Immunohistochemical Localization of Tubulin in the Somite of the Early Chick Embryo

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Immunohistochemical Localization of Tubulin in the Somite of the Early Chick Embryo

Submitted in Partial Fulfillment of the Requirements for Graduation with Honors to the Department of Biology at Carroll College, Helena, Montana

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This thesis for honors recognition has been approved for the Department of Biology.

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ABSTRACT

In this experiment I attempted to devise a procedure for the localization of the cytoskeletal protein tubulin in the somite of the early chick embryo. I also checked the distribution of tubulin in the early chick somite for any possible relationship to the epithelial-mesenchymal transition that occurs in these somitic cells. Newly fertilized chicken eggs were incubated for approximately 48 hr; then the embryos were removed from the egg, fixed, embedded, and frozen for cryostat sectioning. After sectioning the embryos, I attempted to localize tubulin by attaching a primary antibody to the tubulin molecule followed by a secondary antibody and an enzyme (peroxidase). A peroxidase substrate was then introduced to the tissue, and the enzyme reacted with it to produce a colored reaction product which served as the indicator for the presence and distribution of the tubulin.
INTRODUCTION

The somites of the early chick embryo have long been studied for their significant role in embryogenesis. Of particular interest is a morphogenetic phenomenon that occurs during the early stages of embryonic development in which the epithelial cells of the somite undergo a transformation and take on the characteristics of mesenchymal cells. The cause of this unusual change is a source of mystery for researchers today. Furthermore, recent studies have shown that cancer cells undergo a very similar transformation during their transition to malignancy. Approximately 85 percent of human tumors is carcinomas derived from epithelia, and many carcinomas are characterized by morphological changes in cell polarity (6). Consequently, any insights into this intriguing transformation may prove to be inherently beneficial to cancer research and treatment.

Functional differences among cells that arise during development in multicellular organisms are often reflected at the level of changes in components of the cytoskeleton, such as use of different forms of cytoskeletal proteins, changes in microtubule or microfilament stability, or assembly of specialized microtubule arrays (10). Also, researchers have recently proposed that the presence or absence of certain cell surface junctions, such as gap or tight junctions, and their associated molecules plays an important role in this unique transformation. It is therefore logical to speculate that the cytoskeletal protein tubulin, the major constituent of
microtubules, may affect morphological changes in somitic cells. Insight into the concentration and distribution of tubulin in these somitic cells also may prove helpful in the understanding of their role in the epithelial-mesenchymal transformation.

Through the use of immunohistochemical staining techniques, a protocol for localization of tubulin was worked out, and the distribution of tubulin in the somites of early embryonic chicks were studied as these somitic cells responded to determinative signals during development.
Tubulin

Tubulin is the major protein constituent of microtubules found in the cellular cytoplasm, including those of the mitotic spindle (5). Each molecule is a heterodimer of two tightly bound globular units called alpha-tubulin and beta-tubulin, each containing approximately 450 amino acid residues and weighing about 60 kDa (1). Tubulin's molecular arrangement is unique. A complete microtubule consists of 13 "protofilaments" which are a linear arrangement of alpha-tubulin subunits, each next to a beta-tubulin subunit set around an apparently empty central core (1).

Tubulin, like most cytoskeletal proteins, is encoded by a group of genes that are closely related in most organisms (1). A recent study of the plant genus Arabidopsis, which has genes very similar to those of animal tubulin, showed that there are eight beta-tubulin genes of varying lengths, each containing two introns. It was also discovered that one alpha-tubulin gene contains two introns, one contains three introns, and the remaining six alpha-tubulin genes have four introns each (7). Raff proposes that these multiple genes facilitate precise control of tubulin synthesis and that differential expression of tubulin is important for morphogenesis (7).

Structurally, tubulin associates with many related proteins which function to modify the properties of microtubules and attach them to other
structures in the cell (1). When mixed in a test tube, all tubulins will form the same microtubules (1). Researchers think variations of tubulins within organisms may be functional (1). For example, studies have shown that the carboxyl-terminus of alpha-tubulin and beta-tubulin molecules contain hundreds of amino acids which show distinct differences in various tissues (1). It is believed that this terminus is involved in binding to microtubule accessory proteins (MAPs), and changes in the amino acid sequence of this terminus may alter the functions by altering this binding (1).

However, tubulin's function is another story. This aspect is necessarily linked to that of the microtubules since tubulin is the major component of these flexible fibers. Microtubules serve a variety of purposes in such cellular movements as the placement of chromosomes on the spindle formed during mitosis and are the major structural components of cilia and flagella (9). Along with their role in cell movements, microtubules play a critical role in regulating cell shape (1). For example, it is well known that microtubules are aligned with the long axis of elongated cells and are necessary for the maintenance of the cell's elongated shape (1). It has also been found that microtubules determine the location of the Golgi apparatus and the endoplasmic reticulum, as well as influencing the distribution of intermediate filaments and actin filaments (1). Recently, evidence has been uncovered that seems to indicate that communication between the cytoskeletons of adjacent cells underlies a fundamental process in animal morphogenesis—the folding of cell sheets, and it is often microtubules that guide these morphogenetic movements (1).
Somites and Somitic Formation

For many years after its recognition, the segmental plate mesoderm was thought to have elusive functions that somehow led to the formation of somites later in the embryogenetic process (2). Through the use of stereo scanning electron microscopy, regular segmentation of the segmental plate has been observed (2). These segments, called somitomeres, begin in the rostral part of the head and are laid down as the primitive streak regresses caudad (2). The most recently laid down pair of somitomeres is located just caudal to Hensen’s node (2).

The somitomeres are eventually transformed into somites with the first pair forming from the eighth pair of somitomeres (2). For some unknown reason the first seven pairs of somitomeres remain primitively organized (2). During early formation, one pair of somites forms about every hour until 50 pairs are formed (2). As somitomeres form somites, the cells become more compactly arranged around a central cavity, their apical surfaces become cemented together by continuous tight junctions, and a basal lamina is laid down around the outer surface of the somite (2). The acquisition of these epithelial characteristics is the key indicator that somitomeres have been transformed into somites.

Once a somite has been formed, the ventromedial wall of the somite loses its epithelial characteristics through a burst of mitosis and becomes secondary mesenchyme (2). This morphological change is induced by the notochord and neural tube as proven by the extirpation experiments of Holtzer and Detwiler in 1953 (2). These newly formed mesenchymal cells are derived from a region of the somite called the sclerotome. Shortly,
these cells move away from the somite, eventually surrounding the notochord and the ventral portion of the neural tube (2). Once in place, they secrete great quantities of chondroitin sulfate and other materials found in cartilage matrix (2). Ultimately, the vertebrae, the ribs, and the scapulae are all formed from cells of the sclerotome (2).

**Epithelial-Mesenchymal Transformation**

Epithelial tissue is the first tissue to form in early vertebrate embryos (4). Epithelium is the main tissue type in the bodies of our chordate ancestors and is characterized by the close apposition of its constituent cells and their presence at a free surface (4,8). Epithelial cells have apical-basal polarity and adhere through lateral cell junctions (4). The most common junctions present are gap junctions, which assemble at the eight-cell stage, and tight junctions, which appear anywhere from the eight- to thirty-two-cell stage (3). Epithelial cells also sit on a basal lamina and do not invade collagenous matrices when placed on top of them (4).

Mesenchyme, known as "the embryonic connective tissue," consists of stellate cells, delicate reticular fibers, and ground substance (8). Mesenchymal cells eventually form fibroblasts, chondroblasts, and similar cells (4). Arising from embryonic epithelium, they gradually lose epithelial cell polarity and acquire the ability to invade and move through extracellular matrix (4).

The transformation from epithelium to mesenchyme in the embryo is carefully regulated and occurs only in predicted regions at expected times (4). The genetic sequence responsible for this highly controlled phenotypic
change in the tissue seems to be inactivated or not present in the embryo and adult (4). It has been suggested, however, that epithelia found in the lens and certain neoplasms give rise to fibroblast-like cells and/or collagen fibrils, indicating that some epithelia retain activatable programs for forming mesenchyme (4).

In their study of rat thyroid follicles, Greenburg and Hay found that, without exception, epithelial-mesenchymal transformation occurs from the basal surface of the epithelium (4). Before mesenchymal cells are formed, the basal lamina disappears, but intercellular spaces increase and the staining pattern of the presumptive mesenchyme changes before this point (4). Greenburg and Hay believe that a genetic program switches on, causing changes in the cell surface, cytoskeleton, and polarity prior to the disappearance of the basal lamina (4). Greenburg and Hay’s research also indicates that the confrontation of definitive epithelium with a three-dimensional collagen gel triggers the epithelial-mesenchymal transformation in a normally stable epithelium, and, although this theory is disputed, the researchers believe that the gel is the key to the initiation of the genetic program that causes epithelial-mesenchymal transformation (4).

Why then, would the distribution of tubulin be relevant to understanding the epithelial-mesenchymal transition? The answer lies in the dynamic instability of microtubules. The average lifetime of a microtubules is less than 10 minutes (1). This inherent instability of microtubules helps to explain how they can be induced to grow in specific directions in a cell—toward the leading edge of a crawling cell, for example, or toward a condensed chromosome in a dividing cell (1). The continual formation and loss of microtubules is characteristic of cells undergoing a major internal
reorganization, such as cells undergoing the epithelial-mesenchymal transformation (1). When cells have become a part of an established tissue, the microtubules they contain become relatively permanent features (1). The study of the distribution of tubulin in early somitic cells may give clues as to the microtubule arrangement in these cells which, in turn, may indicate whether the cell is in the process of epithelial-mesenchymal transition, or whether it has become an established mesenchymal cell.
MATERIALS & METHODS

Eggs and Incubation

Newly fertilized chicken eggs obtained from commercial suppliers, were placed into an incubator immediately upon reception of shipment. The eggs were incubated approximately 48-65 hr at 38.5°C and turned 180° every 12-24 hr. A flow diagram of the general procedure for immunohistochemical localization of tubulin in the somite of the embryonic chick is represented in Figure 1.

Fixation

The eggs were removed from the incubator, and the shell of each was carefully cracked open to reveal the embryo underneath. A small paper ring was placed around the embryo and, using fine scissors, the embryo was removed from the surface of the egg. Once freed from the egg, the embryo was rinsed briefly in phosphate buffered saline (PBS), then immersed in a fixative solution consisting of 2% formaldehyde (prepared from paraformaldehyde) in PBS at a final pH of 7.2. The embryos were fixed for 1-2 hr at room temperature.

After fixation, the embryos were removed from the fixative solution and rinsed three times with PBS. Then the paper rings, along with any
excess membranes, were cut off using fine scissors. The embryos were then placed in rectangular aluminum or plastic molds, embedded with OCT embedding compound (Miles), and placed in the freezer at -20°C until sectioning.

Sectioning

A frozen embryo was removed from the freezer and the aluminum mold. It was then mounted on a cryostat sectioning plate with OCT compound. Beginning with the tail end, the embryo was slowly sectioned at 5-10 μm intervals through the caudal portion of the body. Once cut, the sections were placed on microscope slides that had been previously treated with a 0.5% gelatin and 0.05% CrK(SO₄)₂·12 H₂O subbing solution for adherence. The slides were then returned to the freezer for storage until immunohistochemical staining.

Immunohistochemical Staining

The slides were removed from the freezer, placed section side up in a moist chamber, and allowed to warm to room temperature. Before the sections became air dry, they were covered with phosphate buffered saline (PBS) and allowed to stand for 5 min while a permeabilizing and blocking solution of 2.0% nonimmune goat serum/0.3% Triton X-100/PBS (GTP) was prepared. The PBS was then aspirated off, and the permeabilizing/blocking solution was layered over the slides which were then allowed to sit at room temperature for 10 min.
A primary antibody solution of anti-tubulin antibody from rabbit serum was prepared in a dilution of GTP. At the same time a similar solution of nonimmune antibody from rabbit was prepared using similar protein concentrations. Following this procedure, the permeabilizing/blocking solution was removed from the slides, and the primary antibody dilution was layered over them, after which, they were incubated for 3 hr at room temperature.

After the appropriate incubation, the slides were washed three times with PBS at 5-min intervals. During this incubation period, a secondary antibody, a biotin conjugate, was diluted in the same manner as the primary antibody using GTP. The secondary antibody was then layered over the sections which were incubated for 1.5 hr at room temperature.

During this second incubation period, ABC reagent (Vector) was prepared by adding one drop of reagent A to 5 ml of GTP; then one drop of reagent B was added to the buffer, and the mixture was allowed to stand at room temperature for 30 min.

Once again the sections were washed three times with PBS at 5-min intervals after removal of the secondary antibody solution. Afterwards, the ABC reagent was added to the slides, and they were allowed to incubate for 1.5 hr at room temperature.

Finally, a peroxidase substrate solution of 0.1% DAB, 0.02% hydrogen peroxide (freshly diluted from 30% stock), and 0.1 M Tris-HCl was prepared and brought to a final pH of 7.2. The sections were washed three times at 5-min intervals in PBS after which they were incubated in the peroxidase substrate solution for 5-7 min, then washed with tap water for 5 min.
To preserve the sections, a small drop of glycerol was placed on each of the sections, and coverslips were placed over the entire slide. The sections were then scanned microscopically for the presence of colored reaction product. A flow chart for the immunohistochemical staining procedure is represented in Figure 2, and a diagram illustrating the immunological reactions occurring in the immunohistochemical staining is in Figure 3.
Fig. 1. General procedure for the localization of tubulin in the somite of the embryonic chick.
REHYDRATE SECTIONS (PBS)

PERMEABILIZING/BLOCKING SOLUTION

PRIMARY ANTIBODY (3 HR)

WASH (PBS)

SECONDARY ANTIBODY (1.5 HR)

WASH (PBS)

ABC REAGENT (1.5 HR)

WASH (PBS)

PEROXIDASE SUBSTRATE SOLUTION (2-7 MIN)

WASH (TAP WATER)

COVER SECTIONS (GLYCEROL)

Fig. 2. Procedure for Immunohistochemical staining of tubulin in the somite of the embryonic chick.
Fig. 3. Diagram for the immunological reactions occurring in the immunohistochemical staining of tubulin in the somite of the embryonic chick.
RESULTS & DISCUSSION

Following several trials of the immunohistochemical localization procedure, an efficient protocol was achieved, but the appearance of the distribution of tubulin was inconclusive in determining the role of microtubules in cells undergoing the epithelial-mesenchymal transformation. Although some colored reaction product appeared in the cells, it was randomly distributed and present in insignificant amounts.

Several modifications in the staining technique were made during the study. First, it was discovered that a higher concentration of Triton X-100 detergent was needed in the permeabilizing/blocking solution (GTP). Consequently, a 0.3% dilution of Triton X-100 was used, instead of a 0.1% dilution, along with the nonimmune goat serum and PBS, in the GTP.

The second major adjustment made was to change the incubation times for the antibodies and ABC reagent from 1 hr each to 3 hr for the primary antibody incubation, and 1.5 hr for the other two incubations. This extended amount of time allowed for greater probability of the primary antibody’s binding to the tubulin and the secondary antibody’s and ABC reagent’s binding as well.

As a result of the present experiment, I have modified and improved a procedure that can now be applied to the study of tubulin distribution in the somites of early embryonic chicks. This protocol has proven to be efficient and effective and will undoubtedly yield conclusive information.
about tubulin’s role in the epithelial-mesenchymal transition of embryonic chick somites in future experiments.
LITERATURE CITED


