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# Immunohistochemical Detection of Fibrillar Collagens in Tissue Sections and in Culture Cells

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**Abstract**

We evaluated conditions for optimal immunostaining for Type I procollagen and Type III collagen in tissues with high content of these fibrillar collagens. We tested polyclonal and monoclonal antibodies on formalin fixed, paraffin embedded sections and frozen sections from avian tendons and gizzard and on tendon cell cultures grown on glass slides. We also evaluated procedures for antigen unmasking. We obtained the strongest specific staining with monoclonal antibodies to both collagen types using frozen tissue sections or methanol-acetone fixed tendon cell cultures. The use of antigen unmasking was not only unnecessary, but also undesirable as it led to the detachment of sections. (*The J Histotechnol* 23:333, 2000)

**Key words:** avian tissues, frozen sections, monoclonal antibody, Type 1 procollagen, Type III collagen

## Introduction

Type I and III fibrillar collagens are integral components of numerous connective tissues, such as tendons, bone, skin, and gastric wall, or gizzard (1,2). Type I collagen predominates, especially in the bone and tendon; whereas low quantities of Type III collagen are usually present in non-embryonal tendons (3). Immunohistochemical visualization facilitates studies on changing distribution of collagens during physiological and pathological conditions in tissues.

The use of formalin fixed, paraffin embedded tissues has become commonplace in immunohistochemical labeling. However, extensive crosslinking occurring between formalin and collagen leads to inaccessibility of collagen epitopes to antibodies and, thus, to inconsistent immunostaining, especially in tissues with high collagen content such as the tendon or gizzard. We evaluated the effect of fixation and some other conditions on immunodetection of fibrillar collagens in the avian tendon.

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## Materials and Methods

### *Tissue Collection and Processing*

Gastrocnemius tendons and gizzard wall from 6–8 wk old broiler chickens were removed from the animals immediately after death and either snap-frozen at –70°C or fixed in 10% formalin or 4% paraformaldehyde. The formalin fixed tissue was processed for routine histology and embedded in paraffin. Frozen sections were cut using a standard cryostat.

### *Tendon Cell Culture*

We established short-term tendon cell cultures from 18-day-old chicken embryonal gastrocnemius tendons following a modified method of Kempka et al (4). Steriley dissected gastrocnemius tendons were cut, under sterile conditions, in a laminar flow hood with scissors into 1–2 mm long pieces. These were digested with 3 mg Type I or Type II collagenase/ml Dulbecco modified Eagle medium (DMEM, Sigma Chemical Co, St. Louis, MS) at 37°C for 12 to 18 hr. The cell suspension was filtered through a 70 μm nylon mesh, washed in phosphate buffered saline (PBS), and suspended in DMEM supplemented with 13% fetal bovine serum (FBS). The tendon cells were grown on UV sterilized histology glass slides placed into 100 mm tissue culture plates containing 10 ml DMEM with 13% FBS. When covered with cell monolayer, the glass slides were lifted from the culture plates and fixed in cold methanol (–20°C for 5 sec) and acetone (4°C, 3 × 5 sec) (5). The

slides were air-dried and either stained immediately or stored at –20°C until ready to use.

### *Immunohistochemical Protocol*

Rabbit polyclonal antibodies (IgG) to chicken Type I collagen and chicken Type III collagen were obtained from Biodesign International (Kennebunk, ME). After dehydration of formalin fixed, paraffin embedded sections and overnight air-drying at room temperature (RT) of frozen sections, endogenous peroxidase was quenched for 30 min in 0.3% hydrogen peroxide ($H_2O_2$) in methanol. Nonspecific binding was blocked by 2% normal goat serum in PBS applied for 1 hr at RT. Antigen retrieval methods were not used because both microwaving (2 × 5 min in PBS) and exposure to 2.5% trypsin (30 min at RT) led to tendon or gizzard section detachment. After 1 hr incubation at RT or overnight incubation at 4°C with a primary antibody diluted 1:10 or 1:100, the slides were incubated with a biotinylated secondary goat antirabbit antibody (1:1,000 dilution, Vector Laboratories Inc, Burlingame, CA) for 1 hr at RT, then in avidin-biotin complex solution prepared according to manufacturer's recommendation. Vectastain Elite ABC kit with the DAB substrate kit was used to detect the presence of antibody-antigen complexes. In both modifications, 2 drops of reagent A and 2 drops of reagent B were added to 5 ml of PBS and thoroughly mixed. The sections were counterstained with hematoxylin.

We also analyzed mouse monoclonal antibodies to human Type I procollagen and chicken Type III collagen (6,7). The monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank maintained by The University of Iowa, Department of Biological Sciences, Iowa City under contract NO1-HD-7-3263 from the NICHD. The monoclonal antibody to Type I procollagen crossreacts with chicken Type I procollagen. After dehydration of formalin fixed, paraffin embedded sections and overnight air-drying at RT of frozen sections, endogenous peroxidase was quenched for 30 min in 0.3% $H_2O_2$ in methanol. Nonspecific binding was blocked by incubation in 10% normal horse serum in PBS for monoclonal antibodies for 1 hr at RT. Antigen retrieval methods were not used for reasons specified above. After 1 hr incubation at RT (1:10) or overnight incubation at 4°C, with a primary antibody (1:100 dilution), the slides were incubated with a biotinylated secondary antimouse antibody (1:1,000 dilution, Vector Laboratories) for 1 hr at RT, then in avidin-biotin complex solution prepared according to manufacturer's recommendation. Vectastain Elite ABC kit with the DAB substrate kit was used to detect the presence of antibody-antigen complexes. The sections were counterstained with hematoxylin.

Double staining on frozen tendon sections was done for both collagen types. After overnight incubation with the monoclonal antibody to Type I procollagen, the secondary antibody and the ABC reagent were applied. The antibody-antigen complexes were visualized with the Vector SG substrate kit. Immediately following the color development, the sections were incubated with the monoclonal antibody to Type III collagen overnight. After incubation with the secondary antibody and the ABC reagent, the antibody-antigen complexes were visualized with the Vector VIP substrate kit. No counterstaining was performed.

Nonimmune (normal rabbit IgG) or irrelevant antibody (mouse monoclonal trpE antibody; Oncogene Science, Uniondale, NY) was used as a negative control in all experiments.

## Results

We tested all 4 (polyclonal and monoclonal) antibodies on formalin fixed, paraffin embedded sections or frozen sections of chicken gizzard and tendon and fixed cultured tendon cells.

Polyclonal antibodies to either collagen type gave no specific staining when used on sections of formalin fixed tissues, even when used at 1:10 dilutions, and only weak specific staining when used on frozen sections, even with longer incubation time (overnight vs 1 hr staining). The nonimmune rabbit IgG (used as a negative control) gave relatively strong background staining, indistinguishable from the specific staining, at the dilutions used (1:10 or 1:100).

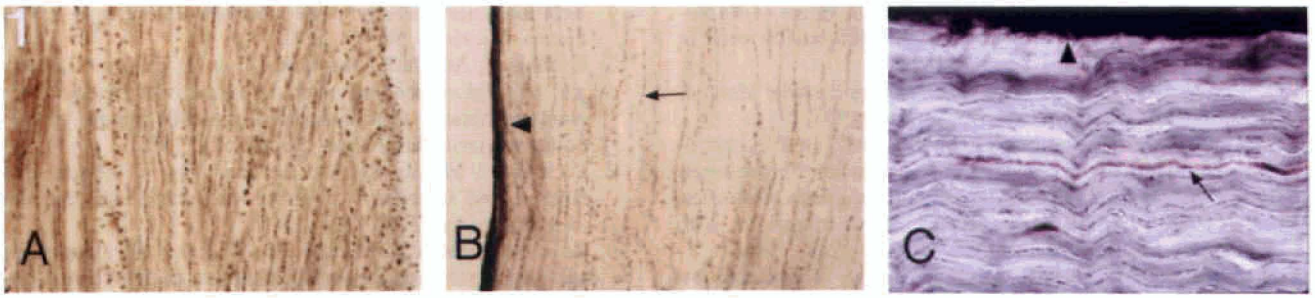
The use of monoclonal antibodies on formalin or 4% paraformaldehyde fixed tissue led to minimal specific staining. The use of frozen sections and monoclonal antibodies (1:100 dilution overnight) resulted in prominent specific staining for Type I procollagen (Figure 1A) and less prominent staining for Type III collagen (Figure 1B). No staining was observed with an irrelevant primary antibody (data not shown). In addition, overnight incubation at 4°C not only gave more intense staining than 1 hr incubation at RT, but it also allowed higher dilution than that recommended by the supplier (1:100 vs 1:5). Similarly, immunostaining of chicken gizzard worked the best when monoclonal antibodies to Type I procollagen and Type III collagen were used on frozen sections in overnight incubation at 4°C (Figure 2).

We also double-stained frozen tendon sections for Type I procollagen and Type III collagen using the monoclonal antibodies and the Vector SG and VIP substrate kits. The Type I procollagen appeared as thick gray fibers. The Type III collagen stained as thin purple fibers in the tendon proper, and it was widely distributed in the tendon sheath (Figure 1C). We found this sequence of primary antibodies with this color combination to give the best visual effects and color discrimination. Moreover, no counterstaining was necessary. The use of antibody to Type I procollagen following the antibody to Type III collagen led to almost exclusive visualization of Type I procollagen mainly because of its predominant expression in the tendon.

Collagen in cultured tendon cells was best detected by monoclonal antibodies using the protocol developed for frozen sections. The immunostaining for Type I procollagen and Type III collagen was strong, with no background in methanol-acetone fixed cell cultures (Figure 3), and no background staining occurred with an irrelevant primary antibody (data not shown).

## Conclusions

We conclude that the use of frozen sections of tissues with large collagen content in immunostaining for fibrillar collagen is preferable over the use of sections of formalin fixed, paraffin embedded tissues. This method is sensitive, gives low background, does not require antigen retrieval processes, and is suitable for simultaneous visualization of 2 antigens using commercially available substrate kits. The weak specific staining by the polyclonal rabbit antibodies





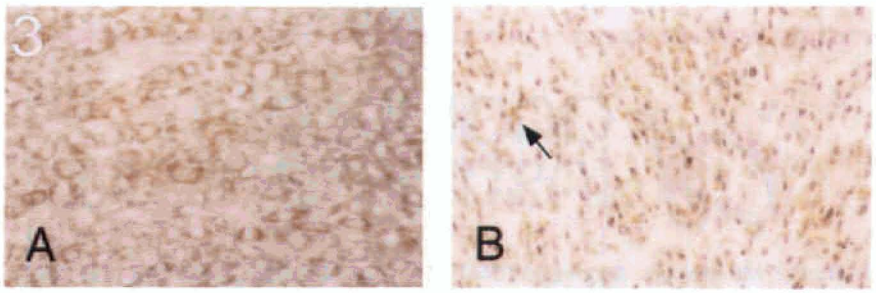



**Figure 1.** Immunohistochemical staining of avian tendon. Monoclonal antibodies visualized Type I procollagen as thick fibers **(A)** and Type III collagen as thin fibers (arrow, **B**) in frozen sections of avian tendon. Dual staining allows simultaneous staining for both collagens, Type III collagen stained as thin red strands (arrow, **C**). Arrowhead: tendon sheath staining intensely for Type III collagen **(B,C)**. Original magnifications ×200 **(A,B)** and ×375 **(C)**.
**Figure 2.** Immunohistochemical staining of chicken gizzard. Monoclonal antibodies visualized Type I procollagen as thick fibers (arrow, **B**) and Type III collagen as thin fibers (arrow, **C**) in frozen sections of chicken gizzard. No specific staining was observed when irrelevant trpE was used in place of the specific antibodies **(A)**. Original magnifications × 100.
**Figure 3.** Immunohistochemical staining of tendon cell cultures. Monoclonal antibodies were used to visualize large quantities of Type I procollagen **(A)** and significantly smaller amounts of Type III collagen fibers (arrow, **B**) in primary tendon cell cultures. Original magnification ×300.

was even more diminished by the strong background obtained with the nonimmune rabbit IgG used as a negative control. Our results, thus, make the use of polyclonal rabbit antibodies unsuitable for collagen detection.

Our findings are in agreement with literature reports documenting decreased immunoreactivity for collagen in formalin fixed tissues when compared with frozen sections (8). Most likely, blocking of amino groups on the collagen molecule by formaldehyde is responsible for the decreased immunoreactivity (9). Yoshida et al also observed that immunoreactivity for Type IV collagen decreases proportionately with the length of fixation in 10% formalin (8). Enzymatic treatment, or incubation with the primary antibody at 4°C rather than RT, helped restore the epitope to a certain extent and, thus, enhanced collagen immunoreactivity (8,10). As noted in our findings, no significant difference was observed between 10% formalin and paraformaldehyde fixation (8).

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