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Preservation of Actin Immunoreactivity in Paraffin Section by Tannic Acid Fixation on Chick Embryonic Heart: Comparison with Phalloidin Staining on Frozen Section

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ABSTRACT

Degree of binding reactivity to actin in variously fixed tissues was histochemically examined by using phalloidin and antiactin staining on paraffin or frozen sections of 5-day chick embryonic heart. Fluorescein-labeled phalloidin staining on frozen sections of the specimens fixed with 4% formaldehyde showed intense staining of actin in the myocardium and the aorticopulmonary (AP) septum. However, phalloidin did not react to actin on paraffin sections of the specimens fixed with 4% formaldehyde, nor fixed with 4% formaldehyde and 1% tannic acid (TA). FITC-labeled antiactin staining on paraffin sections of the specimens fixed with 4% formaldehyde and 1% TA showed comparable fluorescent intensity with that of phalloidin staining on frozen sections. However antiactin staining on paraffin sections of the specimens fixed with 4% formaldehyde only showed reduction of fluorescent intensity around the Ap septum. Both phalloidin and antiactin react non-specifically to the tissues fixed with solutions including 1% glutaraldehyde. Since paraffin sections showed much better tissue preservation than frozen sections, we suggest that paraffin embedding with TA fixation is very useful for actin histochemistry using antiactin.

Actin is a component of thin filament, and actin filaments (F-actin) are the most important component of the cytoskeleton. For visualization of actin filaments in the cells, antiactin antibody labeled with fluorescent dye has been generally used. Antiactin, however, also bind globular actin (G-actin) that is a monomer of F-actin[1].

Phalloidin is an useful tool to visualize F-actin[18,20]. Phalloidin specifically binds F-actin and stabilizes F-actin. Phalloidin, however, does not bind G-actin[17]. Besides phalloidin has an advantage of better permeability than immunoglobulin because of its low molecular weight. However, frozen sections are necessary for phalloidin staining. Detection of F-actin by phalloidin is very difficult on paraffin sections (see our results).

We have reported organized F-actin bundles in the aortico-pulmonary (AP) septum of the chick and rat developing heart by using phalloidin staining on the frozen sections[13,14]. We have also tried antiactin staining of the AP septum on the paraffin sections. However F-actin images by antiactin staining of the AP septum on the paraffin sections are somewhat weak and obscure compared with that by phalloidin staining on the frozen sections (see our results).

In the past decade, the fixative including tannic acid (TA) has been used for electron microscopy of actin fibers[5,9]. TA preserves actin filaments and gives a high contrast staining of F-actin. TA has also been shown to have good ability to preserve F-actin in histochemical study[2].

Since paraffin embedding is better to preserve tissue structure and to keep tissue orientation than freezing method, paraffin embedding is better method for studying organ structure such as developing heart than freezing method. The purpose of this study is to test the actin preservation by TA for antiactin staining on the paraffin sections.

MATERIALS AND METHODS

White Leghorn chick embryos incubated for 5 days at 38°C in humidified atmosphere were used.

Immunofluorescent staining

The chick embryos were fixed in 4% formaldehyde only, or in 4% formaldehyde and 1% TA, or in 4% formaldehyde, 1% glutaraldehyde and 1% TA for 1 hr at room temperature. The fixative were dissolved in 0.1M phosphate buffer. The specimens were dehydrated in graded series of ethanol, and embedded in paraffin.

Deparafinized 6 μm sections on glass slides were incubated with 30 times diluted antiactin rabbit serum (purchased from Bio-Yeda, Israel) for one hour at room temperature. For control, non-immune rabbit IgG was employed. The sections were rinsed in phosphate buffered saline (PBS) for 15 min with at least three times changing of PBS. The sections were incubated with 40 times diluted FITC-labeled anti-rabbit IgG for 20 min at room temperature. After rinsing, the sections were coverslipped with 1:1 solution of glycerol and PBS.

Phalloidin staining

To compare with antiactin staining, we performed phalloidin staining. For phalloidin staining, embryos which were fixed in 4% formaldehyde with 0.1M phosphate buffer for 30 min at room temperature, were immersed in 30% sucrose solution in the same buffer for 10 min. The specimens were oriented in Tissue-Tek embedding compound in small alminum foil boats and rapidly frozen in liquid nitrogen.

About 8 μm frozen sections were incubated with 0.16 μg/ml of Fluorescein (F1)-labeled phalloidin (purchased from Molecular Probes Inc., USA) in PBS for 40 min. The sections which were preincubated with 5 μg/ml of non-labeled phalloidin (Sigma, USA) for 40 min, were used for control. After rinsing, the sections were covered as described above. In some cases, phalloidin staining were performed on the paraffin sections.

Histological examination

After fluorescent study, the coverslips were removed in PBS, and the sections were stained with Hematoxilin and Eosin (H.E.).

RESULTS

Results of antiactin staining on paraffin sections and phalloidin staining on frozen sections are shown in Table 1 and in Fig. 1. Tissues were histologically preserved better in paraffin section (Fig. 1 a,c,e) than in frozen sections (Fig. 1g). The myocardium surrounding the truncus (MC), the presumptive tunica media (TM), the cellular condensation within the Ap septum (arrowheads) and cushion mesenchymal cells (CM) were clearly distinguishable in paraffin sections. In contrast,

Table 1. Fluorescent intensity of FITC-antiactin and F1-phalloidin on various tissues in 5 day chick embryonic truncus

		Ap Septum	Myocarium	Cushion Tissue	Tunica Media
antiactin	Paraffin sections				
	4% Formaldehyde	+	+ + +	±	−
	4% Formaldehyde + 1% TA	+ +	+ + +	±	±
	4% Formaldehyde + 1% TA + 1% Glutaraldehyde	+ +	+ +	+ +	+ +
Phalloidin	4% Folmaldehyde	−	−	−	−
	4% Folmaldehyde + 1% TA	−	−	−	−
	4% Formaldehyde + 1% TA + 1% Glutaraldehyde	+ +	+ +	+ +	+ +
	Frozen Section	+ +	+ +	±	±

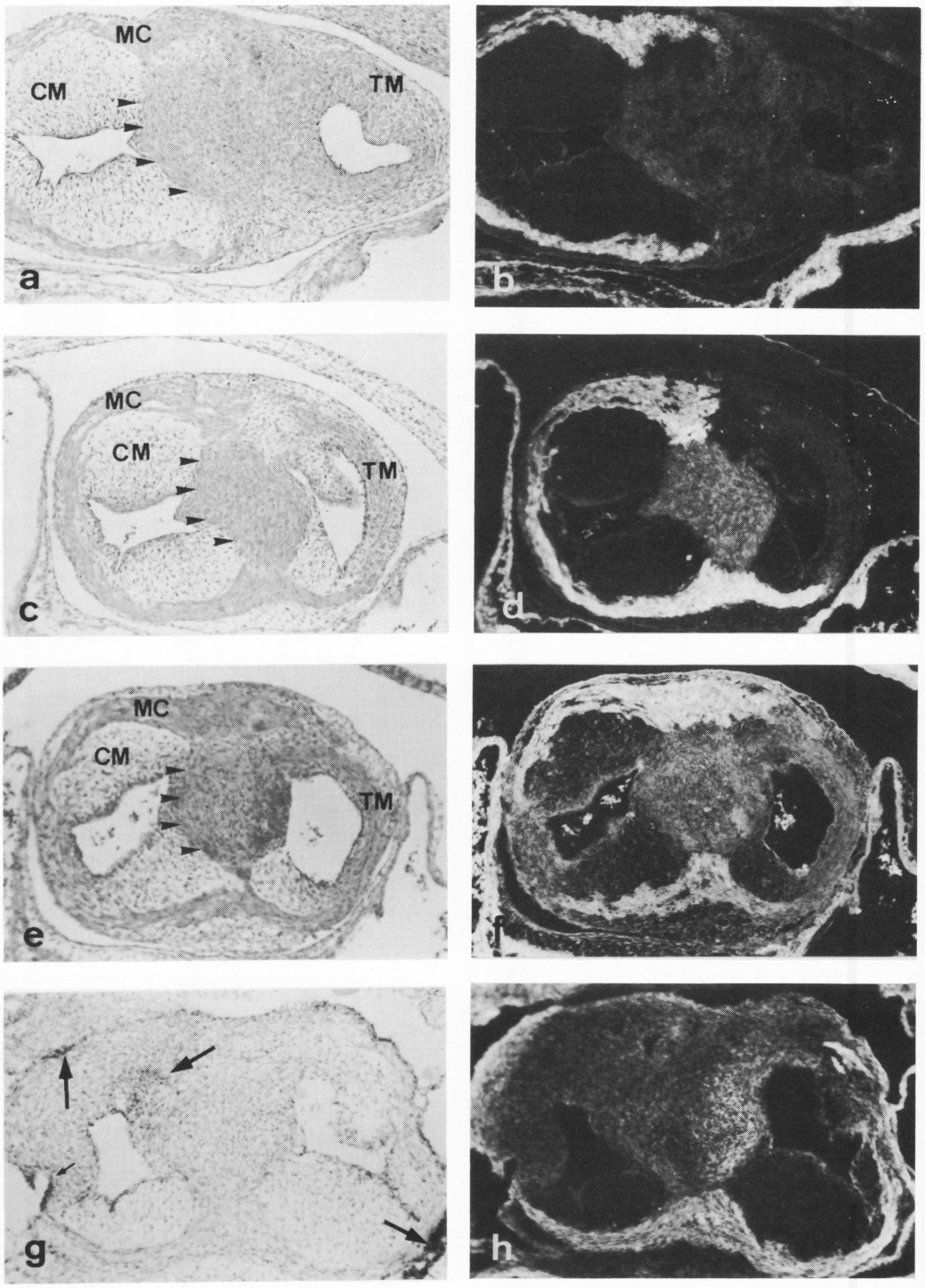

Fig. 1. Light micrographs and immunofluorescent micrographs of antiactin staining on paraffin sections of the specimens with 4% formaldehyde (a,b), 4% formaldehyde and 1% TA (c,d), 4% formaldehyde, 1% glutaraldehyde and 1% TA fixation (e,f), and light micrograph and fluorescent micrograph of phalloidin staining on frozen section (g,h). Arrowheads show outline of the cellular condensation of the Ap septum. Large arrows in (g) show uneven thickness of section, and small arrow shows artificial damage. CM: cushion mesenchymal cells MC: myocardium surrounding the truncus. TM: presumptive tunica media. × 200.

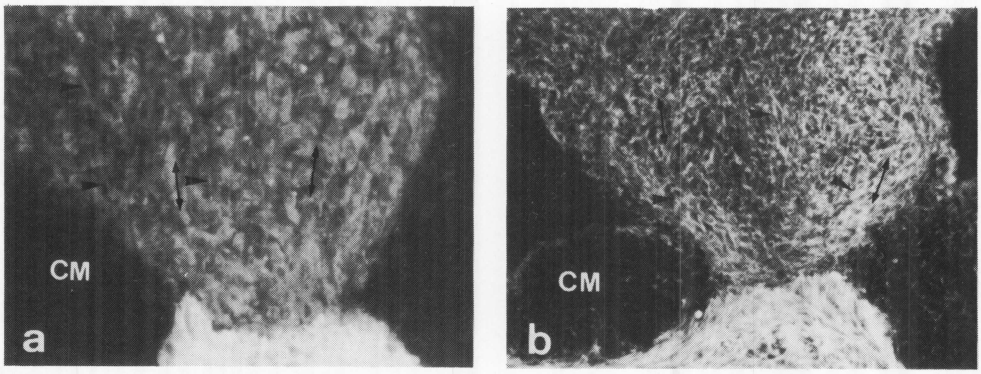

Fig. 2. Higher magnification of fluorescent micrographs of antiactin staining on paraffin section of the specimen with 4% formaldehyde and 1% TA fixation (a) and phalloidin staining on frozen section (b). Actin fibers (arrowheads) with consistent direction (double heads arrows) are clearly seen in (a) and (b). No such fiber is recognizable in other cushion mesenchymal cells (CM). × 450.

image of the tissue in frozen sections were obscure. Uneven thickness and artificial damage were seen in frozen sections (Fig. 1g). In fluorescent micrograph, the myocardium surrounding the truncus and the atrium were intensely stained in each sections. The actin in the Ap septum was well preserved in frozen sections (Table 1, Fig. 1h).

Fluorescent image of actin in the myocardium by antiactin staining on paraffin sections of the specimens fixed with 4% formaldehyde was comparable with phalloidin staining on frozen sections. However intensity in the Ap septum area in the paraffin sections (arrowheads) was reduced (Table 1, Fig. 1b) compared with that in the frozen sections.

Well preserved F-actin in the Ap septum was shown in specimens which was fixed with 4% formaldehyde and 1% TA (Table 1, Fig. 1d). Intensity around the Ap septum was much higher than that in the specimens fixed with 4% formaldehyde. In the specimens fixed with 4% formaldehyde, fluorescent image of actin was foggy around the cellular condensation of the Ap septum, but fluorescent image showed definite outline of the Ap septum in the specimens fixed with 4% formaldehyde and 1% TA. The cushion mesenchymal cells (CM in Fig. 1) were not stained in both cases. At high magnification, actin fibers were well preserved on paraffin sections of the specimens fixed with 4% formaldehyde and 1% TA (Fig. 2a). Actin bundles (arrowheads) with consistent direction (double-heads arrows) within the Ap septum were recognizable as shown by phalloidin staining on the frozen sections (Fig. 2b). No organized actin bundle was recognized in the other cushion mesenchymal cells (CM) in both stainings. Fluorescent images of actin by antiactin staining were more obscure than those by phalloidin staining, since this antibody binds G-actin well.

The specimens which fixed with 4% formaldehyde, 1% glutaraldehyde and 1% TA also showed strong fluorescence around the Ap septum (Fig. 1f), but cushion mesenchymal cells were also stained strongly (Table 1). In the control, employed non-immune rabbit IgG instead of antiactin and fixed with same solution, the

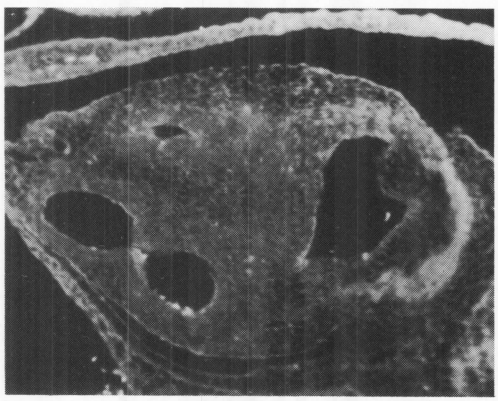

Fig. 3. Control micrograph of actin staining on paraffin section of the specimens with 4% formaldehyde, 1% glutaraldehyde and 1% TA fixation. This photograph shows non-specific staining. × 200.

fluorescence was ubiquitously distributed in the tissue, showing non-specific staining in the section (Table 1, Fig. 3). All of the control cases without glutaraldehyde fixation showed negligible fluorescent intensity.

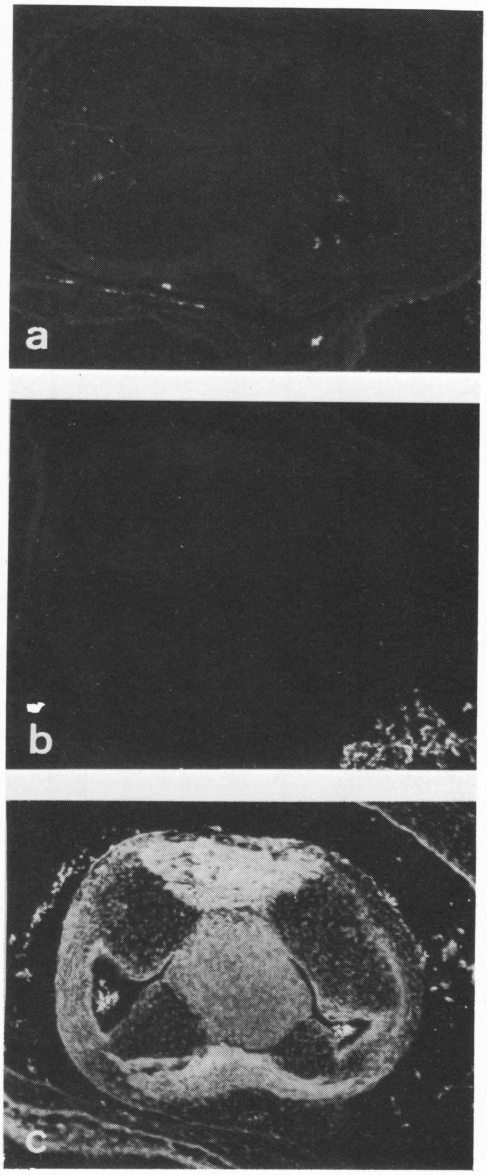

Fig. 4. Fluorescent micrographs of phalloidin staining on paraffin sections of the specimens with 4% formaldehyde (a), 4% formaldehyde and 1% TA (b), and 4% formaldehyde, 1% glutaraldehyde and 1% TA (c). No fluorescence on the tissue is seen in (a) and (b). (c) shows non-specific staining. × 200.

We tried phalloidin staining on the paraffin sections (Table 1, Fig. 4). No fluorescent image was seen in the tissue in the cases which were fixed in 4% formaldehyde (Table 1, Fig. 4a) or in 4% formaldehyde and 1% TA (Table 1, Fig. 4b). However, very strong non-specific fluorescence was seen when specimens were fixed with 4% formaldehyde, 1% glutaraldehyde and 1% TA (Table 1, Fig. 4c). In this section, the cushion mesenchymal cells were strongly stained as well as myocardial cells in the truncus and the atrium.

DISCUSSION

Frozen sections are very useful in histochemical studies for high detection sensitivity, however frozen sections have disadvantage of their poor tissue preseration. Fixation with 4% formaldehyde did not work to preserve tissue for freezing sectioning (see Fig. 1). At light microscopic observation of frozen sections stained with H.E., it was difficult to recognize the tissue type. Poor tissue preservation is major disadvantage to study localization of target materials on frozen sections. In contrast, tissues were well preserved in paraffin sections (see Fig. 1). However, F-actin, our target material, is depolymerized because of high temperature during embedding[6,8]. Probably, reduction of fluorescent intensity around the Ap septum area in paraffin sections is due to this reason.

On the other hand, fluorescent intensity of the cardiac muscle was not so reduced. At least six actin isoforms have been shown by Vandekerckhove and Weber[15,16]. α-cardiac actin is isoform of cardiac muscle actin, and probably α- and γ-smooth muscle actin or ß- and γ-cytoplasmic nonmuscle actin are isoforms of the actin of the cells in the Ap septum. Since the cells within the Ap septum contain desmin, muscle type intermediate filament (unpublished data), α- and γ-smooth muscle actin can be considered as the isoforms of the actin within the Ap septum. Some of these isoforms are immunologically different[10,11]. Antiserum to cardiac actin bind skeletal actin but failed to bind brain actin[10]. The antibody which we used, was prepared against chicken backmuscle actin[12]. This antibody should have higher affinity to cardiac muscle than to smooth muscle since the major component of α-cardiac actin is very similar to

α-skeletal actin[15,16]. Furthermore, F-actin in cardiac muscle is well organized by association with myosin. These immunological affinity and degree of dissociation of actin are possible reasons of difference in fluorescent intensity between the cardiac muscle and the cellular condensation within the Ap septum.

In the present study, the actin in the Ap septum is well preserved in paraffin sections of the sepecimens fixed with 4% formaldehyde and 1% TA fixation. Tannic acid protects F-actin from disruption by OsO_4 for electron microscopy[5,9]. And image contrast of microfilaments is enhanced because of high affinity of OsO_4 to TA binded actin. TA have usually been used for observation of stress fibers. Probably TA works to preserve actin in stress fiber more clearly than actin in cardiac and skeletal muscle fibers by reasons as follows. The skeletal or cardiac muscle actin filaments are well organized in muscle fibers. In contrast, actin filaments in stress fiber are easily anchored to membrane by vinculin, α-actin in and unknown molecule[7,19].

Tissues fixed with solutions including 1% glutaraldehyde show flourescence all over the specimens. Although glutaraldehyde has advantage for its good tissue preservation[3], our results suggest that fixation without glutaraldehyde is good for antiactin and phalloidin staining.

Compared with Ap septum, not so much actin was shown in developing smooth muscle of tunica media. Electron microscopy[4] has shown no microfilament bundle in developing smooth muscle cells in the tunica media of the proximal truncus at this stage. These findings support that the cells in the Ap septum are different from the developing smooth muscle cells in the tunica media[13,14].

In our study, phalloidin staining was totally negative on paraffin sections even when the specimens were fixed with TA fixation which allowed good antiactin staining. This result indicates that TA does not preserve non-immunoreactional binding site of actin for phalloidin.

In the present study, image of actin bundles by antiactin staining on paraffin sections with TA fixation was comparable with phalloidin staining on frozen sections, and showed better tissue preservation than frozen sections. We suggest that our method is very useful to study localization of actin in situ and to observe tissue structure of same section stained with H.E. after fluorescent examination.

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