A Scanning Electron Microscopic Study on Hepatic Changes Induced by Mouse Hepatitis Virus-2

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OIKAWA, K. A Scanning Electron Microscopic Study on Hepatic Changes Induced by Mouse Hepatitis Virus-2. Tohoku J. exp. Med., 1979, 129 (4), 389-404 — No significant changes in the structure of the liver were seen until 9 hr after the inoculation of mouse hepatitis virus-2 (MHV-2) into mice. At the 24 hr stage, distinct swelling of hepatocytes and narrowing of sinusoidal lumina were observed from the middle to the central area of the hepatic lobules. Most of the Kupffer cells were swollen. Their villous projections were decreased in number, and the remaining projections became like blebs. Virus particles appeared from this stage in the hepatocytes, the Kupffer cells and the space of Disse. At the 48 hr stage, parenchymal necrotic foci were present in the central and the middle area of the lobules. The necrotic change was increased from 72 hr after inoculation, and was followed by submassive or massive necrosis. It is suggested that hepatic necrosis in both the central and the middle area, or in either area, of the lobules was advanced by aggravation of the sinusoidal microcirculation, as a result of the swelling of the hepatocytes and the Kupffer cells in addition to the direct affection by virus. Fine granulation was observed on the surface of most of the central flagella of the bile duct. Some flagella were degenerated, and came in part to be a fibrillar net. ——— SEM; mouse liver; Kupffer cell; sinusoid; MHV-2

A fulminant form of human hepatitis is the acutest and severest among various liver diseases, and reveals high mortality, and is caused generally by hepatitis viruses (Trey 1972). A number of studies have been made on its pathogenesis. Various experimental studies also have been performed on mice infected by mouse hepatitis virus as a model of human hepatitis. For morphological studies, light microscopy (LM) or transmission electron microscopy (TEM) has been applied hitherto. The scanning electron microscopic (SEM) study has not been reported on the experimental study of mouse hepatitis. The three-dimensional features of the cellular surface can be clearly recognized by SEM. The purpose of the present study is to show the morphological changes, especially the changes of the cellular surface occurring in the liver of mice infected by mouse hepatitis virus-2.

MATERIALS AND METHODS

Male mice of the ICR strain, aged 5 weeks and weighing 20 to 25 g, were used. Mouse hepatitis virus-2 (MHV-2) was used in the present experiment. MHV-2 in the
dose of $10^3$ times LD$_{50}$ was injected intraperitoneally in each mouse. Fifteen mice were examined at the following stages: 5, 20 min, 9, 24, 48, 72 and 96 hr after inoculation of MHV-2. Four untreated mice were also used as controls, the findings of which were previously reported (Oikawa 1979).

Laparotomy was carried out under anesthesia with ether. The liver was perfused with phosphate buffer solution (pH 7.4, 300 mOsm) through the portal vein at a pressure of 50 cmH$_2$O for 5 min, followed by perfusion with a 2.8% glutaraldehyde solution (pH 7.4, 300 mOsm) at the same pressure for 20 min. The extirpated liver was cut into small blocks, and they were divided into the following three groups; blocks of about $5 \times 5 \times 2$ mm size for SEM, about $1 \times 1 \times 1$ mm for TEM, and about $10 \times 5 \times 3$ mm for LM.

For SEM, the blocks were fixed further in the glutaraldehyde solution at $4^\circ C$ for 24 hr. Then, they were immersed in a mixture of glycine, sucrose, sodium glutamate and arginine hydrochloride, 2 g of each in 100 ml of distilled water, at $4^\circ C$ for 16 hr, followed by immersion in 2% tannic acid solution at the same temperature for 24 hr. After the rinse in distilled water, the blocks were postfixed in 2% osmium tetroxide at room temperature for 6 hr (Murakami 1974). Then, after being rinsed again in distilled water, they were dehydrated with ethanol, fractured in liquid nitrogen (Tokunaga et al. 1974), and dried at the critical point in CO$_2$. Finally, they were coated with gold by the ion sputtering method. The samples were examined in a scanning electron microscope (Hitachi, HSM-2B) at 25 kV of the accelerating voltage.

For TEM, the blocks were fixed in the 2.8% glutaraldehyde solution for 2 hr, followed by postfixation in 1% osmium tetroxide for 2 hr. Then, they were dehydrated with a graded series of ethanol, and embedded in Epon-812. Thin sections obtained were stained doubly with uranyl acetate and lead citrate. The sections were examined in a transmission electron microscope (Hitachi HU-11A, or JEOL JEM-100B).

For LM, the blocks were embedded in carbowax and sectioned at 4 $\mu$m. The sections were stained with hematoxylin-eosin. Carbowax used for embedding was removed from the blocks after sectioning. Then, the blocks were postfixed in 2% osmium tetroxide for 2 hr, and treated by the same procedure as mentioned already for SEM, except that fracturing in liquid nitrogen was omitted. Finally, the cut-surface of the blocks was examined in the SEM. The LM features of the sections and the SEM features of the cut-surface of the blocks were carefully compared in order to confirm the interpretation of both features.

**RESULTS**

Most mice died 60 to 96 hr after inoculation of MHV-2. None of them could survive over 120 hr after inoculation. No significant changes were observed by SEM, TEM and LM until 9 hr after inoculation.

24 hr after inoculation. At this stage, a number of hepatocytes were swollen like balloons (Fig. 1), whereas hepatocytes in normal mice showed a polyhedral outline (Fig. 2). The Kupffer cells were also swollen. The sinusoidal lumina were remarkably narrowed, and occasionally obstructed, because of the swelling of hepatocytes and Kupffer cells. The villous projections of the Kupffer cells were decreased in number. Some remaining projections became like blebs (Figs. 3 and 4). Virus particles, about 100 nm in diameter, were observed in hepatocytes, the Kupffer cells, the space of Disse, and in the intercellular recesses by TEM (Figs. 5 and 6). By SEM, the particles were found on the surface of hepatocytes facing to the intercellular recesses (Fig. 7). The bile canaliculi between the swollen hepatocytes were irregularly dilated. Microvilli of the bile canaliculi were shortened in length and decreased in number (Fig. 8). Shortening and decrease of
the microvilli were, however, not so distinct at the junctional line of the semicanaliculi, where two cells were connected to form the lumen of the bile canaliculus.

**48 hr after inoculation.** From this stage necrotic foci appeared in the central and the middle area of the lobules (Figs. 9 and 10). The sinusoids became irregular, some large and some narrow, around the necrotic foci. Erythrocytes were occasionally found to be grasped by the Kupffer cells which were swollen and protruded into the sinusoidal lumen (Fig. 11). A plate of hepatocytes was broken up into cells, and the hepatocytes became spherical in form (Fig. 12). Then, the bile canaliculi could not be recognized in some areas. Microvilli of the hepatocytes were decreased in number at the sinusoidal surface. Furthermore, globules were observed in hepatocytes (Figs. 13 and 14). They were from 2 to 15 μm in size, and were thought to be the Councilman-like bodies (acidophilic bodies). Their surface was usually rugged. They were separated from the hepatocytic cytoplasm by a space, when they were found within hepatocytes. Most globules found in the space of Disse were smaller than in hepatocytes. Similar globules were also seen in the Kupffer cells (Figs. 15 and 16). The endothelial cells were injured. Their fenestrations were fused, and irregularly widened with jaggy fringe (Fig. 17).

**From 72 to 96 hr after inoculation.** Submassive or massive necrosis occurred throughout the liver. Most mice died at this stage. Villous projections of the Kupffer cells were further decreased in number. The remaining projections became withered (Fig. 18). Hepatocytes disappeared in collapsed areas. The ground framework composed of reticulin fibers and degenerative endothelial cells was, however, reserved (Figs. 19 and 20). Some degenerating endothelial cells disclosed the filamentous inner structure forming a meshwork which resembled a fine cobweb (Figs. 21 and 22). The filaments of the endothelial cells were thought to be microtubules which acted as cytoskeleton. Fine granulations became clear on the surface of the central flagella of the interlobular bile duct (Fig. 23). Some flagella changed into a fibrillar net (Fig. 24), whereas no significant changes occurred in the microvilli of the bile duct.

**DISCUSSION**

The present experimental hepatitis was planned to study as a model of human fulminant hepatitis, which is thought to be caused mainly by virus infection. Hitherto, the morphological studies have been made mostly by LM or TEM. In the present study, the three-dimensional features of the altered hepatic components were examined by SEM. Additionally, LM and TEM were used to confirm the SEM findings.

In the present study, virus particles were definitely recognized from 24 hr after inoculation. They were found simultaneously in the hepatocytes, the space of Disse and the Kupffer cells. Two possible routes were reported of the inoculated viruses to hepatocytes. One is that the viruses are taken up first by the Kupffer
cells (Ruebner and Miyai 1962; Sabesin and Koff 1974) and transferred from the Kupffer cells to the hepatocyte. This observation was also supported by the immunofluorescent study (Boss and Jones 1963). The other is that the viruses transfer directly from the blood stream to the hepatocyte (Watanabe 1969). After multiplication, the viruses are released to the space of Disse. In the present study, most of the virus particles were observed in the hepatocytes and the space of Disse. The hepatocytes have been considered to be the major sites for multiplication of MHV-2. No multiplication of virus in the Kupffer cell has been suggested. It is more acceptable that the viral progeny is released from the hepatocytes to the space of Disse, then taken up by the Kupffer cells (Watanabe 1969).

Concerning the Kupffer cell, the villous projections were decreased in number as the first stage of the change 24 hr after the inoculation of MHV-2. A few remaining projections were shortened in length, and became like blebs. Finally, they withered. Such findings were also observed on the cultured cells infected by various kinds of virus through the SEM studies (Porter et al. 1973; Panem and Kirsten 1974; Ambros et al. 1975; Czajkowski and Heneen 1977). The projections of the peritoneal macrophages have been known to become longer by stimulation with triolein (Carr 1967). On the other hand, it was reported that the projections of the Kupffer cells disappeared by blockade with colloidal carbon (Satodate et al. 1977). The changes of the projections of the Kupffer cells, observed in the present study, have been concluded to reveal a degenerative process. It has also been suggested from the study by the carbon clearance method that the phagocytic activity is decreased by the infection of MHV-2 (Hatakeyama 1977).

The changes of the hepatocytes were revealed concomitantly with the appearance of virus particles. The first change was swelling of the hepatocytes. Then, the hepatocellular plate was broken up into cells. Each separate hepatocyte became round, and the characteristic form of polyhedra found in normal hepatocytes was lost. The microvilli became atrophic on their surface facing to the space of Disse. The Councilman-like bodies were seen in such degenerative hepatocytes. The Councilman-like bodies were also found in the Kupffer cells. Concerning the origin of the Councilman-like body, there have been the following three different reports: It is originally produced in (1) the Kupffer cell (Ruebner and Miyai 1962; Smetana 1962), (2) the hepatocyte (Mallory 1947; Klion and Schaffner 1966), and (3) both the Kupffer cell and the hepatocyte (Ruebner et al. 1967). However, the occurrence of the Councilman-like bodies in the Kupffer cells was much rarer than in the hepatocytes in the present study. In hepatocytes, the bodies were recognized as an aggregation of degenerating organelles. However, they were observed in the phagosome, when they appeared in the Kupffer cells. Therefore, the Councilman-like bodies in the Kupffer cells are considered to be phagocytosed after released from the hepatocytes to the sinusoids, as described by Nakazawa (1974).

From 48 hr after inoculation, necrotic foci were seen close to the central vein.
The necrotic foci were composed of various degenerating hepatic components. Focal necrosis tended to occur in the centrilobular area in various hepatic injuries. Many views were presented concerning the centrilobular occurrence of the hepatic necrosis (Warkim and Mann 1942; Glynn and Himsworth 1948; Brauer 1963; Hase 1966; Nakata and Higaki 1969; McClugage and McCuskey 1971; Stefenelli and Gericke 1972; Itoshima et al. 1974). The larger fenestrations of the endothelial cells were found more often in the central area than in the periphery of the lobule in the guinea pig (Itoshima et al. 1974). Therefore, the noxae and germs may easily reach the hepatocytes in the central area through the larger fenestrations. However, no difference was seen in the distribution of the centrilobular occurrence of the endothelial fenestrations in mice as described already in the previous report (Oikawa 1979). Muto (1975) also observed no difference in the distribution of the fenestrations in rats. The centrilobular occurrence of necrosis has not sufficiently been explained only by the larger endothelial fenestrations in the centrilobular area. On the other hand, the circulatory disturbance, which usually appear in the central and the middle areas, should not be overlooked on CCl₄ intoxication (Hase 1966; Nakata and Higaki 1969; McClugage and McCuskey 1971).

The in vivo microscopical study showed that swelling of the hepatocytes caused the circulatory disturbance in CCl₄ intoxication. In addition to the direct damage with CCl₄, hypoxia caused by the circulatory disturbance enhanced hepatic necrosis. In the present study, narrowing of the sinusoidal lumina by swelling of the hepatocytes was more remarkable in the central and the middle area than in the peripheral area. Furthermore, there were also seen swelling of the Kupffer cells and adhesion of erythrocytes to the Kupffer cells. It is thought, in conclusion, that the circulatory disturbance accelerates the necrotic change.

It was reported on the experiment of frog virus-3 that the endothelial cells were severely damaged parallel to the Kupffer cells, but the damage appeared earlier in the endothelial cells than in the hepatocytes (Gendrault et al. 1977). The degenerative changes of the hepatocytes were assumed to be accelerated by the endothelial destruction (Fiume 1975). In the present study, the enlargement of the endothelial fenestrations was first found in the necrotic foci 48 hr after inoculation. The changes of the endothelial cells were, however, revealed after the alteration of the hepatocytes. The endothelial cells were so much destroyed that they barely remained as a mesh-work of microtubules. Then, the hepatocytes were exposed almost directly to the blood stream. The necrotic change of the hepatocytes is considered to have followed the endothelial destruction. Then the alteration of the endothelial cells seemed to accelerate hepatic necrosis in conspiracy with the circulatory disturbance.

From the TEM study, Grisham (1963) reported that cilia were rarely found in the epithelial cells of the bile duct in rats. Motta and Fumagalli (1974) contrarily noted from the SEM study that the cilia were often seen in rats. However, the present author already mentioned, in the previous report, that epithelial cell of the bile duct has a single central flagellum (Oikawa 1979). Three
possibilities have been proposed on the single cilium of the pancreatic islets of mice from the TEM study; (1) an embryologic remnant without any function, (2) a chemoreceptor, and (3) an agitator for the extracellular fluid with the result of increasing the transport (Munger 1958). Concerning the central flagellum of the bile duct of rat, the first and the third interpretation described here were supported in general (Grisham 1963; Motta and Fumagalli 1975). The central flagellum of the bile duct revealed the degenerative change showing fine granulations 48 hr after inoculation of MHV-2 in the present study. Later it became like a fibrillar net in consequence of the advanced degeneration. However, no virus particles could be found in the bile duct or in its epithelial cells. The flagellum was considered to be very sensitive to the pathogenic condition, while the microvilli of the biliary epithelial cells remained intact. The changes of the flagellum were induced possibly not directly by virus, but indirectly by the altered biliary fluid or the hepatocytic damage.

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References


Fig. 1. The middle area of the lobule at the 24 hr stage after inoculation of MHV-2. Hepatocytes are swollen, and the sinusoidal lumina (Sin) become remarkably narrow. BC, bile canaliculus; HCP, hepatocellular plate. × 950.

Fig. 2. The middle area of the lobule in normal mice is presented in contrast with Fig. 1. Figs. 1 and 2 are at the same magnification. BC, bile canaliculus; HCP, hepatocellular plate; Sin, sinusoid. × 950.

Fig. 3. The Kupffer cell (KC) at the 24 hr stage. The villous projections of the Kupffer cell are decreased in number, and the remaining projections become like blebs. Sin, sinusoid. × 8,600.

Fig. 4. TEM of the Kupffer cell (KC) corresponding to Fig. 3 at the 24 hr stage. The projections of the Kupffer cell become like blebs. × 14,300.
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Fig. 5. TEM of the space of Disse at the 24 hr stage after inoculation of MHV-2. Many virus particles are seen in the space of Disse. HC, hepatocyte; KC, Kupffer cell. × 26,000.

Fig. 6. TEM of the Kupffer cell (KC) at the 24 hr stage. Virus particles (arrows) are taken up into the Kupffer cell. HC, hepatocyte. × 25,500.

Fig. 7. The hepatocellular surface facing to the intercellular recess at the 24 hr stage. Numerous virus particles, which are spherical and about 100 nm in diameter, are also seen on the surface. Some particles are indicated with arrows. D, the space of Disse; Sin, sinusoid. × 20,000.

Fig. 8. A bile canaliculus (BC) at the 24 hr stage. Its lumen is dilated. It is observed at the canalicular groove (BC) that the microvilli are shortened in length and decreased in number. The microvilli still remain, however, intact at the edge of the canalicular groove to the intercellular surface (IS). × 10,000.

Fig. 9. An SEM picture of the cut-surface of the specimen embedded in carbowax. The cut-surface was examined in the SEM after removing carbowax. The central area of the lobule at the 48 hr stage after inoculation of MHV-2. Necrotic focus (arrow) is revealed near by the central vein (CV). × 200.

Fig. 10. LM corresponding to Fig. 9. Carbowax section stained with hematoxylin-eosin. Necrotic focus is indicated with an arrow. CV, central vein. × 200.
Fig. 11. Two Kupffer cells (arrows) at the 48 hr stage after inoculation of MHV-2. Erythrocytes are grasped by the Kupffer cells. HC, hepatocyte; Sin, sinusoid. × 1,900.

Fig. 12. Hepatocyte at the 48 hr stage. Hepatocyte becomes spherical. Two surfaces, sinusoidal (SS) and intercellular (IS), can, however, still be recognized in this picture. × 6,000.

Fig. 13. At the 48 hr stage after inoculation of MHV-2. A large spherical body corresponding to the Councilman-like body (CB) is seen in a hepatocyte. It is separated from the hepatocytic cytoplasm by a narrow space. × 3,600.

Fig. 14. At the 48 hr stage. Some Councilman-like bodies (CB) of various sizes are seen in a hepatocyte. KC, Kupffer cell; Sin, sinusoid. × 4,500.

Fig. 15. The Kupffer cell (KC) at the 48 hr stage after inoculation of MHV-2. The Councilman-like body (CB) is also observed in the swelling Kupffer cell through the chink made by destruction of the cell membrane. HC, hepatocyte; Sin, sinusoid. × 2,400.

Fig. 16. TEM of the Kupffer cell (KC) corresponding to Fig. 15. The Councilman-like body (CB) is taken by the Kupffer cell. × 12,500.
Fig. 17. Endothelial cell at the 48 hr stage after inoculation of MHV-2. Fenestrations of the endothelial cell are fused, and irregularly widened. The fringe of the fenestrations is jaggy. ×10,000.

Fig. 18. The Kupffer cell (KC) at the 72 hr stage after inoculation of MHV-2. The villous projections of the Kupffer cell are decreased in number. The remaining projections become withered. HC, hepatocyte. × 4,700.

Fig. 19. The necrotic collapsed area in the lobule at the 72 hr stage. Most hepatocytes disappeared. The ground framework composed of reticulin fibers and degenerative endothelial cell is, however, reserved. × 3,400.

Fig. 20. High magnification of the collapsed area at the 72 hr stage. × 5,000.
Fig. 21. Endothelial cell (EC) at the 72 hr stage after inoculation of MHV-2. The endothelial cell disclosed the filamentous inner structure forming a fine cobweb. The filaments are thought to be microtubules. × 17,000.

Fig. 22. Endothelial cells (EC) at the 72 hr stage. Some of the filaments are dispersed outside of the cells. × 4,700.

Fig. 23. Two flagella of the epithelial cells of the bile duct at the 72 hr stage after inoculation of MHV-2. The surface of the flagella is finely granulated. × 26,000.

Fig. 24. A flagellum of the ductal epithelial cell at the 72 hr stage. The flagellum partly becomes swollen and turns into a fibrillar net. However, no significant changes occur on the microvilli. × 8,400.