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Abstract

Phalloidin, a toxin from the plant Amanita phalloides, irreversibly polymerizes actin filaments and causes cholestasis. Three-dimensional structural changes induced by phalloidin in the bile canaliculi and the intra-acinar localization of these changes were studied in the rat liver by scanning and transmission electron microscopy. After 3 days of treatment, canalicular changes appeared mainly in zones 2 and 3 of Rappaport's acinus, but after 7 days of treatment changes occurred in bile canaliculi of the whole acinus. The changes in the bile canaliculi included tortuosity, saccular dilatation, loss of microvilli, bleb formation and elongation of canalicular side branches. Some side branches extended near to Disse's space, leaving only a thin cytoplasmic rim between the canalicular lumen and Disse's space. Kupffer cells were occasionally situated near such extended bile canaliculi and protruded their processes into the hepatic cord. These results suggest that bile canaliculi in zone 3 are more susceptible to phalloidin toxicity than those in zone 1 and that biliary constituents may leak from such altered bile canaliculi.

KEYWORDS: phalloidin, bile canaliculi, choletasis

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Phalloidin-Induced Alterations of Bile Canaliculi

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Phalloidin, a toxin from the plant *Amanita phalloides*, irreversibly polymerizes actin filaments and causes cholestasis. Three-dimensional structural changes induced by phalloidin in the bile canaliculi and the intra-acinar localization of these changes were studied in the rat liver by scanning and transmission electron microscopy. After 3 days of treatment, canalicular changes appeared mainly in zones 2 and 3 of Rappaport's acinus, but after 7 days of treatment changes occurred in bile canaliculi of the whole acinus. The changes in the bile canaliculi included tortuosity, saccular dilatation, loss of microvilli, bleb formation and elongation of canalicular side branches. Some side branches extended near to Disse's space, leaving only a thin cytoplasmic rim between the canalicular lumen and Disse's space. Kupffer cells were occasionally situated near such extended bile canaliculi and protruded their processes into the hepatic cord. These results suggest that bile canaliculi in zone 3 are more susceptible to phalloidin toxicity than those in zone 1 and that biliary constituents may leak from such altered bile canaliculi.

Key words : **phalloidin, bile canaliculi, cholestasis**

Actin filaments of hepatocytes are especially rich around bile canaliculi (1, 2) and are thought to play an important role in bile secretion (1-4). Various drugs which affect the organization of actin filaments have been utilized to study the function of pericanalicular actin filaments. Among these drugs, cytochalasin B and phalloidin are the most well studied. The former depolymerizes actin filaments and causes cholestasis (5). The latter irreversibly polymerizes actin filaments especially around bile canaliculi, and also results in cholestasis (6-11). Cytochalasin B induced changes in bile canaliculi have been extensively studied by scanning and transmission electron microscopy (5) but changes induced by phalloidin have not been studied so extensively. The three-dimensional arrangement and intra-acinar distribution of phalloidin induced changes in bile canaliculi are of interest in considering the pathogenesis of phalloidin-induced cholestasis. Phalloidin-induced changes in bile canaliculi were studied by scanning and

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transmission electron microscopy.

Materials and Methods

Nine male Wistar rats weighing 200-250 g were divided into three groups of three animals each. Rats of Group 1 (control group) were not treated. Rats of Groups 2 and 3 were sacrificed on the 4th and 8th day after 3 and 7 days of treatment, respectively. The phalloidin (Boehringer Manheim, West Germany) was dissolved in 0.9% NaCl and a dose of 500 μg/kg body weight was injected daily into the peritoneal cavity (7,9).

The liver was perfused with Ringer's solution and perfusion-fixed with 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) through a catheter inserted retrogradely into the abdominal aorta. Just after the perfusion was started, the aorta was clamped above the celiac artery and the inferior vena cava was cut above the diaphragm.

For scanning electron microscopy (SEM), small samples were further fixed in the same fixative and cracked into halves under a dissecting microscope. The both matched surfaces were examined. The specimens were dehydrated in a graded series of ethanol, dried in a critical point dryer and mounted on metal stubs. After ion sputter coating, the specimens were observed with a JSM-35 SEM at an accelerating voltage of 15-20 kV. Stereo-pictures were taken for three-dimensional analysis.

For transmission electron microscopy (TEM), small 1×1×1 mm samples were further fixed in a solution of 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h. After post fixation in 2% osmium tetroxide for 2 h, the samples were dehydrated through a graded series of ethanol and embedded in Epon. Serial sections were cut and placed on a single slot grid coated with formvar. After staining with uranium acetate and lead citrate, the specimens were observed with a Philips 300 TEM at an accelerating voltage of 60 kV.

Results

Control. Scanning electron microscopy (SEM) provided a clear three-dimensional image of bile canaliculi as was found in the previous study (10). Bile canaliculi with a constant diameter of about 1 μm and numerous microvilli were situated in the middle of the hepatic cord. A few side branches were observed protruding to Disse's space.

Three-day treatment. In the group of rats treated with phalloidin for 3 days, changes in the bile canaliculi were mainly observed in zone 2 and 3 hepatocytes of Rappaport's acinus. The periportal bile canaliculi remained almost normal (Fig. 1) except for slight dilatation. The following morphological changes of bile canaliculi were observed (Fig. 2); tortuosity with loss of microvilli, saccular dilatation, bleb formation into the canalicular lumen and extension of side branches. However, the severity of changes differed from one hepatocyte to another.

Seven-day treatment. The changes in bile canaliculi increased in severity, and the periportal bile canaliculi also began to show changes (Fig. 3). Tortuous bile canaliculi were not situated in the middle of the hepatic cord. Blebs protruded into the canalicular lumen like casts, resulting in obstruction of the canaliculus (Figs. 3,4). The blebs were observed only in the canalicular lumen, not on lateral or sinusoidal surfaces. In transmission electron micrographs (TEM), filaments appeared to be more sparse and more granular in the blebs than around bile canaliculi (Fig. 5). Dilated side branches extended near to Disse's space (Figs. 6,7). In transmission electron micrographs of serial sections, such side branches appeared to be separated from Disse's space with a thin cytoplasmic rim of hepatocytes (Fig. 6). There were fewer actin filaments at the tip of the side branches than around bile canaliculi. Near the extended side branches, Kupffer cells were occasionally observed. Kupffer cells had processes which protruded into the

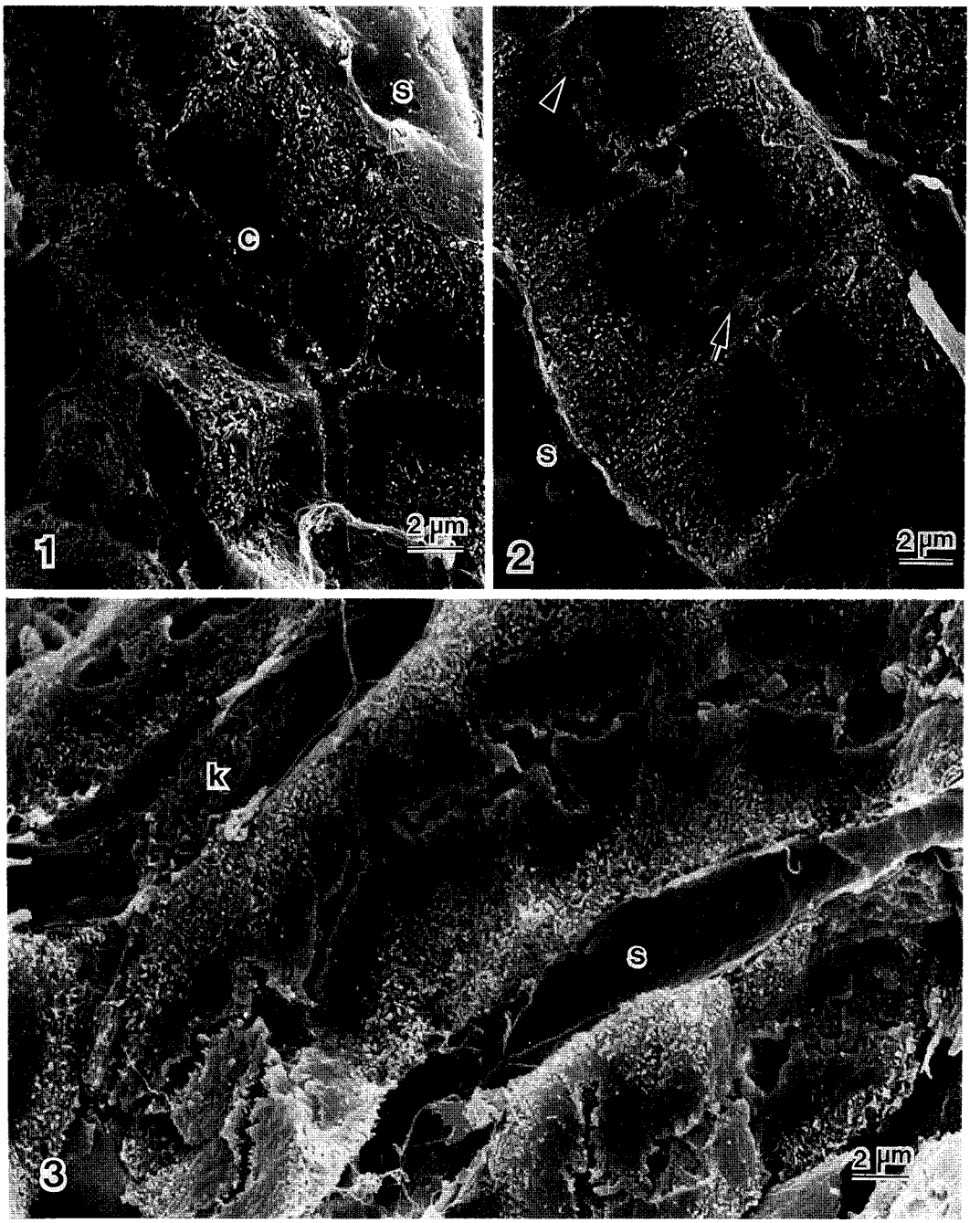

Fig. 1 Scanning electron micrograph (SEM) of periportal bile canaliculi treated with phalloidin for 3 days. Bile canaliculi (c) are almost normal except for slight dilatation. s, Sinusoid. ×4,400.

Fig. 2 SEM of zone 3 bile canaliculi of the 3-day treatment group. Note tortuous bile canaliculi with saccular dilatation (arrow), loss of microvilli and bleb formation (arrowhead). s, Sinusoid. ×4,400.

Fig. 3 SEM of periportal bile canaliculi of the 7-day treatment group. Note more severe canalicular changes than those in Fig. 2. A bleb (arrowhead) obstructs the canalicular lumen. s, Sinusoid ; k, Kupffer cells. ×4,500.

hepatic cord (Fig. 8).

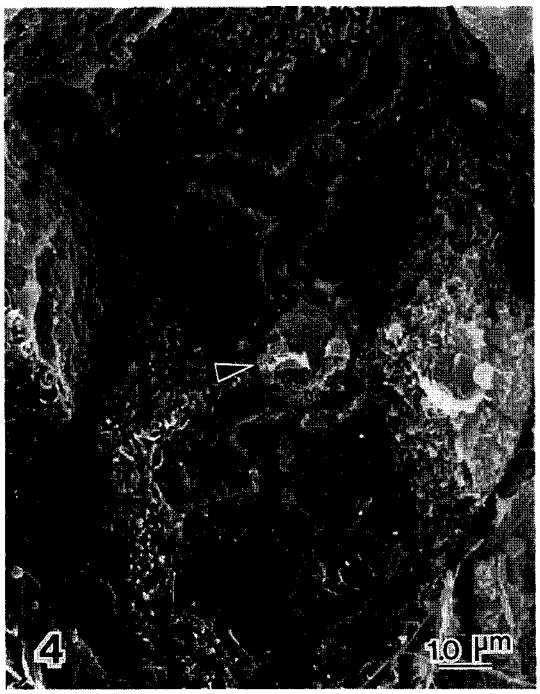

Fig. 4 Higher magnification of altered bile canaliculi in zone 1 in the 7-day treatment group. Note marked dilatation, loss of microvilli and bleb formation (arrowhead). ×7,100.

Discussion

Recent morphological and functional studies (1-3,5,12,13) suggest that pericanalicular actin filaments may play an important role in bile secretion, but the exact mechanism of bile secretion and the role of actin filaments remains unclarified. Phalloidin is a toxin which polymerizes actin filaments, and cytochalasin B is a toxin which depolymerizes them. Both toxins are known to cause experimental cholestasis. This study is the first to show by SEM the detailed changes in bile canaliculi induced by phalloidin, especially with regard to the intraacinar distribution and three-dimensional morphology of the changes.

Although actin filaments were increased in thickness as reported earlier (6,9), the distribution was uneven around the bile canaliculi. The filaments in blebs were granular and sparse and were different from those surrounding bile canaliculi. These findings suggest that phalloidin not only increases the polymerization of actin filaments, but also disturbs the normal organization of filaments. Interestingly, the blebs induced by phalloidin *in vivo* appear only in bile canaliculi and contain only filamentous structures. In contrast, blebs of isolated hepatocytes treated with phalloidin were observed all around the cell surface (5) and contained intracellular organelles. Such differences may be related to the redistribution of actin filaments in isolated hepatocytes following loss of polarity.

SEM and TEM showed extension of side branches of bile canaliculi to Disse's space where only a thin cytoplasmic rim separated the canalicular lumen from Disse's space. Such extension of side branches may also be explained by disorganized actin filaments around the bile canaliculi. Although no direct continuity was demonstrated, this findings suggests that bile contents might leak through the altered tight junction (11) or through a rupture in the thin cytoplasm. Kupffer cells occasionally seen near the extended side branches may take a part in clearing such leaked materials. The finding that processes of Kupffer cells protrude into the hepatic cord near the extended bile canaliculi suggests that they might be stimulated by such materials.

In the 3 day-treatment group, the changes in bile canaliculi showed zonal heterogeneity ; zone 3 > zone 1 (14). Zonal heterogeneity was first recognized by Gabbiani *et al.* (6) and was confirmed in this study. A recent study (15) suggests that phalloidin uptake into hepatocytes is

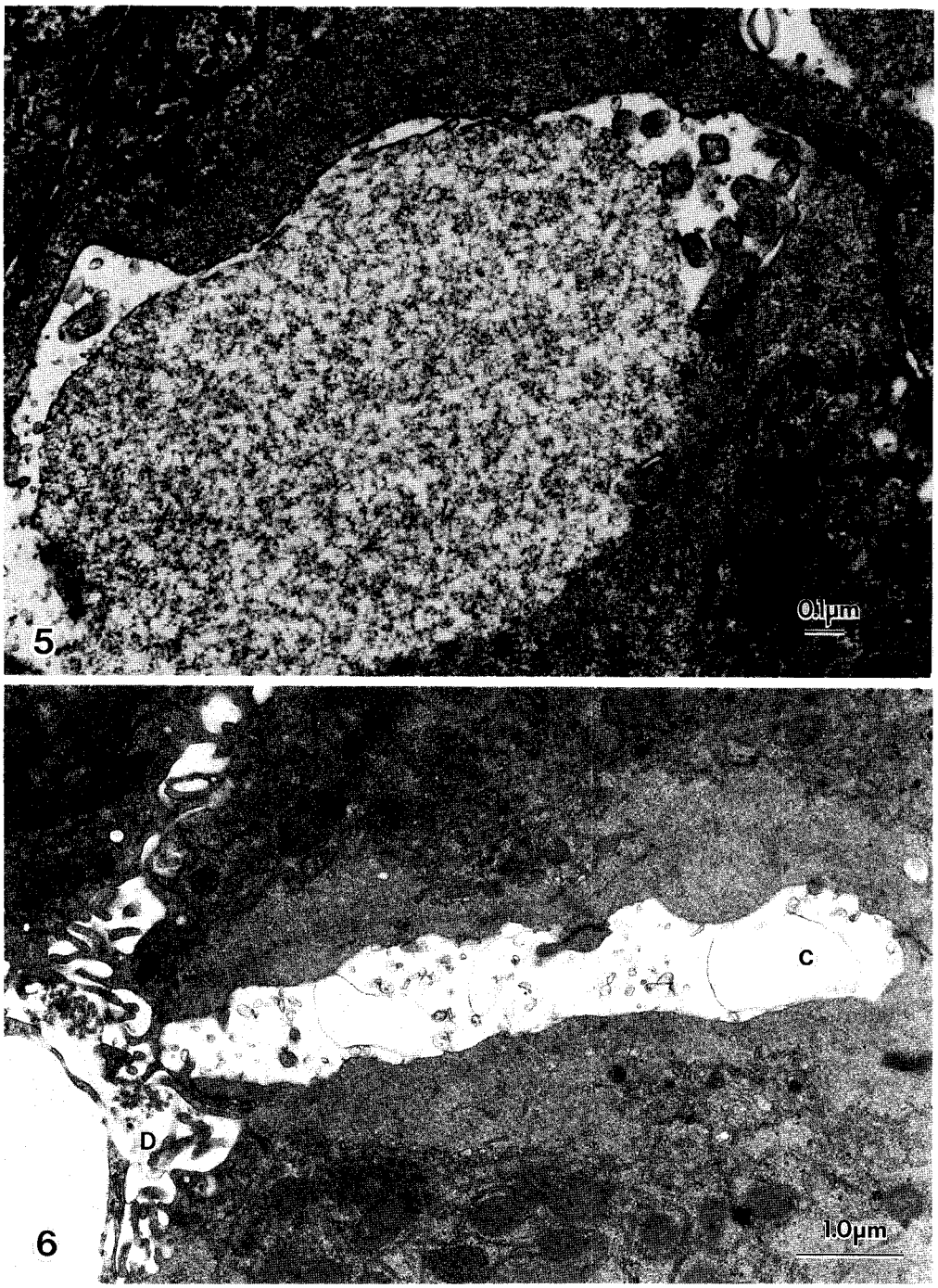

Fig. 5 Transmission electron micrograph (TEM) of a bleb in the periportal bile canaliculus containing sparse and granular filaments. 7-day treatment. ×62,000.

Fig. 6 TEM of a bile canaliculus in the 7-day treatment group. The canalicular lumen (c) is separated from Disse's space (D) with a thin stretch of cytoplasm. ×16,600.

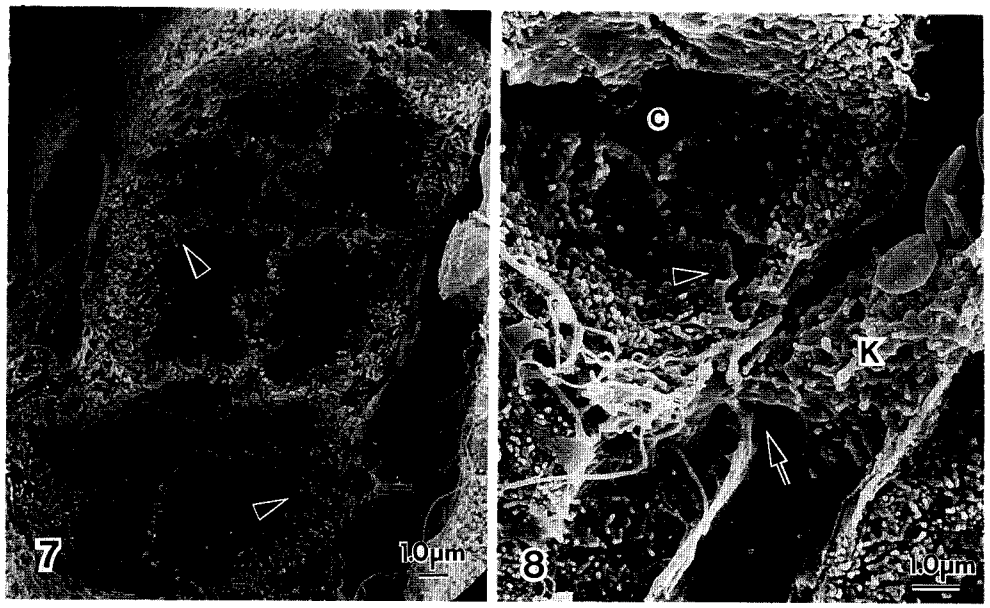

Fig. 7 SEM showing extension of side branches of bile canaliculi close to Disse's space (arrowheads). Zone 2 hepatocytes in the 7-day treatment group. ×4,500.

Fig. 8 SEM of a Kupffer cell (K) with a process that protrudes into the hepatic cord (arrow). A side branch of the bile canaliculus extends close to Disse's space (arrowhead). ×7,100. c, Bile canaliculi.

receptor-mediated and that phalloidin may share a common receptor with bile acid. This concept may explain intra-acinar heterogeneity of phalloidin induced changes in bile canaliculi. Bile acid uptake into hepatocytes follows the intra-acinar gradient, high in zone 1 and low in zone 3 (16). A high concentration of bile acid might protect zone 1 hepatocytes from phalloidin intoxication due to the competitive binding to their receptors. As a result, zone 3 hepatocytes may receive phalloidin at a higher concentration. In fact, bile acid is shown to protect phalloidin toxicity *in vitro* (17). Further experiments in which the bile acid concentration are controlled are necessary to clarify the role of bile acid in protection from phalloidin toxicity.

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