization and apoptosis and to determine whether annexin A5...
translocation was occurring in the border zone, we assessed annexin A5 localization (Fig. 3, middle column) on serial sections used for TUNEL assay (Fig. 3, left column) and compared it to that of Na\(^{+}\)-K\(^{-}\)-ATPase, a sarcolemmal marker (Fig. 3, right column). The fields in Fig. 3, B–D, were representative of border and perfused area. They have been elicited according to their localization in Evans blue serial section (Fig. 1C). In sham-operated animals (Fig. 3A), annexin A5 was

![Figure 3](image)

**Fig. 3.** Distribution of TUNEL-positive nuclei and localization of annexin A5 and Na\(^{+}\)-K\(^{-}\)-ATPase in the area adjacent to infarcted tissue. TUNEL, annexin A5, and Na\(^{+}\)-K\(^{-}\)-ATPase labelings were performed on serial cryostat sections from sham-operated animals (A) and 0.5 h (B), 4 h (C), and 8 h (D) after MI. Fields were representative of the border and perfused areas identified with the red fluorescence of Evans Blue as described in Fig. 1, B and C. TUNEL-positive nuclei were brownish and TUNEL-negative nuclei were blue. Position of TUNEL-positive cardiomyocytes were identified by asterisks on annexin A5 immunolabeled sections and were found in border areas with translocation of annexin A5. Arrowheads depicted the usual labeling of annexin A5 in perfused areas. Sarcolemma was identified by Na\(^{+}\)-K\(^{-}\)-ATPase immunolabeling. Bar, 50 μm.
found in the cytosol and at the level of sarcolemma, T-tubules, and intercalated disks of cardiomyocytes, whereas Na\(^+\)/K\(^+\)-ATPase was absent from cytosol and strictly restricted to membranes. After MI, annexin A5 localization remained unchanged in the perfused areas, whereas in the border zone annexin A5 was translocated to the sarcolemma as early as 0.5 h after coronary occlusion, and areas of translocation increased with ischemia periods. At 4 and 8 h, annexin A5 translocation was found in border zone where TUNEL-positive cardiomyocytes were detected (Fig. 3, C and D, asterisks). In contrast, Na\(^+\)/K\(^+\)-ATPase labeling was not affected in the same fields (Fig. 3, right column). Moreover, restricted localization of annexin A5 to the sarcolemma and intercalated disks was also detected in the infarcted area (data not shown) but could be due to membrane leakiness after permanent ischemia, as reported by Kajstura et al. (23).

To examine whether the process of annexin A5 translocation was limited to the apoptotic areas after short-time ischemia or prolonged during ventricular remodeling, we studied the localization of annexin A5 in the remodeling tissue around the infarcted area 1 mo after MI. As shown in Fig. 4, bottom left, tissue disorganization of the infarcted area was evidenced by a decrease in cellularity (hematoxylin staining) and by the absence of ordinary Na\(^+\)/K\(^+\)-ATPase or annexin A5 labeling. By contrast, annexin A5 and Na\(^+\)/K\(^+\)-ATPase localization in cardiomyocytes were not affected in the remote area, and serial sections showed the absence of TUNEL-positive nuclei, suggesting that apoptosis was not present 1 mo after MI.

**Quantification of Annexin A5 After MI**

To determine whether translocation and externalization of annexin A5 was accompanied by changes in protein levels, immunoblot analysis was performed for annexin A5 and \(\alpha\)-actinin in homogenates from sham-operated hearts and from the three different areas of hearts after MI. Figure 5A shows that the homogenates from perfused, border, and infarcted zones contained both annexin A5 and \(\alpha\)-actinin. Quantitative results (Fig. 5B) showed that the amounts of annexin A5 were similar in the border zone and the perfused area, whatever the time of infarction. Unlike border zone, annexin A5 amount in the infarcted zone was significantly decreased after 1 h (77.1 ± 4.8%; \(P < 0.01\) vs. perfused area) and remained significantly lower throughout the study. By comparison, the amount of \(\alpha\)-actinin remained unchanged.

![Fig. 4. TUNEL, annexin A5, and Na\(^+\)/K\(^+\)-ATPase labelings in fields adjacent to the infarcted area 1 mo after MI. Representative field from serial cryostat sections depicts the infarcted area (bottom left) and adjacent tissue. Tissue organization was evidenced by hematoxylin and eosin (H/E) coloration. In cardiomyocytes, annexin A5 localization in the remote area was similar to sections from sham-operated animals. Bar, 50 μm.](image)

![Fig. 5. Quantification of annexin A5 and \(\alpha\)-actinin in the perfused, border, and infarcted areas. A: representative immunoblots of annexin A5 and \(\alpha\)-actinin were performed with equal amounts (20 μg/lane) of total protein extracts from the sham-operated animals and the perfused (P), border (B), and infarcted (I) areas at various times after MI. B: relative amounts of annexin A5 and \(\alpha\)-actinin in the sham-operated animals (hatched bars) and in the perfused (open bars), border (shaded bars), and infarcted (solid bars) areas (\(n = 5\) experiments for each area at separate time). *\(P < 0.05\), **\(P < 0.01\) vs. corresponding perfused area.](image)
DISCUSSION

In this study, we reported that very early in MI, border areas were characterized by a relocation of annexin A5 from cytosol to sarcolemma and intercalated disks of cardiomyocytes without loss of annexin A5 from the tissue. At 8 h after MI the area of relocation was extended, and the TUNEL-positive cardiomyocytes were colocalized in this area suggesting a relationship between annexin A5 relocation and apoptosis. These observations agree well with the early externalization of annexin A5 previously reported in vitro in apoptotic cardiomyocytes and are in favor of a proapoptotic role of annexin A5 in vivo like that demonstrated in vitro (20, 28, 39).

Evidence for apoptotic cell death has been widely reported in response to both prolonged ischemia and ischemia followed by reperfusion (5, 10, 15, 19, 23). Apoptosis occurs preferentially in the border zone around the infarcted area (10, 23, 30, 41), whereas in the infarcted area it is well known that MI induced both necrotic and apoptotic cell deaths and development of inflammatory processes after 24 to 48 h (10, 15, 23). Because we were interested only in apoptotic cell death, we decided to carefully identify the border zone and to study annexin A5 localization in this area (Fig. 1, B and C). In this study, the border zone accounted for 42.3 ± 5.5% of the area at risk in agreement with a previous study (37). Moreover, this zone was the only one exhibiting the characteristics of an apoptotic area with increases in caspase 3 activity together with TUNEL-positive cardiomyocytes and presence of DNA ladder. Therefore, we are rather confident that we were dealing with the border zone, but we cannot totally exclude a minute contamination.

By contrast, the infarcted area has an unchanged caspase 3 activity and an attenuated DNA ladder. Moreover, infarcted area has been repeatedly characterized by immediate loss of plasma membrane integrity, major tissue disorganization (23), and release of myocyte proteins in the setting of myonecrosis (1). In line with this last result, annexin A5 was found decreased in the infarcted area (Fig. 5) and reported to be increased in plasma 3 h after MI in humans (24) supporting the issue that in this area, the decrease of annexin A5 was associated with necrosis.

Unlike in the infarcted area, the specific translocation of annexin A5 to sarcolemma and intercalated disks in the border zone was occurring without release of annexin A5. Accordingly, annexin A5 translocation and exposition onto the cell surface have been previously reported in vitro in cardiomyocytes during apoptosis and did not lead to annexin A5 release (28). Together, the results of these studies suggest that annexin A5 translocation is important in the modulation of apoptosis.

However, the common trigger of annexin A5 translocation and apoptosis is not yet established, but there are evidences that both depend on Ca2+. The binding of Ca2+ to calcium-binding sites of annexin A5 was critical for membrane binding of the protein and during ischemia, and elevation of intracellular Ca2+ concentration has been widely reported to be an important trigger of apoptosis (13, 31). Thus it is reasonable to expect that after MI the sustained elevation of Ca2+ triggering the initiation phase of apoptosis and leading to mitochondrial dysfunction through sarcoplasmic reticulum activation (35) could likely be responsible for annexin A5 translocation to sarcolemma. However, in this study, unlike in vitro (28) some data are not completely in concert: we found that the area of annexin A5 translocation was larger than that of TUNEL-positive cardiomyocytes, whereas in vitro, annexin A5 externalization has been observed in all TUNEL-positive cardiomyocytes. This apparent discrepancy could be accounted for by multiple reasons that might be combined. First, the apoptosis process involved in ischemia is multifactorial, whereas in vitro the trigger is unique and the isolated cardiomyocytes are more susceptible. Second, the time frame of our model may have been too short to detect DNA fragmentation and thus the number of TUNEL-positive cells was underestimated. Although others (10, 23) have found that TUNEL positivity increased during the first 6 h to 2 days, the number of TUNEL-positive cardiomyocytes found in this study at 8 h is in agreement with other reports and could only slightly increase (40). However, it seems highly unlikely that the area of TUNEL-positive cells could reach that of annexin A5 translocation without leading to death. Third, we and others (20, 28, 39) have found that apoptosis required externalized annexin A5 to proceed and was occurring in 10% cardiomyocytes in the border zone (40). Although differences between membrane binding and/or externalization cannot be established in vivo, we can assume by combining these arguments that only part of the relocated annexin A5 was externalized and by participating in Ca2+ influx might represent an important signal for the activation of the apoptotic process (16, 39). As a Ca2+ binding protein, the remaining annexin A5 associated to the membrane might buffer Ca2+ and thus delay the initiation phase of apoptosis. Increase of Ca2+ beyond the buffering capacity of annexin A5 might be responsible for activation of the subsequent steps.

In conclusion, consistent with the essential proapoptotic role of annexin A5 in vitro, we suggested that annexin A5 translocation and externalization may also play an essential role in apoptosis mechanism in vivo. The early translocation of annexin A5 might be an important feature of the border area after MI and could be of relevance concerning a preapoptotic state suspected by Communal et al. (11). Maintaining this state and blocking annexin A5 externalization could therefore attenuate apoptosis and, in turn, postischemic damages.

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