Experimental Study on the Fine Structure of Chicken Liver Parenchyme with Special References to Extrasinusoidal Macrophages and Sinusoidal Blood Cells. Part 2. Sinusoidal Blood Cells in Normal and India Ink Perfused Livers

Masako OHATA and Toshio ITO

Department of Anatomy (Prof. K. UCHIDA and Prof. T. ITO), Teikyo University School of Medicine, Tokyo, Japan

Received February 3, 1986

Summary. Leucocytes and thrombocytes in the chicken liver sinusoids were observed under normal conditions and after intravenous India ink perfusion. The monocytes exhibited conspicuous phagocytic activity. At 30 min or earlier and 4 hr after the perfusion, they ingested considerable amounts of the carbon particles, which were deposited in small vacuoles and/or lysosomes. In this study we revealed two transitional forms of the monocyte changing into the Kupffer cell. In one transitional form, which already at 15 min after the perfusion stored considerable amounts of the particles, the ectoplasmic layer was partly differentiated and projected many pseudopodia into the sinusoid. At 48 hr after the perfusion, the other transitional form was attached by its wide basal surface to the endothelial lining and projected well-developed pseudopodia into the sinusoid like the Kupffer cell without, however, storing the carbon particles. These findings are thought to suggest the transformation of the monocytes into the Kupffer cells. Thus we came to the assumption that the Kupffer cells might be replenished: 1) by self-proliferation; 2) by the macrophages from the hepatic parenchyme into the sinusoid; or 3) by transformation from the monocytes circulating into the sinusoid (the “triple origin” as opposed to the “dual origin” of the Kupffer cell).

In the earliest stage after India ink perfusion, the thrombocytes exhibited the most striking reaction comparable to the Kupffer cells toward which they were assembled. The India ink particles were taken up into the “surface connected canicular system” (SCS), which thickened and made vacuolar expansions as the amount of the particles was increased. At 4 hr after perfusion, the particles disappeared from the majority of the thrombocytes, leaving an empty SCS. The India ink particle uptake and storage by the thrombocyte were thought to be temporary phenomena, different from the true phagocytosis of the macrophages.

In our previous paper we studied the sinusoidal cells and extrasinusoidal macrophages scattered in the parenchyme and in the lymphoid tissue of both normal and India ink perfused chicken livers, seeking to broaden knowledge on their participation in the renewal and replenishment of the Kupffer cells in the sinusoid. The role of sinusoidal
blood cells, especially monocytes, which is an important topic when attempting a thorough criticism of van Furth's theory of the "mononuclear phagocyte system," has been essentially left untouched. In this study, therefore, we observed monocytes together with other leucocytes and thrombocytes in chicken liver sinusoids under normal conditions and after intravenous India ink perfusion with the aim of elucidating the relationship between monocytes and Kupffer cells.

MATERIALS AND METHODS

Electron microscope preparations of livers from the chickens (male adult white Leghorn) prepared for the previous study were used. For the strict distinction of monocytes from Kupffer cells as well as from transitional forms between these cells, the cytochemical demonstration of the endogenous peroxidase activity was deemed essential, and we followed the procedures proposed by Deimann (1984) and Wisse (1974a, b). However, these methods devised for rodent cells have yet to exhibit peroxidase activities in cells of chicken livers in spite of positive reactions in the nuclear envelope and cisterns of rough endoplasmic reticulum (RER) in Kupffer cells of the rat, simultaneously used as the control. We are presently continuing cytochemical examinations for the purpose of preparing a suitable method for cells of the chicken liver.

To examine phagocytic activities of the sinusoidal leucocytes and thrombocytes, a large dose of India ink was intravenously perfused, and the livers were fixed in the same way as in the previous investigation at 15, 30 min, 1, 4 and 48 hr (2 days) after perfusion. Evidence that these intravenous applications of a large dose of India ink might also stimulate the RES has been presented in the previous paper.

RESULTS

1. Leucocytes and thrombocytes in normal chicken liver

In the normal chicken liver, heterophil leucocytes (Fig. 1a) were most frequently encountered in the sinusoid. They contained numerous electron dense specific granules of variable shapes and sizes, among which spindle-shaped ones with pointed ends or rod-shaped ones predominated. Some of them contained electron dense spots. Large masses of heterochromatin were seen beneath the nuclear membrane.

Eosinophils were rarely found in the sinusoid. Two lobes of the nucleus were usually found to be eccentric and rich in heterochromatin beneath the nuclear membrane (Fig. 1b). Electron dense specific granules varied considerably in size.

Basophils were rare leucocytes in the sinusoid. The specific granules were variable in number and shape from cell to cell. Granules of the highest electron density were generally small, and between the granules and the limiting membranes there was either a wide or narrow empty halo, as if showing the appearance of dense granules contained in a vacuole. Less electron dense granules presented a densely packed fibrillar structure, and arrangements of electron dense fibrils varied from one granule to another (Fig. 1c). In the case of nearly parallel arrangement, the granules appeared as finely striped; in the case of irregular arrangement, mottled or webbed appearances were observed. The granules of a fibrillar structure were partly or entirely vacuolated because of the lowering electron density of the fibrils.

Lymphocytes were classified roughly into large and small types, both projecting
lobopodia and pseudopodia (Fig. 1d, e). Heterochromatin masses along the nuclear membrane were conspicuous. The large lymphocytes comparable in size to monocytes contained a considerable number of round or oval mitochondria, but few small lysosomes.

Monocytes (Fig. 2a) were of about the same size as large lymphocytes. They contained an indented or kidney-shaped eccentric nucleus. Heterochromatin masses were
smaller and arranged at long intervals along the nuclear membrane. In the deeper indentations of the nucleus there was a Golgi complex. Several round, oval and rod-shaped mitochondria were distributed in the cytoplasm. The monocytes were characterized by a well-developed RER; its long tortuous cisterns extended along the nuclear and plasma membrane, while shorter ones were distributed at random. They were rich in small round or elongated lysosomes of moderate electron density which were distributed throughout the cytoplasm either solitarily or forming small, loose clusters. On the cell surface, finger-shaped pseudopodia and lobopodia were generally scarce. Monocytes were rather often encountered in the chicken liver sinusoid.

Thrombocytes (Fig. 2b, c) were small nucleated spindle-shaped cells. In contrast to the description by Maxwell and Trejo (1970), they projected neither finger-shaped pseudopodia nor lobopodia in the hepatic sinusoid from the cell surface. The oval nucleus with large masses of heterochromatin distributed on the nuclear membrane. In addition to the small round mitochondria and cisterns of RER in the cytoplasm, larger vacuoles and small vesicles are conspicuous. In b some of the small vesicles are attached on the plasma membrane or open into the sinusoid. EC endothelial cell, PS Disse's space. Normal condition. a-c: ×8,000

**Fig. 2.** a. Monocyte with a kidney-shaped nucleus. The Golgi complex (arrowhead) is present opposite to the deeper indentation of the nucleus. Along the nuclear membrane, many long tortuous cisterns of RER, many small lysosomes of low electron density and sparse mitochondria (M) are distributed. E erythrocyte, EL endothelial lining. b. and c. Thrombocytes (TH) in the sinusoid (SN). Thrombocytes in b and c possess an oval nucleus rich in large masses of heterochromatin distributed on the nuclear membrane. In addition to the small round mitochondria and cisterns of RER in the cytoplasm, larger vacuoles and small vesicles are conspicuous. In b some of the small vesicles are attached on the plasma membrane or open into the sinusoid.
2. Reactions of sinusoidal leucocytes and thrombocytes after intravenous India ink perfusion

At the earliest stage after India ink perfusion, the most conspicuous changes found in the hepatic sinusoid were those in the thrombocytes, aside from the early endocytic activity of the Kupffer cells. It is possible that thrombocytes are temporarily assembled in the neighborhood of the Kupffer cells in the sinusoid to take up India ink particles. At 30 min or earlier after perfusion, the particles were observed closely crowded around the thrombocytes (Fig. 3a), and some of them began to enter cells with a network of...
particle-containing canalicules (Fig. 3b). Vacuolar expansions filled with particles along the canalicular network then appeared (Fig. 3c, d); they were enlarged with the increase of their contents (Fig. 3e).

At 4 hr after perfusion, the particles had already disappeared from the majority of thrombocytes, leaving behind empty vacuoles (vesicles) (Fig. 3f). At 48 hr the thrombocytes returned to their normal cytological state.

Fig. 4. Legend on the opposite page.
Monocytes exhibited conspicuous though unstable, endocytic activity against the India ink particles. At 30 min or earlier after India ink perfusion, monocytes endocytosed the particles into small vacuoles and lysosomes (Fig. 4a). At 4 hr the particles were deposited in vacuoles and/or lysosomes in monocytes (Fig. 4b, c). At this stage, elongated, curved, or tortuous cisterns of RER became prominent.

The monocyte shown in Figure 4d seemed to be especially worthy of attention, as a homogeneous ectoplasmic layer was partly differentiated in this cell, as is the case in Kupffer cells; the cell protruded many pseudopodia of variable shapes and sizes, one of which was attached to the endothelial lining. In the endoplasm, well-developed tortuous cisterns of RER were revealed. Furthermore, a considerable amount of the particles was stored in vacuoles and probably also in lysosomes at 15 min after perfusion. On the basis of the above findings, this monocyte must be considered to represent an early stage of the monocyte's transformation into a Kupffer cell. In addition, at 48 hr after the India ink perfusion, another transitional form from monocyte to Kupffer cell was detected close to a Kupffer cell containing a large vacuole filled with India ink particles (Fig. 4e). Although this transitional form exhibited cytological features suggestive of a monocytic origin, it projected numerous well-developed and complicated pseudopodia into the sinusoid and was attached by a wide basal surface to the endothelial lining. It did not, however, ingest any India ink particles, probably because they would be unlikely to circulate into the sinusoid at this late stage.

Heterophils exhibited no conspicuous signs of India ink uptake. At 30 min, signs of possible particle uptake were rarely demonstrated (Fig. 5a). A reliable image of particle uptake was obtained at 4 hr (Fig. 5b).

Basophils, though more frequently found, showed no endocytic activity against the India ink particles. At 30 min after perfusion, the majority of their basophilic granules turned small, highly electron dense and compact (Fig. 5c), while at 4 hr, the normal granules were gradually increased (Fig. 5d) in proportion with the decrease in small, dense ones; at 48 hr the basophilic granules returned to their normal state.

Lymphocytes showed no distinct morphological signs of an endocytic activity against the India ink particles. The following reactions were occasionally observed after the India ink perfusion: at 30 min, the particles were occasionally accumulated...
on the surface of a lymphocyte, and smooth-surfaced curved tubles were seen radiating from the nuclear surface toward the cell membrane (Fig. 5e).

DISCUSSION

The ultrastructure of leucocytes in the chicken liver has been described in detail by Dhingra et al. (1969), while Maxwell and Trejo (1970) observed ultrathin sections of
leucocytes and thrombocytes collected from a chicken blood-anticoagulant mixture by centrifugation. With reference to their descriptions, we have observed blood cells in the chicken liver sinusoids. As DHINGRA et al. (1969) described, the heterophils were the most numerous granular leucocytes in the chicken hepatic sinusoids. These cells exhibited only a weak endocytic activity. Wisse (1974b) observing at 3 hr after Thorotrast injection, noted degranulated neutrophils in the rat liver sinusoid, and confirmed their endocytosis against Thorotrast particles.

Relatively numerous monocytes were detected in the chicken liver sinusoid. Although a demonstration of the endogeneous peroxidase activity turned out unsuccessful in the chicken liver in the present study, we were able to clearly distinguish between Kupffer cells and monocytes by their ultrastructural characteristics. Monocytes found in chicken liver sinusoids were characterized by their eccentric, kidney-shaped nucleus with relatively small and scanty heterochromatin masses disposed along the nuclear membrane at wide intervals, by well-developed long curved cisterns of the RER mainly oriented both along the nuclear and cytoplasmic membrane, by relatively numerous small lysosomes, and by several oval mitochondria. The cytoplasm of the monocytes generally appeared somewhat muddy, lacking a well-defined homogeneous ectoplasmic layer.

After the perfusion of India ink, monocytes evidenced the most conspicuous, though unstable, endocytic activities. At 30 min and 4 hr after perfusion, the particles were endocytosed in fairly large amounts and deposited in small vacuoles and/or lysosomes. DAIMON and UCHIDA (1978) have revealed in their in vitro experiment that the monocyte phagocytosed ferritin particles, forming a densely packed aggregate in the phagocytic vacuole. As a noteworthy finding in the present study, we have demonstrated transitional forms from monocyte into Kupffer cell. In the case of the transitional monocyte observed at 15 min, an ectoplasmic layer, as in the Kupffer cell, was partly differentiated, projecting pseudopodia and having stored a considerable amount of the carbon particles in vacuoles and, probably, in lysosomes. Another transitional monocyte observed at 48 hr projected numerous, complicated pseudopodia into the sinusoid, being attached by a wide basal surface to the endothelial lining, although this cell retained the ultrastructural features of a monocyte. This transitional form stored no particles, most probably because in later stages of India ink perfusion, the particles to be endocytosed are already absent from the sinusoid. In his experimental study on Kupffer cell reactions under various conditions including Thorotrast stimulation, Wisse (1974b) has asserted the absence of transitional stages between monocytes and Kupffer cells in rat liver sinusoids. The attachment of the monocytes to the sinusoidal wall was thought to be important, because it has been evidenced by the experimental studies by BODEL et al. (1977, 1978) and BEELEN et al. (1978) that the localization of peroxidase activity of the cultured monocytes and peritoneal exudate macrophages of man and rodents changed in agreement with that of the Kupffer cell after they adhered to the wall of the culture dish.

The nucleated thrombocytes in chicken liver sinusoids showed a strong reaction to the intravenously introduced India ink. The ultrastructure of the chicken thrombocytes was studied by MAXWELL and TREJO (1970); as for the thrombocytes and blood platelets of submammalian species inclusive of the chicken and mammalian species, DAIMON (1980) performed a comprehensive comparative study. Furthermore, DAIMON and UCHIDA (1978, 1982) carried out electron microscopic and cytochemical studies on chicken thrombocytes collected from a blood-anticoagulant mixture by centrifugation. In the present electron microscopic observation of the chicken thrombocytes, the
existence of several larger and smaller vacuoles or vesicles in the cytoplasm was confirmed, the larger ones probably corresponding to the vacuoles containing peripherally osmiophilic dense granules described by Maxwell and Trejo (1970). Osmiophilic granules were cytochemically demonstrated by Daimon and Uchida (1982) to contain monoamine (5-hydroxytryptamine or serotonin), whereas the numerous small vacuoles, some of which opened into sinusoid or attached to the plasma membrane, were referred to as a "surface connected canalicular system" (SCS) observed on section planes of the electron microscopic preparation. This SCS has been vividly visualized by means of the ruthenium red staining method as proved by Daimon and Uchida (1978). It has been proven that between the monoamine-containing vacuoles and the SCS there are no connections, and that the two organelles are distinctly different from one another (Daimon and Uchida, 1982). Takahashi and Daimon (1979) demonstrated by in vitro experiments that platelets of the rabbit and rat, and thrombocytes of the chicken, frog and carp phagocytosed large latex particles (1 μm in diameter) by means of a mechanism called "sinking" and not by means of pseudopodia (Wissee, 1977). In contrast, similar in vitro experiments had ferritin particles taken up by the SCS (Daimon and Uchida, 1978). Wissee (1974), however, reported that intravenously perfused Thorotrast particles were never phagocytosed by blood platelets in rat hepatic sinusoids.

In the earliest stage after the intravenous perfusion of India ink, thrombocytes and Kupffer cells in chicken hepatic sinusoid showed the most striking reactions. The former were assembled to the Kupffer cells. Wissee (1974) also showed that after a Thorotrast injection, many thrombocytes were assembled in the vicinity of the Kupffer cells in the rat hepatic sinusoid. At 30 min or earlier, India ink particles were crowded around the thrombocytes, to then be taken up into them. The particles internalized into the thrombocytes were contained in networks of canalicles, which clearly corresponded to the SCS by Daimon and Uchida (1978). Paralleling the increase of the particles filling this system, a thickening of the canalicles and enlargement of the vacuolar expansions along them are brought about in order to store larger amounts of the particles in them. The larger vacuoles containing dense, osmiophilic monoamine granules along their limiting membrane can be distinguished from the particle-containing expansions without any connection lying between (Daimon and Uchida, 1982). At 4 hr after perfusion, the carbon particles had already disappeared from the majority of the thrombocytes, leaving empty small vacuoles or vesicles derived from the SCS, which had completely released the particles. At 48 hr the thrombocytes in the chicken liver sinusoid returned to their normal state. It might subsequently be concluded that the thrombocytes found in the chicken hepatic sinusoid may possess no such genuine phagocytic activity as that of the macrophages, as proposed by Daimon and Uchida (1978). Wissee (1974b) reported that rat blood platelets in liver sinusoids showed no phagocytic activity of taking up Thorotrast particles. Nevertheless, the chicken thrombocytes are thought to be responsible for the removal of particulates or colloidal substances as they ingest them into their canalicular system for temporary storage. The cells are thus presumed to play an important role in the "clean-up" of the foreign substances in the blood.

Eosinophils, basophils and lymphocytes in the chicken hepatic sinusoid barely showed any ultrastructural reactions suggestive of phagocytic activities against the carbon particles of intravenously perfused India ink.
REFERENCES

Beelen, R. H. J., D. M. Brockhuis-Fluitsma, C. Korn and E. C. M. Hoefsmit: Identification of exudate-resident macrophages on the basis of peroxidatic activity. J. Reticuloendothel. Soc. 23: 103–110 (1978).

Bodel, P. T., B. A. Nicholes and D. F. Bainton: Appearance of peroxidase reactivity within rough endoplasmic reticulum of blood monocytes after surface adherence. J. exp. Med. 145: 264–274 (1977).

Daimon, T.: Differences in peroxidase localization of rabbit peritoneal macrophages after surface adherence. Amer. J. Pathol. 91: 107–117 (1978).

Daimon, T. and K. Uchida: Electron microscopic and cytochemical observations on the membrane systems of the chicken thrombocyte. J. Anat. 125: 11–21 (1978).

Deimann, W.: Endogeneous peroxidase activity in mononuclear phagocytes. Progr. Histochem. Cytochem. 15 (2): 1–56 (1984).

Dhingra, L. D., W. B. Parrish and W. G. Zenzke: Electron microscopy of granular leucocytes of chicken (Gallus domesticus). Amer. J. Vet. Res. 30: 637–642 (1969).

Maxwell, M. H. and F. Trejo: The ultrastructure of white blood cells and thrombocytes of the domestic fowl. Brit. Vet. J. 126: 583–592 (1970).

Takahashi, I. and T. Daimon: Phagocytosis of thrombocyte: Electron microscopic studies on the phagocytosis of latex particles in vitro (Japanese text with English abst.). Teikyo Med. J. 2: 75–83 (1979).

Wisse, E.: Observations on the fine structure and peroxidase cytochemistry of normal rat liver Kupffer cells. J. Ultrastr. Res. 46: 393–426 (1974a).

———: Kupffer cell reactions in rat liver under various conditions as observed in the electron microscope. J. Ultrastr. Res. 46: 499–520 (1974b).

———: Ultrastructure and function of Kupffer cells and other sinusoidal cells in the liver. In (ed. by) E. Wisse and D. L. Knook: Kupffer cells and other liver sinusoidal cells. Elsevier Biomedical Press, Amsterdam, 1977 (p. 33–60).