Circulating α-Actin in Angina Pectoris

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*Basic Cardiovascular Research Section, Department of Morphological Sciences, School of Medicine, University of Granada, E-18071 Granada, Spain, and †Cardiology Service, Hospital General de Especialidades Virgen de las Nieves, E-18014 Granada, Spain

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A. E. ARÁÑEGA, A. REINA, C. VELEZ, L. ALVAREZ, C. MELGUIZO AND A. ARÁÑEGA. Circulating α-Actin in Angina Pectoris. Journal of Molecular and Cellular Cardiology (1993) 25, 15-22. We used Western blot, a highly sensitive technique that detects amounts of protein as low as 0.1 to 1.0 ng, to investigate the possible presence in the blood stream of the contractile protein α-actin in 29 patients diagnosed with angina pectoris (Braunwald's classification). Circulating protein was identified with a monoclonal antibody specific for cardiac α-actin. Of the 20 control samples of blood, the immunoblot results were negative for α-actin in 19. Of the 30 patients with skeletal muscle damage caused by surgery, 27 were negative for circulating α-actin. Of the 29 patients with angina pectoris, circulating α-actin was found in 19 as a 43 kDa band in immunoblots. Of the four patients with anterior acute myocardial infarction, mean concentration of circulating α-actin was 38 mg/l. Among the patients with angina pectoris, the highest circulating concentrations (mean 40 mg/l) was found in those with prolonged angina (class III B, according to Braunwald's classification). In the entire group of individuals with angina pectoris α-actin was detectable in serum for up to 175 h after the onset of pain, and showed two peaks, one at 1 h (112 mg/l) and one at 50 h (82 mg/l) after the onset of pain. These findings reinforce the notion that unstable angina should be considered a serious condition.

KEY WORDS: Angina pectoris; α-Actin; Immunoblotting.

Introduction

The introduction by Towbin et al. [1] of the immunoblotting technique—involving electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets for the subsequent demonstration of antibody binding—was a major contribution to the techniques available to identify and characterize proteins in complex mixtures [2]. The last 15 years have seen the development and application of Western blot techniques to the serological diagnosis of clinical entities involving immunocomplexes. This method is sensitive enough to detect amounts of protein as low as 0.1 to 1 ng [3, 4].

Efforts in recent years have been directed toward the search for a new, specific cardiac marker for the diagnosis of ischemic heart disease. Research on these new cardiac markers has centered on different contractile proteins in the serum of post-infarction patients. In 1978, Khaw et al. [5] used RIA to demonstrate an increase in circulating cardiac myosin light chain (CM-LC) after experimentally induced and pathologically confirmed acute myocardial infarction (AMI) in dogs. Traherne et al. [6] independently reported similar findings in man.

More recently, radioimmunoassay (RIA) has been used to demonstrate the presence of circulating cardiac tropomyosin [7], CM-LC [8] and troponin I [9] in patients with AMI. Enzyme-linked immunoassay (ELISA) has also been used to detect cardiac troponin T after AMI [10, 11].

In patients diagnosed with angina pectoris (AP), studies of the circulating levels of contractile proteins have shown no increase in CM-LC in patients with stable angina, but have detected increases in patients with un-
stable angina [8]. However, troponin I fails to surpass normal levels in patients with either type of angina pectoris. [9].

According to Korn et al. [12] and Aránegua et al. [13], actin, a molecule consisting of a single 43 kDa polypeptide, is one of the two largest proteins in striated muscle tissue, representing more than 20% of all cellular protein. Actin is one of the most important proteins in heart contraction, and is the major component of thin filaments.

**Material and Methods**

**Control group**

Control assays were performed in 20 serum samples from the Regional Granada Province Blood Bank: 16 (80%) men, 4 (20%) women, mean age 30 ± 7 years.

**Group with muscle damage**

Blood samples were collected from 30 patients with skeletal muscle damage caused by surgery: 21 men (70%), 9 women (30%), mean age 50 ± 20 years.

**Cardiac, skeletal and smooth muscle tissues**

Intraoperative biopsies of ventricular heart muscle from patients undergoing valvuloplasty, of the femoral biceps in patients undergoing general surgery and of chicken gizzard (Shaver Starcross 288) were washed in PBS, cut into pieces approximately 2mm in diameter and processed according to previously published methods [14]. One milliliter of buffer F [2 mmol/l Tris (Merck, Darmstadt, Germany), 0.2 mmol/l ATP (Sigma, St. Louis, MO, USA) and 0.2 mmol/l 2-mercaptoethanol (2-ME) (Biorad, Irvine, CA, USA), pH 8.0, containing 0.1% Triton X-100 (Sigma)] was added and the tissue was homogenized in a glass-glass manual homogenizer set in crushed ice (4°C) for 30 s at 60 strokes/min. Suspensions were centrifuged (Labofuge AE, Heraeus, German) for 5 min at 13,000 g in a microcentrifuge (BHG-Hermle, Gosheim, Germany) at room temperature. The Triton X-100-soluble fraction of the supernatant (cytoplasmic protein) was used.

Pellet fractions obtained by centrifuging the extracts (Triton X-100-insoluble fractions) were washed 5 times in 500 μl buffer F and centrifuged at room temperature for 5 min at 30,000 g. The supernatant was discarded and the residue resuspended in 500 μl diluted Laemmli's sample buffer [15] without bromophenol blue or 2 ME, and heated to 100°C for 5 min. Finally, the mixture was centrifuged at 13,000 g for 5 min to obtain the SDS-soluble protein (cytoplasmic) fraction.

Protein content in the cellular subfractions was determined in 100 μl aliquots by the methods of Lowry et al. [16].

**Patients**

We studied patients with angina pectoris; myocardial necrosis was ruled out by the finding of normal creatine kinase (CK) and lactate dehydrogenase (LDH) values, and the absence Q-waves in the electrocardiographic analysis. Of the 29 patients who participated (22 men, 7 women, mean age 58 ± 20 years), the 5 who had post-infarction angina were analyzed separately (group III; Table I).

The 24 patients who satisfied the above-

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number</th>
<th>Type of ischemic heart disease</th>
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<tbody>
<tr>
<td>Group I</td>
<td>22</td>
<td>Class III B</td>
</tr>
<tr>
<td>Group II</td>
<td>2</td>
<td>Class I A</td>
</tr>
<tr>
<td>Group III</td>
<td>5</td>
<td>Post-inf. angina*</td>
</tr>
<tr>
<td>Group IV</td>
<td>4</td>
<td>Anterior AMI*</td>
</tr>
</tbody>
</table>

* Post-inf. angina = postinfarction angina; Anterior AMI = acute anterior myocardial infarction.

Class III B and I A patients with unstable angina pectoris according to Braunwald's classification.
mentioned electrocardiographic and analytical criteria were classified into two groups according to Braunwald's system [17] (Table 1).

Group I consisted of 22 class III B patients (Braunwald) with prolonged angina in the last 48 h.

Group II consisted of 2 class I A patients with secondary unstable angina.

Blood samples (4 ml) were obtained every 4 h on the first day, and once daily until day 10 after the onset of pain. Of the 29 patients in the study, the Killip score [18] was 1 in 21 (72.4%), Killip 2 in 5 (17.2%) and Killip 3 in 3 patients (10.3%). Twenty-three patients (80%) had a Forrester score [19] of 1 and 6 (20%) had a Forrester score of 2. Eighteen of the 29 patients were smokers, 7 had hypercholesterolemia, 6 had diabetes, 9 had hypertension and 14 had a previous history of some type of cardiac ischemia. Complications (cardiac insufficiency, acute pulmonary edema, angina or death) occurred in 10 of the 29 patients.

**Patients with AMI**

Blood samples were collected from four patients diagnosed on the basis of electrocardiographic and laboratory criteria as having suffered acute anterior myocardial infarction (group IV, Table 1).

All blood samples were centrifuged at 500 g for 5 min to obtain approximately 1 ml of serum, to which was added 15 μl thimerosal to prevent contamination. The serum samples were frozen at −20°C in aliquots of 500 μl until analysis.

**SDS-PAGE**

Cytoplasmic and cytoskeletal fractions of cardiac, skeletal and smooth muscle, and all serum samples from all patient and control groups were resuspended in Laemmli's sample buffer [15] and analyzed by SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) in a Mini Protean II cell (Biorad) at 60 mA for 1 h at room temperature. The gels with samples of serum from all groups were run in duplicate. One gel from each sample was used for densitometric analysis, and the other for immunoblot analysis. The gels for densitometric analysis were stained with Coomassie brilliant blue R-250 and destained according to Laemmli et al. [15] and Leiva et al. [20] (all reagents Biorad). The gels were dried on filter paper (Whatman, Maldstone, England) with a Biorad 583 drier.

**Immunoblotting**

The gels for immunoblot analyses were separated electrophoretically and transferred to nitrocellulose paper by applying a current of 30 V at room temperature for 12 h. The blots were treated with a blocking solution (20 mM Tris, 0.9% NaCl, 10% non-fat milk) and then reacted with a 1:200 dilution of anti-α-sarco-meric actin monoclonal antibody (clone 5C5, Sigma). To visualize specific antigen-antibody reactions, a commercial enzyme immunoassay kit and protocol were used (Biorad Immunoblot AGR-HRP Assay Kit).

**Densitometric analysis**

Once dry, the gels were laid in plastic film, and the bands in SDS-PAGE were analyzed with a densitometer (Beckman Appraise, Brea, CA, USA (slit width 4 mm, wavelength 540 nm)) with the help of an integral computing device. The protein bands revealed by immunoblotting were also quantified densitometrically, to derive the standard curve (Fig. 1). The amount of α-actin in each SDS-PAGE band (in mg) was plotted on the ordinate, and

![FIGURE 1. Standard curve obtained from the amount of α-actin in 12% SDS-PAGE (mg/l) and percentage of densitometric value of immunoblotting results. Ordinate, amount of α-actin in each SDS-PAGE band (in mg); abscissa, percentage densitometric value yielded by the corresponding band obtained by immunoblotting. All data are means of four measurements. The analytical sensitivity (detection limit) in our test system was 1.3 mg/l.](image)
the percentage densitometric value yielded by the corresponding band obtained by immunoblotting was plotted on the abscissa.

Statistical analysis

Analysis of variance (ANOVA model I) was used for the amounts of $\alpha$-actin obtained at different times, and Student's $t$ test was used to compare the concentrations of $\alpha$-actin in patients with different types of AP, in patients with and without specific antecedents, and in patients whose clinical course was complicated or uncomplicated.

Results

Of the 20 control samples, 19 were negative for $\alpha$-actin, and only one produced a single, faint band at the 43 kDa level in the immunoblotting assay.

Of the 30 patients with skeletal muscle damage, 27 were negative for $\alpha$-actin, and a faint band appeared in the three remaining samples.

Immunoblotting of the cytoplasmic and cytoskeletal fractions of cardiac and skeletal muscle was positive, yielding a 43 kDa band in both subfractions. However, both subfractions of smooth muscle were negative (Fig. 2).

Immunoblotting assay detected circulating $\alpha$-actin as a single 43 kDa band in 19 (66%) of the 29 patients with angina (Fig. 3). Of the positive cases, 14 were patients with prolonged angina in the last 48 h (class III B of Braunwald's classification). Serum from all five patients with post-infarction angina (group III) was positive for $\alpha$-actin, whereas this protein was not detected in serum from any of the patients with secondary unstable angina (class I A of Braunwald's classification) (Table 2).

Mean $\alpha$-actin levels in the group with angina pectoris was 26 mg/l ($9.76% \pm 11.73\%$ of the densitometric value), with a range of 2 (2.1%)–112.6 mg/l (45.6%). The value of circulating $\alpha$-actin in the positive control patients was 1.3 mg/l (1%). In the 30 patients with skeletal muscle damage, $\alpha$-actin was elevated in 3 (range 2.5–4.1 mg/l), mean 3.2 mg/l.

![FIGURE 2. Immunoblotting assay of cardiac, skeletal and smooth muscle with anti $\alpha$-sarcomeric actin clone 5c5 Sigma). Lane 1, molecular weight control; lane 2, positive immunoblotting in the cytoskeletal fraction of heart muscle; lane 3, positive immunoblotting in the cytoplasmic fraction of cardiac muscle; lane 4, positive immunoblotting in the cytoskeletal fraction of skeletal muscle; lane 5, positive immunoblotting in the cytoplasmic fraction of skeletal muscle; lanes 6 and 7, negative immunoblotting in cytoplasmic and cytoskeletal fractions of smooth muscle.](image-url)
FIGURE 3. Detection of serum α-actin in different types of angina pectoris. Lane 1, molecular weight control; lane 2, positive immunoblotting assay with serum from a patient with prolonged angina (class III B in Braunwald's classification) 1 h after the onset of pain. The anti-sarcometric α-actin mAb detected a single band at 43 kDa. Lane 3, positive immunoblotting assay with serum from a patient with prolonged angina 50 h after the onset of pain; lane 4, positive immunoblotting assay with serum from a patient with post-infarction angina; lane 5, negative immunoblotting assay with serum from a patient with hemodynamic angina (class I A in Braunwald's classification); lane 6, positive immunoblotting assay with serum from a patient with anterior AMI.

TABLE 2. Circulating cardiac α-actin in sera of various patients

<table>
<thead>
<tr>
<th>Group</th>
<th>Positive</th>
<th>Negative</th>
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<tbody>
<tr>
<td>Group I</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>Group II</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Group III</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Group IV</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

In this group, serum CK activity, measured after incubation at 37°C, ranged from 160 to 5850 IU/l, with a mean value of 1931 IU/l, which was 10-fold greater than the upper limit of normality (normal range 24–195 IU/l).

Circulating α-actin was detected by immunoblotting in all four of the patients with anterior acute myocardial infarction (Table 2) with a band at the 43 kDa level (Fig. 2). The mean concentration of α-actin in this group was 58 mg/l (29.13%), with a range of 10 mg/l (1.3% of the densitometric value) to 122 mg/l (51%).

Mean circulating values of α-actin were 40 mg/l (15.28%) in patients with prolonged angina (class II B), and 36 mg/l (13.98%) in patients with post-infarction angina.

Our immunoblot data for cardiac α-actin in patients with angina pectoris suggest that this protein is released for as long as 175 h after the onset of pain, during which time two peaks in circulating levels were detectable. The first peak was recorded 1 h post-episode...
(112 mg/l), and the second peak at 50 h after the onset of pain (82 mg/l) (Fig. 4). Thereafter, α-actin decreased slowly over the following 125 h, returning to normal levels at approximately 175 h post-episode. Analysis of variance showed the changes in α-actin values at different times post-episode to be significant (significance level 0.93). That is, the amounts of α-actin detected at different times were significantly related with the time elapsed since the onset of pain.

Contrast of the means (95% confidence limit) showed no significant differences in the mean serum values of α-actin between the different types of angina pectoris, between smokers and non-smokers, or between patients with antecedents of hypertension, hypercholesterolemia, diabetes or previous ischemic cardiopathy. Mean concentration of circulating α-actin in patients with an uneventful post-episode course and with complications did not differ significantly.

Discussion

In this century, the clinical pathologic features of two of the principal manifestations of ischemic heart disease – acute myocardial infarction and chronic stable angina – have been well described. It has taken much longer to define a syndrome that is intermediate in severity between these two conditions. Patients with neither acute myocardial infarction nor stable angina are difficult to classify, because of the heterogeneous nature of the syndrome and the lack of agreement as to its precise definition. This syndrome is usually diagnosed retrospectively in patients with prolonged ischemia or acute myocardial infarction, especially when electrocardiographic and analytical data (serum enzymes) are not compatible with the suspicion of AMI [17].

Because contractile proteins are released in myocardial cell injury [7–11] we suspected that circulating levels of cardiac α-actin might also be detectable in patients with angina pectoris. This notion was suggested by immunological and morphological studies showing that the actin protein subunit is present in the cytoskeleton and cytoplasm of muscle and cardiac cells [13, 21].

According to our findings, myocardial cell injury occurs in angina pectoris, and leads to the release of α-actin. When heart muscle cells are subjected to anoxia or ischemia, they release an intracytoplasmic material secondary to the primary cell membrane lesion [22–24]; 24 h after lesioning; the striated bands of affected myofibrils become undetectable [25].

Among patients with prolonged angina (class III B) or post-infarction angina 63.6% and 100%, respectively, were positive for circulating α-actin. These findings are compatible with data published by Katus et al. [8]; who reported significant increases in serum levels of the contractile protein CM-LC in one out of 15 patients diagnosed with stable angina, and in 8 of 15 with unstable angina. Cummins et al. [9] detected slight increases in cardiac troponin I over minimum detectable levels in 21 patients with stable or unstable angina.

In our control group, circulating α-actin was weakly detected in only one subject out of 20 (1.3 mg/l); this value was far below the lowest concentration of circulating α-actin found in patients with angina pectoris (26 mg/l). Of the 30 patients with skeletal muscle damage, circulating α-actin was weakly detected in only three subjects (mean 3.2 mg/l), a finding comparable to that of Katus et al. [10], who used mAb to test 24 patients with skeletal muscle damage, and found positive reactions in only four.

The assay developed in this study is an informative source of data on myocardial cell suffering in patients with different types of angina pectoris. The highest circulating levels of α-actin (40 mg/l) were found in prolonged
angina (class III B). However, in patients
with myocardial necrosis (anterior AMI), the
mean concentration of circulating α-actin
(58 mg/l) was much higher.

The two peaks in circulating α-actin in our
group of patients with angina pectoris are
consonant with previous findings for CM-LC
in patients with AMI. According to Katus et
al. [8], the initial appearance of CM-LC in
plasma reflects the loss of the soluble pool,
whereas the persistence of elevated plasma
concentration probably reflects the break-
down of myosin myofilaments. This notion
may account for the two successive peaks we
recorded in serum levels of α-actin.

These results suggest that in patients with
angina pectoris, the presence of circulating α-
actin may well reflect a process of myocardial
cell injury. The peak values of circulating α-
actin, and the prolonged period during which
this protein can be detected (up to 175 h) are
findings of interest that reinforce the concept
of unstable angina pectoris as a severe entity.

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