Application of an NaOH Maceration Method to a Scanning Electron Microscopic Observation of Ito Cells in the Rat Liver

Hiromi TAKAHASHI-IWANAGA and Tsuneo FUJITA

Department of Anatomy (Prof. T. FUJITA), Niigata University School of Medicine, Niigata, Japan

Received June 23, 1986

Summary. A three-dimensional observation of the Ito cells in the rat liver was made by scanning electron microscopy (SEM) with an NaOH maceration method. The treatment with NaOH facilitated the intercellular separation of the specimen and completely removed reticular fibers around the sinusoid. In this way, the entire shapes of the Ito cells located in the perisinusoidal spaces or between the hepatocytes, were exposed under the SEM.

The Ito cells were disposed at regular intervals of 30–55 μm all over the hepatic lobule, and surrounded the whole sinusoid with their subendothelial processes. SEM revealed a pattern of branching for the primary processes into secondary and tertiary processes. Furthermore, previously unknown thorn-like processes of the fourth order were also demonstrated. The Ito cells showed sparse microvilli on their surfaces confronting the hepatocytes, and occasional round elevations, these probably corresponding to lipid droplets.

SEM also exhibited the relationship between the Ito cells and other types of cells in the liver. The Kupffer cells often interposed between the sinusoidal wall and the subendothelial processes of the Ito cells.

The fat-storing cell of Ito (1951) is a stellate cell characteristically housed in the perisinusoidal space of Disse in the liver. The Ito cell is known to have a vitamin A-storing ability (NAKANE, 1963; BRONFENMAJER et al., 1966; KOBAYASHI and TAKAHASHI, 1971; WAKE, 1971; HIROSAWA and YAMADA, 1973), and to share fibrogenetic functions with fibroblasts; in hepatic fibrosis, they transform into fibroblasts (SCHNACK et al., 1966; ITO and SHIBASAKI, 1968; McGee and PATRICK, 1972; KAWANAMI, 1973; TANIKAWA, 1975).

ITO and NEMOTO (1952) precisely described the Ito cells by light microscopy as being situated outside the sinusoid and evenly scattered in the whole hepatic lobule. They failed to visualize the entire shapes of the Ito cells because there were then no methods to selectively stain these cells.

The extrasinusoidal localization of the Ito cells was confirmed by transmission electron microscopy (TEM) by Ito and his co-workers (YAMAGISHI, 1959; ITO and SHIBASAKI, 1968; TANUMA and ITO, 1978). Furthermore, these TEM studies revealed that the Ito cells extended several attenuated processes immediately beneath the sinusoidal endothelium. They also found that the subendothelial processes occasionally surrounded the sinusoid almost completely (TANUMA and ITO, 1978; Fig. 19). Although it has appeared difficult to conceive of the three-dimensional extensions of these processes from the fragmental TEM images, GEMMELL and HEATH (1972) presumed that the
processes might form a network around the sinusoid, Tanuma and Ito (1978) assumed that the Ito cell processes might mechanically protect the sinusoidal wall and have a contractile ability with which they could regulate the caliber of the sinusoid.

On the other hand, early investigators reported on perisinusoidal cells which stained with various metal impregnation methods. Von Kupffer (1876) showed numerous "Sternzellen" (stellate cells) impregnated with gold outside the liver sinusoid. A century later, Wake (1971) re-evaluated the gold impregnation study and stated that the "Sternzellen" in the initial report by von Kupffer (1876), but not in his later papers, are identical to the Ito cells. The gold-impregnated Ito cells as shown by von Kupffer (1876) and by Wake (1971) extended from one to five, horn-like processes with very few branches. These processes appeared far smaller in number and simpler in shape than those expected from the TEM study by Tanuma and Ito (1978), who found numerous profiles of the processes of the Ito cells around the sinusoids of the bat liver.

Zimmermann (1923) observed flattened cells surrounding the sinusoid with their branching processes by means of Golgi-Kopsch's impregnation method, and referred to these cells as "Perizyten" of the liver. Based on their perisinusoidal localization, Tanuma and Ito (1978) assumed that the "Perizyten" corresponded to the Ito cells. The question as to whether the "Perizyten" might be identical to the Ito cells remains to be settled.

In order to resolve these discrepancies between the TEM and the light microscopic findings concerning the three-dimensional structure of the Ito cells, scanning electron microscopy (SEM) of these cells is quite naturally required. However, there is great difficulty in viewing the entire shapes of these cells by SEM. By routine specimen preparation procedures, the hepatocytes and the sinusoidal endothelium are rarely separated from each other. Moreover, the Ito cells are covered with reticular fibers in the Disse's space.

Several investigators have attempted to remove the cellular and intercellular elements obscuring the objects of interest in various tissues, utilizing treatment with chemical agents and enzymes; e.g., HCl, NaOH, KOH, NaClO, collagenase and trypsin (Evan et al., 1976; Fujwara and Uehara, 1980; Miller et al., 1982; Shimada et al., 1983). Aoki et al. (1985a) treated the liver with 6N HCl in order to remove collagen in the Disse's space, and then cracked the specimen after critical-point drying. They were able to discern almost the entire shape of a single Ito cell. However, their method could not concurrently expose several Ito cells along the course of the sinusoidal capillary.

The present paper introduces an improved method to simultaneously expose numerous Ito cells with consistency and minimal artifacts.

MATERIALS AND METHODS

Adult Wistar rats of both sexes weighing 180-210 g were examined in this study. The rats were anesthetized with sodium pentobarbital and perfused through the ascending aorta with Locke's solution followed by 2.0% glutaraldehyde in 0.1 M phosphate buffer,
Fig. 1. Legend on the opposite page.
pH 7.3. The liver was excised and cut into small cubes, about 2 mm on each side, and immersed in the same fixative for 3 hr or more at room temperature. The tissue pieces were rinsed in phosphate buffer (pH 7.3) and placed in 6N NaOH for 20–30 min at 60°C. After the NaOH maceration, the tissue was thoroughly rinsed in the phosphate buffer and conductive-stained by the tannin-osmium method by Murakami (1974). Throughout the staining procedure, the phosphate buffer was used in place of distilled water which was written in the original method. The osmicated tissue blocks were dehydrated through a graded series of ethanol, transferred to isoamyl acetate and critical-point-dried using liquid CO₂. After the critical-point drying, the tissue pieces were lightly pricked with a thin needle and fractured into several fragments. The factured surface of the specimen was evaporation-coated with gold-palladium and examined in a Hitachi HFS-2 SEM at an acceleration voltage of 10 kV.

RESULTS

The rat liver treated with NaOH was, after being dried, favorably fractured along the boundaries of the hepatocytes and along the Disse's spaces, thus exposing the intercellular surfaces of the hepatocytes or the outer aspects of the sinusoidal capillary (Fig. 1). Reticular fibers in the hepatic lobules were found to be completely removed by the treatment with NaOH. These maceration effects enabled us to observe by SEM the entire shapes of the Ito cells surrounding the sinusoidal capillary.

The Ito cells occurred all over the hepatic lobules at regular intervals of 30–55 μm. The cells were located in the perisinusoidal spaces or in the parasinusoidal spaces (Motta et al., 1978) (Fig. 2a, b). The latter were deep recesses between adjacent hepatocytes continuous to the former. The surfaces of the hepatocytes confronting both of these spaces were densely covered with microvilli. The Ito cells issued many branching processes, which surrounded the sinusoid along its whole extent (Fig. 1).

The Ito cells possessed a round and flattened perikaryon. From its margin radiated 8–10 cell processes, also flattened in shape (Fig. 1, 2a, b). Some of these processes, here called major processes, were longer and thicker than others, accordingly called minor processes. The major processes arising from each cell varied in number from one to four, and measured 2–3 μm in width at the proximal portion, becoming thinner to the distal portion. They were extended longitudinally along the sinusoid for lengths of 15–20 μm, and occasionally bifurcated at a fork of the sinusoid. The major processes issued many secondary branches bilaterally and at right angles, these measuring 0.5–1.0 μm in width. Each of these side branches pursued a semicircular course along the sinusoidal wall and, together with its counterpart extending on the opposite side, surrounded almost the entire circumference of the sinusoid. The outlines of the side branches were double serrated as they extended short tertiary processes of 0.5 μm width, which bore thorn-like processes of the fourth order of various lengths. The minor processes issuing directly from the cell body corresponded to the side or secondary branches of the major processes in their manner of surrounding the sinusoid circularly and in their double serrated contours (Fig. 2c).

The side branches of the major processes, and the minor processes themselves were disposed at regular intervals, only rarely crossing or overlapping with each other (Fig. 1, 2a, b). Some of the processes terminated upon contact with those of neighboring Ito cells, while others terminated in free ends. There was no interdigitation between the processes from different cells. The cell processes sometimes passed through the
SEM Observation of Ito Cells in Rat Liver

plate of hepatocytes via the parasinusoidal spaces and enclosed the next sinusoid (Fig. 1).

The surface of the body and processes of the Ito cells showed sparse, short microvilli on their aspect facing the hepatocytes (Fig. 1, 2a, b). Small rounded elevations, which probably corresponded to lipid droplets, were occasionally encountered on the perikarya or on the major processes (Fig. 3). Such cytoplasmic elevations often emitted an excessive amount of secondary electrons and were recognized as bright spots.

The Kupffer cells or their parts were often found protruding into the Disse's space in the present study (Fig. 2a). They were readily distinguishable from the Ito cells, as their cell processes branched less frequently than those of the latter cells and as their surfaces were densely covered with microprojections of various size and shape. The Kupffer cells usually interposed between the sinusoidal endothelium and the processes of the Ito cells, which were often infolded by microprojections of the Kupffer cells (Fig. 2c).

The fine structures of the cell surfaces in the present specimens were well preserved even after the rigorous treatment with NaOH, although treatment for a longer period caused erosion of the cell surfaces and artificial gaps between the hepatocytes. The sinusoidal endothelium showed numerous small fenestrations arranged in clusters (sieve plates), and large oval gaps which occurred only sparsely (Fig. 1, 2c). The surfaces of the hepatocytes facing adjacent hepatocytes were rather smooth, while those facing the Disse's space were covered with microvilli (Fig. 1). Bile capillaries were about 0.5 μm in width, and provided with microvilli.

DISCUSSION

Although SEM observation of the Ito cells has been reported by some researchers (Fujita et al., 1981; Aoki et al., 1985a, b; Shibasaki, 1985), few satisfactory results have been obtained, due to their complex cell shape and their being surrounded by other cellular and non-cellular elements. The use of 6N NaOH in the present study facilitated the separation between hepatocytes and between hepatocytes and the sinusoidal wall; furthermore, this treatment completely removed reticular fibers. We could thus succeed in exposing intact Ito cells with their long elaborate processes which pursued tortuous courses in the hepatic lobule.

The present SEM observations corroborate the evenness in intralobular distribution of the Ito cells, and the perisinusoidal and interhepatocytic localization of these cells, two points which were demonstrated light microscopically by Ito and Nemoto (1952).

Furthermore, SEM reveals that the sinusoid is surrounded by the circular processes of the Ito cells almost completely along its whole length, while previous TEM observations only occasionally demonstrated such processes. This finding by SEM supports the assumption by Tanuma and Ito (1978) that the subendothelial processes of the Ito cells may reinforce the sinusoidal wall. The present study provides no information with regard to the possible contractility of the Ito cell processes, a proposition by Tanuma and Ito (1978).

The occurrence of the processes of the Ito cells appears to favor the uptake of vitamin A from the blood. The cell processes are located immediately beneath the sinusoidal endothelium with numerous fenestrations. In addition, their branches are extended without overlapping each other, keeping their entire surface area in contact with the endothelium.
Fig. 2. Legend on the opposite page.
SEM Observation of Ito Cells in Rat Liver

FuJITA et al. (1986) examined the liver of the cod, a teleostean fish, and found three-dimensional networks composed of Ito cells which anastomosed with each other by their processes. They assumed that this network of Ito cells served as a mechanical support of the tissue. The Ito cells in the rat liver appear to play a less important role as a tissue framework than those in the fish, as their processes are thin and often terminate in free ends. It is probable that the well-developed networks of reticular fibers, which are only poorly developed in the fish, support the liver in mammals.

The "Sternzellen" described by von Kupffer (1876) and Wake (1971) issued less numerous cell processes than the Ito cells observed by SEM, probably because only the proximal trunks of their major processes were stained with the gold impregnation method by von Kupffer. These investigators pointed out that some of the processes of the Ito cells were extended into the spaces between neighboring hepatocytes and that these processes occasionally arrived at the lumen of the bile capillary. In the present study, however, the intercellular processes of the Ito cells were confined to the para-sinusoidal spaces, which never come into continuity with the bile capillary under the physiological conditions as reported by Motta et al. (1978).

The present SEM study strongly suggests that the "Perizyten" of Zimmermann (1923) correspond to the Ito cells, because these cells appear closely similar to each other

Fig. 2. a. An Ito cell with one major process situated in a para-sinusoidal space. Arrows: extrasinusoidal Kupffer cells. ×2,700 b. An Ito cell in a perisinusoidal space extending two major processes in opposite directions. ×2,500. c. High magnification of a sinusoidal wall. A Kupffer cell (red) interposes between the endothelium and processes of an Ito cell (yellow). Arrow: a microprojection of the Kupffer cell infolding an Ito cell process. P double serrated contour of an Ito cell process. ×10,000

Fig. 3. Round elevations on surfaces of Ito cells. a. Two hemispheric projections on a perikaryon of an Ito cell are recognized as bright spots. b. An Ito cell shows similar round bright spots on the trunk of a major process. a, b: ×5,200
in their perisinusoidal localization, in their flattened shape and in the branching pattern of their processes. ZIMMERMANN successfully revealed the tertiary processes of his cells, though he was unable to see the processes of the fourth order or thorns which the present SEM observation disclosed.

The SEM exhibited that the Kupffer cells occur in the Disse's space more frequently than expected, even under the physiological conditions. The intimate association of the Kupffer cells with the Ito cells may suggest a functional relationship between these cells. WAKE (1971) assumed that vitamin A might be delivered by the Kupffer cells to the Ito cells.

The NaOH maceration method appears to cause very little damage to the specimen. The findings obtained by the present method coincide with those obtained by the freeze-cracking method (MUOTO, 1975; MOTTA et al., 1978; FUJITA et al., 1981), especially concerning the surface morphology of the hepatocytes and the sinusoidal fenestrations. It is thus reasonable to assume that the fine surface structures of the Ito cells demonstrated in the present study well reflect the actual images of the cells.

The NaOH maceration method is highly useful for SEM observation of the Ito cells in the liver, and is expected to serve for the elucidation of their normal and pathological structures as well as their relations to Kupffer and other kinds of cells.

Acknowledgement. The authors wish to thank Mr. K. ADACHI for his technical assistance in operating the SEM.

REFERENCES

Aoki, T., K. Taira and S. Shibasaki: Scanning electron microscope observations on Ito's (fat-storing) cells in rats. I, II (Japanese abstract). Acta anat. nippon. 60:138, 403 (1985a, b).

Bronfenmajer, S., F. Schaffner and H. Popper: Fat-storing cells (lipocytes) in human liver. Arch. Pathol. 82: 447–453 (1966).

Evan, A. P., W. G. Dail, D. Damrose and C. Palmer: Scanning electron microscopy of cell surfaces following removal of extracellular material. Anat. Rec. 185: 433–446 (1976).

Fujita, H., H. Tatsumi, T. Ban and S. Tamura: Fine-structural characteristics of the liver of the cod (Gadus morhua macrocephalus), with special regard to the concept of a hepatoskeletal system formed by Ito cells. Cell Tiss. Res. 244: 63–67 (1986).

Fujita, T., K. Tanaka and J. Tokunaga: SEM atlas of cells and tissues. Igaku-Shoin, Ltd., Tokyo, 1981 (cf. p. 150–157).

Fujiwara, T. and Y. Uehara: Scanning electron microscopy of myenteric plexus: A preliminary communication. J. Electron Microsc. 29: 397–400 (1980).

Gemmell, R. T. and T. Heath: Fine structure of sinusoids and portal capillaries in the liver of the adult sheep and the newborn lamb. Anat. Rec. 172: 57–70 (1972).

Hirosawa, K. and E. Yamada: The localization of the vitamin A in the mouse liver as revealed by electron microscope radioautography. J. Electron Microsc. 22: 337–346 (1973).

Ito, T.: Cytological studies on stellate cells of Kupffer and fat storing cells in the capillary wall of the human liver (Japanese abstract). Acta anat. nippon. 26: 42 (1951).

Ito, T. and M. Nemoto: Über die Kupfferschen Sternzellen und die “Fettspiecherungszellen” (“fat-storing cells”) in der Blutkapillarenwand der menschlichen Leber. Okajima’s Fol. anat. jap. 24: 243–258 (1952).

Ito, T. and S. Shibasaki: Electron microscopic study on the hepatic sinusoidal wall and the fat-storing cells in the normal human liver. Arch. histol. jap. 29: 137–192 (1968).

Kawanami, O.: Electron microscopic study of mammalian liver with periodic acid methenamine
silver stain—Basement membrane structure and fibrogenesis in space of Disse. Acta pathol. jap. 23: 717-738 (1973).

Kobayashi, K. and Y. Takahashi: Effect of the administration of large doses of vitamin A on the fine structure of rat liver with special reference to changes in the fat-storing cell. Arch. histol. jap. 33: 421-443 (1971).

Kupffer, C. von: Über Sternzellen der Leber. Briefliche Mittheilung an Prof. Waldeyer. Arch. mikrosk. Anat. 12: 353-358 (1876).

McGee, J. O'D. and R. S. Patrick: The role of perisinusoidal cells in hepatic fibrogenesis. An electron microscopic study of acute carbon tetrachloride liver injury. Lab. Invest. 26: 429-440 (1972).

Miller, B. G., R. I. Woods, H. G. Bohlen and A. P. Evan: A new morphological procedure of viewing microvessels: A scanning electron microscopic study of the vasculature of small intestine. Anat. Rec. 203: 493-503 (1982).

Motta, P., M. Muto and T. Fujita: The liver. An atlas of scanning electron microscopy. Igakushoin, Ltd., Tokyo, 1978 (cf. p. 30-49, 100-107).

Murakami, T.: A revised tannin-osmium method for non-coated scanning electron microscope specimens. Arch. histol. jap. 36: 189-193 (1974).

Muto, M.: A scanning electron microscopic study on endothelial cells and Kupffer cells in rat liver sinusoids. Arch. histol. jap. 37: 369-386 (1975).

Nakane, P. K.: Ito’s “fat-storing cell” of the mouse liver. Anat. Rec. 145: 265-286 (1963).

Schnack, H., L. Stockinger and F. Wewalka: Die Bindegewebszellen des Disseschen Raumes in der menschlichen Leber bei Normalfällen und pathologischen Zuständen. Wien. klin. Wochenschr. 78: 715-724 (1966).

Shibasaki, S.: Scanning electron microscope observations on the Ito cell, with special remarks to the subendothelial process (Abstract). J. clin. Electron Microsc. 18: 724 (1985).

Shimada, T., M. Nakamura, Y. Kitahara and M. Sachi: Surface morphology of chemically-digested Purkinje fibers of the goat heart. J. Electron Microsc. 32: 187-196 (1983).

Tanikawa, K.: Ultrastructure of hepatic fibrosis and fat-storing cells. In: (ed. by) H. Popper and K. Becker: Collagen metabolism in the liver. Stratton Intercontinental Medical Book Corp, New York, 1975 (p. 93-99).

Tanuma, Y. and T. Ito: Electron microscope study on the hepatic sinusoidal wall and fat-storing cells in the bat. Arch. histol. jap. 41: 1-39 (1978).

Wake, K.: “Sternzellen” in the liver: Perisinusoidal cells with special reference to storage of vitamin A. Amer. J. Anat. 132: 429-463 (1971).

Yamagishi, M.: Electron microscope studies on the fine structure of the sinusoidal wall and fat-storing cells of rabbit livers. (Japanese text with English abst.) Arch. histol. jap. 18: 223-261 (1959).

Zimmermann, K. W.: Der feinere Bau der Blutcapillaren. Z. Anat. Entw.-Gesch. 68: 29-109 (1923).

岩永ひろみ
〒951 新潟市旭町通1
新潟大学医学部
第三解剖学教室

Dr. Hiromi TAKAHASHI-IWANAGA
Department of Anatomy
Niigata University School of Medicine
Asahimachi, Niigata
951 Japan