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Secretions of the biliary mucosa in experimental clonorchiasis

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Abstract: The histological change of the biliary mucosa in clonorchiasis is characterized as adenomatous hyperplasia, and cross-sectioned mucosa looks like intestinal mucosa. In addition to the glandular hyperplasia, the metaplasia of mucin secreting cells is also known. The present study investigated the presence of intestinal secretion from the biliary mucosal cells of rabbits and rats with *Clonorchis sinensis* infection. The rabbit was infected with 300 and the rat was infected with 100 metacercariae of *C. sinensis*. A part of the animals were followed up after praziquantel treatment. The rabbit livers were prepared for histochemistry to observe any endocrine secretion and the bile duct mucosa of the mice was processed for the activity of brush border membrane (BBM)-bound enzymes of the small intestine. Immunohistochemistry with the polyclonal antibodies and biotin-streptavidin-peroxidase staining kit showed no positive cells for gastrin and secretin, but a few cells were positive for serotonin. The proliferated biliary mucosa of the mice revealed no activity of dissaccharidases and aminopeptidase. Only alkaline phosphatase activity was found both in the control and the infected. The hyperplastic biliary mucosal cells showed no gastrointestinal secretory functions. The serotonin secreting cells may be one of the inflammatory cells.

Key words: *Clonorchis sinensis*, biliary epithelial cells, gastrin, secretin, serotonin, brush border membrane enzymes, alkaline phosphatase

INTRODUCTION

Clonorchiasis is a liver fluke disease caused by *Clonorchis sinensis* which is prevalent in China, North Indochina, Japan and Korea. At present, it is the most commonly detected parasite of human by fecal examination (Chai, 1990), and the infected population is now estimated about one million in Korea. This is a remarkably decreased number when compared with previous data but still its prevalence is high along the rivers (Kim *et al.*, 1990). Praziquantel is the drug of choice for fluke diseases, and also highly effective in clonorchiasis (Rim *et al.*, 1981; Seo *et al.*,

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1983; Lee, 1984). The decrease of clonorchiasis owes mainly to the introduction of praziquantel in early 1980s. However, its prevalence is rather high in contrast to rapid and distinctive declining of the soil-transmitted helminthiases in Korea. The situation may be due to the obstinate habit of raw consumption of fresh water fish (MHSA & KAH, 1992).

The epithelium of the infected bile ducts showed marked glandular hyperplasia and goblet cell metaplasia (Lee *et al.*, 1978a & 1978b; Song *et al.*, 1989). The changes persisted more than 6 months after praziquantel treatment (Lee *et al.*, 1987 & 1988). More residual changes were in the bile ducts with severe histopathological changes (Lee *et al.*, 1987). Mucin secretion disappeared but the hyperplasia was still found in mild degree even 12 months after treatment (Hong *et al.*, 1990).

Some sections of the heavily infected bile ducts simulate gross appearance of the cross sectioned small intestine because of the characteristic hyperplasia of the biliary mucosa. Furthermore, goblet cells appear at the base of the epithelium. Those findings suggest that *Clonorchis* worms make the epithelium of bile ducts proliferate in shape of the small intestine. Is the change confined only in the morphology?

The small intestine has some secretory functions other than mucin production. Major secretions are gastrointestinal hormones and digestive enzymes. The APUD (amine precursor uptake and decarboxylation) cells secrete gastrin, secretin, serotonin, glucagon, and somatostatin to control the functions of the digestive tract (McGuigan, 1989). As for the final digestion in the intestine, the brush border membrane (BBM) retains several disaccharidases, aminopeptidases and phosphatases (Anderson *et al.*, 1983).

The bile duct and the small intestine share their ancestral origin in the development. Therefore, a hypothesis can be proposed that some kind of stimulations from the fluke make the bile ducts change into the intestine. The present study was executed to observe any secretory functions of the small intestine from the infected biliary mucosa, and to reevaluate the pathogenesis of clonorchiasis.

MATERIALS AND METHODS

1. Infection of animals with *C. sinensis*

Rabbits and rats were experimentally infected by introducing the metacercariae of *C. sinensis* into the stomach, 300 and 100 respectively. The metacercariae were collected from peptic digested material of *Pseudorasbora parva* which were captured at the endemic area, the Nakdong River, Kyongsangnam-do. The animals were reared in the laboratory by feeding commercial diet and tap water for 14 weeks. Total 8 rabbits were infected, and half of them were treated with praziquantel 100 mg/kg × 2 days. The treated rabbits were sacrificed 6 or 12 months after the treatment.

2. Immunohistochemical staining for the endocrine functions

Normal control, infected and treated rabbits were prepared for routine histopathological observation by sectioning their livers in paraffin blocks. The ABC staining kits with polyclonal antibodies and biotinylated anti-IgG antibody, peroxidase conjugated streptavidin (AtrAviGen, BioGenex Lab., U.S.A.) were used for detection of gastrin, secretin, and serotonin. Following the packaged procedure, the sections were deparaffinized and hydrated. Endogenous peroxidase was blocked by 10 minute incubation in PBS (pH 7.6) containing 3% H_2O_2. Polyclonal antibodies were reacted 30 minutes at room temperature with one negative serum control. After thorough rinsing with PBS, the link solution of biotinylated anti-IgG antibody was incubated for 30 minutes at room temperature. Washed several times, and peroxidase labeled streptavidin was to the sections for 20 minutes. The color reaction was developed by 3-amino-9-ethylcarbazole (AEC) in N,N-dimethylformamide, and controlled the intensity by the reaction time from 7 to 10 minutes. Counter stain used Mayer's hematoxylin.

3. Activities of BBM enzymes

The common bile ducts of the infected rats and normal controls were isolated.Their mucosal layer was scraped on ice and stored at

-70°C in 0.05 M mannitol 2 mM Tris HCl (pH 7.0). The tissue was homogenated with a teflon tipped homogenizer (Stir-R model S63C Tri-R Inst. Inc., U.S.A.) and then with an ultrasonic sonicator (Sonicator W-385, Heat Systems-Ultrasonics Inc., U.S.A.). Their protein contents were quantitated following modified Lowry method. The absorbances were checked through a UV/VIS spectrophotometer (Hewlett-Packard Co., U.S.A.). The mucosal homogenates were reacted with the substrates of disaccharides (sucrose, lactose, trehalose), p-nitrophenyl phosphate (pH 10.0), and leucine naphtylamide HCl. The enzyme activities were estimated by the absorbances after color reaction. The activities of the infected rats were compared with those of the control.

RESULTS

1. Endocrine secretions

ABC staining showed several gastrin positive cells in the stomach wall as a positive control, but no cells were stained in the bile ducts of the control, infected or treated rabbits (Figs. 1 & 2). Secretin staining was positive only in the small intestine as a positive control, and negative in the bile ducts of all group of rabbits (Figs. 3 & 4). The ABC staining for serotonin detected a few positively stained cells among the epithelial cells of infected rabbits. The cells were slender with a round nucleus, and included brown granules in their cytoplasm (Figs. 5-10). No cell was positive in the treated rabbits (Figs. 11-12). Also the edematous stroma layer of the hyperlastic tissue was stained for serotonin. These findings are summarized in Table 1.

2. BBM enzyme activities

The activities are expressed in units as one unit corresponds to hydrolysis of 1 M substrate per minute at 37°C. Sucrase activities were from 2.6 to 12.1 mu/mg protein while 4.4 mu/mg protein in the control. Lactase activities ranged from 0 to 34.4 mu/mg protein and that of control was 0. Trehalase activities were in the range of 0 to 32.9 mu/mg protein (control 6.9 mu/mg protein). Alkaline phosphatase showed higher activities than other enzymes, control 63.8 mu/mg protein and the infected rats 30.6-156.1 mu/mg protein. Leucine aminopeptidase activities were 11.6 mu/mg protein in the control, and 6.4 to 18.1 mu/mg protein in the infected rats. The activities are presented in Table 2.

DISCUSSION

As observed in the bile ducts of rabbits infected with *C. sinensis*, the hyperplastic biliary mucosa is known to secrete neutral and acid mucopolysaccharide (Chou and Gibson, 1970; Hong *et al.*, 1990). The mucopolysaccharide producing cells are normally absent in the biliary mucosa. The mucopolysaccharide in the intestinal tract is known to play a role in protection of the host by two ways. The one is covering the brush border membrane, and the other is expediting expulsion of foreign materials especially well-known in nematode infections (Roitt, 1988). The goblet cells increase by parasitic infections in the intestine, and the function is associated with cellular or humoral immunity (Tse and Chadee, 1991).

Little has been revealed for the cell reaction at the biliary mucosa in clonorchiasis. Hong *et al.* (1990) suspected any role of hypersensitive reaction in the pathogenesis of adenomatous hyperplasia and goblet cell metaplasia in clonorchiasis. In fascioliasis, proline secreted from the fluke was known to induce proliferation of the biliary mucosa (Isseroff *et al.*, 1977).

Other than mucopolysaccharides, the glandular structured mucosa contains a few serotonin secreting cells and the activity of alkaline phosphatase. Serotonin (5-hydroxytryptamine) is one of major neurotransmitters in the central nervous system and the vasoconstricters. The majority of its content is stored in the enterochromaffin cells of the small intestine. The mast cell of rodents harbours it, but not the cell of humans (Douglas, 1987). The serotonin containing cells were one or two on a section of the biliary tree, and not found after treatment though mucin secretion and the hyperplasia lasted more than 6 months after treatment (Hong *et al.*, 1990). The serotonin positive cells seem to be mast

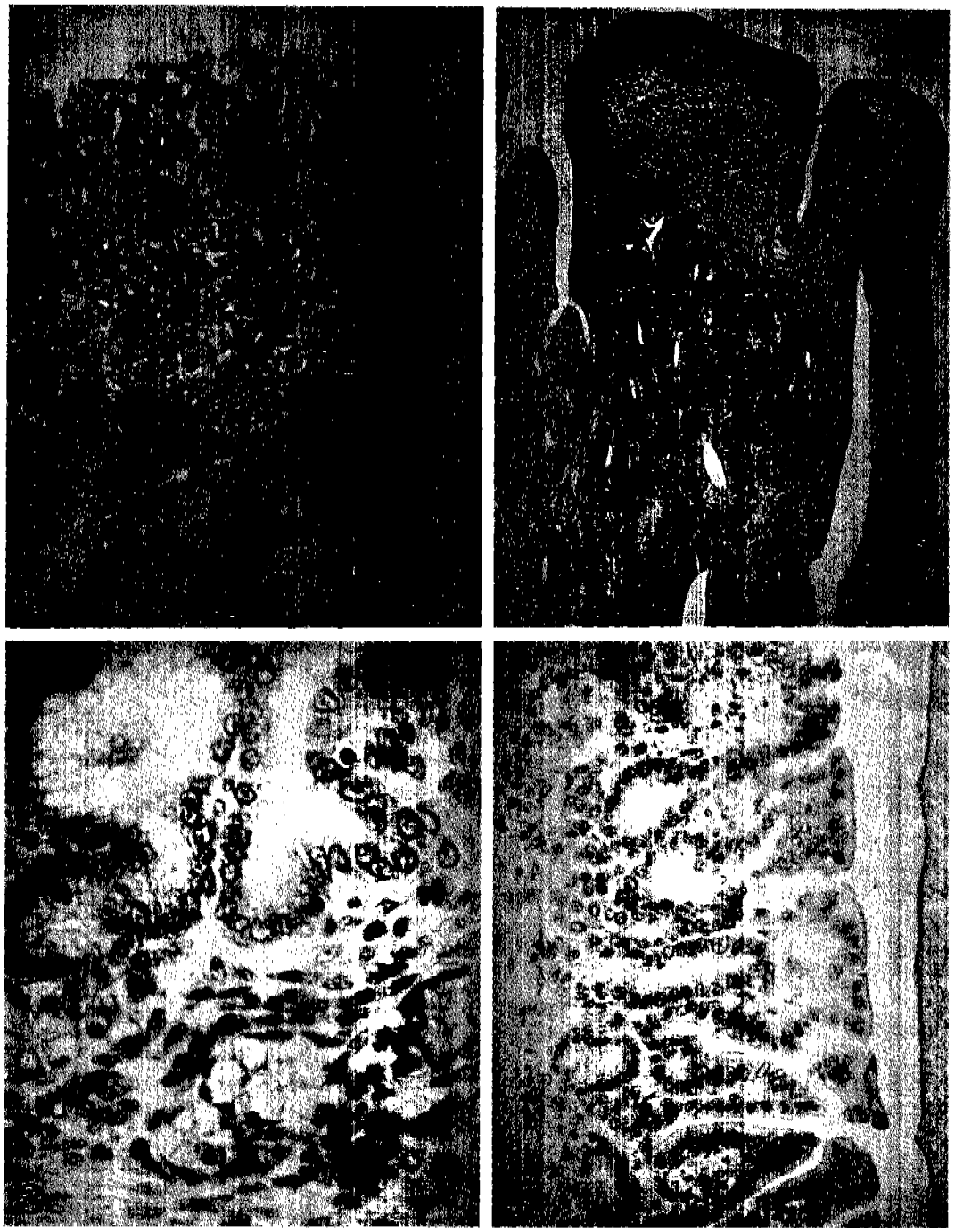

Fig. 1. Mucosa of the stomach as a positive staining for gastrin, × 200. **Fig. 2.** The proximal intrahepatic bile duct of an infected rabbit with negative gastrin staining, × 100. **Fig. 3.** Mucosa of the jejunum as a positive staining for secretin, × 400. **Fig. 4.** Negative secretin staining in the biliary mucosa of an infected rabbit, × 200.

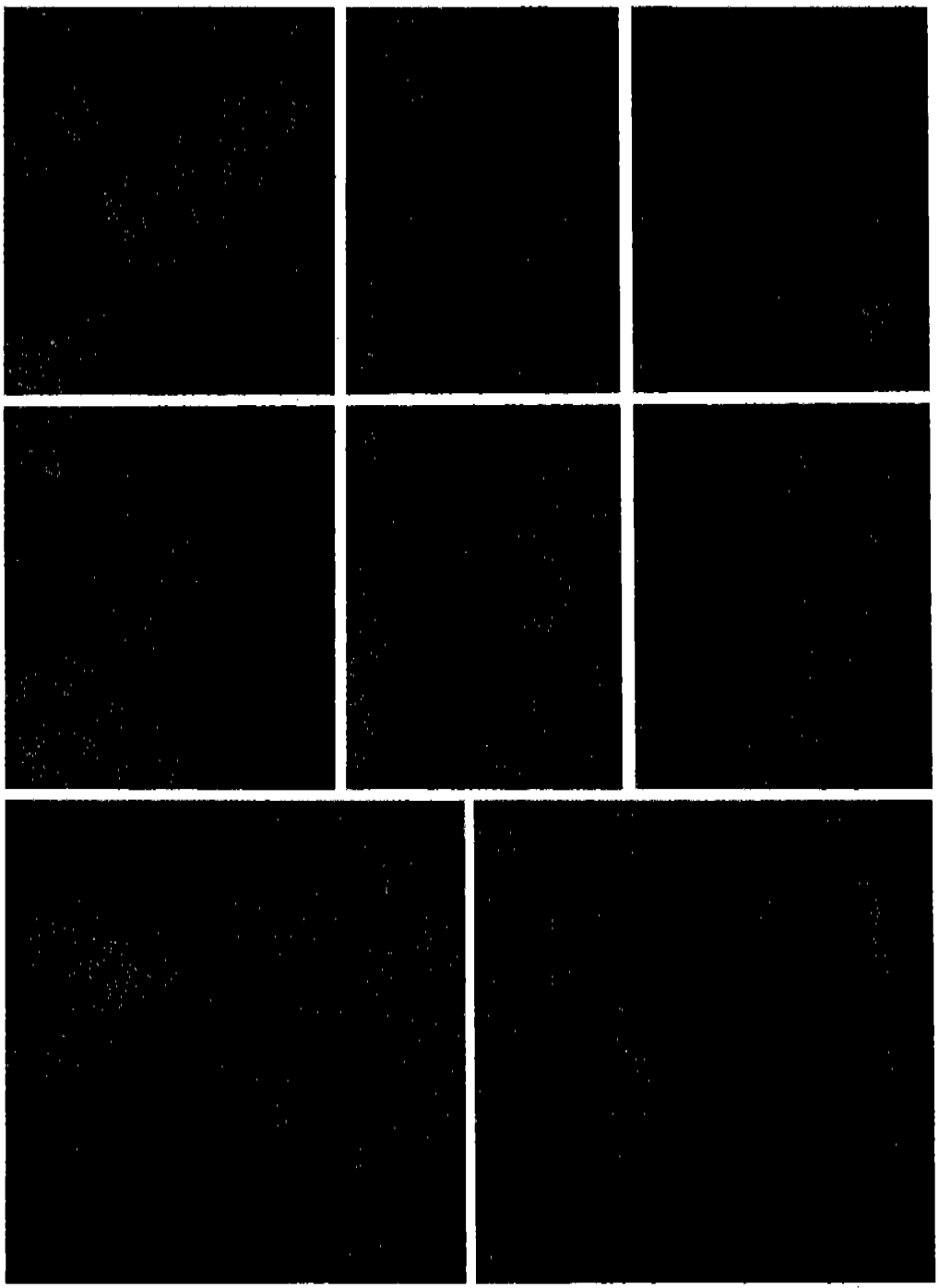

Fig. 5. Serotonin positive cells in the jejunum as a positive reference staining, × 400. **Figs. 6-10.** Serotonin positive cells (arrows) in the biliary mucosa of the infected rabbits, × 400. **Fig. 11.** Negative serotonin staining in the biliary mucosa of the infected rabbits 6 months after treatment, × 200. **Fig. 12.** Negative serotonin staining in the biliary mucosa of the infected rabbits 12 months after treatment. × 100.

Table 1. Immunohistochemical findings of the biliary mucosa in rabbit clonorchiais

Group	Location	ABC staining for		
		gastrin	secretin	serotonin
Uninfected control	Intrahepatic duct	−	−	−
	Extrahepatic duct	+*	−	+
	Stomach	+++	nd	nd
	Jejunum	−	++	++
Infected	Intrahepatic duct	−	−	+
6M treatment	Intrahepatic duct	−	−	−
12M treatment	Intrahepatic duct	−	−	−

*+: minimal changes found in less than 1/4 field of a section duct
++: moderate changes found in 1/2 field of a sectioned duct
+++: heavy changes found in more than 3/4 field of a sectioned duct
nd: not done

Table 2. Activities of the brush border membrane bound enzymes in the biliary mucosa of rats infected with *C. sinensis*

unit: mu/mg protein

Enzymes	Uninfected control	Infected
Sucrase	4.4	4.9± 3.1
Lactase	0.0	4.3±12.7
Trehalase	6.9	2.9±19.6
Alkaline phosphatase	63.8	67.3±46.6
Leucine aminopeptidase	11.6	10.1± 4.8

cells or other inflammatory cells rather than metaplastic enterochromaffin cells.

Still any information on mast cell reaction in clonorchiasis is not available, however, the cells are known to increase locally in the intestine by parasite infections (Woodbury *et al.*, 1984; Guy-Grand *et al.*, 1984; Novak and Nombrando, 1988; Kho *et al.*, 1990). Mast cells can be infiltrated into the mucosa with other inflammatory cells by the irritation of the liver fluke. This reaction should be evaluated further.

The activity of alkaline phosphatase was found in the proliferated epithelium, but not increased compared to that of the control. The enzyme is universal in the epithelium-lined tubular organs of mammals, such as the stomach, intestine, bile duct, kidney tubule, *etc.* (Miura *et al.*, 1982). Since the enzyme activity was not changed by the infection though the epithelial cells proliferated severely, the infection and associated hyperplasia might not influence the cell maturity. Other BBM enzymes than alkaline phosphatase were of negligible activity in the biliary mucosa.

The present findings confirm that the mucosal change of the biliary mucosa in clonorchiasis confines to the morphological intestinalization. Though cross sections of the bile ducts give the appearance of the sectioned intestine, the ducts showed no secretory functions of the gastrointestinal tract except production of mucopolysaccharide. The epithelial cells lining the biliary mucosa seem to differentiate enough not to convert to the intestinal epithelial cells. The change of biliary mucosa in clonorchiasis is not the metamorphosis to the small intestine.

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=국문초록=

간흡충에 감염된 토끼 및 흰쥐 담관상피의 분비기능

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간흡충에 감염되었을 때 나타나는 조직병리학적 소견의 변화로는 담관 직경이 확장되고 담관벽이 비후되며, 담관 상피층에 선종성 증식과 배세포 화생이 관찰되었다. 치료 후에는 이러한 변화 중에서 화생이 소실되나 선종성 증식은 상당히 완화되어 잔존하고 담관벽의 비후는 지속된다. 이러한 형태학적인 변화는 선종성인 특징이 있어 어떠한 분비기능이 예상되었다. 이 연구에서는 특히 소장의 분비물이 담관 점막층에서 분비되는가를 확인하고자 하였다. 간흡충 피낭유충을 300개씩 8마리의 토끼에 감염시킨 후, 14주에 프라지콴텔로 치료하여 구충 후 6개월 및 1년에 간을 적출하고, 파라핀 포매하여 절편을 만들어 분비물에 대하여 면역조직화학법을 시행하였다. Gastrin과 secretin은 정상토끼의 간내 담관 및 감염된 담관에서 나타나지 않았다. Serotonin은 비감염 간 내 담관에서는 관찰되지 않았으나, 감염 대조군의 담관상피층에서 몇 개의 양성 세포가 관찰되었다. 그러나 치료 후 6개월에는 관찰되지 않았다. 또한 흰쥐에 100개씩 간흡충 피낭유충을 감염시키고 증식된 상피층에서 소장 미소융모막 효소의 활성을 관찰한 바, alkaline phosphatase의 활성이 감염군에서 대조군과 같은 정도로 관찰되었으나 sucrase, trehalase, lactase, leucine aminopeptidase의 활성은 나타나지 않았다. 이 결과를 통하여 간흡충 감염시에 증식된 담관상피에 소수의 serotonin 분비세포가 있음을 관찰하였고 다른 소장의 분비기능은 생기지 않음을 확인하였다. 이 serotonin을 포함한 세포는 비만세포로 추정된다.

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