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Immunohistochemical reaction of myocardial fibers with actin antiserum in autopsy cases of myocardial infarction

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Summary. The purpose of this study was to determine the immunoreactivity of myocardial actin filaments with actin antiserum and to examine the significance of its application to diseased human cardiac muscle.

The actin was extracted and purified from chicken gizzards. Anti-actin rabbit serum was prepared and purified by affinity chromatography and defined by an immunoblotting test.

Using the avidin-biotin-peroxidase complex (ABC) method, the actin antiserum was applied to paraffin sections prepared from hearts taken from routine autopsies of patients who had died of myocardial infarction.

Reactivity was shown to be completely lost, not only in necrotized fibers, but also in non-specific degenerative fibers which could be identified by their eosinophilic cytoplasm with pyknotic nuclei, and clearly remaining and/or diminished cross-striations stained with hematoxylin-eosin. In contrast, hypertrophic myocardial fibers adjacent to granulation or scar tissue and those adjacent to infarcted foci exhibited a more intense reaction.

These results indicated that the immunohistochemical reaction of actin filaments can be used for the easy detection of very mild degrees of degeneration of cardial muscle fibers, and for hypertrophic fibers adjacent to diseased foci.

Studies of the immunoreactivity of actin protein suggestive of alteration at the molecular level might yield morphological clues regarding the nature of functional activity in the contraction of cardiac fibers.

Key words: Myocardial fibers-Degeneration-Ischemia-Actin-Immunohistochemistry

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Introduction

Since immunoreactivity depends on the antigenic determinant sites on an antigen molecule, disintegration or chemical alteration of such molecules may be reflected by changes in their immunoreactivity. We therefore postulate that even very mild changes in myocardial fibers might be revealed by changes in their immunoreactivity.

This report concerns the immunohistochemical reaction of myocardial fibers with actin antiserum in autopsy cases diagnosed as myocardial infarction, with specific reference to very mildly degenerated eosinophilic fibers recognized by routine hematoxylin-eosin (H-E) staining.

Materials and methods

The subjects were 16 autopsied cases diagnosed as myocardial infarction. A description of the cases is given in Table 1. The patients had died between 2 hours and 30 days after clinical onset; all autopsies were performed about 1 to 2 hours after death.

Specimens were routinely taken from the hearts and fixed in 10% neutral formalin followed by paraffin embedding. For the purpose of exact histological comparison between cardiac fibers stained by routine methods and fibers reacted with actin antiserum, serial or semi-serial sections were stained with H-E, Azan-Mallory (Azan), acid fuchsin (Poley et al., 1964), hematoxylin-basic fuchsin-picric acid (HBFP) (Lie et al., 1971; Nayar et al., 1974), and the avidin-biotin-peroxidase complex (ABC) method for actin antiserum.

Heart slices from three cases (Case Nos. AN - 1561, 1603 and 1617) were stained by the triphenyltetrazolium chloride (TTC) reaction (Lie et al., 1957; Sandritter and Jestadt, 1958; Jestadt and Sandritter, 1959). After staining and photography, the sections obtained were stained with actin antiserum using the ABC method, together with

H-E, Azan, acid fuchsin and HBFP. In order to compare the extent of loss of the TTC reaction with the diminution of the actin antiserum reaction, areas giving a reaction in sections with actin antiserum were plotted on the photographs of the slices stained by the TTC reaction.

Irnrnunohistochernical study

a) Preparation of anti-actin antibody.

Actin was prepared from fresh chicken gizzard muscle according to the method described by Suzuki et al. (1978), followed by actin purification (MW 42 K dalton) according to Spudich and Watt (1971). The actin preparations were confirmed to be pure by sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis. The actin band in the polyacrylamide gel was used as the antigen. Rabbits were immunized by injecting them intracutaneously 3 times a week with 500 μg of actin conjugated with Freund's complete adjuvant. Blood was then collected from the rabbits 10 days after the last injection and the serum was clarified by centrifugation at 10,000 rpm. The gamma-globulin fraction was purified twice using precipitation with 40% saturated ammonium sulfate, followed by dialysis into 0.01 M phosphate buffer solution (PBS) (pH 7.4). The pure actin antiserum was prepared by affinity chromatography using purified actin bound to CNBr-activated sepharose 4B. These mono-specific antibodies were then checked by an immunoblotting test to ensure that they reacted only with the pure actin. The actin antiserum was diluted 1:100 in PBS for use.

b) Routinely prepared 5 nm thick paraffin sections of the hearts, including the infarction and the adjacent regions, were deparaffinized and immersed in methanol containing 0.03% H_2O_2 to inhibit endogenous peroxidase activity.

The sections were dehydrated with graded alcohols and equilibrated with buffered saline (pH 7.4).

In order to visualize the binding of actin antiserum to the tissue section, the avidin-biotin-peroxidase complex method (Hsu et al., 1981) was performed.

In order to estimate the effect of autolysis on the reactivity of cardiac fibers with the actin antiserum, 5 autopsy cases which were from the Osaka Medical Examiner's Office, and dead from causes other than heart disease, were examined. They had been autopsied at 4, 10 in 2 cases, 21 and 28 hours after death. The staining characteristics of the sections obtained from these cases were compared, and they did not show an intensity dependent on the length of time after death before 28 hours.

As a control reaction, normal rabbit serum (NRS) was applied to all tissue sections.

Results

Gross findings

According to the comparison of gross findings of TTC reactions with actin antiserum, the extent of loss of the actin reaction was larger than the area of loss of the TTC reaction (Fig. 1). This disparity between the extents of loss of the TTC and actin reactions was more pronounced in fresh cases of infarction.

Microscopic findings

The sections of the myocardium stained with H-E, Azan for scar tissue, acid fuchsin, and HBFP revealed various findings. Predominantly degenerative myocardial fibers had eosinophilic cytoplasm with pyknotic nuclei in H-E-stained preparations. Cross-striations of the eosinophilic fibers showed various degrees of preservation or homogenization. Completely necrotized fibers had lost their nuclei.

These degrees of change were also very easily identified by both acid fuchsin and HBFP staining. The degenerative and necrotized fibers including those with contraction band necrosis (Bulkley and Hutchins, 1977) showed very intensive "fuchsinophilia" (Poley et al., 1964) and "fuchsinorrhagia" (Lie et al., 1971).

As for the reaction of actin antiserum on the sections, the smooth muscle cells of the vascular walls in the myocardium were the most significantly stained, appearing intensely brown (Fig. 2). Myocardial fibers were pale brown to dark brown and fairly diffusely stained with remarkable striations in the intact areas. Intercalated discs showed a more remarkable staining reaction, but to a lesser degree than that in the vascular smooth muscle cells. The degree of intensity of the reaction with myocardial fibers was not dependent on the time of the post-mortem before 28 hours (Fig. 2). However, the reactivity of the actin antiserum on muscle fibers which showed necrosis was completely lost (Fig. 3). Apparently focal intact muscle fibers within the necrotic foci which were infiltrated by neutrophils in H-E preparations were clearly visible by their intense dark brown positive reaction with actin antiserum (Fig. 3).

Loss of reactivity was also revealed in the degenerative muscle fibers which contained eosinophilic cytoplasm and slightly pyknotic nuclei (Fig. 4). The various degrees of eosinophilia shown by H-E in the cytoplasm included not only homogeneous fibers showing diminution of striations in muscle fibers but also one of slight eosinophilia with clear striations (Figs. 5, 6). This was identical with the "fuchsinophilia" and the "fuchsinorrhagia" described above. The latter was positive in completely necrotized fibers, while the former was positive in degenerative fibers including necrotized fibers.

Another finding in the preparation reacted with actin antiserum was that there was a very strong positive reaction of material deposited in basophilic degeneration of the cardiac fibers (Figs. 5, 6).

The muscle fibers adjacent to granulation or scar tissue, and to completely infarcted foci, revealed a dark brown reaction, more significant to, that in more distant areas, but the eosinophilic fibers in these areas did not show reactivity with actin antiserum (Fig. 7).

Control staining reactions to NRS applied to all the preparations in this series were completely negative.

Table 1. Clinical description of the cases.

time course after the episode	Case No.	post mortem	age. sex	location of the infarction	scar	past history
2 hours (h)	AN-1423	and 35 m	70 m	antero-lateral and septum	(+) anterior	hypertension (HT)
4 h and 46 minutes (m)	AN-1393	1 h and 41 m	57 f	mid-anterior	antero-septal	[illegible]
8 h	AN-1374	1 h and 51 m	71 m	lateral	(−)	(−)
11 h	AN-1436	1 h and 15 m	81 f	antero-septal, rupture	(−)	HT cholelithiasis
18 h and 40 m	AN-1375	1 h and 10 m	80 m	anterior and septum	(+) anterior	gastric ulcer pneumonia
1 day (d)	AN-1433	1 h and 30 m	83 m	posterior and right wall	(+) anterior	HT
1 d	AN-1468	1 h and 11 m	72 f	anterior, rupture	lateral	HT chronic rheumatoid arthritis
1 d	AN-1561	2 h and 9 m	74 f	antero-septal	(−)	HT
2 d	AN-1522	2 h and 22 m	67 f	subendocardial circumference and partial transmural	(−)	DM HT angina pectoris
3 d	AN-1109	57 m	85 f	posterior	(−)	asthma bronchiale Parkinson disease
4 d	AN-1617	2 h and 32 m	61 f	anterior, lateral, septum and rupture	(+) posterior	(−)
5 d	AN-1543	1 h and 55 m	66 f	septum and circumference	(−)	rnyorna uteri DM HT cerebral thrombosis
6 d	AN-1238	1 h and 38 m	67 m	lateral	(−)	cerebral apoplexy
19 d	AN-1603	1 h and 7 m	88 f	posterior	(+) posterior and septum	HT
24 d	AN-1537	1 h and 55 m	54 m	postero-lateral and septum	(−)	tuberculosis
30 d	AN-1396	2 h and 6 m	49 f	antero-septal and posterior	(+) anterior	HT hyperlipemia

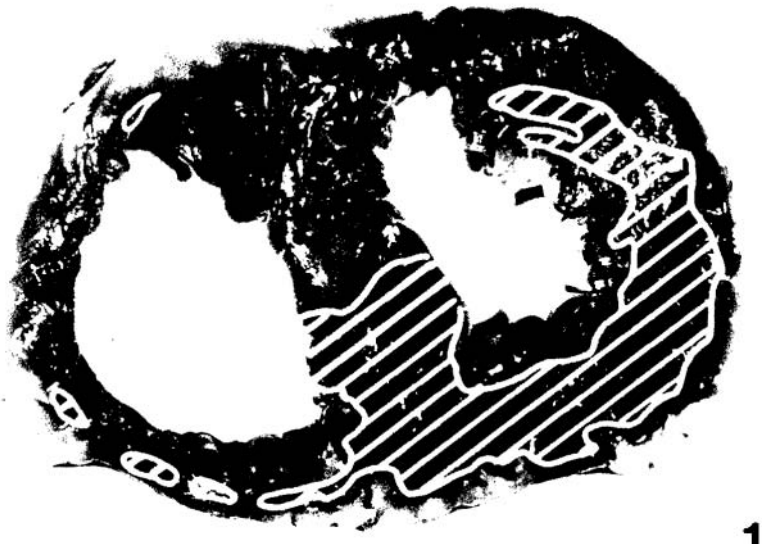

Fig. 1.' Extent of loss of reaction with actin antiserum is indicated by unshaded areas. (The shading indicates areas of positive reaction). The dark area of the myocardium is positive and the pale area shows loss of reaction with TTC. Note that extent of loss of actin reaction is larger than area of loss of TTC reaction (Case No. 1561i.

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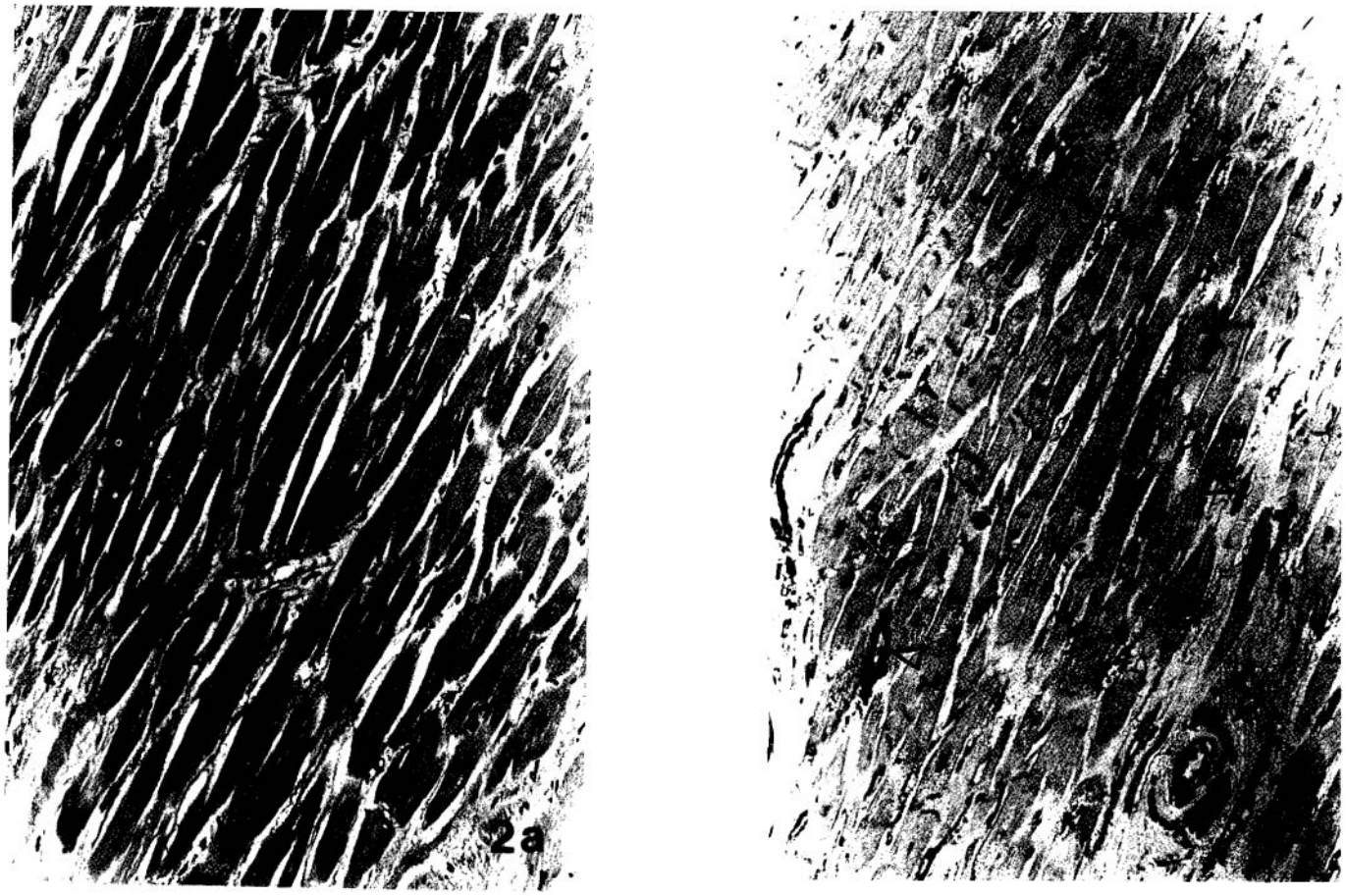


Fig. 2. Effect of post-mortem time on reactivity with actin antiserum. Myocardium from an autopsy case, *28* hours after death due to a cause other than myocardial infarction. a. H-E stain. x *200* b. Actin antiserum reaction of a section, almost corresponding to H-E preparation. Vascular smooth muscle is intensely positive (arrowheads). Intercalated discs (arrows) are clearly visible, but the cytoplasm shows significant reduction in the reactions for this post-mortem time. x *200*

Fig. 3. **a.** H-E stain of a focus of myocardial infarction with hemorrhage and diffuse neutrophil infiltration (Case No. 1375). b. The corresponding section reacted with actin antiserum, showing complete loss of reaction on the necrotic fibers (N). However, in the center of the field, intense reactivity is clearly visible on the intact fibers even in the focus of infarction.

Fig. **4.** a. Degenerative cardiac muscle fiber (arrows) showing eosinophilic cytoplasm with slightly pyknotic nuclei by H-E stain (Case No. 1325). x 200 **b.** The corresponding section reacted with actin antiserum, showing complete loss of reaction on degenerative fibers (arrows) and in area (d). Smooth muscle of the blood vessels is intensely positive (arrowheads). x 200

Fig. 5. **a.** Eosinophilic degenerative cardiac fibers with clearly preserved cross-striations and basophilic deposited material (arrow) in one of the fibers in an H-E preparation (Case No. 1109). x 400 **b.** Actin antiserum reaction on the corresponding section. The fibers show almost no reaction, but very strong reactivity is visible on the deposited material (arrow). x 400

Fig. **6.** a. An area other than one of infarction with eosinophilic degenerative fibers (arrowheads) and basophilic material (arrow) in one of the fibers (H-E, Case No. 1396). x 200 b. The corresponding section reacted with actin antiserum. Complete loss of reaction on the eosinophilic fibers (arrowheads) and an intense positive reaction (arrow) on the deposited material are observed.

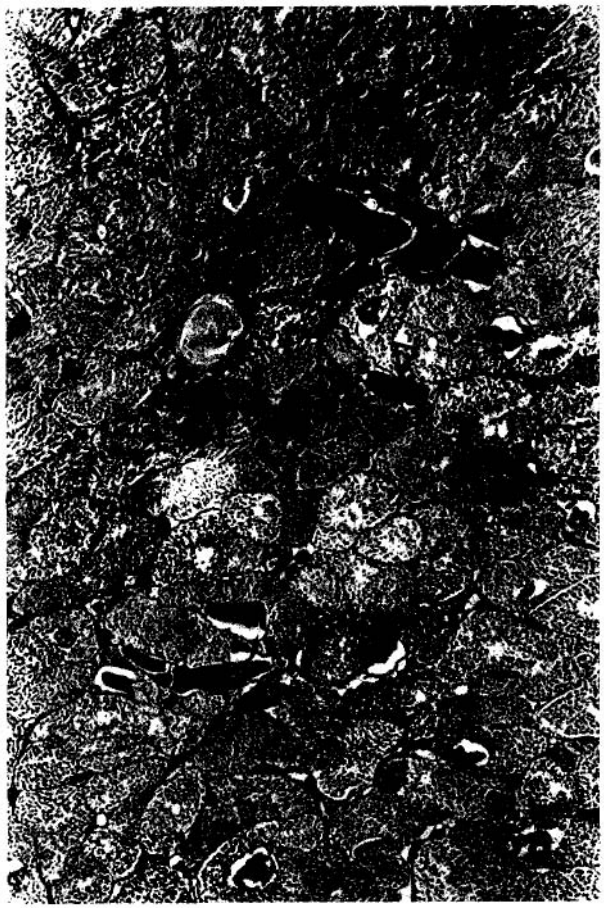
Fig. **7.** a. Muscle fibers adjacent to the scar, some appearing intact and others hypertrophied. However, eosinophilic degenerative changes can be identified in this field by the darkness of the cytoplasm with pyknotic nuclei (H-E, Case No. 1396). x 200 b. The corresponding section reacted with antiserum, revealing a more significant intense reaction (arrow) on the fibers adjacent to the scar, although eosinophilic fibers in the area have also lost their reaction (L).


4a


4b


5a


5b

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Discussion

At least six kinds of actin, according to differences in their amino-terminals, exist in higher animals. Among these actins the differences in the amino acid sequences are very minor. Four of the total 368 amino acid residues account for the differences between skeletal and cardiac actin, and 8 residues are responsible for the difference between skeletal alpha-actin and the smooth muscle gamma-actin of chicken gizzard. From the amino acid substitution pattern, actin divergence involves tissue rather than species specificity among higher vertebrates, and smooth muscle actin is very similar to non-muscle cell actin. Cardiac muscle actin lies between skeletal and smooth muscle actin in its amino acid substitution pattern (Vandekerckhove and Weber, 1979).

Antigenic differences in actins between cardiac muscle and skeletal muscle have also been examined (Morgan et al., 1980). Substitutions in only a few residues of amino acid-sequence data may alter the antigenic properties.

In the present report, taking into consideration these previous findings on the molecular structure and antigenicity of actins involving wide similarities among tissues, and not involving animal species specificity, antiserum from rabbit, immunized by smooth muscle actin extracted from chicken gizzards and prepared and purified in our laboratory, was applied to tissue samples of human myocardium.

Several pilot studies were first conducted on routine autopsy preparations embedded in paraffin which had been taken from both fresh and old infarcted tissues, and on the effect of time after death. Their appearance was not dependent on the post-mortem time before 28 hours, so that the preparations showed constant, diffuse and reproducible patterns of reaction to the actin antiserum. We therefore considered that, as far as could be determined by comparison between average normal and diseased cells in the same tissue preparation, the actin antiserum reaction with myocardium tissue by the ABC method was reasonably reliable.

Although there have been many fundamental studies conducted on actin, reports of its application to pathomorphological research on various human and experimental diseases are relatively scarce.

As for the heart, Moalic et al. (1984) described that overloading of rat hearts increased the rate of actin and myosin synthesis. Harmjanz and Reale (1981) reported an increase of actin filaments in human hypertrophic cardiomyopathy, and Chuaqui and Garrido (1982) examined actin and myosin in hypertrophied human myocardium.

In order to examine cytoplasmic actin from a morphological point of view, the heavy mero-myosin method (Ishikawa et al., 1969) involving electron microscopy and the immunofluorescence of FITC or rhodamine-phalloidin (Andrews and Bates, 1984) is commonly utilized. Immunofluorescence is the most sensitive method of light microscopic detection. Even electron microscopy without any specific method has been utilized for the quantitative estimation of actin filaments. Gulbenkian (1985) recently utilized an immunogold staining technique.

In the present study, however, we applied the ABC method for revealing the actin antiserum reaction on the myocardial fibers and took advantage of the easy identification of the cells under light microscopy using permanent preparations and the possibility of subsequent electron microscopic examination.

Specific attention was paid to the reactivity of the myocardial fibers with the actin antiserum in relation to the staining characteristics of the fibers following routine staining.

The tissues used were from cases in which the age of the infarction ranged from 2 hours to 30 days. However, the reactivity of the antiserum was characteristic, being dependent not on the age of the infarction, but on the staining features of the individual cardiac fibers.

One of the distinct findings was that basophilic degeneration of the fibers produced a very strong reaction to the actin antiserum. Although such degeneration is not a specific one for ischemic change, several points concerning it have been mentioned in the literature (Gregory et al., 1982; Kawamoto and Wakabayashi, 1983). Roy (1975) described a filamentous material which was different from contractile filaments in its accumulation of glycoprotein. However, the possibility was not denied that the material was a thread-like polymer produced by aberration in the synthesis of actin and myosin filaments. From our results showing a strong positive reaction to actin antiserum, it was clarified that actin material was present in the basophilic degenerative material.

Necrotic fibers and ischemic changes in cardiac fibers which can be detected in the very early stage of infarction are well described in many papers and textbooks. As for immunohistochemical methods among these, myoglobin has been morphologically investigated by indirect immunofluorescence (Kent, 1982). However, as actin antiserum is directly related to the contraction protein, it might be effectively used to detect earlier stages of ischemia. The present authors have some experimental data to suggest this, although it has not yet been published.

The most important finding to be emphasized in this study is the complete disappearance of reaction to actin antiserum not only in completely necrotized muscle fibers in the infarction, but also even in fibers showing eosinophilic cytoplasm retaining cross-striations with very slightly pyknotic nuclei.

In the present study, hypertrophic muscle fibers adjacent to granulation and scar tissue showed a more intense positive reaction to the antiserum. This may reflect the increase of actin fibers in overcontracting and hypertrophied muscle fibers (Harmjanz and Reale, 1981).

On the other hand, the complete loss of reactivity to actin antiserum of muscle fibers may reflect complete disappearance or alteration at a molecular level, such as those of the amino acid residues of the actin filaments.

Although eosinophilic degenerative fibers are not pathognomonic, it is necessary to clarify their morphological details and functional condition in considering the cause of death not only in cases of heart disease but also in other kinds of disease.

The reactivities of actin protein with actin antiserum

presented in this report are more directly suggestive of the functional condition of the cardiac fibers than other morphological methods which have been reported in the literature. A chronological experimental study involving functional data should be performed in order to clarify this.

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