

# How Do Lampreys Avoid Cholestasis After Bile Duct Degeneration?

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## 1. Introduction

Biliary atresia (BA) is the most common cause of cholestasis during infancy for which an etiology remains undetermined. Patients require hepatic portoenterostomy within the first 2-3 months of life in order to restore bile flow from the liver into the intestinal tract. Even with successful surgery, in most patients the disease advances to end-stage cirrhosis due to the progressive destruction of bile ducts, and requires liver transplantation in order for long term survival to be viable (Mack & Sokol, 2005). Without a better understanding of the etiology and pathogenesis of the progressive cholangitic mechanisms in BA, improvement in the prognosis of non-transplantation patients should not be expected. Despite the importance of understanding these underlying mechanisms, research of BA has been hindered by the lack of a suitable animal model.

The lamprey is the only vertebrate in which bile duct disappearance occurs spontaneously during metamorphosis. Multiple reports have described a histological similarity between human and lamprey bile duct loss and suggested that the lamprey be used as an animal model in the study of BA (Youson & Sidon, 1978; Boomer et al., 2010; Morii et al., 2010). While the lamprey differs greatly from humans in both appearance and character, striking parallels exist between the behavior of their hepatocytes and biliary epithelial cells. Understanding the mechanism that controls cholestasis in the lamprey liver during metamorphosis might contribute to the development of treatments for obstructive cholangiopathy in human BA.

## 2. Lamprey biology

The lampreys, which include 38 species, are considered the ancestral representatives of vertebrates, and studies regarding their genealogy, phylogeny and biology have all been made in detail (Hardisty & Potter, 1971).

## 2.1 Lamprey life cycle

All lampreys have a complex life cycle that consists of both larval and adults stages with a full metamorphosis in between. Eggs laid in freshwater streams hatch, giving rise to young larvae which burrow into the mud and feed on detritus. After a period of 4 to 7 years, larvae undergo metamorphosis into an adult lamprey.

Adult lampreys can be divided into two categories, parasitic (anadromous) and non-parasitic (fluvial), based on their adult life history immediately following metamorphosis. After metamorphosis, parasitic lampreys migrate to lakes or oceans in order to parasitize fish. After several years, sexually mature adults migrate back to freshwater streams to reproduce. Non-parasitic lampreys, on the other hand, stop feeding before the commencement of metamorphosis, spawn and die without ever resuming feeding.

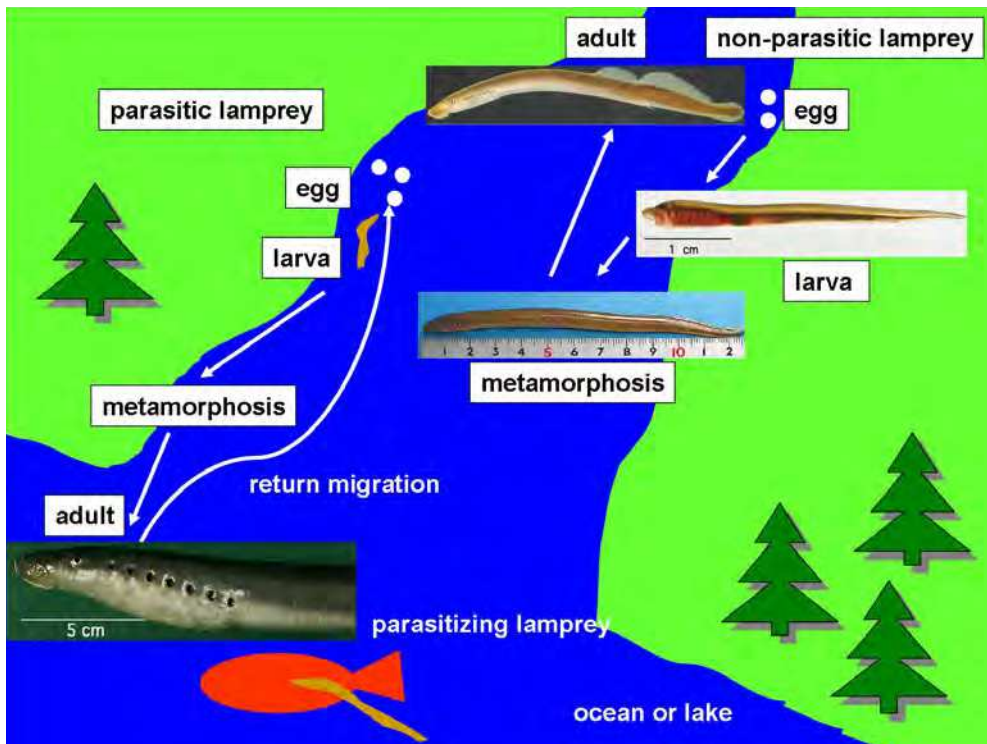


Fig. 1. Reproductive life cycle of lampreys.

Figure 1 illustrates the reproductive life cycle of lampreys with photographs taken during different stages of development. An undeveloped larva has no eyes, and the green bile juice of its gall bladder is visible through the abdominal skin. At the onset of metamorphosis, a small eye begins to appear. The top and bottom photographs show parasitic and non-parasitic adult lampreys, each with a prominent oral disc.

## 2.2 The lamprey in Japan

The occurrence of three *Lethenteron* species, *L. japonicum* (parasitic), *L. kessleri* (non-parasitic) and *L. reissneri* (non-parasitic), have been reported in Japan (Yamazaki & Goto, 1998). Taxonomically, these species are closely related to one another and may constitute a group of satellite species, a term used to describe ancestor-descendant pairs in lampreys. Morphological similarities between parasitic and non-parasitic species have supported evidence that different non-parasitic species evolved multiple times from parasitic species. Current hypotheses postulate that the evolution of non-parasitism in lampreys may be the result of a protracted larval stage (Yamazaki et al., 2001).

## 2.3 Metamorphosis of the lamprey

During metamorphosis, lampreys develop functional eyes and a prominent oral disk with teeth. Internal organs, such as the alimentary canal and the liver, also undergo significant changes during this process. A non-parasitic lamprey in Japan, *L. reissneri*, stops feeding prior to the commencement of metamorphosis and never resume feeding because major alterations to the digestive system, including loss of the gall bladder and biliary tree in the liver and prominent atrophy of the intestinal canal, have occurred. A parasitic lamprey in Japan, *L. japonicum*, parasitizes fish in order to feed on blood and tissue which is then digested by its potent saliva. This change in feeding behavior makes digestive requirements of the adult parasite much different than those of the larval stage lamprey. In order to accommodate these changes, bile ducts disappear entirely and the intestinal canal becomes specialized for nutrient absorption (Higashi et al., 2005).

## 2.4 Bile salts of lampreys

Bile salts are the major end-metabolites of cholesterol and serve an important function in digestion. For many species of fish, they are also used as potent olfactory stimulants. The increased use of cholesterol by vertebrates as compared to invertebrates is believed to have been the cause of a major evolutionary shift, allowing vertebrates to better regulate cell membrane fluidity, insulate nerve fibers and synthesize steroid hormones. The increased levels of cholesterol seen in vertebrates require tightly regulated systems for controlling the synthesis of cholesterol and its elimination from the body. The ability to remove cholesterol from the body in a regulated fashion is accomplished by the parallel development of a hepatobiliary tract.

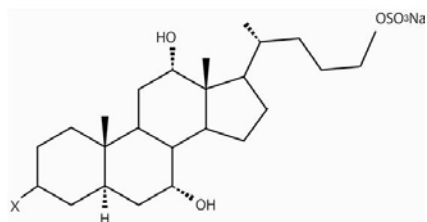
Sea lampreys are known to produce, release or contain four kinds of sulfated bile salts (Fig. 2). Three kinds of bile salts (petromyzonol sulfate, petromyzonamine disulfate, and petromyzosterol disulfate) in particular are known to be produced by larval lampreys and excreted in feces so that they may function as migratory pheromones for adults (Fig. 2). 3-ketopetromyzonol sulfate, an oxidized analog of petromyzonol sulfate, is produced and released by sexually mature male sea lampreys, and functions as a sex pheromone that attracts ovulating females (Burns et al., 2011).

## 3. Hepatobiliary structure of larval lampreys

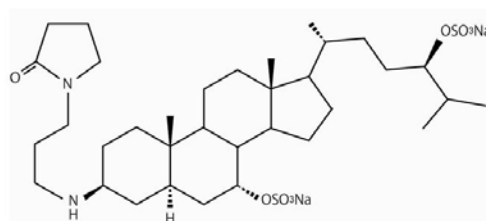
During the larval period, lampreys have a biliary system that is similar to that of humans. Bile acids are synthesized from cholesterol in hepatocytes and secreted into the alimentary

petromyzonol sulfate (X is  $\alpha$ -OH)

3-ketopetromyzonol sulfate (X is =O)



petromyzonamine disulfate



petromyosterol disulfate

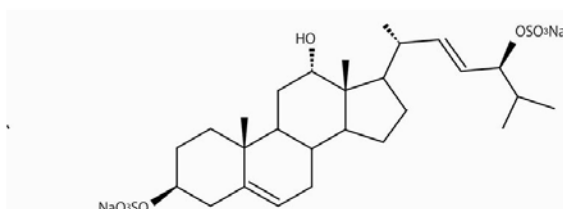


Fig. 2. Principal bile salts of the sea lamprey.

canal through the bile duct. We analyzed the morphology of *L. reissneri*, a non-parasitic lamprey, as they do not migrate, allowing larvae, metamorphosing animals and adults to be collected from the same waters (Yamazaki & Goto, 2000).

### 3.1 General liver structure of larval lampreys and humans

Larval lampreys have a complete biliary system similar to that of humans. The system contains terminal hepatic venules, a portal triad and sinusoids. The portal triad is composed of a portal vein, a hepatic artery and bile ducts and the interconnecting plates of hepatocytes which surround the sinusoidal channels run between the central vein and the portal triad. The entire liver appears fan-shaped and is similar to the functional unit in the human liver known as the acinus (Fig. 3).

### 3.2 Larval biliary system

Five-micron serial sections were made from the livers of larval lampreys and stained with hematoxylin and eosin (HE). Some slides were recorded digitally using NanoZoomer Digital Pathology (Hamamatsu Photonics, Hamamatsu, Japan). Studies of serial sections confirmed that larvae have a complete biliary system similar to that of humans (Fig. 4).

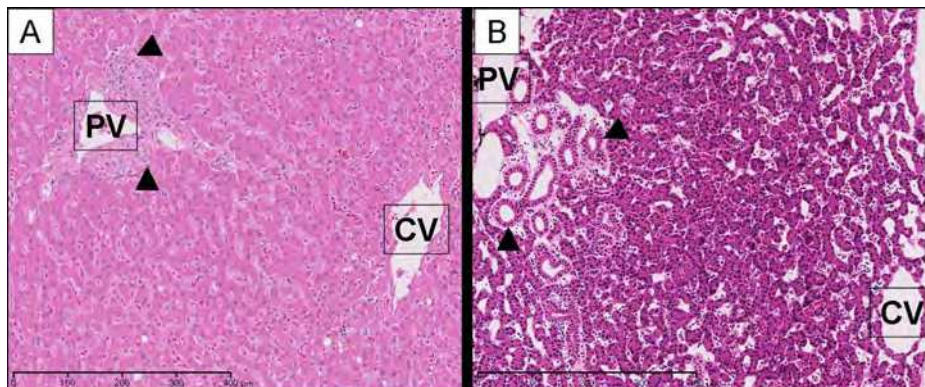


Fig. 3. Histologic analyses of human (panel A) and larval lamprey (panel B) liver specimens using HE staining to show the general liver architecture. PV: portal vein. CV: central vein (branch of hepatic vein). ▲: bile ducts.

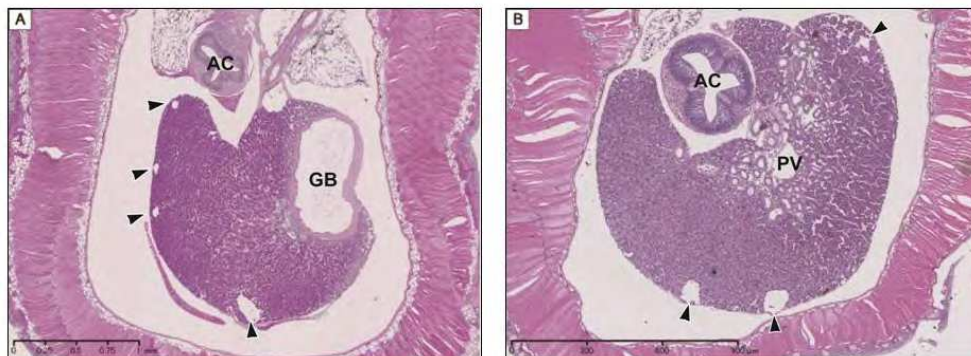
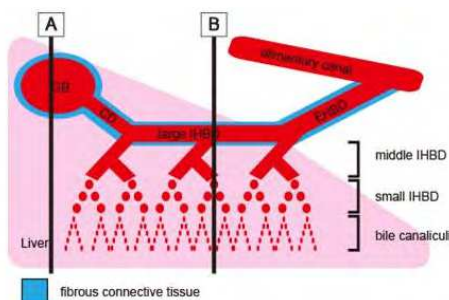


Fig. 4. Schematic diagram of the bile duct system in the liver of a lamprey larva and low-power micrographs of cross-sections taken at points A and B (depicted in panels A and B, respectively). GB: gallbladder. CD: cystic duct. EHBD: extrahepatic bile duct. IHBD: intrahepatic bile duct. AC: alimentary canal. PV: portal vein. ▲: hepatic veins.

The gall bladder (GB) was located at the cranial end and the cystic duct (CD) connected the GB to a large intra-hepatic bile duct (IHBD) that collected bile juice from the liver. The large

IHBD was composed of a simple columnar epithelium surrounded by some fibrous connective tissue. The middle IHBDs were also composed of simple columnar epithelia but they lacked surrounding connective tissue. The small IHBDs consisted of simple cuboidal epithelia. A cross-section of the central region of the liver revealed a single lobe with a large IHBD, portal vein and hepatic artery, as well as hepatic veins present at the edge.

### 3.3 Structure of bile canaliculi

Prior to examination by transmission electron microscopy (TEM), livers of larval lampreys were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 3 h, rinsed with the same buffer (pH 7.4), postfixed in 2% osmium tetroxide for 2 h, dehydrated and then embedded in Epon-812 resin. Ultrathin sections of the livers were cut at 60 nm by an ultramicrotome (LKB 250) and stained with uranylacetate and lead citrate. The stained sections were examined under a transmission electron microscope (JEM-1200EX, JEOL) at an acceleration voltage of 100 kV.

Electron microscopic analyses of specimens during the larval period revealed bile canaliculi within lumina surrounded by three to six hepatocytes. Numerous microvilli were present on the apical membrane of the canaliculi (Fig. 5).

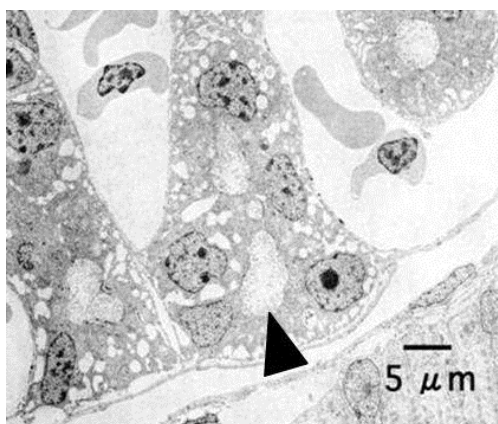


Fig. 5. Transmission electron micrograph of the bile canaliculi of a lamprey larva.  
▲: bile canaliculus.

### 4. Changes in the biliary system at metamorphosis

As part of the normal ontogeny of the sea lamprey, the entire bile-transport apparatus of the larvae disappears completely during metamorphosis; however, the cause of this BA is not known.

Disappearance of the bile ducts accompanied by inflammatory change or necrosis have, until recently, been considered the primary processes occurring during human BA. Several recent studies have reported, however, that apoptosis of biliary epithelial cells takes place during BA (Sasaki et al., 2001; Harada et al., 2008; Funaki et al., 1998). Apoptosis, or programmed cell death, is defined in the narrow sense as the process associated with nucleic



acid fragmentation due to a caspase cascade, which eventually leads to caspase-3 accumulation in the cell nucleus (Alnemri et al., 1996). Cleavage of DNA by caspase cascades can be induced by various factors in many kinds of cells. These factors can include viral infection in biliary epithelial cells, ischemia in the neurocytes of prenatal fetuses, cholestasis in the hepatocytes with intestinal failure-associated liver diseases.

We have previously demonstrated that apoptosis plays a significant role in the degeneration of lamprey bile ducts during metamorphosis (Morii et al., 2010). The involvement of apoptosis in both human BA and lamprey bile duct degeneration during metamorphosis indicates that lampreys could be used as a model animal for studying the etiology and underlying mechanisms of human BA.

#### 4.1 Liver structure in metamorphosing lampreys and humans with BA

The Liver structures of metamorphosing lamprey have some similarities to those of humans with BA at the time of hepatic portoenterostomy during the first 2-3 months of life.

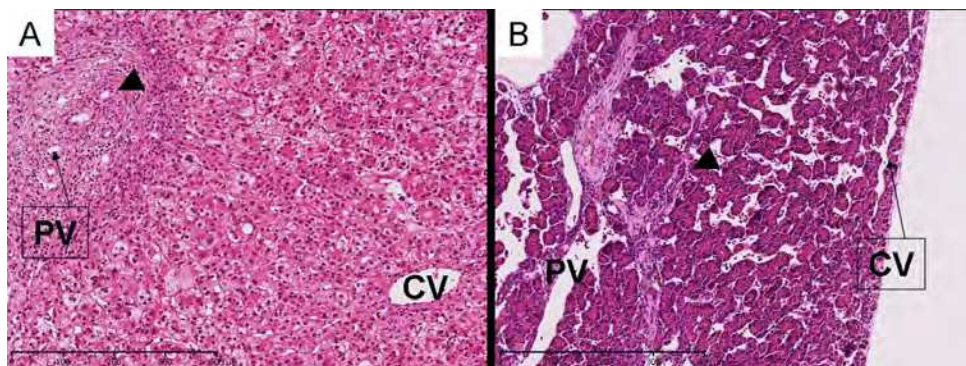


Fig. 6. Histological analyses of liver specimens from a human with BA (panel A) and a metamorphosing lamprey (panel B) using HE staining. The photograph in panel A was taken when the patient underwent initial surgery for treatment of BA. PV: portal vein. CV: central vein (branch of hepatic vein). ▲: bile ducts.

Both micrographs in figure 6 show the marked expansion of portal space with fibrous connective tissue and degeneration of bile ducts with intercanalicular bile stasis or debris. However, the intracellular bile stasis, proliferation of lobular bile ducts and infiltration of polymorphonuclear leukocytes are observed only in the human specimen (Fig. 6), indicating more severe cholestasis in human BA as compared to lamprey biliary degeneration.

#### 4.2 Apoptosis of biliary epithelial cells

Five-micron serial sections of paraffin-embedded blocks were made from the livers of lampreys during the early metamorphosing stage. Alternate sections were stained with HE or apoptotic markers such as terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) or immunohistochemistry using an antibody against active caspase-3.

#### 4.2.1 Light microscopy

Figure 7 shows the cross section of the GB stained with HE. The upper edge of the cross section is the mucosa of the GB, which is composed of biliary epithelial cells. The amount of fibrous connective tissue beneath the mucosa increases, and the biliary epithelial cells degenerate as the height of the epithelial cells is reduced. A muscular layer with myocytes was not present in the mucosa and some basophilic components were observed between the fibrils.

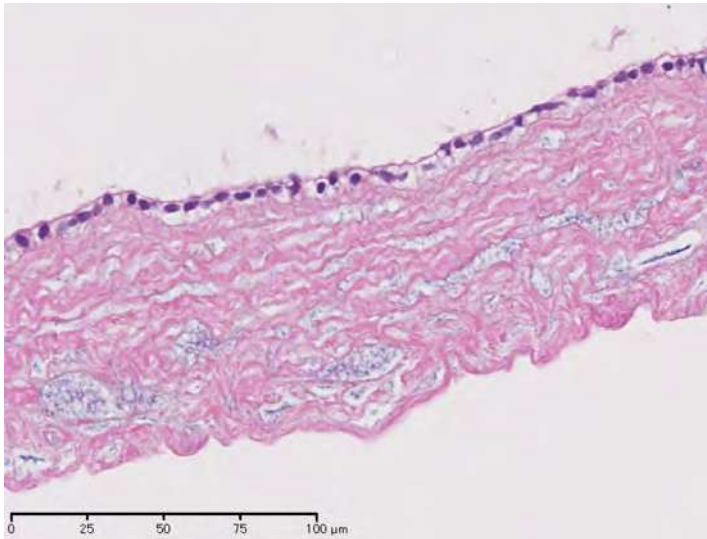


Fig. 7. Cross section of part of the gallbladder of a metamorphosing lamprey.

#### 4.2.2 TUNEL staining

Sections were subjected to TUNEL assay using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI). Chronological TUNEL analysis of metamorphosing lampreys showed that apoptosis started in the region around the CD and then progressed towards both the periphery and the center (Morii et al., 2010).

Figure 8 shows that the epithelial cells of the GB were positive for TUNEL staining. TUNEL-positive staining was also observed within the extracellular debris among the connective tissue of the submucosa. This extracellular debris corresponded to the basophilic components seen in figure 7. In humans, apoptotic cells, which contain basophilic components such as nucleic acids, are processed and degraded promptly by phagocytosis. If intercellular components cannot be processed, the resulting autoimmune diseases lead to morbidity in humans. In lampreys, the activity of phagocytotic cells may not be sufficient for complete degradation of apoptotic cells, leading to the presence of the TUNEL-positive cell debris observed in the submucosa. In larval lampreys, we did not detect any signs of DNA fragmentation of the biliary system, which included hepatocytes, bile canaliculi, the extrahepatic and intrahepatic bile ducts, the CD and the GB (date not shown).



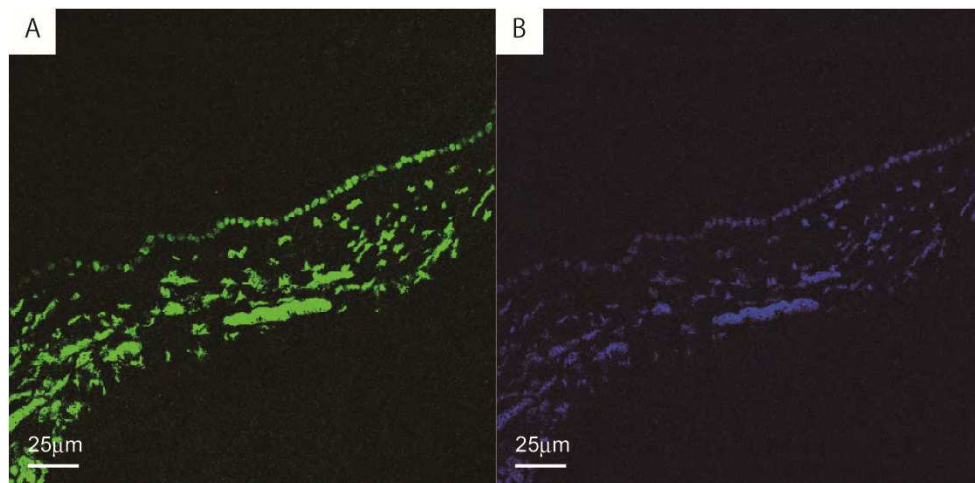


Fig. 8. TUNEL staining of the GB of a metamorphosing lamprey. The upper most layer is the mucosa epithelial cell layer. Most epithelial cells are TUNEL-positive (panel A, green). Nuclei are stained with TO-PRO-3 (panel B, blue).

#### 4.2.3 Active caspase-3 staining

To confirm that these cells were undergoing apoptosis, cross sections of early metamorphosing stage lampreys were stained with an antibody against active caspase-3, a key regulator of the apoptotic cascade activated during the early phase of apoptosis prior to DNA fragmentation.

The deparaffinized sections were treated with 0.2% Triton X-100 in PBS for permeabilization, incubated with a blocking solution (1% bovine serum albumin), and then incubated with 1:50 rabbit anti-active caspase-3 antibody (Cell Signaling Technologies, Danvers, MA) at 4°C overnight. Following washes, the sections underwent incubation with 1:100 biotin SP-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). Finally, the sections were treated with 1:100 streptavidin, Alexa Fluor 488 conjugate (Invitrogen, Carlsbad, CA). Nuclei were stained with TO-PRO-3 (Invitrogen).

Caspase-3 has been identified as a key mediator of apoptosis. In response to various death signals, caspase-3 proenzyme is activated in the cytoplasm before it enters the nucleus, where it leads to the fragmentation of DNA (Woo et al., 1998; Zheng et al., 1998; Kamada et al., 2005).

In our observation, epithelial cells of the CD, GB and part of the large IHBD at the early metamorphosing stage were negative for active caspase-3 staining (data not shown) while they were positive for TUNEL staining, indicating the occurrence of more advanced apoptotic processes in these regions; however, the nuclei of the epithelial cells of middle IHBDs were positive for active caspase-3 staining (Fig. 9). Collectively, these observations indicate that apoptotic cascades in the CD, GB and the large IHBD precede those in the middle IHBDs.

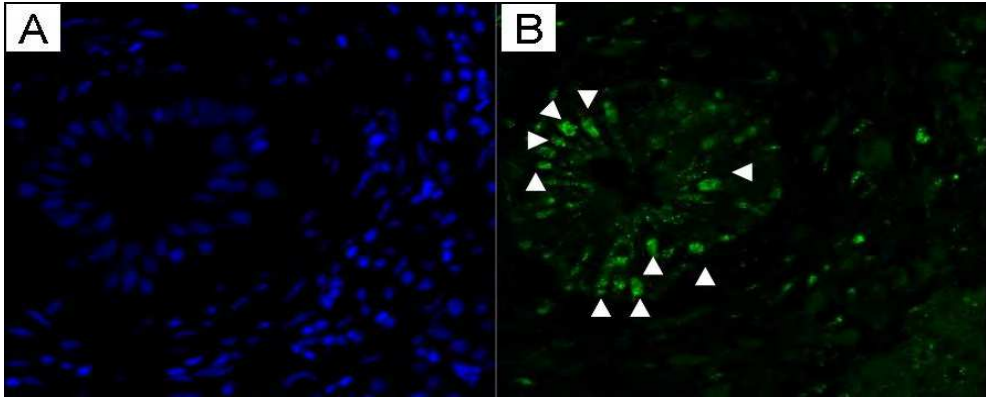


Fig. 9. Immunohistochemical staining of biliary epithelial cells of an early metamorphosing lamprey with an antibody against active caspase-3. The middle IHBDs of metamorphic lampreys were stained with an antibody against active caspase-3 (green, panel B), followed by staining with TO-PRO-3 (blue, panel A) for nuclei.  $\Delta$ : nuclei of biliary epithelial cells.

#### 4.2.4 Cross-reactivity of an antibody against human active caspase-3 to lamprey active caspase-3

In order to confirm the presence of caspase cascades in lampreys and the cross-reactivity of an antibody raised against human active caspase-3 to lamprey active caspase-3, a primary culture of lamprey cells was treated with staurosporine, an apoptosis-inducing reagent, and then analyzed in order to ascertain the expression of active caspase-3 using western blotting.

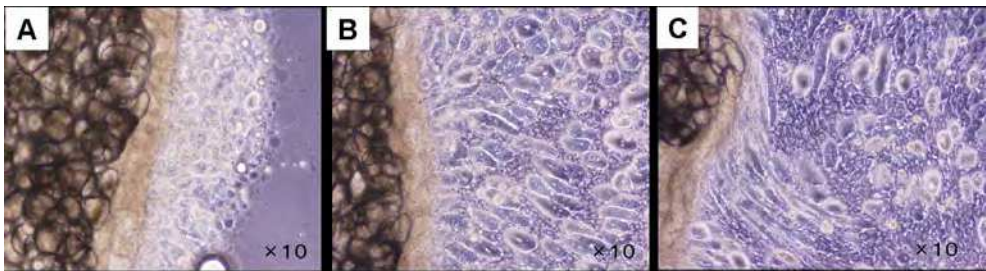


Fig. 10. Migration of cellular patches. Primary cell culture after 5 h (panel A), 24 h (panel B) and 2 days (panel C).

Anesthetized lampreys were washed with phosphate buffered saline (PBS) solution with 100 U/mL penicillin and 100 mg/mL streptomycin. The epidermis was then scraped off using an operating knife blade starting at the tail of lamprey. Scraped tissue was placed onto plastic dishes and cultured for 7 days in L-15 medium (Invitrogen) with 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin in a CO<sub>2</sub> ambient incubator at 20°C. Cellular patches were formed by the migration of tissue from small explants (Fig. 10). Culture medium was changed every 3 days. After 7 days of incubation, we removed the explants from tissue clumps. Cells reaching 30–60% confluence were harvested and washed with the medium and seeded onto new dishes.

Three days later, cells were treated by 1  $\mu$ M staurosporine (Cosmo Bio, Tokyo, Japan) and collected after 4 h. After centrifugation at 1,000  $\times$  g for 5 min, the pellets were washed by PBS and dissolved in SDS gel-loading buffer. Two micrograms of cell pellets were separated by sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western-blotting using rabbit anti-active caspase-3 antibody (Cell signaling Technologies, Danvers, MA).

In mammals, pro-caspase-3, which is about 35 kD, is present in both mitochondria and the cytosol. When a death signal is transmitted to the cells, caspase cascades are activated, resulting in pro-caspase-3 dividing into two activated fragments. Activated fragments accumulate in cell nuclei, leading to nucleic acid fragmentation. The anti-active caspase-3 antibody we used can detect activated fragments of approximately 17 kD. Western blot analysis of lamprey cells from samples treated with staurosporine displayed a band of approximately 17 kD (Fig. 11) which was not present in untreated control cells, indicating that the band in treated cells corresponded to the presence of active caspase-3.

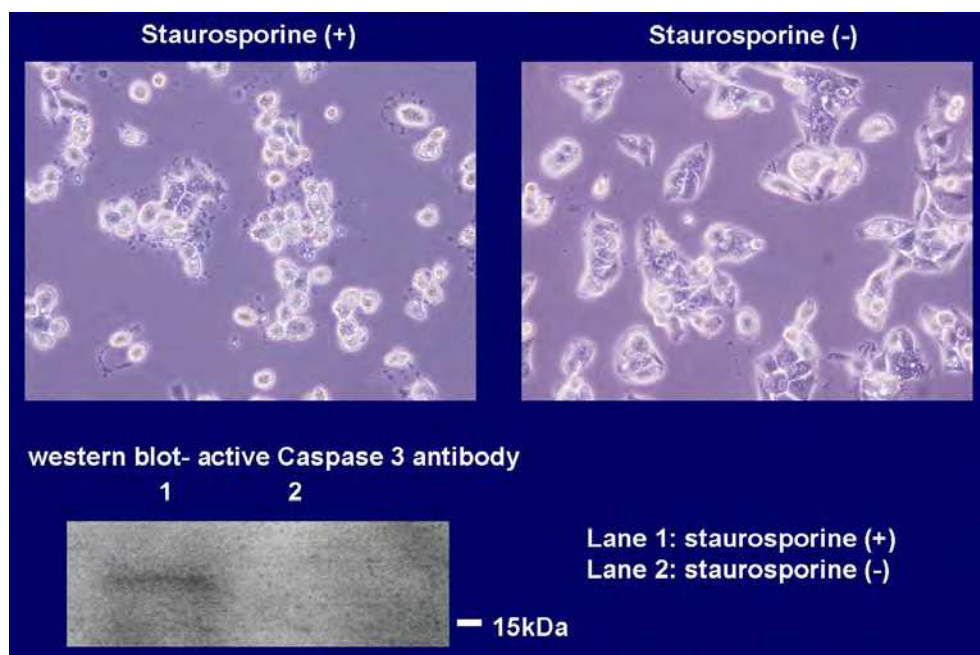


Fig. 11. Western blot analysis of untreated or staurosporine-treated lamprey cell.

#### 4.2.5 Electron microscopy

TEM observation revealed apoptotic features in the biliary epithelial cells of metamorphosing lampreys.

The electron micrograph showed highly electron-dense materials and dispersed chromatin in the nucleus, and the cell surface was characterized by the loss of microvilli and the dome-shaped morphological appearance of the apical side (Fig. 12, panel A). Panel B shows a

higher-magnification view of the apoptotic cell with chromatin condensation occurring at the periphery of the nuclear membrane.

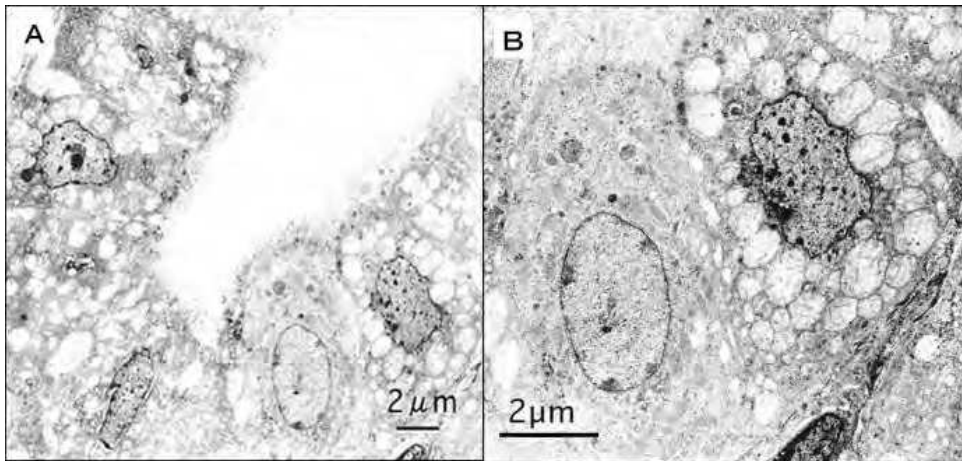


Fig. 12. Transmission electron micrograph of the middle IHBD of a metamorphosing lamprey. Lower right portion of panel A is magnified in panel B, showing characteristic features of apoptotic cells.

## 5. Biliary atresia in adult lampreys

While adult lampreys lacked a bile duct system, fibrous connective tissue remained at the porta hepatis. Electron microscopy also confirmed the absence of the interstitia between hepatocytes, known as bile canaliculi. Despite such obvious BA, lampreys do not develop either biliary cirrhosis or liver dysfunction.

### 5.1 Liver architecture of adult lampreys and humans with BA

Figure 13 shows micrographs of liver specimens from a human with BA at the time of liver transplantation (panel A) as well as an adult lamprey (panel B), both prepared using HE

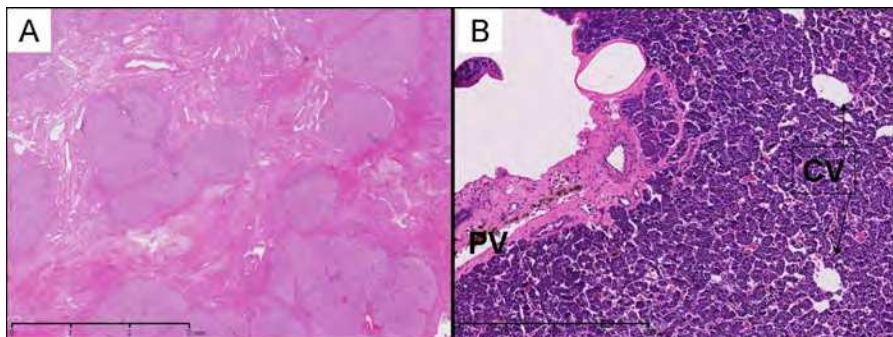


Fig. 13. Micrograph of the cirrhotic liver of a human with BA (panel A) and the liver of an adult lamprey (panel B).



staining. Despite the loss of all bile ducts in both specimens, the prognosis of each organism is much different. In the human specimen, there are nodules of regenerating hepatocytes, but their connections with the vascular supply and bile drainage system are destroyed. Irregular scarring caused by massive increases in fibrocollagenous tissue is also present.

In the adult lamprey specimen, the only remaining fibrous connective tissue can be found at the porta hepatis, and a complete absence of bile ducts can be noted. Some pigments that appear to be vestiges of bile stasis can be observed between the fibrils. The parenchymal cells appear undamaged and seem to keep blood supply abundant through sinusoidal connection.

## 5.2 Disappearance of bile canaliculi

In the adult lamprey specimen, neither bile canaliculi nor tight junctions between hepatocytes could be identified, even when electron microscopy was employed. Hepatocytes in adult lampreys showed a complete loss of their canalicular (apical) membrane (Fig. 14). As adult lampreys lose their entire biliary system including bile canaliculi during metamorphosis, bile acids, if they are synthesized in hepatocytes, must be released into circulation via the basolateral (sinusoidal) plasma membrane in order to serve as endocrine factors. Accumulation of lipid droplets was also observed, along with well-developed granular endoplasmic reticulum in the cytoplasm.

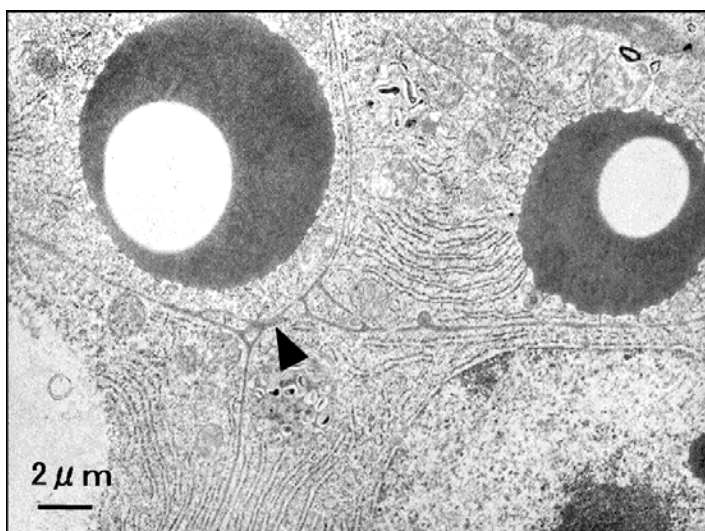


Fig. 14. Transmission electron micrograph of the absence of interstitia between hepatocytes (▲).

## 6. Cholesterol metabolism

In order to determine the organ in which they metabolize cholesterol, we intravenously injected adult male lampreys (*L. japonicum*) with  $^{14}\text{C}$ -cholesterol and then radioassayed the liver, intestine, muscle, gonads (testis), and epidermis after periods of 1, 3 and 16 h had

elapsed. As adult lampreys obtain cholesterol from the blood and tissue of fish on which they feed, they must have a means of metabolizing or accumulating cholesterol. Male lampreys must also use cholesterol in order to synthesize 3-Ketopetromyzonol sulfate for use as a sex pheromone in attracting ovulating females. We recorded levels of radioactivity in the liver and intestine that were approximately 10-times greater than those recorded in the muscle, gonads and epidermis at 3 h after injection. Radioactivity levels measured in the muscle, gonads and epidermis were saturated at 16 h post-injection. These findings indicate that  $^{14}\text{C}$ -cholesterol did accumulate in these tissues; however, that radioactivity levels decreased in the liver and intestine in the period from 3 h to 16 h post-injection suggests that these tissues metabolized and eliminated some cholesterol over time.

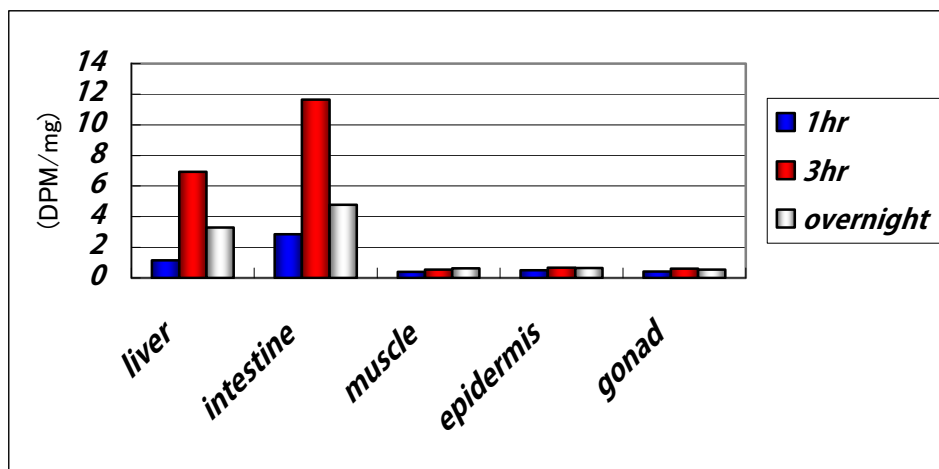


Fig. 15. Radioactivity detected in the liver and intestine was greater than that observed in the muscle, gonads and epidermis.

## 7. Discussion

Human BA can be categorized as either embryonic (fetal) or perinatal (acquired). The embryonic form of BA is believed to be caused by mutations in genes regulating biliary development and, while the etiology of the perinatal form of BA is not completely understood, proposed precipitating factors include viral infections, toxins, vascular and immune mediators (Mack & Sokol, 2005). If the initial surgery used to treat BA can successfully restore bile flow, treatment will still be complicated by progressive destruction of bile ducts resulting in ductopenia. BA in humans is not wholly distinguished by any single disorder during the perinatal period, it is also characterized by the slow and continuous degeneration of biliary epithelial cells. There is no doubt that this destruction of biliary epithelial cells plays a role in the inflammatory processes seen in livers affected by BA. Furthermore, recent studies have reported the occurrence of apoptosis in biliary epithelial cells of humans affected by BA (Sasaki et al., 2001; Harada et al., 2008; Funaki et al., 1998). Two potential causes of the progressive disappearance of biliary epithelial cells observed in human BA are purported: an autoimmune response induced by the initial



disorder in the biliary epithelial cells, and cytotoxicity of the bile acids stasis. These two causes could potentially account for the common behaviors of hepatocytes and biliary epithelial cells in both human and lamprey livers.

Many previous studies have indicated a viral cause of BA, such as reovirus type 3, group C rotavirus, and cytomegalovirus; however, these viruses are not always detected in BA patients (Bangaru et al., 1980; Riepenhoff-Talty et al., 1996). Mack *et al.* proposed that bile duct infection or injury due to rotavirus infection could be inciting factors leading to progressive, autoimmune-mediated damage of the biliary tree after recovery from infection (Mack & Sokol, 2005; Barnes et al., 2009). Incidentally, it has been revealed that programmed cell death in amphibian metamorphosis is induced by a selective auto-immune response, and that the immune system may contribute to the remodeling processes in vertebrate morphogenesis, universally (Mukaigasa et al., 2009). The onset of human BA always occurs during the pre-natal or perinatal periods despite the fact that rotavirus infection with acholic stool and other viral infections are so common among preschool children and infants under one year of age. It is likely that humans are sensitive to the inducement of apoptosis during pre-natal or perinatal periods in which the fetus loses many prenatal structures such as the ductus arteriosus, ductus venosus, webbing between the fingers, and branchial arches. The suppression of autoimmune-mediated apoptosis could be a valuable tool in the treatment of human BA.

Generally, bile salts are synthesized through a many-stepped process starting with cholesterol oxidation and will damage the liver if they are not correctly discharged. The main mechanism of maintaining cholesterol homeostasis in many vertebrates is the enterohepatic circulation, the obstruction of which is fatal due to the cytotoxicity of bile. The adult lamprey, however, loses its entire bile duct system without developing the pathological effects of cholestasis. It was confirmed that the lamprey metabolized cholesterol into bile alcohol within the liver and intestinal canal despite the loss of bile canaliculi in the hepatocytes, which resulted in the loss of the apical membrane used for bile secretion. These results indicate that 3-ketopetromyzonol sulfate, a bile alcohol produced in the adult male lamprey, is probably released into circulation via the basolateral (sinusoidal) plasma membrane. Sulfation is an important metabolic pathway responsible for the detoxification and elimination of bile acids in many species such as hamsters, monkeys, guinea pigs and humans. Sulfated bile acids are less toxic to cells than those without a sulfate group. The sulfation of bile acids also increases their solubility, enhancing their ability to be released into the circulation by ATP-binding cassette transporters via the basolateral (sinusoidal) plasma membrane and to be excreted in urine, especially under cholestatic conditions. Sulfation plays an important role in maintaining bile acid homeostasis under pathologic conditions in mammals. The proportion of sulfated bile acids differs markedly between species, and is very small in humans (Alnouti, 2009). In mammals, the sulfation of bile acids and the expression of the ATP-binding cassette transporters of the basolateral plasma membrane, such as multidrug resistance-associated protein 3 and multidrug resistance-associated protein 4, are regulated by nuclear receptors, such as farnesoid X receptor, for which bile acids are known to be a ligand.

It is still unclear which process occurs first in the metamorphosing lamprey liver, cessation of the synthesis of larval bile alcohols by the hepatocytes or apoptosis of the biliary epithelial cells. The stage by stage features of hepatocyte transformation with a gradual loss

of bile canaliculi (Sidon & Youson, 1983) along with the gradual disappearance of tight junctions at the bile canaliculi and an increase in area occupied by gap junctions (Youson et al., 1987) have been described in previous studies. Moreover, the degeneration of bile ducts has been associated with the development of marked periductal fibrosis due to fibroblast activation caused by leaked biliary materials (Youson et al., 1987; Yamamoto et al., 1986). Our observation of apoptosis in biliary epithelial cells detected prior to changes in hepatocyte morphology (Morii et al., 2010) was consistent with the results of previous reports. Intrahepatic cholestasis may regulate the remodeling of hepatocytes by changing the way cholesterol is metabolized, the expression of transporters in the basolateral membrane and the disappearance of tight junctions in hepatocytes surrounding bile canaliculi. Understanding the means by which adult lampreys are able to avoid cholestasis without bile ducts could allow for valuable progress in the treatment of human obstructive cholangiopathy to be made.

## 8. Conclusions

Lampreys could serve as animal models in the study of cholestasis in humans. Understanding the mechanism that controls bile acid sulfation and the progress of apoptosis in biliary epithelial cells in the lamprey liver during BA could contribute to the development of treatment for obstructive cholangiopathy in humans. Unfortunately, as the molecular biology of the lamprey has not been well studied, these mechanisms have not yet been elucidated.

## 9. Acknowledgment

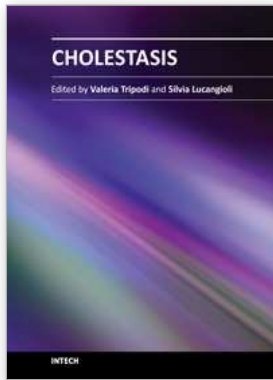
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