Summary. The “liver” of puffer fish had both a hepatic portion and a pancreatic portion. The hepatic portion had the parenchyma and the portal area as mammalian livers such as human or mouse or rat. However, the hepatic lobule was not obvious histologically in the puffer liver. In the hepatic parenchyma, both parenchymal cells (hepatocytes) and non-parenchymal cells such as liver sinusoidal endothelial cells and hepatic stellate cells (vitamin A-storing cells, Ito cells) were demonstrated to exist. Cytoplasm of the parenchymal cells was filled with large fat droplets, namely, the liver was in steatosis, but neither hepatic fibrosis nor liver cirrhosis was identified. In the pancreatic portion, pancreatic acinar cells containing zymogen granules were seen. Tetrodotoxin was demonstrated mainly in the acinar cells of the pancreatic portion; hepatic parenchymal cells, liver sinusoidal endothelial cells, and hepatic stellate cells were also shown to contain tetrodotoxin by immunohistochemistry using specific antibody against tetrodotoxin. This is the first report demonstrating the localization of tetrodotoxin in acinar cells of the pancreatic portion and non-parenchymal cells of the hepatic portion in the hepatopancreas of the puffer fish.

Introduction

There have been many cases of human intoxication due to the ingestion of tetrodotoxin-bearing puffer fish, mainly in Japan, China, and Taiwan, and several victims have died (Hashimoto, 1978; Japanese Ministry of Welfare, 1984; Noguchi, 1996; Noguchi et al., 2006a; Noguchi and Arakawa, 2008). Many puffer fish of the family Tetrodontidae possess a potent neurotoxin, tetrodotoxin. In marine puffer fish species, toxicity is generally high in the liver and ovary, whereas in brackish water and freshwater species, toxicity is higher in the skin (Hashimoto, 1978; Japanese Ministry of Welfare, 1984; Noguchi, 1996; Noguchi et al., 2006a; Noguchi and Arakawa, 2008). However, uptake, biosynthesis, and localization of tetrodotoxin in puffer fish are still controversial (Matsumura, 1995, 1998, 2001; Noguchi, 1996; Noguchi et al., 2006a; Noguchi and Arakawa,
Tetrodotoxin is produced primarily by marine bacteria, and puffer fish accumulate tetrodotoxin via the food chain that begins with these bacteria. Consequently, puffer fish become non-toxic when they are fed tetrodotoxin-free diets in an environment in which the invasion of tetrodotoxin-bearing organisms is completely shut off (Noguchi et al., 2006b). However, Matsumura (1995, 2001) demonstrated that tetrodotoxin was detected in the medium materials for the bacterial culture but not in a bacterium. He also reported that the puffer fish embryos could produce tetrodotoxin (1998).

The hepatic lobule of the mammals consists of hepatic parenchymal cells (hepatocytes) and non-parenchymal cells associated with the liver sinusoids: liver sinusoidal endothelial cells, Kupffer cells, pit cells, dendritic cells, and hepatic stellate cells (also called vitamin A-storing endothelial cells, Kupffer cells, pit cells, dendritic cells, and liver sinusoidal stellate cells or Ito cells) (Wake, 1971, 1980; Senoo et al., 1984, 1990, 1993, 1997, 2007, 2010, 2011a, 2011b, 2012; Senoo and Wake, 1985; Blomhoff et al., 1990, 1991, 1992; Blomhoff and Wake, 1991; Blomhoff, 1994; Bloom and Fawcett, 1994; Kojima et al., 1998; Sato et al., 2003; Senoo, 2004, 2007, 2014; Friedman, 2008; Blaner et al., 2009). Liver sinusoidal endothelial cells (Wisse, 1970, 1972) express lymphocyte co-stimulatory molecules (Kojima et al., 2001) and form the greater part of the extremely thin lining of the sinusoids, which are larger than ordinary capillaries and more irregular in shape. Kupffer cells are tissue macrophages and components of the diffuse mononuclear phagocyte system. They are usually situated on the endothelium with cellular processes extending between the underlying liver sinusoidal endothelial cells. The greater part of their irregular cell surface is exposed to the blood in the lumen of the sinusoid. Pit cells are natural killer cells. Dendritic cells locate in the portal area in human liver (Prickett et al., 1988), and in perportal and central areas in rat liver (Steiniger et al., 1984) that capture and process antigens, migrate to lymphoid organs and secrete cytokines to initiate immune responses (Banchereau and Steinman, 1998).

Previously, tetrodotoxin was reported to be localized in hepatic parenchymal cells in puffer liver by immunohistochemistry (Mahmud et al., 2003a). However, no description on the localization of tetrodotoxin in hepatic non-parenchymal cells, namely, liver sinusoidal endothelial cells, hepatic stellate cells, Kupffer cells, and cells within the portal area was reported. The purpose of this study was to analyze thoroughly the structure of puffer liver at light and electron microscopic levels and to analyze and demonstrate the localization of tetrodotoxin at cellular and cell organellar levels.

Materials and Methods

Materials

Three puffer fishes (Takifugu rubripes Temminck et Schlegel) (Fig. 1Aa) that have been reported containing TTX in the liver, ovary, skin, and other organs (Hashimoto, 1978; Japanese Ministry of Welfare, 1984; Noguchi, 1996; Noguchi et al., 2006a; Noguchi and Arakawa, 2008) were purchased from Akita People’s Market (Akita-City, Akita) (January–April, 2012). Mouse monoclonal antibody against tetrodotoxin (IgG1) (Kawatsu et al., 1997; Tanu et al., 2002, 2004; Mahmud et al., 2003a, 2003b) was used for immunostaining.

Methods

The protocols for animal experimentation described in this paper herein were previously approved by the Animal Research Committee of Akita University Graduate School of Medicine. All subsequent animal experiments adhered to the “Guidelines for Animal Experimentation” of the University.

Small parts (1 cm×1 cm×1 cm) of the liver (Fig. 1Ab) were excised and immersed in 3.7% formaldehyde for light microscopy; smaller blocks (2 mm×2 mm×2 mm) were excised and immersed in 2% glutaraldehyde in 0.062 M cacodylate buffer (pH 7.4) containing 1% sucrose for 15 min for electron microscopy as described previously (Imai and Senoo, 1998; Imai et al., 2000; Senoo et al., 2012). The buffer and fixatives were used at room temperature.

Light microscopy

For hematoxylin and eosin (H&E) staining and azan staining, several blocks were immersed in 3.7% formaldehyde as described above, dehydrated in a series of graded ethanol, and embedded in paraffin. For Sudan III staining and Ishii-Ishii’s silver impregnation (Ishii and Ishii, 1965), several liver blocks were immersed in 3.7% formaldehyde as described and cut at 10 μm thickness with a freezing microtome (Leitz, Germany) as described previously (Wake, 1980; Wold et al., 2004; Senoo et al., 2010, 2012).

Fluorescence microscopy for the detection of autofluorescence of vitamin A

Two blocks (3 cm×3 cm×0.5 cm) were immersed in 3.7% formaldehyde for 24 h at 4°C in total darkness, and 20-μm thick sections were made with the freezing microtome. The sections were examined by a Zeiss Axioskop 20FL
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( excitation filter BP365/12, barrier filter BP495/40) for the detection of rapidly fading green autofluorescence characteristic of vitamin A (Popper, 1941; Wake, 1980; Wake et al., 1986; Higashi and Senoo, 2003; Senoo et al., 2010, 2012). Color photomicrographs were taken.

**Transmission electron microscopy (TEM)**
Blocks from the liver were immersed in 1.5% glutaraldehyde in 0.062 M cacodylate buffer (pH 7.4) containing 1% sucrose for 15 min as described above. Then, the tissue blocks were postfixed in 2% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C, dehydrated in a graded ethanol series, and embedded in Epon 812. Ultrathin sections were made with an ultramicrotome EM-UC6 (LEICA) and stained with 2% uranyl acetate and 0.1% lead citrate. Thin sections were examined under a transmission electron microscope (TEM) (HITACHI H-7650) at an acceleration voltage of 80 kV (Nagy et al., 1997; Imai and Senoo 1998; Imai et al., 2000; Higashi et al., 2005; Senoo et al., 2012). Thick sections were examined under a light microscope after staining with 1% toluidine blue containing 1% borax.

**Immunohistochemistry of tetrodotoxin**
To detect tetrodotoxin in the liver of puffer fish, we performed immunohistochemistry using specific antibody against tetrodotoxin (Kawatsu et al., 1997). Samples of
the liver were fixed, dehydrated, and embedded in paraffin as described above. Immunohistochmical study was performed as reported previously (Tanu et al., 2002, 2004; Mahmud et al., 2003a, 2003b) with a slight modification. Briefly, sections (3 μm in thickness) were treated successively with 10% H₂O₂ in distilled water and 25% goat serum in 0.01 M PBS (Iatron Lab. Inc., USA), and then incubated with monoclonal tetrodotoxin-antibody (Kawatsu et al., 1997; Tanu et al., 2002, 2004; Mahmud et al., 2003a, 2003b) followed by the secondary antibody for 50 min. For negative control, mouse serum was used instead of anti-tetrodotoxin antibody. After being treated with 0.017% 3,3’-diaminobenzidine tetrahydrochloride (DAB; Wako Pure Chemical Industries Ltd, Japan) substrate solution in 0.01 M PBS buffer and 0.01% H₂O₂ for 1–2 min, the sections were counter-stained with Carrazzi’s hematoxylin (Merck, Germany), dipped in ammonia (0.25%) water, and observed under a light microscope. Antigen-antibody complexes were visualized as brown color deposits.

Results
Structure of the hepatopancreas of puffer fish
Surface anatomy of the representative puffer fish, Takifugu rubripes (male, 850 g body weight) showed the characteristic black spots surrounded by white circles on each side in the middle of the body (Fig. 1Aa). The surface of the liver (78 g) was beige and smooth (Fig. 1Ab). The gallbladder contained dark green bile juice.

Differential interference microscopy showed many droplets in the liver section (Fig. 1B). Sudan III staining demonstrated that these droplets were formed by fat (Fig. 1C–F). Autofluorescence of vitamin A emanated from the lipid droplets of hepatic non-parenchymal cells but not from the large fat droplets observed by differential interference microscopy or Sudan III staining (Fig. 1D–F).

Around branches of the portal vein, arteries, and ducts in the portal area, pancreatic tissue was readily discernible (Fig. 2A). Cells having granules in the cytoplasm were pancreatic acinar cells containing zymogen granules (Fig. 2B; Bloom and Fawcett, 1994). Thus, the pancreatic portion was clearly shown that it was the exocrine pancreas.

Cytoplasm of hepatic parenchymal cells (hepatocytes) was occupied by fat droplets (Fig. 2C–F). The place that was occupied by these fat droplets was transparent because of extraction of fat during the preparation procedure using organic solvent such as ethanol and xylol (Fig. 2C–F). No obvious structure of hepatic lobule was observed. Within the cytoplasm of hepatic parenchymal cells, meshwork-like structure of cytoplasmic substances remained even after tissue preparation using ethanol and xylol (Fig. 2C–F); the nucleus was usually pressed to the periphery (cell membrane) of the cell (Fig. 2C–F). Sinusoid contained nucleated blood cells (Fig. 2C–F). In the perisinusoidal space, hepatic non-parenchymal cells containing lipid droplets were observed (Fig. 2C–F). The localization and structure of the cells with cytoplasmic lipid droplets were consistent with that of hepatic stellate cells.

In the H&E preparation, neither inflammation nor fibrosis was demonstrated in the liver even hepatic parenchymal cells were occupied by large fat droplets (Fig. 2A, C–F).

Extracellular matrix (ECM) in the hepatopancreas of puffer fish
No fibrosis was demonstrated in the hepatic parenchyma and the portal area by Ishii-Ishii’s silver impregnation method (Fig. 3A). Fine ECM fibers extended directly from the portal area into the parenchyma, but neither fibrosis nor liver cirrhosis was found (Fig. 3A–F). The fine ECM fibers distributed with the sinusoid and perisinusoidal space (Fig. 3B, D). Usually the nuclei were pressed to the periphery of hepatic parenchymal cells (Fig. 3B, D, F), but sometimes the nucleus was located in the center of the cell. Pancreatic portion existed in the portal area and around a vein (Fig. 3A, C, E).

Histology of the hepatopancreas shown by azan staining was essentially the same with that obtained by Ishii-Ishii’s silver impregnation method (Fig. 3E, F). The cytoplasm of hepatic parenchymal cells was occupied by fat droplets, but neither fibrosis nor cirrhosis was observed (Fig. 3F).

Transmission electron microscopy (TEM) of the hepatopancreas of puffer fish
In the pancreatic portion of the liver, acinar cells were demonstrated containing zymogen granules in the cytoplasm (Fig. 4A). The cytoplasm was filled with zymogen granules containing proenzymes and well-developed rough-surfaced endoplasmic reticulum (Fig. 4B). Large nucleolus was discernible in the nucleus (Fig. 4B).

In the hepatic portion, hepatic parenchymal cells contained large fat droplets in the cytoplasm (Fig. 4C–I). The number and size of these droplets indicated that these morphological characteristics were not inconsistent with the light microscopical findings, namely, differential
Fig. 2. Hematoxylin and eosin (H&E) staining of liver sections of the hepatopancreas in puffer fish. Portal area contains a pancreatic portion (PP) (A). Acinar cells in the pancreatic portion are readily discerned (B). In the hepatic portion, hepatic parenchymal cells (hepatocytes; P) and hepatic stellate cells (HSCs; arrows) containing fat (F) or vitamin A-lipid droplets (L), respectively (C–F). Nuclei of hepatic parenchymal cells are pressed to the periphery of the cytoplasm. Sinusoid (S) formed by liver sinusoidal endothelial cells contains nucleated blood cells (arrowheads) (C–F). Hepatic stellate cells (arrows) contain vitamin A-lipid droplets (L) (C–F). Staining was performed as described in Materials and Methods. Bars: 500 μm (A), 50 μm (B–F).
Fig. 3. Ishii-Ishii’s silver impregnation method (A–D) and azan staining (E, F) of the hepatopancreas of puffer fish. The pancreatic portion (PP) is readily recognized in the portal area (PA) (A, E). Connective tissue fibers (arrows) extend from the portal area (PA) into the hepatic parenchyma (Pa) (B). The hepatopancreas shows neither hepatic fibrosis nor liver cirrhosis. The cytoplasm of hepatic parenchymal cells (P) is occupied by fat droplets (FD) (D, F). Nuclei of hepatic parenchymal cells are pressed to the periphery of the cytoplasm (D, F). Ishii-Ishii’s silver impregnation method and azan staining were performed as described in Materials and Methods. Bar: 500 μm (A, C, E), 50 μm (B, D, F).
Fig. 4. Transmission electron micrographs of the hepatopancreas of puffer fish. Acinar cells in the pancreatic portion contain zymogen granules (g) (A, B). The nucleus of the acinar cell contains large nucleolus (n) (B). In the hepatic portion, the cytoplasm of a hepatic parenchymal cell is filled with fat droplets (FD) (C–I). Nucleus (N) of a hepatic parenchymal cell (P) is indented (arrow) and pressed to the periphery of the cytoplasm by the fat droplets (FD) (D). One bile canaliculus is formed by four hepatic parenchymal cells (P) (E). Nucleated blood cells (arrowhead) are shown within the sinusoid (S) (F, G). Liver sinusoidal endothelial cells (asterisks) forming sinusoid (S) (G–I). Hepatic stellate cells (SC) are located in the perisinusoidal space (painted blue) between hepatic parenchymal cells (P) and liver sinusoidal endothelial cells (asterisks). J, junctional complex; L, lipid droplets in hepatic stellate cells; mv, microvilli; rER, rough-surfaced endoplasmic reticulum. Transmission electron microscopy was performed as described in Materials and Methods. Bar: 25 μm (A), 10 μm (F), 5 μm (D, G, H), 4 μm (C), 2 μm (B, I), 1 μm (E).
interference (Fig. 1B), Sudan III (Fig. 1C), H&E (Fig. 2C–F), Ishii-Ishii’s silver impregnation (Fig. 3A–D), and azan (Fig. 3E, F) staining. The nucleus containing well-developed nucleolus was compressed to the periphery of the cytoplasm (Fig. 4D).

Bile canaliculus was formed by hepatic parenchymal cells (Fig. 4E). Here, four hepatic parenchymal cells formed one bile canaliculus. The number of hepatic parenchymal cells forming the canaliculus is different from that of mammals such as human or rat. Microvilli of the bile canaliculus extended from the apical cell membrane of hepatic parenchymal cells into the lumen of the bile canaliculus. Junctional complexes were demonstrated to exist between hepatic parenchymal cells forming the bile canaliculus (Fig. 4E). Within the sinusoid, nucleated blood cells were shown (Fig. 4F, G). The blood cells indicated that there was a bloodstream in the sinusoid.

Liver sinusoidal endothelial cells were demonstrated between the sinusoid and perisinusoidal space (the space of Disse) (Fig. 4G–I). Hepatic stellate cells were located between liver sinusoidal endothelial cells and hepatic parenchymal cells (Fig. 4G–I). Hepatic stellate cells contained lipid droplets within the cytoplasm (Fig. 4G, I). The structure and localization of the lipid droplets were similar to that of the vitamin A-lipid droplets in hepatic stellate cells of the mammals such as human or rat.

**Localization of tetrodotoxin in the liver of puffer fish**

The distribution of tetrodotoxin was visualized as brown color in the pancreatic portion of the puffer liver (Fig. 5A–C). Zymogen granules and nuclei of the acinar cells were stained brown (Fig. 5C). Hepatic parenchymal cells in the hepatic portion were stained also positively (Fig. 5A, B, D). In the cytoplasm of hepatic parenchymal cells, network of remained substances that were not extracted by tissue preparation process using organic solvent was stained positively (Fig. 5B, D). The nuclei of hepatic parenchymal cells were also stained brown (Fig. 5D). The cytoplasm and nucleus of endothelial cells were stained also brown (Fig. 5B).

The cytoplasm and nucleus of hepatic stellate cells were stained brown (Fig. 5D). These cells were identified because the cytoplasm contained lipid droplets. Tetrodotoxin antigen was not shown in the control section using non-immune mouse serum instead of the antibody against tetrodotoxin (Fig. 5E, F).

In summary, tetrodotoxin existed in the zymogen granules and nuclei of pancreatic acinar cells, and cytoplasm and nucleus of hepatic parenchymal cells, hepatic stellate cells, and liver sinusoidal endothelial cells in the hepatic portion of the hepatopancreas.

**Discussion**

There are two general basic types of fish livers: those that contain pancreatic tissue versus that do not (Genten et al., 2009). Fish livers with exocrine pancreas are often called “hepatopancreas.” The pancreas is generally diffusely spread within the fat and mesenteries that connect the intestine, stomach, liver and gallbladder. It can also form a discrete organ (Genten et al., 2009). It is composed of exocrine and endocrine tissues. The exocrine pancreas that localizes in the hepatopancreas consists of clusters of pyramidal cells mostly organized in acini as observed in mammals. In the present study, the pancreatic portion of the hepatopancreas of puffer fish (Takifugu rubripes Temminck et Schlegel) contained acini. The acini contain acinar cells that have a dark basophilic cytoplasm, distinct basal nuclei, and many large eosinophilic zymogen granules containing proenzymes responsible for the digestion of proteins, carbohydrates, fats, and nucleotides.

The present study is the first report demonstrating the localization of non-parenchymal cells in the hepatic portion of hepatopancreas of puffer fish, namely, liver sinusoidal endothelial cells and hepatic stellate cells, and the fat accumulation (steatosis) in the cytoplasm and nuclear deviation to the periphery of hepatic parenchymal cells.

In alcoholic liver disease (ALD) (Clark et al., 2002), non-alcoholic fatty liver disease (NAFLD), and non-alcoholic steatohepatitis (NASH) (Ludwig et al., 1980; Diehl, 1999; Newuschwander-tetri and Caldwell 2003), fat accumulates in hepatic parenchymal cells. The responsible cell causing hepatic fibrosis and liver cirrhosis in these diseases has been suggested to be hepatic stellate cells.

Although there is influence of species, strain, and sex difference on the degree of hepatic steatosis, inflammation, and fibrosis, animal models have greatly contributed to the understanding of NASH and NAFLD (Kirsch et al., 2003). In the present study, even large fat droplets accumulated in hepatic parenchymal cells, neither fibrosis nor liver cirrhosis was seen in puffer fish liver. This kind of fish might be useful for investigation of molecular and cellular mechanisms of NASH and NAFLD, and further useful for the search of the therapy of such liver diseases, because all the genome has been revealed in DNA of the puffer fish (Brenner et al., 1993; Aparicio et al., 2002).

Hepatic parenchymal cells of puffer fish were reported to contain tetrodotoxin (Mahmud et al., 2003a). Our present study further demonstrated that the puffer “liver” is hepatopancreas and tetrodotoxin mainly localizes in the pancreatic portion of the hepatopancreas by using the same immunohistochemical method as that of the
Fig. 5. Immunohistochemistry of tetrodotoxin using specific antibody against tetrodotoxin. Acinar cells (ac) in the pancreatic portion are positively stained by immunohistochemistry of tetrodotoxin (A–C). Zymogen granules in the cytoplasm, the nucleus and the area surrounding the nucleus are positively stained (C). The pancreatic portion (PP), endothelium (arrowheads) (B), and the network of the remaining substance (asterisk) in the cytoplasm of hepatic parenchymal cells (P) of hepatic portion show the positive reaction (D). Hepatic stellate cells (arrows) containing lipid droplets in the cytoplasm show also positive reaction. Control sections that mouse serum was used instead of anti-tetrodotoxin antibody (E, F). Bar: 500 μm (A), 50 μm (B–F).
previous study (Mahmud et al., 2003a). In the present study, tetrodotoxin was detected in the nuclei in acinar cells of the pancreatic portion, parenchymal cells, liver sinusoidal endothelial cells, and hepatic stellate cells of the hepatic portion. These data is consistent with the report by Nagashima et al. (1999) demonstrating that fractions of blood cells, nucleus, mitochondria, cytosol obtained from the liver homogenate of puffer fish contained tetrodotoxin. They showed by HPLC analysis that the amount of tetrodotoxin was high (82.2±8.7%) in the cytosol fraction and some (5.4±4.2%) in the nuclear fraction of the total tetrodotoxin amounts of puffer fish liver. However, the route of exogeneous tetrodotoxin reaching the nucleus and function of the molecule in the nucleus is still unknown.

As reported previously, tetrodotoxin is localized in skin (Mahmud et al., 2003b; Tanu et al., 2002, 2004) and ovary (Mahmud et al., 2003a; Tanu et al., 2002, 2004) of puffer fish. It was inferred that when tetrodotoxin from the blood plasm enters the undifferentiated basal skin cells through diffusion, it is taken by the lysosomes by phagocytosis and exists there through binding with internal constituents of the cell organelles (Tanu et al., 2002; Mahmud et al., 2003b). This tetrodotoxin is probably secreted from the body surface (Tanu et al., 2002; Mahmud et al., 2003b). These molecules might be useful for the body protection from the environment.

In the ovary, tetrodotoxin was reported to be localized in the nuclei of oocytes and in the connective tissue (Mahmud et al., 2003a; Tanu et al. 2002, 2004). The physiological function of tetrodotoxin in puffer eggs remains obscure (Mahmud et al., 2003a), but it has been speculated that the toxin is protective against natural environment.

This is the first report that tetrodotoxin localizes in the pancreatic portion and non-parenchymal cells, namely, liver sinusoidal endothelial cells and hepatic stellate cells of hepatic portion in the hepatopancreas of puffer fish.

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