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Gallstone Formation and Gallbladder Mucosal Changes in Mice fed a Lithogenic Diet

To investigate the pathologic change of gallbladder mucosa related to gallstone formation, 52 mice were fed a lithogenic diet containing 1% cholesterol and 0.5% cholic acid and we evaluated the sequential morphologic changes in the gallbladder from two days to 40 weeks. Cholesterol gallstones began to appear after two weeks and all the mice had gallstones after eight weeks. At two days, the mitotic index was at its highest. The gallbladder mucosa showed progressive hyperplastic change with earlier papillary projection of the folds and later inward proliferation. At the same time of stone formation, mucous cells forming glands appeared. Their histochemical profile of mucin was different from that of normal epithelium. Numbers of mucous cells increased gradually until 24 weeks but slightly decreased afterward. These results suggest hyperplasia and metaplasia are closely related to the gallstone formation. Hyperplasia is probably reactive to irritating effect of lithogenic bile or stone. Metaplasia and cholesterol gallstone may develop simultaneously, and act synergistically.

Key Words: *Mice; Cholelithiasis; Gallbladder; Mucous membrane; Hyperplasia; Metaplasia*

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INTRODUCTION

Cholelithiasis is the most prevalent disorder of the biliary tract (1). It can cause cholecystitis and may be associated with the appearance of hyperplasia, metaplasia and carcinoma in the gallbladder (2-5). Metaplastic changes of the epithelial cells are common adaptive response and usually associated with chronic irritation. In the gallbladder, gallstone and subsequent inflammation may induce epithelial injury and resultant metaplastic changes (2, 4). It has been suggested that the metaplastic epithelium is more susceptible to malignant transformation than normal mucosa (6) and intestinal metaplasia-dysplasia-carcinoma sequence exists in the gallbladder (2, 4, 7, 8).

Since the development of experimental lithogenesis in mice by feeding a cholesterol-cholic acid containing diet was established (9), many authors have studied the mechanism of gallstone formation and the pathophysiologic effect of lithogenic diet to the gallbladder epithelium (9-19). Their results have revealed an early increase in the proliferative activity of the epithelial cells and mucus hypersecretion within a week and stone formation after four weeks (12, 15, 18, 20). But the histologic changes of gallbladder mucosa in the experimental lithogenesis are not well known and epithelial change after stone formation with its relation to the development of hyperplasia or metaplasia had not been studied in detail.

In the present study, we examined the morphologic change of the gallbladder mucosal epithelium in the mice fed a lithogenic diet before and after stone formation and tried to elucidate the developmental process of hyperplasia and metaplasia in association with the gallstone formation.

MATERIALS AND METHODS

Experimental animals and tissue sampling

Fifty-two, male black mice (C57BL) weighing 15-18 g were kept for various periods of time on a standard pellet diet to which 1% cholesterol and 0.5% cholic acid had been added. Four control animals received the standard pellet diet. All animals were kept under normal laboratory conditions with free access to water. Groups of 6, 5, 5, 6, 9, 8, 5, and 4 mice were sacrificed under ether anesthesia at the intervals of 2 days, 1, 2, 4, 8, 12, 24, and 40 weeks, respectively. The gallbladders were quickly removed and their length was measured from the border of cystic duct and neck to the apex of fundus.

They were immediately fixed in 10% buffered neutral formalin. After fixation, they were opened and the bile was examined macroscopically and microscopically. The longitudinally bisected gallbladders were embedded in paraffin, sectioned at a thickness of 3 μm. H&E, PAS, alcian blue at pH 1.0 and 2.5 stains were done.

Histological analysis

Histologic changes of epithelia, glands and folds were examined microscopically. All mitotic figures observed in the entire wall were counted and the percentage of mitotic number in total number of epithelial cells (mitotic index) was estimated. The presence of metaplasia or hyperplasia was also checked. The cells with large intracytoplasmic mucin vacuoles and basally located, flat nuclei were defined as mucous cells. The presence of mucous glands at body or fundus was regarded as a metaplastic change because normal mice gallbladders do not contain mucous glands in these foci (12). The diagnostic criteria of hyperplasia was papillary proliferation, villous elongation or spongioid coalescence of the mucosal folds covered by a single layer of tall columnar cells with basally located nuclei (21, 22).

RESULTS

Control group

The gallbladders were small and measured 5 mm in average length. Stones were not present. Microscopically, the wall was thin and mucosal folds were short and smooth (Fig. 1). The epithelium consisted of a single layer of uniformly low columnar cells without intracytoplasmic mucin vacuoles. But their apical borders were partly covered by thin mucus which was positive for PAS and pH 2.5 alcian blue stain but negative for pH 1.0 alcian blue stain. Mitotic figures were not observed.

Lithogenic group

Stones were identified from two weeks after feeding a lithogenic diet. After eight weeks, all animals had whitish granular cholesterol gallstones. According to the degree of stone formation, the results could be divided into three phases: prelithiasic (2 days and 1 week), stone-forming (2 and 4 weeks) and postlithiasic (8, 12, 24 and 40 weeks) phases. The morphologic findings are summarized in Table 1.

Prelithiasic phase

At two days after feeding the diet, the gallbladders were distended (mean length: 7.2 mm) and more dilated at 1 week (mean length: 8.2 mm). At two days, the epithelium was same as that of the control group. Although the mucosal folds were largely flattened, they focally showed minute foci of spongioid hyperplasia (16.7%). Mean mitotic index was $0.91 \pm 1.15\%$, which was at its highest in all experimental groups (Fig. 2).

Fig. 1. The gallbladder mucosa of a control shows thin and low folds with smooth margin (H&E, ×100).

At a week, the mucosal folds were partly flat and partly showed micropapillary projection with focal hyperplastic change (20%) (Fig. 3). The mucosa consisted of pseudostratified tall columnar cells. Mitotic index was somewhat decreased (mean $0.61 \pm 0.66\%$). Intracytoplasmic mucin production or metaplastic change did not appear in this phase.

Stone-forming phase

Twenty percent of the animals had stones at two weeks. The average size of the gallbladders was similar to that in the prelithiasic group (mean 7.6 mm). Hyperplastic change was also more extensively identified (60%). The mucosal folds were elongated and proliferated with more prominent papillary projection. The epithelium revealed more pronounced pseudostratification. In contrast, the mitotic index decreased (mean $0.27 \pm 0.32\%$). During this phase, a small number of mucous cells began to appear (mean number 18.8 ± 17.7). They were individually scattered between the normal epithelial cells or focally formed the alveolar glands which were present in 20%. Their intracytoplasmic mucin vacuoles were positive for PAS, pH 2.5 and pH 1.0 alcian blue stain (Fig. 4).

At four weeks, 83% of the animals had stones. Mean

Table 1. Morphologic findings of the gallbladder in the control and lithogenic diet group

	Case No.	Size (Length)	Stone* (%)	Mucosal morphology	Hyperplasia* (%)	Metaplasia* (%)
Control	4	5 mm	0 (0)	Simple fold	0 (0)	0 (0)
Prelithiasic phase						
2 days	6	7.2 mm	0 (0)	Flat	1 (17)	0 (0)
1 week	5	8.2 mm	0 (0)	Flat or micropapillary fold	1 (20)	0 (0)
Stone-forming phase						
2 weeks	5	7.6 mm	2 (40)	Papillary fold	3 (60)	1 (20)
4 weeks	6	8.3 mm	5 (83)	Papillary fold	2 (33)	2 (33)
Postlithiasic phase						
8 weeks	9	9.7 mm	9 (100)	Papillary fold with glandular budding	0 (0)	6 (67)
12 weeks	8	8.6 mm	8 (100)	Papillary or villous fold Inward glandular proliferation	3 (38)	8 (100)
24 weeks	5	8.2 mm	5 (100)	Simple fold Inward glandular proliferation	2 (40)	5 (100)
40 weeks	4	9.5 mm	4 (100)	Flat or simple fold Inward glandular proliferation Sinus formation	1 (25)	4 (100)

* Numbers of the positive cases

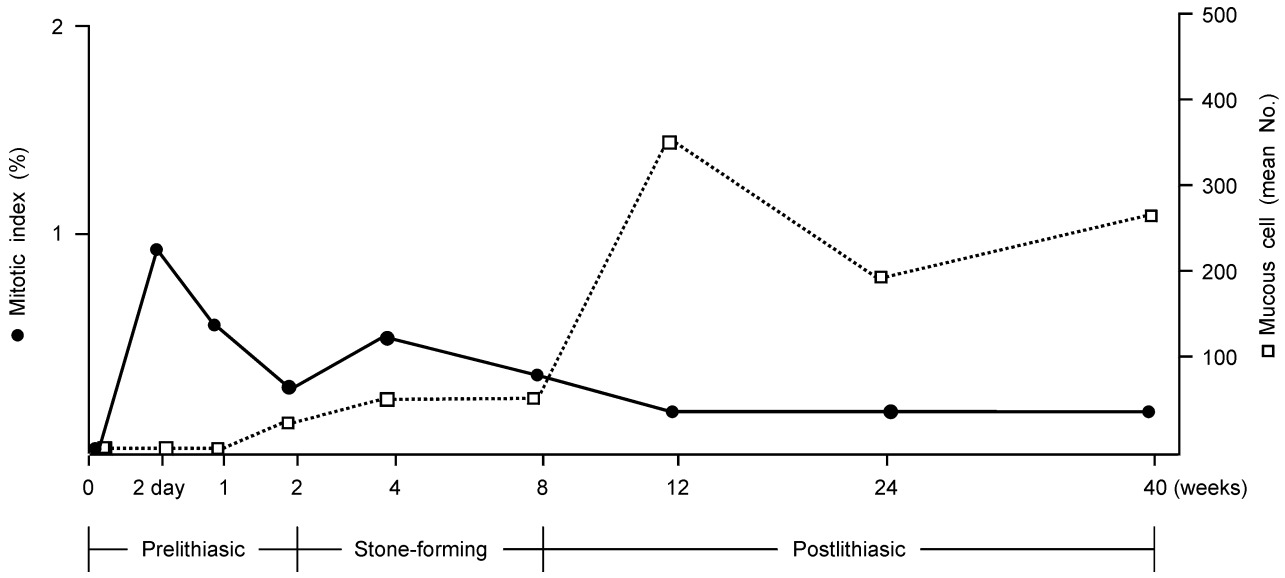

Fig. 2. Changes in mitotic indices and numbers of mucous cells after feeding of lithogenic diet.

length of the gallbladder was 8.3 mm. The mucosal fold pattern and epithelium were same as those of the animals studied at two weeks, except for a decreased hyperplastic change (33.3%) and increased mitotic index (mean 0.65 ±0.54%). Mucous glands were found in 33.3% and the number of mucous cells also increased (mean number 36.17±57.15).

Postlithiasic phase

At eight weeks, the distension of the gallbladders was

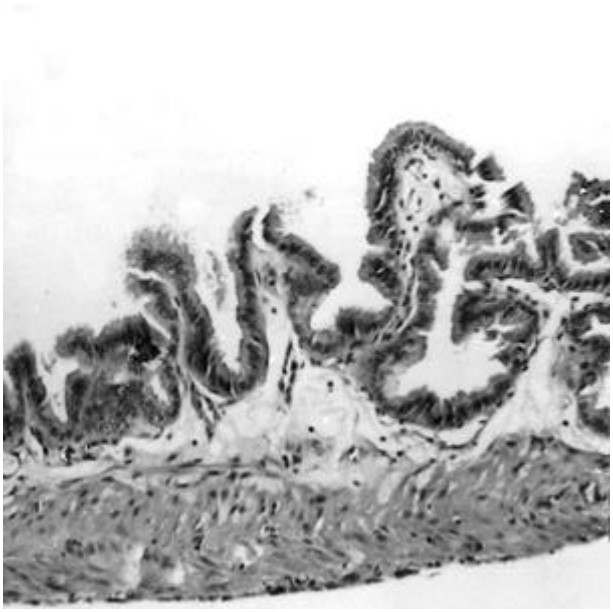

Fig. 3. At 1 week, the gallbladder mucosa shows papillary proliferation and elongation of the folds (H&E, ×100).

Fig. 5. Comparison of the control group (upper) with the experimental group resected at 8 weeks (lower).

maximal (mean length 9.7 mm) (Fig. 5). The mucosa showed formation of secondary glands with budding, in addition to papillary projection of the folds (Fig. 6). But hyperplastic change was not observed. The degree of the epithelial pseudostratification and mitotic figures decreased (mean 0.3±0.35%). However, mucous glands were more frequently identified (66.7%), and the total number of mucous cells increased (mean number: 63.44 ±59.09).

At 12 and 24 weeks, the size of gallbladders decreased slightly. But there was no significant change in the mean lengths of the gallbladders between 12 and 24 weeks (12 weeks: 8.6 mm, 24 weeks: 8.2 mm). The morphologic findings of the mucosa and epithelium were generally similar to those at eight weeks but inward proliferation with budding and branching of the secondary glands was identified and the folds were somewhat simplified after 24 weeks. Hyperplastic change reappeared (12 weeks:

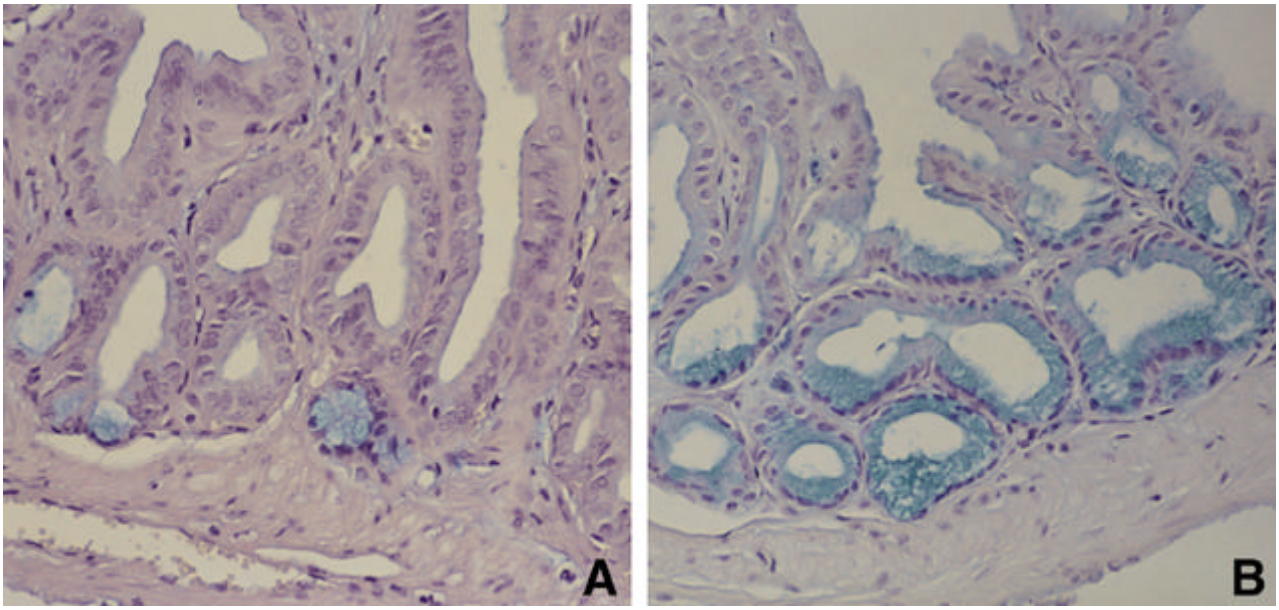

Fig. 4. At 2 weeks, a few metaplastic mucous glands shows intracytoplasmic mucin vacuoles positive for pH 1.0 Alcian-blue stain (A). At 40 weeks, the frequency is increased (B) (Alcian-blue, ×200).

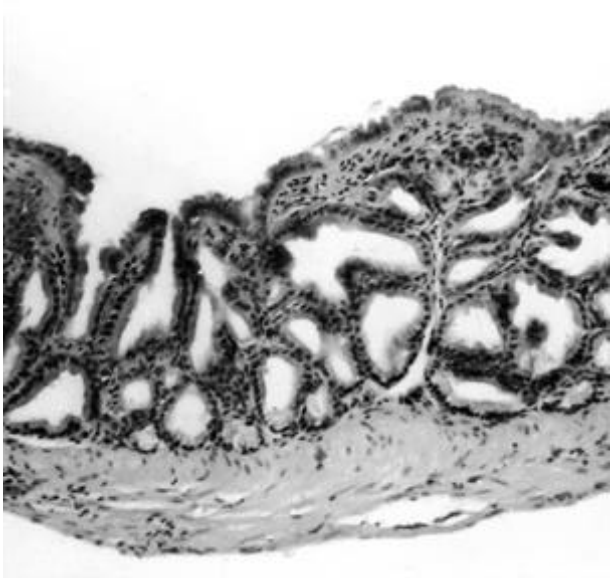

Fig. 6. At 8 weeks, the mucosa shows glandular budding and secondary gland formation with metaplastic change (H&E, × 100).

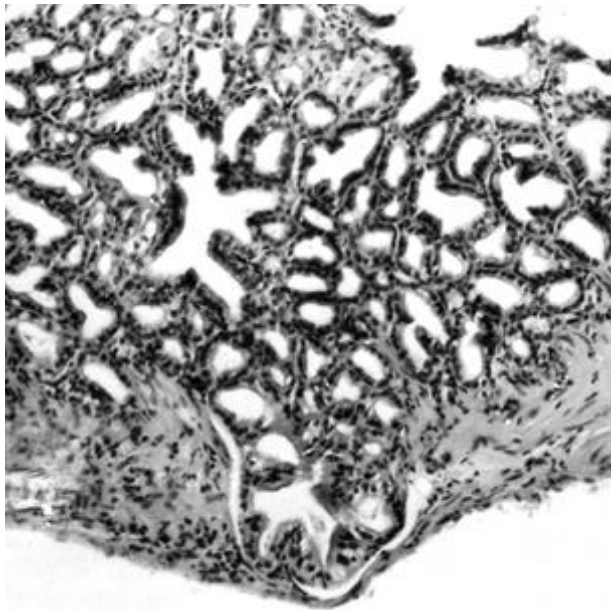

Fig. 7. At 40 weeks, marked inward proliferation of the gland with focal sinus-like invagination is observed (H&E, ×100).

37.5%, 24 weeks: 40%). Mean mitotic indices were 0.15 % in both groups (12 weeks: 0.15±0.21%, 24 weeks: 0.15±0.11%). Metaplastic changes were more marked. All of them contained many mucous glands. The number of mucous cells was maximal at 12 weeks (mean number: 349.38±217.87), but it decreased slightly at 24 weeks (mean number: 200.2±173.71).

At 40 weeks, the gallbladders were rather distended (mean length: 9.5 mm) and hyperplastic change decreased (25%). The mucosa showed focal sinus-like glandular invagination into the wall (25%) (Fig. 7). Mean mitotic index was similar to those of the previous groups (mean 0.16±0.22%). Mucous glands were still present in 100% with slightly increased mucous cells (mean number: 272.75±291.35).

DISCUSSION

Lithogenic diet is known to induce changes in bile components with an increase in the substances such as arachidonyl lecithin and dehydrocholesterol which are highly damaging to gallbladder epithelial cells with membrane injury to the platelets and red cells (16). The changes in the bile components also enhance cell turnover as an adaptive change compensating for the environment (17). According to previous studies of experimental cholelithiasis induced by lithogenic diet, the gallbladder epithelium showed an early, striking increase in the thymidine labelling index between the second day and the first week (12, 17, 18, 20, 23, 24).

The present study showed rapid increase in the mitotic index at the second day of prelithiasic phase. This result indicates that feeding a lithogenic diet can induce immediate biliary change and resultant epithelial injury with regeneration before stone formation. Putz and Willems suggested that distension of the gallbladder could be a factor accelerating the cell turnover and increasing the cellular proliferating activity (24). But in this study, the mitotic index decreased and hyperplastic change disappeared when the gallbladder was dilated at its maximum. Therefore, their result with increased proliferative activity in the epithelium of the distended gallbladder might be due to modification in the bile composition or inflammation.

Hyperplastic changes developed from the early prelithiasic phase, peaked at the second week of stone-forming phase and temporarily disappeared in the postlithiasic phase when the distension of the gallbladder was maximized. But it appeared again after the gallbladder became smaller. In the postlithiasic phase, the gallbladder also showed deepening of the folds with inward proliferation, glandular budding and sinus-like invagination. Human gallbladders with cholecystitis or cholelithiasis usually demonstrate mucosal hyperplasia or Rokitansky-Aschoff sinus formation with epithelial herniation into the underlying wall (5, 25, 26). The sequential hyperplastic changes in this study suggest hyperplasia is a reactive process related to mucosal irritation and regeneration and have a reverse correlation with the dis-

tension of the gallbladder. Gallstone formation with the resultant increase of intraluminal pressure and weakening of the wall by distension might cause inward proliferation and glandular invagination.

Contrary to the previous studies focused on the role of mucus in stone formation (13-15, 17), we put the stress on the developmental process of metaplasia related to gallstone formation. Changes in the bile components induced by a lithogenic diet is known to enhance mucus hypersecretion which protects the mucosa from the stimulus of bile (17). It plays a critical role in cholesterol stone formation as a nucleating agent (14, 15, 27) and promotes stone growth (13). The previous results showed the beginning of mucus hypersecretion from 18 hr after the feeding and it peaked between the first and third week, followed by a gradual decrease (13, 15, 17).

In our result, mucous cells began to appear at two weeks, concurrently with stone formation. Their abnormal location with different histochemical profile of mucin suggests their appearance is a kind of metaplastic change. Gallstones and subsequent inflammation can induce repetitive mucosal defect and repair (2). Metaplasia of the gallbladder mucosal epithelium may arise in association with the decreasing ability to differentiate for normal epithelium (2). As time passed, the numbers of mucous cells increased until the 12th week and afterward they decreased. Initially, the mucous cells may enhance stone formation by mucus hypersecretion but after stone formation, the stone itself can also stimulate metaplastic change of the epithelium along with inflammation and physical injury to the cells. The decrease of mucous cells after 24 weeks supports the metaplastic change is a reversible process. After stabilization of the epithelium with adaptation to the stone and healing of the inflammation, metaplastic change may disappear.

In human gallbladders harboring stones, pseudopyloric or intestinal metaplasia is more frequently seen than in those without gallstones (2). Intestinal metaplasia is regarded as a precancerous lesion based on the same mucin component of intestinal metaplasia and dysplasia (2, 7), features of intestinal differentiation in the invasive gallbladder carcinoma cells and the adjacent normal mucosa (2, 6, 28). In this study, we extended the experiment to 40 weeks, far longer than previous studies. But we could not observe a distinct intestinal metaplasia or dysplasia. To verify intestinal metaplasia-dysplasia-carcinoma sequence in mice, it might be necessary to exposure the mice longer to the stone with interposition of some factors provocating intestinal metaplasia and progressing to the dysplasia.

In conclusion, the results of this study suggest hyperplasia and metaplasia are related to gallstone formation. Hyperplasia is reactive to the irritating effect of lithogenic bile or gallstone. Metaplasia and gallstone develop simultaneously and act synergistically.

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