

Microscopic Observation of Ito Cells Present in the Livers of Several Species of Teleosts

Mitsuko ASADA¹, Hajime NAKABAYASHI² and Yuichi SASAYAMA^{3*}

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Abstract

Ito cells with cytoplasmic protrusions were clearly stained in the livers of 7 species of teleosts by Otsuka's silver staining, which is usually used to detect reticular fibers in neurons and connective tissues. Among those species, in medaka and black scraper, immunostaining was conducted using an anti- β -tubulin antibody (TU27), which recognizes all isotypes of β -tubulin distributed from protozoa to mammals. This antibody reacted to Ito cells in both species. Since β -tubulin is one of the components of microtubules of the axon in neurons, this result suggests that Ito cells express β -tubulin similarly to neurons. In addition, another antibody (TU20), which recognizes Class III β -tubulin was applied. This type of β -tubulin is expressed only in neurons. The antibody stained some cells, which appeared to be Ito cells, in black scraper but not in medaka. On the basis of these results, at this point, at least, in black scraper, Ito cells may have similar functions to neurons, although it is certain that they express some isotypes of β -tubulin as in neurons.

Key Words: Ito cells, teleost liver, β -tubulin, neuronal function

I. Introduction

In the space of Disse of mammalian liver, unique cells exist, which are different from hepatic parenchymal cells and Kupffer cells (Ito and Nemoto, 1956). These cells are called Ito cells after Dr. Ito, who identified them. They are also described as fat-storing cells, since many fat droplets exist in the cytoplasm (Ito and Shibasaki, 1968). Furthermore, because of the distinctive morphology of these cells, they are called stellate cells as well. Ito cells have plural protrusions of cytoplasm and

contacts with hepatocytes in one direction and endothelial cells of the blood vessel in another (Wake, 1971). They contain a large quantity of vitamin A in the fat droplets and participate in the metabolism of vitamin A (Wake, 1971). Furthermore, Ito cells secrete collagen in great quantities in the processes of liver regeneration (Ramadori, 1991). In human, it has been thought that these cells are related to liver diseases because they produce α -smooth muscle actin even under healthy conditions. In chronic liver diseases, therefore, Ito cells cause fiberization of liver (Hautekeete and Geerts, 1997).

¹Division of Biological Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa, 920-1192 Japan

²Research Fellow, Research Adviser, Division of Biological Measurement and Applications, Institute of Nature and Environmental Technology, Kanazawa University, Kakuma-machi, Kanazawa, 920-1192 Japan

³Marine Laboratory, Division of Biodiversity, Institute of Nature and Environmental Technology, Kanazawa University, Kakuma-machi, Kanazawa, 920-1192 Japan

*Correspondence author

Taking these facts into consideration, the origin of Ito cells has been regarded to be myogenic cells, such as smooth muscle cells or muscular fiber blast cells (Yokoi *et al.*, 1984).

During the past ten years, however, the origin of Ito cells has been debated. It is known that Ito cells express the neural cell adhesion molecule and the glial fibrillary acidic protein (Nakatani *et al.*, 1996; Neubauer *et al.*, 1996). Nishi *et al.* (1999) conducted cloning of a new GTP-binding protein from the spinal cord of rats and found that it is expressed in Ito cells as well. Synaptophysin is known as a marker molecule of the nervous-endocrine system (Edelmann *et al.*, 1995). Ito cells express synaptophysin (Cassiman *et al.*, 1999). Furthermore, Cassiman *et al.* (1999) noted that Ito cells have synaptic vesicles and suggested the possibility that they may have unknown functions in neighboring cells. Ito cells have receptors for the nerve growth factor (Trim *et al.*, 2000). Binding with the growth factor causes apoptosis of Ito cells specifically. Trim *et al.* (2000) have suggested that Ito cells are a selective target of the nervous system. Although nestin originally consists of intermediate filaments expressed specifically in neurons, the same filaments have also been found in Ito cells (Niki *et al.*, 2003). Therefore, it has been argued that Ito cells originate from the neural crest because their genetic expression patterns resemble those of nerve cells (Sato *et al.*, 2003). These facts raise questions about their embryonic origin and differentiation processes (Cassiman *et al.*, 1999).

On the other hand, phylogenetically, Ito cells have already been found in lamprey liver (*Lampetra japonica*) (Wake *et al.*, 1987). Wake *et al.* pointed out that these cells have contractility on the basis of electron microscopic observations. In goldfish liver (*Carassius auratus*) and stone flounder liver (*Kareius bicoloratus*), desmosomes develop in all cells composing the livers, and are remarkably abundant around Ito cells (Fujita *et al.*, 1980; Tanuma *et al.*, 1982). Observations of the livers of 19 species of freshwater and seawater fishes revealed that Ito cells remarkably develop desmosomes (Sakano and Fujita 1982). The hepatocytes of cod (*Gadus morhua macrocephalus*) contained a large number of fat

droplets (Fujita *et al.*, 1986). Ito cells have long protrusions of cytoplasm and adhere to each other by the desmosomes present at the tip of the protrusions. Fujita *et al.* (1986) suggested that, in cod, this morphological characteristic among Ito cells constitutes a skeletal system that has the function of maintaining the form of the liver because of the accumulation of fat. In the Pacific halibut (*Atheresthes evermanni*), Ito cells with fine and long protrusions of cytoplasm were stained by gold chloride staining (Yoshikawa *et al.*, 2006).

As mentioned above, in Ito cells of teleosts, long and elongated protrusions of cytoplasm are emphasized, in addition to the development of desmosomes. These facts suggest that, in teleosts, Ito cells have the function of maintaining the form of the liver.

Because teleosts occupy a phylogenetically fundamental position among vertebrates, they are an interesting target in the study of the origin of Ito cells. However, there has been no research about this point so far. In the present study, in 7 species of teleosts, we applied silver staining to detect the Ito cells. Furthermore, we conducted immunostaining with two kinds of anti- β -tubulin antibodies to examine whether Ito cells have neural elements or not. This is the first report in which antibodies of β -tubulin were used to detect Ito cells.

II. Materials and methods

Freshwater medaka (*Oryzias latipes*) was obtained from a commercial source. Six species of seawater fishes, damsel fish (*Chromis notata*), greenfish (*Girella punctata*), fine-patterned puffer (*Takifugu poecilonotus*), bamboo-leaf wrasse (*Pseudolabrus japonicus*), puddingwife wrasse (*Halichoeres poecilopterus*), and black scraper (*Thamnaconus modestus*), were collected from the marine station of our university. The liver of each species of fish was anatomized after having completely anesthetized the fish in a solution with a suitable concentration of tricaine methanesulfonate. As the entire liver of each species was quite large, it was cut to a size of around 1cm³ and fixed.

In hematoxylin and eosin staining, livers were fixed

with a 4% paraformaldehyde solution for 3.5 hr on a shaker, dehydrated with an alcohol series, and embedded in paraffin following a routine method. Paraffin blocks were cut into serial sections with 8 μm thickness in all staining methods.

We adopted Otsuka's silver staining (1962). For this staining, livers were fixed with an 80% ethyl alcohol solution containing 0.5 % glacial acetic acid as well as 0.5% formalin for 48 hr on a shaker. Sections were hydrated with Millipore water and incubated with a 20% silver nitrate solution for 4 hr at 37°C under conditions of shielding from light. After that, the sections were again incubated with a silver nitrate buffer solution for 18 hr under the same conditions. Using a sodium sulfite and hydroquinone mix solution, the sections were deoxidized. A gold chloride solution was used for plating the sections. After treatment with 2% oxalic acid, a sodium thiosulfate solution was used to fix the reaction of the sections.

In immunostaining, 2 species, medaka and black scraper, were used as the representatives of freshwater fish and seawater fish. Their livers were fixed with 4% paraformaldehyde for 3.5 - 5 hr on a shaker. We used 2 kinds of antibody as a primary antibody. One is an anti- β -tubulin monoclonal antibody (clone name TU27, Convance Co. Ltd., USA) which recognizes all isotypes of β -tubulin distributed from protozoa to mammals. The other is a monoclonal antibody (clone name TU20, Acris Antibodies Co. Ltd., Germany) which recognizes only class III β -tubulin expressed in the neurons of fish and mammals. The TU27 antibody was diluted to 2 $\mu\text{g}/\text{ml}$ with 0.5% blocking solution. The TU20 antibody was diluted to 20 $\mu\text{g}/\text{ml}$ with the same blocking solution. In both immunostainings, a reaction with the primary antibody was conducted overnight at room temperature. In the case of immunostaining using the TU20 antibody, to activate the surface of sections, the section was processed with proteinase K (20 $\mu\text{g}/\text{ml}$) diluted with a PBS solution containing 0.1 % Tween20 for 10 min before handling with the primary antibody.

III. Results and discussion

In hematoxylin-eosin staining, Ito cells could not be

identified in every species examined. Therefore, general staining is not suitable for detecting these cells. On the other hand, silver staining revealed the presence of Ito cells in the livers of every species. Plate Figures 1 and 2 exhibit those cells in medaka and bamboo-leaf wrasse, respectively. Ito cells of these fishes closely resembled those found in lamprey liver stained using the improved von Kupffer's gold chloride method (Wake *et al.*, 1987). Fujita *et al.* (1986) observed Ito cell in cod liver using a scanning electron microscope. The 3-dimensional morphology of the cell is in complete agreement with that of Ito cells stained in the present study. In addition, the characteristics of Ito cells observed with silver staining were in good agreement with those of Pacific halibut stained using the gold chloride method (Yoshikawa *et al.*, 2006). In every species examined in the present study, the nucleus of Ito cells had a triangular shape, whereas the nucleus of hepatic parenchymal cells is either spherical or oval in shape. The cytoplasmic protrusion of Ito cells was 10-20 μm long. The number of protrusions was usually 2-4, as observed in the puddingwife wrasse of Plate Figure 3.

On the other hand, Ito cells reacted positively with the TU27 antibody in the livers of medaka and black scraper (Plate Figures 4 and 5, respectively). The protein β -tubulin is usually expressed as a component of a microtubule in the axons of nerve cells. Therefore, this result suggests that Ito cells possess an element of a nerve cell. However, it cannot be said with certainty that Ito cells function as nerve cells do because β -tubulin is distributed over protista and plants as well as nerve cells in animals and plays an important role in cell movement and mitotic cell division. Therefore, we tried to stain Ito cell with another antibody which recognizes the Class III β -tubulin specifically expressed in nerve cells. However, the reaction of this antibody was restricted. A few cells which seemed to be Ito cell showed a positive reaction in black scraper (Plate Figure 6). In medaka, no cells reacted positively. The positive cell in Plate Figure 6 in black scraper does not appear to have cytoplasmic protrusions, as typical Ito cells do, although the section might not have contained parts of the protrusions by accident. However, the number of

Ito cells that reacted with the TU20 was too low when compared with that of cells reacting with the TU27 antibody. Therefore, not all Ito cells, but, rather, only cells in specific stages, might react with the TU20 antibody. Ito cells may express the Class III β -tubulin only under some restricted conditions. To examine the expression of Class III β -tubulin in detail, in teleosts, we should investigate livers at various stages of the reproductive cycle of gonads, of nourishment of the body, or of regeneration from injury to liver.

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Explanations of Plate Figures

Fig. 1 Arrows show Ito cells with cytoplasmic protrusions in medaka. Silver staining.

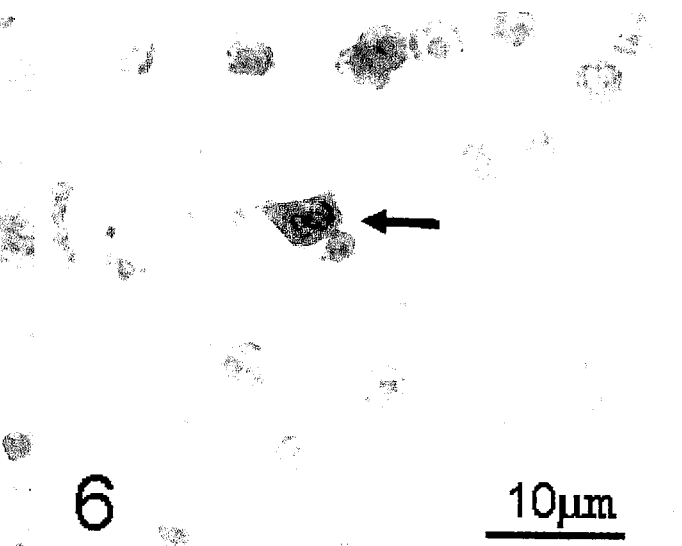
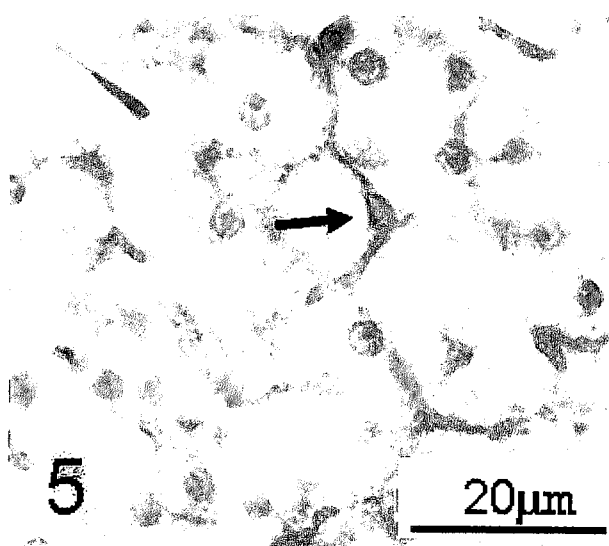
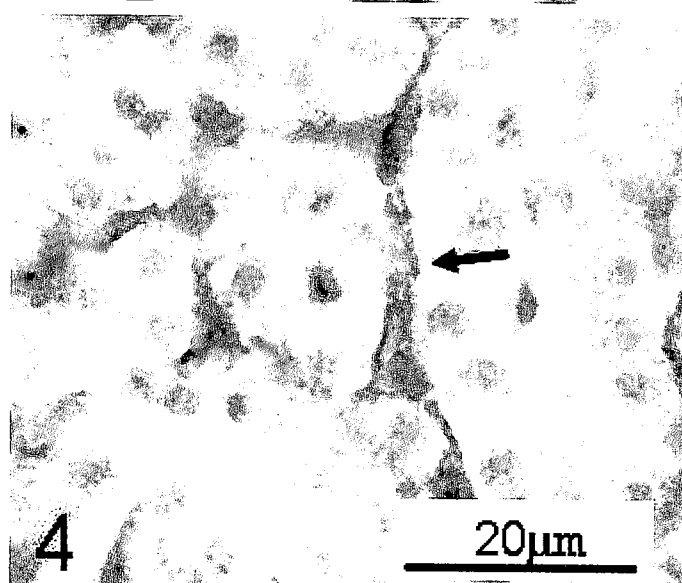
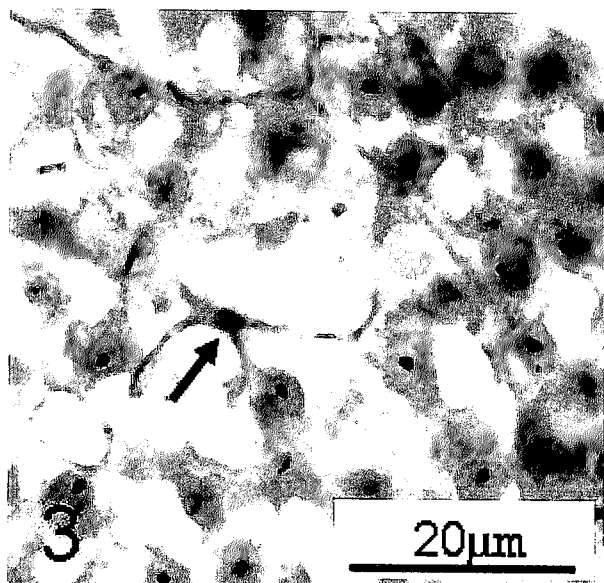
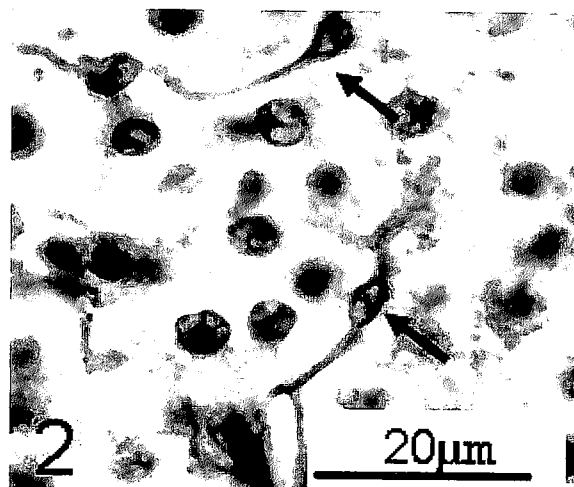
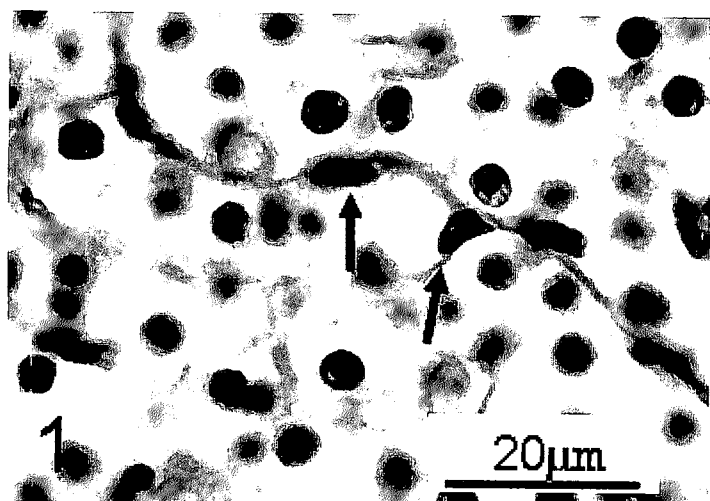
Fig. 2 Arrows show Ito cells with cytoplasmic protrusions in bamboo-leaf wrasse. Silver staining.

Fig. 3 Arrows show Ito cells with cytoplasmic protrusions in puddingwife wrasse. Silver staining.

Fig. 4 An arrow shows the mutual connection of the cytoplasmic protrusions of Ito cells in immunostaining with the TU 27 antibody in medaka.

Fig. 5 An arrow shows Ito cells connected with each other with cytoplasmic protrusions in immunostaining with the TU27 antibody in black scraper.

Fig. 6 An arrow shows an immunopositive cell which seems to be an Ito cell in immunostaining with the TU20 antibody in black scraper.



数種の真骨魚の肝臓に存在する伊東細胞の観察

浅田光子¹・中林 肇²・笹山雄一³

要 旨

7種の真骨魚の肝臓を大塚の渡銀法を用いて調べた。この染色法は、本来、神経細胞や結合組織に存在する細網繊維の検出に用いられる。その結果、調べた全ての種において細胞質突起を持った伊東細胞が鮮明に染色された。これらの魚種の中で、メダカとウマヅラハギについては、 β -チューブリンに対する抗体 (TU27) を用いて免疫染色を行った。この抗体は、原生動物から哺乳類まで存在するすべての β -チューブリンのアイソタイプを認識する。この2種の魚の伊東細胞は、この抗体に対して陽性の反応を示した。本来、 β -チューブリンは、神経細胞の軸索にある微小管の構成要素であるので、この結果は、伊東細胞が β -チューブリンを発現させていることを示唆している。本研究では、さらに神経細胞でのみ発現している Class III β -チューブリンを認識する抗体 (TU20) を用いて、再び、免疫染色を行った。その結果、ウマヅラハギの肝臓においてのみ、少数の伊東細胞様の細胞が陽性の反応を示した。特定の段階にある伊東細胞だけが、Class III β -チューブリンを産生するのかもしれない。したがって、現時点では、真骨魚の肝臓に存在する伊東細胞が、ある種の β -チューブリンを産生していることは確かであるが、少なくともウマヅラハギにおいて、伊東細胞は、神経細胞に似た機能を有しているのかもしれない。

¹金沢大学大学院自然科学研究科生物科学専攻 〒920-1192 石川県金沢市角間町

²金沢大学環日本海域環境研究センター・連携研究員，研究アドバイザー 〒920-1192 石川県金沢市角間町

³金沢大学環日本海域環境研究センター 〒920-1192 石川県金沢市角間町