Scanning Electron Microscopic Examination of Acetaminophen-Induced Hepatotoxicity and Congestion in Mice

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Acetaminophen-induced hepatotoxicity and associated hepatic congestion were investigated by scanning and correlative transmission electron microscopy. Acetaminophen (750 mg/kg orally) causes changes in cell surface morphology and the relationship between hepatocytes and sinusoidal lining cells. There is endocytic vacuolation at lateral and sinusoidal margins of centrilobular hepatocytes, loss of microvilli, Disse space enlargement, dilation of bile canaliculi, and disappearance of the studlike projections from hepatocyte lateral surfaces. Erythrocytes enter the enlarged Disse space and endocytic vacuoles via enlarged pores in sinusoidal lining cells, thereby collapsing the sinusoids. Lining cells are not lost, but apparently held in position by preservation of intercellular junctions, cytoplasmic projections from hepatocytes, and anchorage by fat-storing cells within the Disse space. Congestion can abate by 24 hours, indicating that erythrocytes can return to the general circulation from the Disse space. (Am J Pathol 1983, 113:321-330)

ACETAMINOPHEN (paracetamol, N-acetyl-p-aminophenol) is a widely used analgesic and antipyretic drug that is considered to be relatively safe when taken at therapeutic doses. However, an overdose can cause life-threatening hepatotoxicity in human beings1-3 and experimental animals.4-6

Acetaminophen is metabolized in the liver primarily to glucuronide and sulfate conjugates.7,8 A small fraction of any dose, however, is converted by the cytochrome-P-450-linked mixed-function oxidase system to a reactive metabolite(s) that can combine with glutathione and ultimately be excreted in the urine as a mercapturic acid.9,10 At high doses of acetaminophen, glutathione is depleted, thereby allowing the reactive metabolite to bind covalently to liver macromolecules, principally protein. The magnitude of the covalent binding usually correlates with the extent and severity of liver injury.

We have previously shown, by transmission electron microscopy (TEM), that acetaminophen-induced hepatotoxicity is characterized by alterations of cell surface morphology6,11 and changes in the relationship between hepatocytes and the sinusoidal lining cells which result in the development of massive centrilobular congestion before the appearance of necrosis.6 Such phenomena are particularly amenable to study by scanning electron microscopy (SEM). Therefore, we have conducted a scanning electron microscopic and correlative transmission electron microscopic study of acetaminophen-induced hepatotoxicity in mice in order to better understand the development of liver injury, with particular emphasis on the pathogenesis and abatement of the associated congestion.

Materials and Methods

Animals and Drug Treatments

Male CD-1 mice (Charles River Canada Inc.) weighing approximately 25 g were provided with Purina Laboratory Chow and water ad libitum for 1 week. Food, but not water, was removed 12 hours

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before drug administration and withheld from all animals except the 24-hour group, in which it was restored at 9 hour after acetaminophen administration. Acetaminophen (Sigma Chemical Company) was dissolved in warm distilled water and administered orally by gavage at a dose of 750 mg/kg in 1.0 ml. Controls received 1.0 ml of distilled water. A total of 22 animals were examined, of which 4 were controls. The animals were killed under pentobarbital anaesthesia (100 mg/kg) at various times up to 24 hours after they had been given acetaminophen.

Morphology

Liver was perfusion fixed in situ via the portal vein at pressures of 10–25 mm Hg with the use of 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) as described previously. Immediately before introduction of fixative, the liver was briefly flushed with isotonic saline for the prevention of precipitation of plasma proteins. After approximately 10 minutes of perfusion, liver tissue for scanning electron microscopic study was cut into 2–3-mm wide slices and immersed in fresh fixative for 18 hours. Samples for transmission electron microscopic study were minced and immersed in fresh fixative for 1.5 hours. Tissue for light microscopic study was transferred to 10% neutral buffered formalin. Exposure of surfaces for scanning electron microscopic examination was effected by manual fracture of the slices while they were immersed in 0.1 M phosphate buffer (pH 7.4). Some samples for SEM and all tissue for TEM were post-fixed in 2% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 1.5 hours. Samples for SEM were then dehydrated in acetone, critical-point-dried with Freon in a Polaron E3000 critical-point-drying apparatus, coated with gold or gold–palladium in a Polaron E5100 sputter coater, and viewed on a Hitachi 450 scanning electron microscope. Samples for TEM were dehydrated in ethanol, infiltrated with propylene oxide–Epon, and embedded in fresh Epon mixture. Toluidine-blue-stained semithin sections were examined for determination of orientation within the liver lobule. Thin sections were cut from centrilobular regions, stained with uranyl acetate and lead citrate, and viewed on a Hitachi HS-9 transmission electron microscope. All pictures are from centrilobular regions.

Results

Controls

Liver ultrastructure as revealed by SEM correlates well with corresponding TEM and is similar to that described for other mouse strains. Scanning electron microscopic study of control animals shows the liver to be comprised of 1-cell-thick hepatocellular cords (Figure 1). The sinusoidal surfaces of hepatocytes are covered by numerous microvilli, which fill the narrow Disse space (Figures 2 and 3). Also present within the Disse space are fat-storing, or Ito, cells which are embedded within the hepatocellular plates and have long cytoplasmic processes that extend through the Disse space (Figures 2 and 3). The smooth lateral surfaces of hepatocytes are traversed along their midlines by bile canaliculi that contain microvilli (Figures 2 and 3). The lateral surfaces also possess numerous buds and shallow pits, which can be shown by TEM to interlock in adjacent cells (Figures 2 and 3). The sinusoidal lining cells or endothelial cells are perforated by large numbers of fenestrations about 0.1–0.2 μ in diameter. No large pores were seen in controls. Collagen is infrequently seen in the Disse space but covers the endothelium of central veins (Figure 1) and portal vessels in a dense network of interwoven bundles.

Acetaminophen-Treated

Administration of acetaminophen causes the development of numerous cytoplasmic lesions in centrilobular hepatocytes, which we have described previously. Cell surface changes are evident in centrilobular areas by SEM and TEM. Among the earliest changes is the appearance of large pores (0.5–3 μ in diameter) in the sinusoidal lining cells, which are evident at 1.5 hours after acetaminophen administration and become larger and more extensive with time (Figures 4–6). The intercellular junctions of the sinusoidal endothelium remain intact, even when large pores are immediately adjacent (Figures 4 and 5). These junctions appear as seams in the sinusoidal endothelium by SEM (Figure 5). Under TEM the junctions can be distinguished from fenestrations by an increase in the electron density of the plasma membrane and underlying cytoplasm (Figure 4).

Development of endothelial cell pores is accompanied in hepatocytes by endocytic vacuolation and loss of microvilli from sinusoidal margins and bile canaliculi (Figures 4 and 6). TEM shows that the endocytic vacuoles are lined by unit membranes and connect with sinusoidal and lateral surfaces of hepatocytes (Figure 4). Scanning electron microscopic examination of lateral surfaces shows dilation of bile canaliculi with reduced numbers of microvilli, loss of the studs and shallow pits, and the presence of openings to some of the endocytic vacuoles (Figures 6 and 7).
Figure 1—Control, SEM. The liver is comprised of polyhedral hepatocytes. The field is traversed diagonally by a central vein (CV), a portion of which has been removed in sample preparation to reveal a smooth inner surface (arrowhead) in contrast to the rough outer surface, which is covered by an extensive network of collagen bundles of different sizes. (x400)

Figure 2—Control, SEM. Sinusoids (S) are lined by endothelial cells, which are perforated by many small fenestrations. A fat-storing cell (F) lies in the Disse space and is embedded within a hepatocellular plate. The largely smooth lateral surfaces of hepatocytes are traversed along their midline by bile canaliculi (B), which are filled with microvilli. Lateral surfaces also possess studs (arrowheads) and shallow pits (arrow). (x4000)

Figure 3—Control, SEM. Endothelial lining cells have been removed, revealing the large number of microvilli which occupy the Disse space at the sinusoidal (S) poles of hepatocytes. Embedded within the microvilli are irregularly shaped processes from fat-storing cells (arrows). (x3600)

The stud and pit structures on lateral surfaces can be seen by TEM to interlock in adjacent hepatocytes. (Inset, x19,400)
Figure 4 — One and a half hours after acetaminophen, TEM. There are few microvilli within the slightly enlarged Disse space. Note the late gaps in the sinusoidal lining cells (arrows). An intact intercellular junction (arrowhead) is enlarged in the inset. (x 80,000) Some of the large endocytic vacuoles (V) can be seen to communicate with sinusoidal or lateral surfaces of hepatocytes. (Uranyl acetate and lead citrate, x 8300)

Figure 5 — One and a half hours after acetaminophen, SEM. From the same animal as Figure 4. Intercellular junctions (arrows) remain intact while there are large pores (P) to either side. Note the underlying fat-storing cell process (arrowheads) in the Disse space. (x 15,000)

Figure 6 — Two hours after acetaminophen, SEM. Large pores (arrows) are evident in sinusoidal lining cells, and the Disse space is enlarged (D). There is loss of microvilli from the sinusoidal surfaces as well as the bile canaliculi (B), which are also dilated. At the lateral surfaces of hepatocytes there are openings to deep pits (arrowheads) and an absence of studs. (x 3300)

Figure 7 — Three hours acetaminophen, SEM. Cytoplasmic extensions from hepatocytes stretch through the enlarged Disse space to the sinusoidal lining cells (arrows). A long, irregularly shaped process (arrowheads) from a fat-storing cell (F) traverses the Disse space and lies adjacent to the outer surfaces of sinusoidal lining cells. (x 3600)
The above changes are concomitant with a progressive enlargement of the Disse space as the sinusoidal lining cells separate from underlying hepatocytes (Figures 4, 6, and 7). The separation is not complete, however, because processes from hepatocytes extend through the Disse space to the outer surfaces of the sinusoidal lining cells (Figures 4, 6, and 7). Fat-storing cells continue to maintain close contact with hepatocytes and sinusoidal lining cells, the processes remaining adjacent to the lining cells (Figure 7). Neither the lining cells nor the fat-storing cells show any cytoplasmic disease by TEM.

Centrilobular congestion is increasingly evident from 3 to 6 hours after acetaminophen administration; large numbers of red blood cells occupy the Disse space and endocytic vacuoles. Initially, sinusoidal lumens remain patent, but eventually they are collapsed to a fraction of their original caliber (Figures 8, 11, and 12). The large pores disappear from collapsed sinusoidal lining cells (Figure 11). The appearance of erythrocytes constricted by the sinusoidal lining cell pores suggest that this is the route whereby they gain access to the Disse space (Figure 9). Small pockets of red blood cells in the hepatocellular plates accumulate where there is “dropout” or disintegration of hydropic hepatocytes (Figure 12). At the same time that congestion is developing and present, platelets are seen adjacent to the Disse space side of the sinusoidal lining cells by both SEM and TEM (Figures 9 and 10).

Congestion usually abates between 9 and 24 hours after acetaminophen administration in surviving animals. Proportionately fewer red blood cells are seen in the Disse space, and more are found within large vacuoles of necrotic hepatocytes (Figure 13), which never show any connection with the cell surface. Necrotic cells, viewed under SEM, fracture through their interiors to reveal a spongy, finely vacuolated cytoplasm (Figures 13 and 14). There are few large pores in sinusoidal lining cells at 12 and 24 hours (Figures 13 and 14). Sinusoidal lumens often show a more normal patent appearance at 12 hours (Figure 13), and by 24 hours the lining cells adopt a relatively normal fenestrated appearance overlying necrotic hepatocytes (Figure 14).

**Discussion**

Acetaminophen-induced hepatotoxicity and the pathogenesis of the associated hepatic congestion have been studied by SEM and correlative TEM. This toxicity is particularly well suited to study by SEM because it involves alterations to cell surface morphology and the relationship between parenchymal and sinusoidal cells.

The mouse was chosen as the experimental model to facilitate comparison with previous work.6,17,18 A dose of 750 mg/kg acetaminophen was used because it causes consistently severe liver damage in all mice fasted for 12 hours.17,18 The perfusion fixation pressure (via portal vein) was kept as low as possible because it has been shown that even pressures within the normal arterial range can cause enlargement of sinusoidal lining cell fenestrations, thereby forming large pores.19,20 In control animals perfused at 10–25 mm Hg large pores were never seen. Perfusion pressures of 20–25 mm Hg were necessary for the achievement of good fixation in some of the more congested livers.

Early morphologic changes in the surface of centrilobular hepatocytes after acetaminophen administration occur concomitantly with cytoplasmic lesions, which we have discussed previously,6 and invariably accompany formation of large pores in sinusoidal lining cells (Figures 4–6). The large hepatocytic vacuoles that develop are usually as electron-lucent as the sinusoidal lumens and occasionally show connections with either the lateral surface or sinusoidal margin, thereby indicating their endocytic origin (Figure 4). Endocytic vacuoles at lateral surfaces may be derived from the shallow pits from which the corresponding studs always disappear after acetaminophen administration (Figures 6 and 7). Also at the lateral surfaces, there is dilation of bile canaliculi and loss of microvilli, suggesting early impairment to biliary function. Acetaminophen has been reported to inhibit excretion of conjugated bilirubin into the bile of rats.21

Vacuole formation is accompanied by the loss of microvilli from the Disse space. We suggest that these hepatocyte surface changes are responsible for the concomitant development of large pores in the sinusoidal lining cells and separation of the lining cells from underlying hepatocytes, thereby producing an enlarged Disse space. The lining cells themselves never show any ultrastructural abnormality of the cytoplasm. It is unlikely that increased sinusoidal pressure causes the formation of large pores in centrilobular areas because the endothelium retains its normal fenestrated appearance in undamaged periportal regions.

These changes, while not necessarily directly related to the development of cell death, nevertheless have important consequences in the pathogenesis of hepatic congestion. Congestion is first evident about 3 hours after acetaminophen administration and thus precedes the appearance of necrosis. Congestion develops as a result of red blood cells entering the enlarged Disse space and then moving into the endo-
Figure 8—Three hours after acetaminophen, SEM. Many red blood cells are in the Disse space, and sinusoidal lining cells are riddled with large pores. Sinusoidal lumens (S) are patent. (× 1600) Figure 9—Three hours after acetaminophen, SEM. a—A constricted erythrocyte appears to be squeezing through one of the large pores in a sinusoidal lining cell. (× 9500) b—Four and a half hours after acetaminophen, TEM. A red blood cell seems to be entering the Disse space via a pore in a lining cell (arrow). Note the presence of platelets in the Disse space (arrowheads). (Uranyl acetate and lead citrate, × 4600) Figure 10—Four and a half hours after acetaminophen, SEM. Platelets, characterized by a small irregular shape and the presence of pseudopodia, adhere to the Disse space side of sinusoidal lining cells. (× 3200)
cytic vacuoles and into sites between hepatocytes. As more and more red blood cells accumulate, the congestive enlargement of the Disse space collapses original sinusoidal lumens to a small fraction of their original caliber, a process which is most striking as seen by SEM (Figure 11). Red blood cells appear to enter the Disse space through the large pores in the sinusoidal lining cells. They do not appear to move between lining cells, because the intercellular junctions always remain intact. The importance of the early morphologic changes in the hepatocyte surface, resulting in Disse space enlargement and the development of large pores in lining cells, lies in providing a potential space for red blood cell accumulation and access to that space, respectively. The distinct anatomic design of the liver sinusoids, which lack a basement membrane, serves to maximize serum contact with all hepatocytes through a fenestrated endothelium. However, this renders the liver susceptible to the development of hepatotoxic Disse space congestion due to the resulting fragile relationship between sinusoidal lining cells and hepatocytes.

The appearance of platelets in the Disse space (Figures 9 and 10) and fibrin deposits at later times supports a condition of vascular stasis that may further aggravate congestion. Additional tissue disorganization may result from the loss of the stud and pit structures at the lateral surfaces of hepatocytes. It has been suggested that these structures function in cellular attachment and intercellular communication. However, the location of the stud and pit structures, indicates they may function to provide support in shear to the hepatocellular plates by helping prevent lateral movement of individual cells relative to one another. This subtle form of support may be important in the liver, where there is little in the way of a basement membrane between hepatocytes and sinusoidal lining cells. Thus, disappearance of the stud and pit structures after acetaminophen may contribute to the overall disorganization of hepatic architecture and thereby exacerbate congestion.

Red blood cells also accumulate in small pockets where there has been disintegration or dropout of a hydropic hepatocyte (Figure 12), a finding we have also seen after toxic doses of furosemide. Potentially similar findings with other toxic compounds have been described as a form of peliosis. Congestion gradually abates in surviving animals between 9 and 24 hours after acetaminophen, suggesting that many of the red blood cells which were extrasinusoidal, particularly those in the Disse space, can return to the general circulation. Often there are relatively few red blood cells in the Disse space at 12 and 24 hours, and erythrocytes are still trapped in the vacuoles of damaged and dead cells, which lose their endocytic connections with the surface. The final pattern of necrosis can be quite variable, but often patent sinusoidal lumens and relatively normal-looking sinusoidal lining cells are evident in necrotic centrilobular areas (Figures 13 and 14). We suggest that recovery from congestion can occur, even in necrotic areas, because the network of sinusoidal lining cells remains undamaged and is held in position despite the congestion by preservation of intercellular junctions between lining cells, maintenance of some contacts with hepatocytes via cytoplasmic projections from the latter (ie, separation is not complete), and anchorage by fat-storing cells within the Disse space.

Some clarification of terminology is now desirable, because "congestion" implies increased numbers of red blood cells within the vasculature; but in the congested liver, erythrocytes are present both within the sinusoids and Disse space, as well as in endocytic vacuoles. The term "congestion" is retained to describe this condition, and not hemorrhage, for three reasons. First, the Disse space and endocytic vacuoles contain blood serum, due to the presence of a discontinuous endothelium, and therefore they can be considered a specialized extension of the vasculature. Second, Disse space congestion can occur without rupture of the sinusoidal endothelium. Third, congestion is reversible, as described above.

Liver congestion is associated with damage caused by many toxic agents, but the pattern of early endocytic vacuolation and consequent accumulation of red blood cells after acetaminophen is similar to that caused by phalloidin and cytochalasins. The changes caused by these compounds are thought to arise as a consequence of binding to the microfilament component of the cytoskeleton at cell margins. It is possible that a reactive metabolite(s) of acetaminophen interacts directly, in a way similar to that of the above toxins, or indirectly via glutathione depletion to alter the cytoskeleton and thus cause the cell surface changes.

Congestion is a feature of acetaminophen-induced hepatotoxicity in other experimental models and in human beings; however, its relative importance is not known. Our morphologic results indicate that congestion, particularly if prolonged, could result in impaired liver circulation and consequent hypoxic injury, which would be secondary to the more direct acetaminophen metabolite-mediated damage. We have previously shown that the magnitude of the hepatic congestion is sufficient to cause circulatory changes and may contribute to early mortality after acetaminophen.

In summary, it has been shown by SEM and cor-
relative TEM that acetaminophen-induced hepatotoxicity in mice involves alterations to cell surface morphologic changes and to the relationship between centrilobular hepatocytes and sinusoidal lining cells, which result in massive intratrabecular and sinusoidal congestion. Congestion develops before the appearance of necrosis, as red blood cells leave the sinusoids via large pores in the lining cells and enter the consequently enlarged Disse space. Congestion can be reversible, but is important because of its potential circulatory consequences, and the mechanical effects on the liver sinusoids may result in additional liver damage due to hypoxia. Thus congestion is an important and distinctive aspect of hepatotoxicity and not always a passive epiphenomenon of liver cell necrosis.

References

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Figure 11—Six hours after acetaminophen, SEM. Sinusoids are collapsed as a result of large numbers of red blood cells in the Disse space as seen longitudinally (arrow) and in cross-section (arrowheads). There are no large pores in the sinusoidal lining cells. (x 1600) Figure 12—Six hours after acetaminophen, SEM. A sinusoid (S) is narrowed at its junction with a central vein (CV). A pocket of erythrocytes with bizarre shapes has accumulated where they have packed into a space formerly occupied by a hydropic hepatocyte which has disintegrated. (x 3400) Figure 13—Twelve hours after acetaminophen, SEM. Necrotic hepatocytes have broken through their interiors, which have a spongy appearance, revealing large vacuoles, which contain red blood cells. Notice that there are few erythrocytes in the Disse space, the sinusoids (S) are relatively patent, and there are no large pores in the endothelial lining cells. (x 2000) Figure 14—Twenty-four hours after acetaminophen, SEM. An endothelial cell(s) (E) with a relatively normal appearance overlies a necrotic hepatocyte with a vacuolated interior (arrow) and ruffled surface. (x 4300)


42. Walker RM, Racz WJ, McElligott TF: Acetaminophen-induced hepatotoxic congestion in mice. (Manuscript in preparation)

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