THE ANATOMICAL RECORD 290:862–874 (2007)

Uptake Behavior of Embryonic Chick Liver Cells

MIHARU KANAI,[1]* YOSHIO MURATA,[2] DAMON C. HERBERT,[3] AND TSUYOSHI SOJI[1]

[1]Department of Functional Morphology, Nagoya City University, Graduate School of Medical Sciences, Nagoya, Japan
[2]Department of Chemistry, Faculty of Science, Fukuoka University, Fukuoka, Japan
[3]Department of Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, Texas

ABSTRACT

The capacity of selective uptake by liver cells, focusing particularly on the parenchymal and perisinusoidal stellate cells during chick liver development (8–18 days of incubation), was ultrastructurally examined after injection of 240-nm-diameter lecithin (phosphatidylcholine) -coated or noncoated beads into the extraembryonic circulation. Cytoplasmic projections of both cells as well as extrasinusoidal macrophages reached into the sinusoid-like vascular spaces. The primitive perisinusoidal stellate cells were identified by immunocytochemistry as being rich in desmin-positive cytoplasmic intermediate filaments. The cells demonstrated selective uptake of noncoated beads by means of their cytoplasmic projections. These findings were significant in the early period of incubation, indicating that the phagocytic activity is a characteristic and transient phenomenon of developmental differentiation. Large numbers of coated and a few noncoated beads penetrated into the perivascular spaces. The parenchymal cells incorporated only the coated beads that passed through the endothelial lining, suggesting that these cells express selective but limited phagocytic capacity against large "foreign" substances even long before their maturation. The cell projections were not engaged in uptake function. Extrasinusoidal macrophages, Kupffer cells, and intraluminal primitive macrophages all took up both beads; however, lecithin coating of the beads clearly suppressed their uptake function. These data suggest that the uptake function of large "foreign" substances appears to be intrinsic to liver cells and lecithin coating would be useful for delivering large substances to parenchymal cells. Anat Rec, 290:862–874, 2007.

Key words: phagocytosis; development; hepatic parenchymal cells; perisinusoidal stellate cells; endothelial cells; extrasinusoidal macrophages; lecithin

In the liver, the uptake of large "foreign" substances present in the circulation has been reported, focusing almost exclusively on Kupffer cells and sinusoidal endothelial cells (Jones and Summerfield, 1982; Kirn et al., 1982; Dan and Wake, 1985; Steffan et al., 1986), but few investigators have concentrated on parenchymal (Kirn et al., 1978; Steffan et al., 1978; Soji et al., 1992; Kanai et al., 1996) or perisinusoidal stellate cells (Ito cells, fat-storing cells: Ito and Nemoto, 1952; lipocytes: Bronfenmajer et al., 1966; stellate cells: Wake, 1971). Blood-

*Correspondence to: Miharu Kanai, Department of Functional Morphology, Nagoya City University, Graduate School of Medical Sciences, Nagoya, 467-8601 Japan. Fax: 81-52-842-3210. E-mail: miharu@med.nagoya-cu.ac.jp

Received 29 May 2006; Accepted 31 March 2007
DOI 10.1002/ar.20557
Published online 31 May 2007 in Wiley InterScience (www.interscience.wiley.com).

borne "foreign" substances larger than endothelial fenestrations, which average diameter is 150 nm (Wisse, 1970), have been reported to be prevented from contact with the parenchymal and perisinusoidal stellate cells. It has been observed that many large chylomicrons, which are not "foreign" substances, are taken up by only parenchymal cells in the neonatal rat liver after the onset of suckling, and endothelial fenestrations also have a filtration function by blocking the passage of large chylomicrons (Naito and Wisse, 1978). On the other hand, egg lecithin liposomes, which are being studied as drug carriers, have recently shown to be internalized into the parenchymal cells, even ones as large as 200–400 nm (Daemen et al., 1997, Romero et al., 1999, Moghimi and Patel, 2002). The liposomes were, however, thought to reach the parenchymal regions by means of their mechanical deformation during their passage across the fenestrations (Romero et al., 1999). Using 240-nm-diameter egg lecithin-coated hard polystyrene beads, we previously described the uptake function of adult rat parenchymal cells (Kanai et al., 1996). This uptake function was shown to be facilitated by the endothelial cells, which selectively transcytosed the coated beads from the sinusoids to the space of Disse. It is, however, still unclear whether the parenchymal cells themselves are able to selectively take up large substances. With regard to phagocytosis of the perisinusoidal stellate cells, there have been no reports describing this phenomenon in vivo, however, these cells have recently been observed to take up apoptotic bodies (Canbay et al., 2003) or 1.01μm-diameter latex beads (Viñas et al., 2003) in vitro, when the cells are activated.

As described in the present study, chick embryonic hepatic endothelial cells have few fenestrations and gaps but both parenchymal and perisinusoidal stellate cells occasionally extend their cytoplasmic projections into the sinusoid-like vascular spaces, crossing the endothelial lining. There have been no reports describing the existence of parenchymal cell projections, and only a brief mention of the presence of those from the perisinusoidal stellate cells (Enzan et al., 1983, 1997). While the functional role of the projections is unknown, they resemble in appearance those of extrasinusoidal macrophages, which have been reported to engulf blood-borne "foreign" substances in the adult chicken liver (Ohata et al., 1982). We then examined the uptake behavior of the liver cells in relation to developmental changes using egg lecithin-coated or noncoated beads. The beads have a central void cavity, which makes a conspicuous hole of embedded resin in the electron-microscopic field, and by this feature we can easily avoid missing any beads when accounting for their distribution. To identify the perisinusoidal stellate cells, we used desmin immunocytochemical labeling. Desmin expression is known as a marker of perisinusoidal stellate cells in the adult rodent liver (Yokoi et al., 1984; Tsutsumi, et al., 1987; Geerts, et al., 1991). While this concept is still controversial in rodent fetuses and because it has not yet been well documented in the chicken, we shall discuss its presence in the liver of embryonic chickens.

MATERIALS AND METHODS

For the ultrastructural observations, chick embryos (White Leghorn, DEKALB) at 8, 9, 11, 14, 15, and 18 days of incubation were used, whereas in the experiments on the uptake of beads by liver cells in vivo, 8-, 14-, 15-, and 18-day-old embryos were used. The eggs were obtained from a local supplier (Rikaken Co., Ltd., Nagoya, Japan), whereas the polystyrene latex beads (240 nm in diameter) composed of a polystyrene outer layer and methacrylate inner layer with a central void cavity (140 nm in diameter) were obtained from the Japan Synthetic Rubber Co. Egg lecithin (phosphatidylcholine) -coated or noncoated beads suspended in saline (final concentration was 2 mg/ml) were prepared following a previous report (Kanai et al., 1996). The suspensions were injected into the embryos by means of the umbilical or vitelline vessels in a volume of 1 ml. Chick embryos were removed from the egg and, after decapitation, the liver samples were taken at 2, 3, 7, or 10 min after the injections. All experimental procedures were performed under the guidance of animal protocols approved by Nagoya City University.

Electron Microscopic Preparation

Liver samples were cut into 1 × 1 × 2 mm cubes and fixed for 2 hr with cold 1% OsO_4 in Millonig buffer (pH 7.4; Millonig, 1962). After dehydration by a graded ethanol series, the cubes were placed into propylene oxide and then embedded in Epon 812 (Luft, 1961). Ultrathin sections were prepared, stained with a uranyl acetate (Watson, 1958) and lead mixture (Sato, 1968), and examined using a Hitachi H-7000 electron microscope.

Quantitative analysis of the penetration of coated and noncoated beads through the endothelial lining was performed on ultrathin sections of the liver samples of 8-, 15-, and 18-day-old embryos (three embryos for each group) 10 min after injection of the beads. The numbers of beads located in the perivascular spaces were counted in approximately 20 random sampling fields on each specimen obtained. The lengths of the endothelial lining that bordered the perivascular spaces were measured with a digital curvimeter using electron-microscopic photographs (×1,000 or ×1,500) of the same sample fields. Total length of the lining from each embryo was from 3,000 μm to 5,000 μm. Relative amounts of beads in every 1,000-μm length of the endothelial lining were then estimated and represented in Figure 2 as means ± SEM. The difference among means of "coated beads" groups and that of "noncoated beads" groups were initially tested using one-factor analysis of variance. Then differences between means of different incubation ages of the "coated beads" and the "coated beads" and "noncoated beads" groups were then tested for significance using unpaired Student's *t*-test.

Immunoelectron Microscopic Preparation

Liver samples were cut into 1-mm^3 pieces, fixed for 2 hr in 4% paraformaldehyde–0.5% glutaraldehyde in 0.1 M phosphate buffer, and rinsed overnight in buffer. After dehydration by a graded ethanol series, the samples were embedded in LR White resin (London Resin Company Ltd., Reading, England) and polymerized at 50°C. Ultrathin sections were mounted on nickel grids, exposed to 0.5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) containing 15 mM sodium azide, and incubated for 3 hr in drops of anti-chicken

gizzard desmin serum (Cappel) diluted 1:50 or 1:100 in PBS containing 0.5% BSA and 15 mM sodium azide. After rinsing, the sections were exposed to protein A labeled with 10-nm colloidal gold (Sigma, St. Louis, MO), diluted 1:40 or 1:80 in the same buffer. They were then rinsed in PBS and distilled water. After a 5-min fixation with 1% glutaraldehyde, the grids were counterstained with uranyl acetate and lead citrate and examined with the electron microscope. As negative controls, the grids were incubated in the same drops but without the primary antibody. No significant immunoactivity was observed in the controls.

RESULTS

Structure of Sinusoid-Like Vascular Spaces in the Embryonic Chick Liver

At days 8 and 11 of incubation, the vascular spaces lined by an endothelial layer were irregular in shape and separated by thick, anastomizing hepatic parenchymal cords. Most of the vascular spaces were wide and were covered by one to three perivascular cell layers (Fig. 1a). Within some large parenchymal cords, very narrow vascular spaces were also observed, which were usually covered by only one perivascular cell layer. Parenchymal cells were small at day 8 of incubation and then increased in size with advancing embryonic age. At 11 and 14 days of incubation, only a few central veins were identified, showing the typical arrangement of connections with perpendicular sinusoid-like vascular spaces. Primitive branches of the portal vein were also occasionally identified, which were surrounded by mesenchymal tissue. Thereafter, hepatic cords and sinusoid-like vascular spaces gradually became more regular in size and shape, although no lobular pattern was evident and some sinusoid-like vascular spaces were still wide even at 18 days of incubation. Hematopoiesis was not observed within the parenchymal cords but was noted within the vascular spaces and the mesenchymal sheaths of the vessels.

Endothelial cells of all vascular spaces were characteristically thicker (Fig. 1a,b) as compared with the cells noted after hatching. They had few fenestrations but some gaps (or cell pores) through which the cells under the endothelial lining sometimes extended their cytoplasmic projections, which terminated into the sinusoid-like vascular spaces, as mentioned below. The lateral plates often overlapped each other. They were connected with well-developed intercellular junctions, such as desmosomes and intermediate junctions. Some portions of the lateral plates bulged into the lumen (Fig. 1b). At the base of the bulges, small vesicles were usually arranged in a sheet. Some endothelial cells were round, and the cell bodies themselves also bulged into the sinusoidal lumen (Fig. 1b). Dividing endothelial cells were often revealed throughout the entire incubation period.

Parenchymal cells were polygonal or columnar in shape, with a few microvilli extending along their vascular surfaces (Fig. 1a). Perivascular spaces between the endothelial and parenchymal cells were usually narrow, with the exception of some irregular and wide spaces that were frequently observed at day 8 of incubation. The microvilli slightly increased in number and the perivascular spaces became a little wider at the later periods of incubation (Fig. 1b). A discontinuous basement membrane invested the epithelial and parenchymal cells and amorphous material was often observed in the perivascular spaces at the early periods of incubation. Even at day 18 of incubation, a basement membrane of the parenchymal cells was occasionally seen.

In the perivascular spaces, two cell types were identified: the perisinusoidal stellate cells and extrasinusoidal macrophages (Fig. 1a,b). The former cells widely covered the endothelial cell layer by their long processes, which sometimes overlapped one another and were connected by focal contacts or small intermediate junctions. By contrast, the macrophages were unpaired and scattered. The perisinusoidal stellate cells were rich in intermediate filaments and had well-developed profiles of rough endoplasmic reticulum; both of these are characteristics of the cells in adult mammalian and avian livers. Cytoplasmic lipid inclusions, which are another characteristic of the perisinusoidal stellate cells, were not always noticeable until day 15 of incubation (Fig. 1a,b). Cilia were rarely present, and cell division was seldom observed. Extrasinusoidal macrophages extended their cytoplasmic processes in all directions, running just beneath the endothelial cells, between the perisinusoidal stellate cells and in the spaces along neighboring parenchymal cells. Their processes often reached through the endothelial lining and into the vascular spaces (Fig. 1a). The cells contained many lysosomal structures, even large phagosomes, which was similar to that seen in Kupffer cells. Some appeared to be migrating into the spaces with a major part of the cell bodies located in the spaces and short processes found within the perivascular spaces. Up until day 14 of incubation, there were a few Kupffer cells in the vascular spaces adjacent to numerous, newly formed macrophages. Thereafter, the Kupffer cells increased in number, while primitive macrophages and extrasinusoidal macrophages became less numerous.

One of the most interesting observations of the chick embryonic liver cells was that some parenchymal cells and a few perisinusoidal stellate cells extended their cytoplasmic projections or pseudopodia into the endothelial cell layer; occasionally these projections reached the vascular spaces (Fig. 1a). Close apposition of the projections and the edges of the endothelial cell pores seemed to seal the space. The projections of the parenchymal cells appeared larger than those of the perisinusoidal stellate cells. Small vesicles were never arranged at the bases of either projection, which was dissimilar to the endothelial cell bulges. The frequency with which both cells extended their projections into the vascular space was quite high between days 8 and 15 of incubation, and then decreased. By 18 days of incubation, almost all of the perisinusoidal stellate cell projections disappeared from the vascular spaces.

In Vivo Uptake of the Lecithin-Coated Beads

The lecithin-coated beads easily penetrated through the endothelial layer to the perivascular spaces and into the spaces between the lateral surfaces of the parenchymal cells (Fig. 3a,b,d,e). As shown in Figure 2, the frequency of the coated beads to penetrate was 5.4 to 14 times higher than that of noncoated beads. The beads in the perivascular or lateral spaces were often trapped

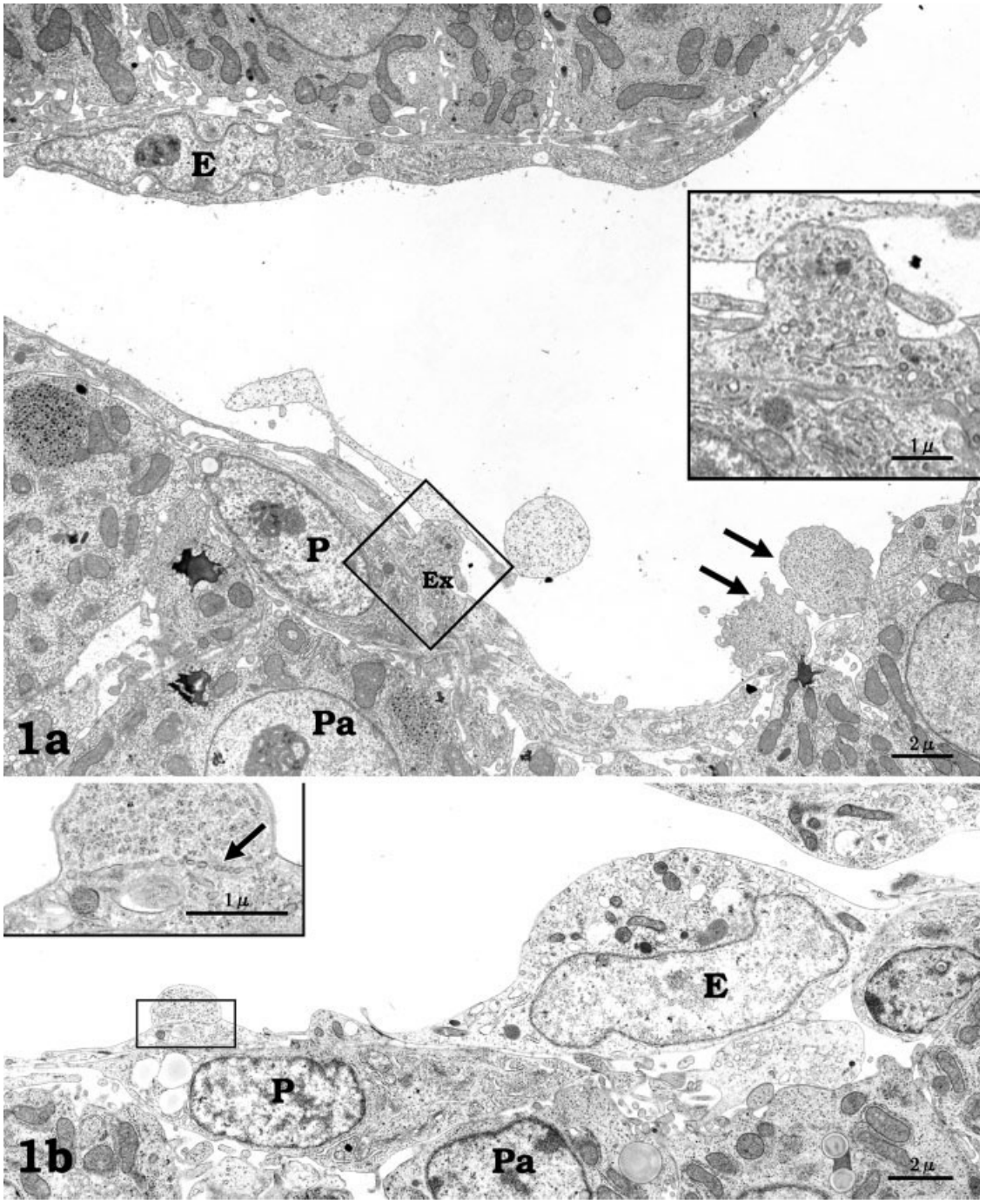

Fig. 1. **a:** Day 11 of incubation. Endothelial cells (E) are noted with no fenestrations and parenchymal cells (Pa) display a few microvilli on their vascular surface. One to three cell layers are found in the perivascular space. A perisinusoidal stellate cell (P) possesses a small lipid droplet. An extrasinusoidal macrophage (Ex) extends its cytoplasmic projection through the endothelial layer, which contains several small vesicles and lysosomes (inset). Two parenchymal cells also extend their pseudopod-like projections into the vascular lumen (arrows). Close apposition between the endothelium and all three projections seems to seal the lumen. **b:** Day 18 of incubation. An endothelial cell (E) has a large, bulging cell body, while others display an enlarged portion of the lateral plate with small vesicles arranged in a sheet (inset, arrow). A perisinusoidal stellate cell (P) has three large lipid droplets in its cytoplasm. Pa, a parenchymal cell.

by the parenchymal cells, particularly through the roots of the cell projections (Fig. 3d,e). Small pores were sometimes recognized between the projections and the endothelial lateral plates, which was in marked contrast to the controls and the group injected with the noncoated beads.

The beads appeared to be incorporated into the parenchymal cell cytoplasm. Neither pseudopodia nor cla-

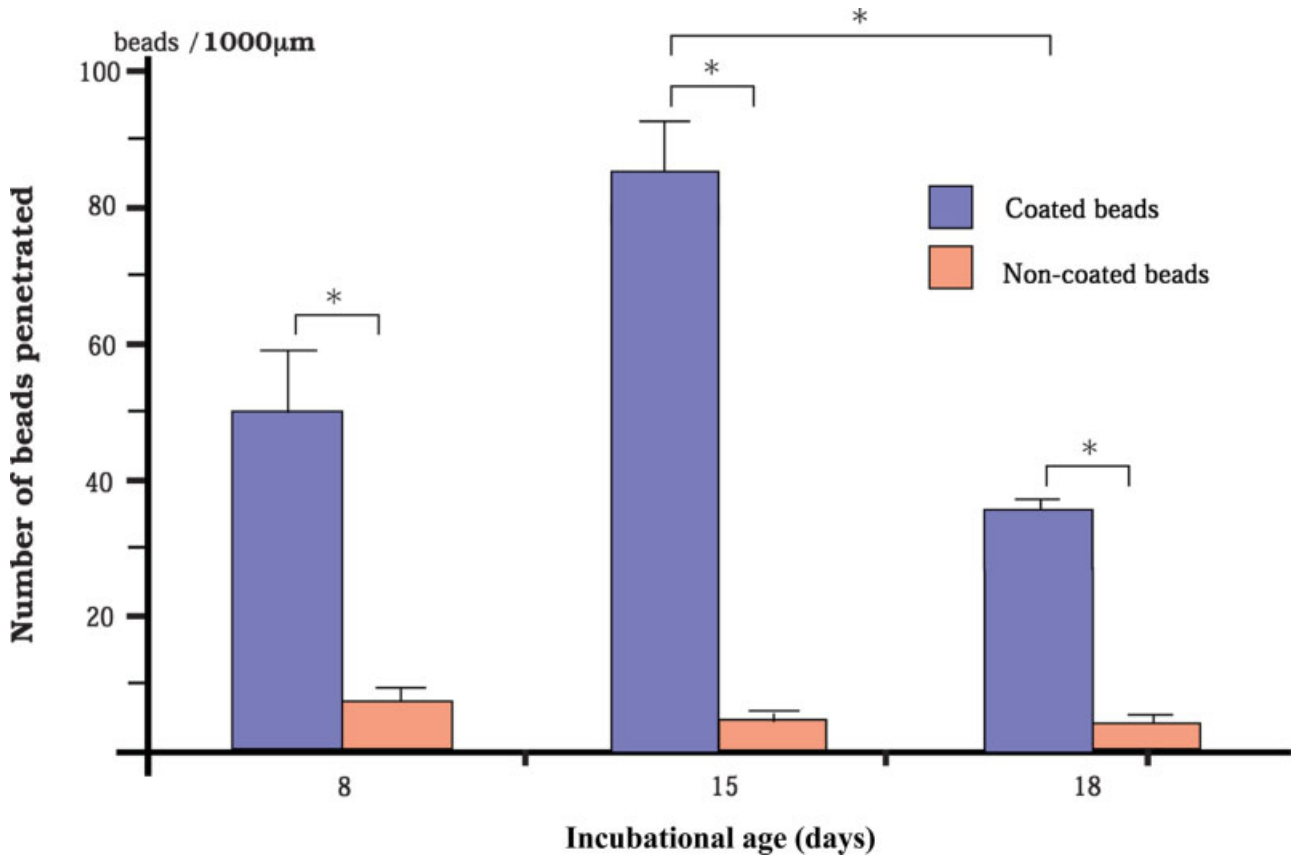

Fig. 2. Relative amounts of coated beads and noncoated beads located in the perivascular spaces every 1,000 μm length of endothelial lining that bordered the spaces. Data were taken on ultrathin sections of the embryonic chick liver of different incubational ages. Values are ± SEM. Asterisks, $P < 0.01$, Student's *t*-test. One-way analysis of variance analysis had a significant difference among the means of "coated beads" groups ($P < 0.05$) but not among that of "noncoated beads" groups ($P > 0.05$).

thrin-coated invaginations were observed to be involved in the incorporation; however, very small clathrin-coated vesicles sometimes connected with the invaginations where the beads were trapped. Phagosomes containing the beads were also observed close to the vascular and lateral surfaces of the cells (Fig. 3e,f). On the other hand, uptake of the beads by the perisinusoidal stellate cells was not found. In the vascular spaces, no uptake was noted by the projections of the parenchymal or perisinusoidal stellate cells, while the projections of the extrasinusoidal macrophages incorporated the beads (Fig. 3c). Kupffer cells, small numbers of intravascular primitive macrophages and endothelial cells also took up the beads (Fig. 3c,e). However, the frequency of bead incorporation by those cells was lower than that of the noncoated beads. Clathrin-coated invaginations in the endothelial cells were involved in almost all the bead incorporation but only occasionally by the Kupffer cells and the extrasinusoidal macrophages, and rarely by the primitive macrophages.

In Vivo Uptake of the Noncoated Beads

The noncoated beads were predominantly taken up from the vascular spaces by Kupffer cells, intravascular primitive macrophages, and extrasinusoidal macrophages (Fig. 4a,d). Endothelial cells also took up the beads (Fig. 4b). The perisinusoidal stellate cells were dramatically activated by the injection of the beads, displaying long cytoplasmic processes that extended into the vascular spaces, and engulfed the beads (Fig. 4c–e). These cells resembled the extrasinusoidal macrophages to the extent that it was difficult to distinguish one from the other. The perisinusoidal stellate cells often shared the trapped beads with other types of the cells, that is, endothelial cells, Kupffer cells, or primitive macrophages (Fig. 4c,d). Penetration of the noncoated beads into the perivascular spaces was much less than that of the coated beads (Fig. 2). While coated beads were randomly scattered in the perivascular spaces, noncoated beads usually clustered in the spaces and endothelial cell gaps were sometimes noted near the noncoated beads. These observations suggested that they penetrated through the gaps. Some penetrated beads were taken up by the perisinusoidal stellate cells (Fig. 4f) or extrasinusoidal macrophages but none were incorporated into the parenchymal cells. The frequency of uptake by perisinusoidal stellate cells from both the vascular lumen and perivascular spaces was higher at day 8 of incubation than days 14 and 15, and the uptake was rarely observed at day 18.

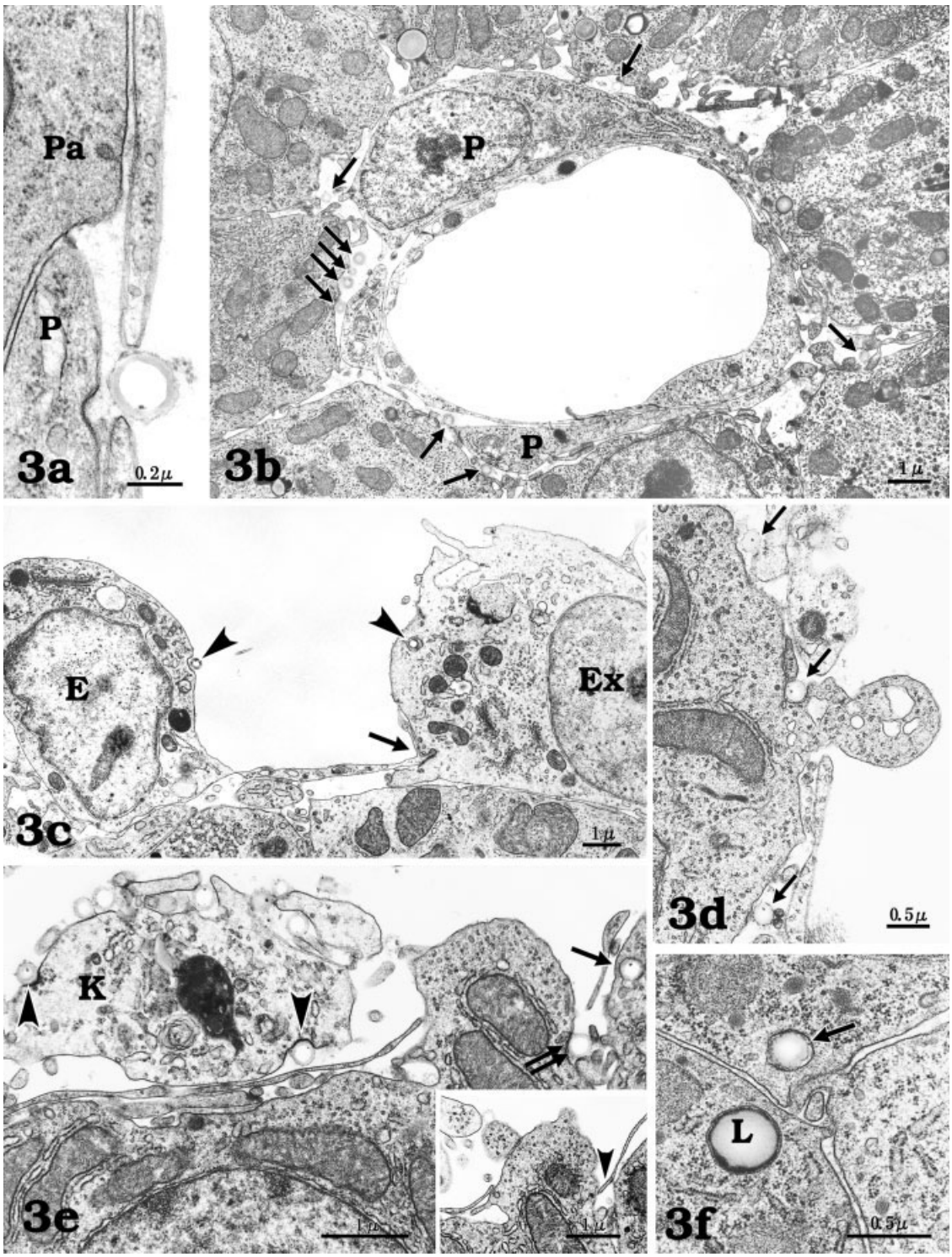

Fig. 3. After injection of lecithin-coated beads. **a:** Two minutes after the injection on day 15 of incubation. A coated bead is passing through an endothelial cell. P, a perisinusoidal stellate cell; Pa, a parenchymal cell. **b:** Ten minutes after the injection on day 8 of incubation. Many beads (arrows) have penetrated into the perivascular space of a narrow sinusoid-like space. Note that no endothelial cell fenestrations are shown. P, perisinusoidal stellate cells. **c:** Three minutes after the injection on day 14 of incubation. A coated bead (left arrowhead) is trapped by a clathrin-coated invagination of an endothelial cell (E). Another (right arrowhead) is incorporated into a phagosome of an extrasinusoidal macrophage (Ex), which extends a part of its cell body through the endothelial layer into the lumen. A tip of the endothelial cell plate inserts into a clathrin-coated invagination of the macrophage (arrow). **d:** Ten minutes after the injection on day 15 of incubation. Three beads have penetrated into the perivascular space (arrows). One is trapped at a root of a parenchymal cell projection by a non–clathrin-coated pit. There are small pores between the projection and the endothelial plate. **e:** Ten minutes after the injection on day 15 of incubation. One bead (arrow) is incorporated into a parenchymal cell, and another (double arrows) is trapped in the perisinusoidal space at the root of a parenchymal cell projection. There is a small pore between the projection and the endothelial plate, which is closed at an adjacent section (inset, arrowhead). A Kupffer cell (K) has trapped some beads; clathrin-coated pits can be observed (arrowheads). **f:** Ten minutes after the injection on day 15 of incubation. A phagosome (arrow) in a parenchymal cell contains a coated bead that was likely taken up from the interparenchymal cell space. L, a lipolysosome.

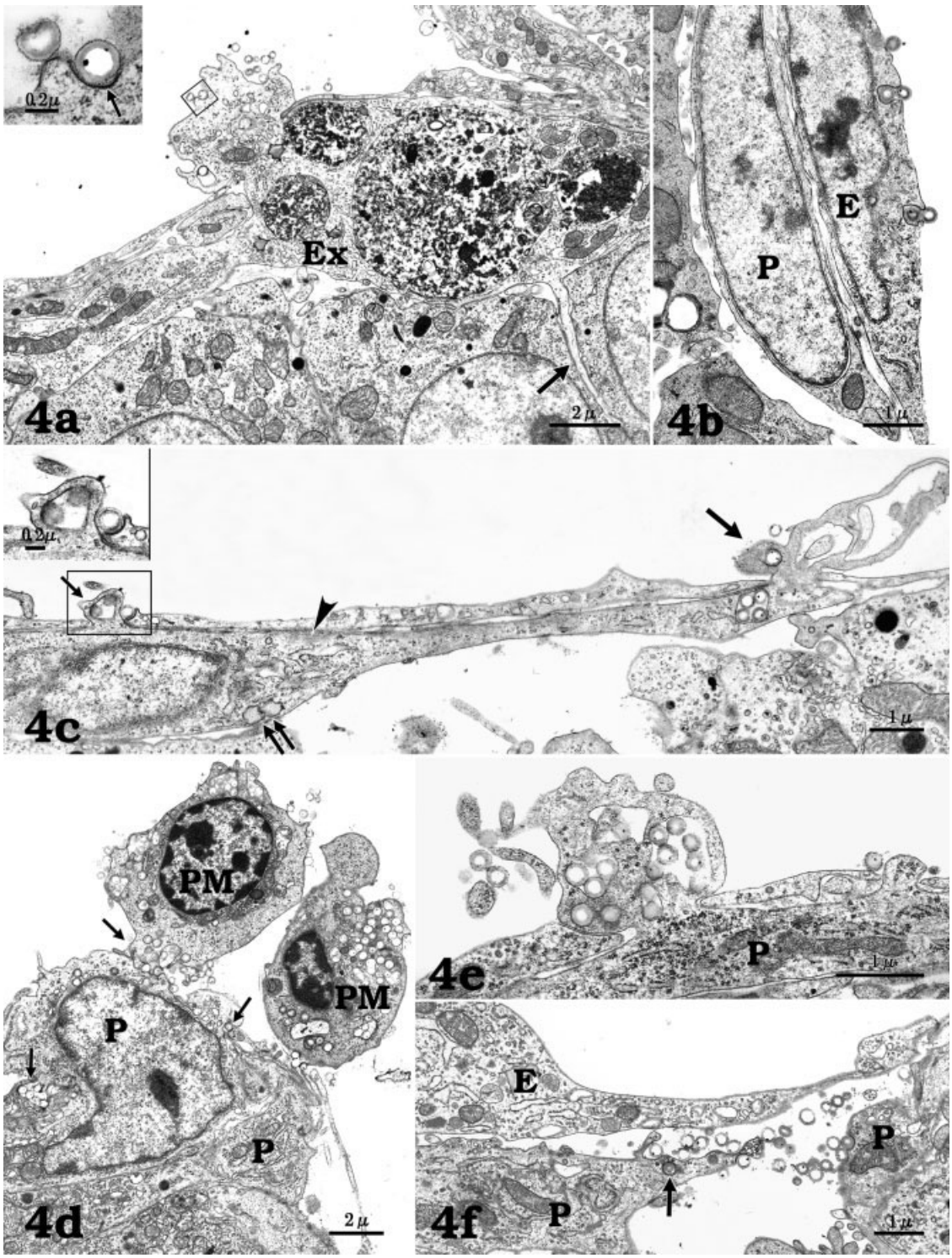

Fig. 4. After the injection of noncoated beads. **a:** Ten minutes after the injection on day 8 of incubation. An extrasinusoidal macrophage (Ex) takes up the beads by a pseudopod, which is extending into the sinusoid-like vascular space. The cell also sends its processes into the perivascular space and the space between parenchymal cells (arrow). Note clathrin-coating on parts of the surface of an invagination (inset, arrow). **b:** Three minutes after the injection on day 15 of incubation. An endothelial cell (E) takes up the beads by clathrin-coated invaginations. P, a perisinusoidal stellate cell. **c:** Ten minutes after the injection on day 8 of incubation. A perisinusoidal stellate cell takes up the beads by two cytoplasmic projections and sequesters it within a phagosome (arrows). One of the projections shares a bead with an endothelial cell invagination, whose surface is coated by clathrin (inset). Intermediate filaments (arrowhead) and lipid droplets (double arrows) exist in the perisinusoidal stellate cell. **d:** Seven minutes after the injection on day 8 of incubation. One of the perisinusoidal stellate cells (P) extends a large part of its cell body into the vascular lumen, and incorporates several beads. Primitive macrophages (PM) also take up some beads. These and the endothelial cells appear to share the trapped beads with one another (arrows). **e:** Seven minutes after the injection on day 8 of incubation. Many noncoated beads are taken up from the vascular lumen by a cytoplasmic projection of a perisinusoidal stellate cell (P), in which intermediate filaments are abundant. **f:** Ten minutes after the injection on day 8 of incubation. An exceptional number of beads are found to penetrate into the perivascular spaces, which become trapped and incorporated (arrow) by perisinusoidal stellate cells (P). E, an endothelial cell.

Clathrin-coated invaginations did not participate in the uptake by the perisinusoidal stellate cells. The invaginations were constantly noted in the endothelial cells, occasionally in the Kupffer cells and extrasinusoidal macrophages, and rarely in the primitive macrophages.

Immunocytochemical Localization of Desmin

The most abundant gold labels were evident in the cytoplasm of the perisinusoidal stellate cells. The labels were mostly localized over the intermediate filaments. These filaments were plentiful in those areas under the surface of the perisinusoidal stellate cells, where they were in close proximity to the endothelial cells, and especially so in similar areas of the cellular projections (Fig. 5). The endothelial and parenchymal cells were less immunopositive, while the Kupffer cells and the extrasinusoidal and primitive macrophages did not stain with the desmin antibody. The labeling was, however, scattering randomly in the cytoplasm of the endothelial and parenchymal cells and showed no particular relationship to the intermediate filaments. Thus, well-labeled cells that trapped or incorporated the noncoated beads were primarily the perisinusoidal stellate cells (Fig. 5b).

DISCUSSION

Before discussing phagocytosis by embryonic liver cells, it is important that we clarify the criteria by which the perisinusoidal stellate cells and extrasinusoidal macrophages present in the perivascular spaces were identified. They formed one to three layers beneath the endothelial lining and both extended cytoplasmic projections through the lining into the sinusoid-like vascular spaces with increased frequency after injection of the noncoated beads. The perisinusoidal stellate cells were noted as being rich in desmin-positive cytoplasmic intermediate filaments. Desmin expression is known to be a marker of the perisinusoidal stellate cells in the adult rodent liver (Yokoi et al., 1984; Tsutsumi et al., 1987; Geerts et al., 1991); however, this finding has not yet been well documented in the chicken. Fujimoto and Singer (1986, 1987) reported that not only the perisinusoidal stellate cells but also the endothelial cells were positive for desmin in the adult chicken liver. Nevertheless, no evidence of colocalization between the intermediate filaments and desmin was shown in their reports. It is also controversial as to whether desmin expression is restricted to the perisinusoidal stellate cells during rodent liver development. According to previous immunohistochemical reports (Vassy et al., 1993; Kiassov et al., 1995), various types of nonhematopoietic liver cells, including parenchymal cells, transiently expressed desmin during the early stages of rat development. In contrast, Nitou et al. (2000) have shown that only the stellate cells immunohistochemically expressed desmin and they differed from hepatoblasts and hepatocytes, which expressed E-cadherin in the fetal mouse liver. There was no description of extrasinusoidal macrophages in those reports.

In the present study, desmin–gold labels localized on the intermediate filaments in the perisinusoidal stellate cells during the developmental stages of the chicken livers. A similar observation was also obtained after the injection of noncoated beads, when the cells, like the extrasinusoidal macrophages, actively extended their cytoplasmic projections into the vascular spaces. On the contrary, the extrasinusoidal macrophages, which accumulated a very sparse number of intermediate filaments, were negative for desmin. Both in the endothelial and parenchymal cells, only small amounts of scattered label were observed without particular topographic relationship to the filaments. Primitive perisinusoidal stellate cells have been reported to possess a latent ability to store vitamin A in the chick embryo starting at 9 days of incubation (Tatsumi and Fujita, 1983) and in the fetal mouse from approximately 15 days of gestation (Matsumoto et al., 1984). Perisinusoidal cells containing cellular retinal-binding protein have also been reported to appear in the embryonic rat liver between days 11 and 13 (Kato et al., 1985). In our study, a few perisinusoidal stellate cells, even at day 8 of incubation, contained small electron-lucent lipid droplets, which were clearly distinct from electron-dense lipid droplets (mainly composed of esterified cholesterol) present in the parenchymal cells (Kanai, 1989; Kanai et al., 1994, 1997). With progressing development, the lipid droplets in the perisinusoidal stellate cells increased in number and size so that identification of these cells became easier. Extrasinusoidal macrophages were first mentioned in the adult avian liver by Ohata et al. (1982); however, there have been only brief descriptions in fetal rat and human livers (Naito and Wisse, 1977, Enzan et al., 1983). Central macrophages within erythroblastic islands have also been reported in fetal mouse hepatic parenchymal cell cords (Sasaki et al., 1993; Sasaki and Iwatsuki, 1997). We did not need to distinguish the extrasinusoidal macrophages from the central ones in chick embryo, because erythropoiesis was not recognized within the parenchymal cords.

The following is a summary of the observations after the injection of coated beads or noncoated beads. The parenchymal and perisinusoidal stellate cells were exposed to both beads in both the vascular and perivascular spaces. By extending their cytoplasmic projections, like extrasinusoidal macrophages, they directly touched the beads in the vascular spaces, even though the cells were situated outside the spaces. Both beads were observed in perivascular space, although 5.4 to 10 times as many coated beads as noncoated beads penetrated into the space. However, these cells show different characteristics in their selection and incorporation of the respective beads. Perisinusoidal stellate cells selectively took up noncoated beads from both spaces, while the parenchymal cells incorporated only lecithin-coated beads but never took them up from the vascular space. Extrasinusoidal macrophages demonstrated phagocytic activity against both beads and favored those that were noncoated, like the Kupffer cells and the primitive macrophages in the vascular spaces. A model based on our observations and the present discussion is shown schematically in Figure 6.

In the chick embryonic liver, we found cytoplasmic projections of the parenchymal and perisinusoidal stellate cells, which reached the vascular spaces across the endothelial lining. Moreover, the noncoated beads induced the perisinusoidal stellate cells to produce these projections, which captured the beads. It seemed that newly formed pores of endothelial cells rather than those

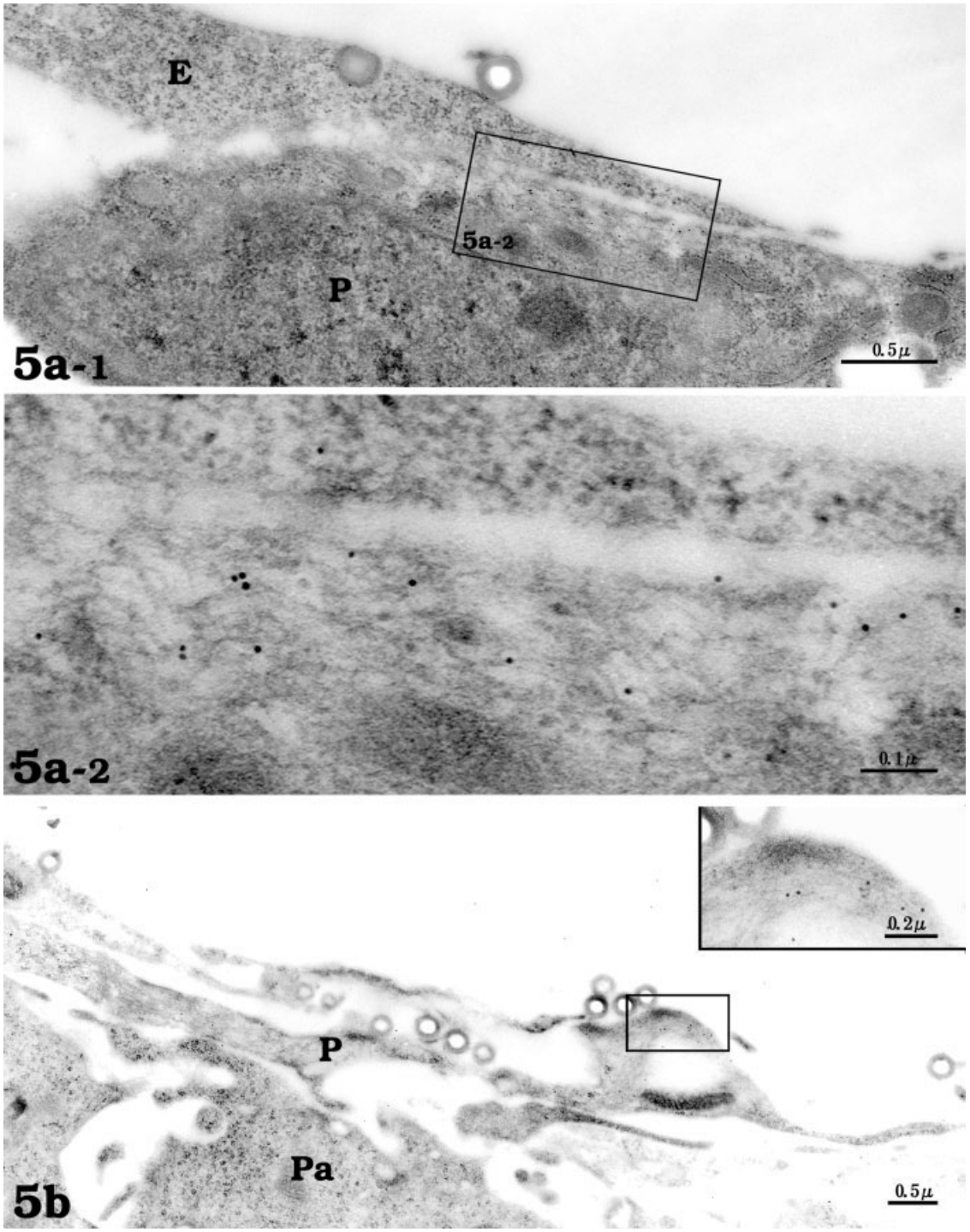

Fig. 5. Immunocytochemical localization of desmin. **a-1:** Three minutes after the injection of the noncoated beads on day 15 of incubation. The 10-nm gold labels are scattered over intermediate filaments in a perisinusoidal stellate cell (P), but are rare in an endothelial cell (E), which can be seen taking up a bead. **a-2:** High magnification of the square in a-1. **b:** Two minutes after the injection of the noncoated beads on day 8 of incubation. Labeled intermediate filaments are observed in a perisinusoidal stellate cell (P), which traps beads both in the perivascular space and in the vascular lumen. Pa, a parenchymal cell. **Inset:** High magnification of the boxed area.

that were preexisting served as the means by which the projections passed. This view was formed from the following findings: the cells sometimes inserted their projections into an endothelial indentation and seemed to then force an out-pouching of the endothelial lining into the vascular spaces. In addition, the pores appeared to form a seal in the vascular spaces by close apposition of the pore edges to the projections. This view on the pass-

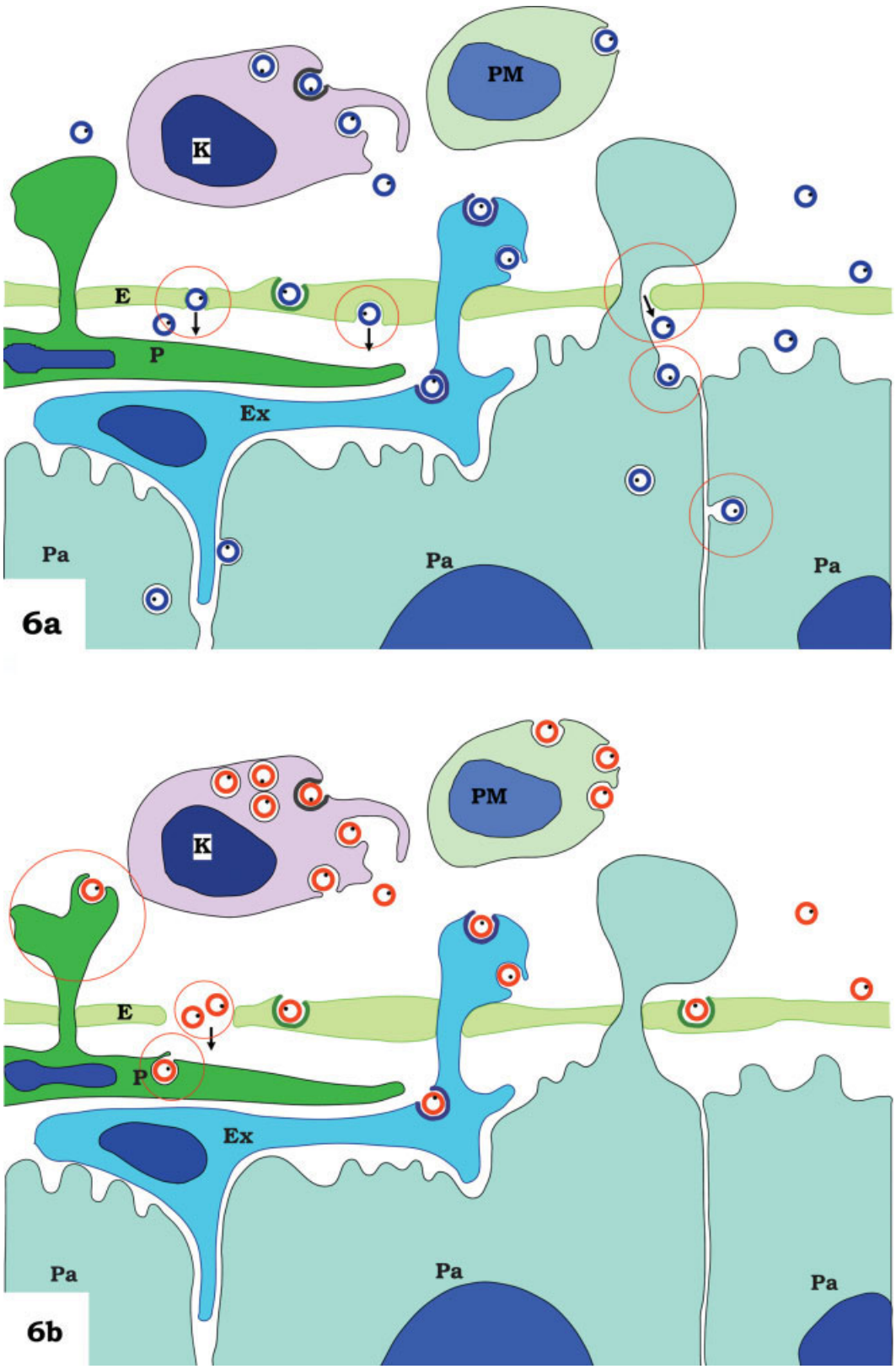

Fig. 6. **a,b:** Different behaviors of chick embryonic liver cells toward lecithin-coated beads (a) and noncoated beads (b). E, endothelial cells. Ex, extrasinusoidal macrophages. K, Kupffer cells. P, perisinusoidal stellate cells. Pa, Parenchymal cells. PM, primitive macrophages. Arrows show main ways that these beads reach the perivascular space. Thick parts of cell membrane designate clathrin-coated invaginations. Note the different behaviors of endothelial cells, perisinusoidal stellate cells, and parenchymal cells toward different beads, which are indicated by red circles.

ing mechanism coincides with that of the transmural diapedesis of newly formed blood cells in the fetal rat liver (Bankston and Pino, 1980). This mechanism also likely applies to the projections of the parenchymal cells and extrasinusoidal macrophages. Engulfing material from the blood vascular system was shown to be a significant function of the projections of the perisinusoidal stellate cells, similar to that of the extrasinusoidal macrophages. However, the functional role of the parenchymal cell projections still remains obscure. Neither beads produced a change in size, shape, nor number of the parenchymal cell projections. The projections did not show any participation in uptake of coated beads from the blood. The cells took up the beads only from the perivascular spaces and the interparenchymal cell spaces. From this point of view, it can be deduced that parenchymal cell uptake of the beads is quite selective but it is still necessary for the beads to pass through the endothelial lining before being sequestered by these cells.

Phagocytic activation of the perisinusoidal stellate cells occurred after injection of only the noncoated beads. This finding was noteworthy as a transient phenomenon of their developmental differentiation. These cells have generally been regarded as inactive phagocytes (Widmann et al., 1972; Yamamoto, 1975; Naito and Wisse, 1978), with the exception of a report by Ohata and Ito (1986), where the cells in the adult chicken liver occasionally ingested a very small amount of India ink particles. Recently, the capacity to internalize extracellular large material, including latex beads, has been described in fibrogenic-activated human hepatic stellate cells in vitro (Viñas et al., 2003). The cells expressed CD68, which belongs to the family of lysosomal/plasma membrane shuttling proteins involving in antigen trafficking. Engulfment of apoptotic bodies by human stellate cell line has also been reported (Canbay et al., 2003). These reports and our results suggest that perisinusoidal stellate cells are potentially "nonprofessional phagocytes" (Rabinovitch, 1995). These cells have been proposed to be a liver-specific pericyte (Pinzani, 1995) and similar properties of phagocytosis have been described in other organ-specific pericytes, such as mesangial cells (Farquhar and Palade, 1962; Savill et al., 1992; Johnson et al., 1992; Hughes et al., 1997) and astrocytes (Watanabe et al., 1989; Vinores and Herman, 1993; Al-Ali and Al-Hussain, 1996). A similar function shared by embryonic perisinusoidal stellate cells and pericyte-related cells should supply one key piece of evidence into the search for an understanding of the developmental origin of these cells. Perisinusoidal stellate cells are classically considered to be differentiated from mesenchymal cells of the septum transversum (Enzan et al., 1997); however, the exact origin of the cells has not yet been established. Some observations have provided speculation that stellate cells could be of neuroectodermal origin (Niki et al., 1999; Friedman, 2000), like astrocytes.

It is important to address the mechanisms that allow considerable amounts of coated beads and fewer noncoated beads to pass through the endothelial lining. These phenomena were conspicuous in the chicken embryo as compared with that of the adult rat, in which only coated beads were, and only occasionally, observed on the transendothelial side of the sinusoid (Kanai et al., 1996). Two possible mechanisms are thought to facilitate transport: transcytosis by endosomes and small pores induced by beads, or leakage through preexisting large gaps. Transcytosis of the coated beads by endosomes were occasionally observed, which was similar to what we have previously reported in the adult rat liver (Kanai et al., 1996). Small pores similar in size to the beads which opened for the coated beads, and seldom for the noncoated beads, to pass through the lining were frequently recognized. Sometimes the pores remained open after the passage of the beads especially at a point adjacent to the roots of the parenchymal cytoplasmic projections. This process seemed to be a common mechanism that was shared with cytoplasmic projections passing the endothelial lining, as mentioned above. Recent evidence has been provided showing that egg lecithin liposomes and the liposomes containing phosphatidylserine (200–400 nm in diameter) accumulated in adult rat parenchymal cells (Daemen et al., 1997; Romero et al., 1999; Moghimi and Patel, 2002). Romero and colleagues showed that liposomes needed to be mechanically deformed when passing through the fenestrations. In addition, Scherphof proposed forced "extrusion" mechanisms documenting that liposomes can be forced through the narrow fenestrations by being squeezed by blood cells in the narrow hepatic sinusoids (Scherphof and Kamps, 2001). However, morphological observation on the passing process was not given in these reports. From our observations, we propose the possible mechanisms of transcytosis when larger rigid particles are selectively allowed to pass. Functional interactions between the endothelial cells and coated beads should be analyzed in future studies. The other possibility of leakage from the gap may have also been the principal means by which noncoated beads entered the space, because these beads were usually clustered and in close proximity to the gaps, while coated beads were diffusely distributed in the space. Arrows in Figure 6 schematically show the main ways that these beads reach the perivascular space.

After coated beads have gained access to the parenchymal cells, they have to interact with these cells, which leads to their selective internalization. In this interaction, the apolipoprotein E-mediated remnant receptor could play a role in this process, because this mechanism has been considered in the case of egg lecithin liposomes with rat parenchymal cells (Bisgaier et al., 1989; Scherphof and Kamps, 2001; Moghimi and Hunter, 2001). On the other hand, the lack of uptake of noncoated beads by these cells is possibly due to an intrinsic inability to recognize the beads. Additionally, it is worth considering mechanisms of phagocytosis by the parenchymal cells and correlating these findings with that of apoptotic cells (Dini et al., 1992, 2002; McVicker et al., 2002). To achieve recognition of the latter cells, signals in the form of molecular modifications of the plasma membrane must occur on the apoptotic cell surface, such as modification of the membrane lipid asymmetry and external exposition of phosphatidylserine (Dini et al., 2002). Lecithin (phosphatidylcholine) is also a component of plasma membrane, and lecithin-coated beads could be selectively recognized by these cells.

As demonstrated in the results and schematically shown in Figure 6, using clathrin-coated invaginations or not was intrinsic to the respective liver cells and was

independent of lecithin coating of the beads. The mode of phagocytosis is different among these cells. Perisinusoidal stellate cells showed clathrin-independent engulfment of noncoated beads; the parenchymal cells also showed clathrin-independent uptake. The coated beads sank directly into the cytoplasm; the endothelial cells incorporated both beads by clathrin-coated invaginations. Moreover, all types of macrophages showed both clathrin-dependent and -independent uptake for both beads. What determined the mode of uptake remains to be elucidated. In addition, lecithin coating of the beads clearly suppressed the uptake function of all types of macrophages, suggesting that the coating might be a useful method for delivering drugs to parenchymal cells and regulating phagocytosis by macrophages.

LITERATURE CITED

Al-Ali SY, Al-Hussain SM. 1996. An ultrastructural study of the phagocytic activity of astrocytes in adult rat brain. J Anat 188:257–262.

Bankston PW, Pino RM. 1980. The development of the sinusoids of fetal liver: morphology of endothelial cells, Kupffer cells, and the transmural migration of blood cells into the sinusoids. Am J Anat 159:1–15.

Bisgaier CL, Siebenkas MV, Williams KJ. 1989. Effects of apolipoproteins A-IV and A-I on the uptake of phospholipid liposomes by hepatocytes. J Biol Chem 264:862–866.

Bronfenmajer S, Schaffner F, Popper H. 1966. Fat-storing cells (lipocytes) in human liver. Arch Pathol Lab Med 82:447–452.

Canbay A, Taimr P, Torok N, Higuchi H, Friedman S, Gores GJ. 2003. Apoptotic body engulfment by human stellate cell line is profibrogenic. Lab Invest 83:655–663.

Daemen T, Velinova M, Regts J, De Jager M, Kalicharan R, Donga J, Van der Want JJL, Scherphof GL. 1997. Different intrahepatic distribution of phosphatidylglycerol and phosphatidylserine liposomes in the rat. Hepatology 26:416–423.

Dan C, Wake K. 1985. Modes of endocytosis of Latex particles in sinusoidal and Kupffer cells of normal and perfused rat liver. Exp Cell Res 158:75–85.

Dini L, Autuori F, Lentini A, Oliverio S, Piacentini M. 1992. The clearance of apoptotic cells in the liver is mediated by the asialoglycoprotein receptor. FEBS Lett 296:174–178.

Dini L, Pagliara P, Carla EC. 2002. Phagocytosis of apoptotic cells by liver: a morphological study. Microsc Res Tech 57:530–540.

Enzan H, Hara H, Yamashita Y, Ohkita T, Yamane T. 1983. Fine structure of hepatic sinusoids and their development in human embryos and fetuses. Acta Pathol Jpn 33:447–466.

Enzan H, Himeno H, Hiroi M, Kioku H, Saibara T. 1997. Development of hepatic sinusoidal structure with special reference to the Ito cells. Microsc Res Tech 39:336–349.

Farquhar MG, Palade GE. 1962. Functional evidence for the existence of a third cell type in the renal glomerulus. J Cell Biol 13:55–87.

Friedman SL. 2000. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. J Biol Chem 275:2247–2250.

Fujimoto T, Singer SJ. 1986. Immunocytochemical studies of endothelial cells in vivo. I. The presence of desmin only, or of desmin plus vimentin, or vimentin only, in the endothelial cells of different capillaries of the adult chicken. J Cell Biol 103:2775–2786.

Fujimoto T, Singer SJ. 1987. Immunocytochemical studies of desmin and vimentin in pericapillary cells of chicken. J Histochem Cytochem 35:1105–1115.

Geerts A, Lazou JM, De Bleser P, Wisse E. 1991. Tissue distribution, quantitation and proliferation kinetics of fat-storing cells in carbon tetrachloride-injured rat liver. Hepatology 13:1193–1202.

Hughes J, Liu Y, Van Damme J, Savil J. 1997. Human glomerular mesangial cell phagocytosis of apoptotic neutrophils. J Immunol 158:4389–4397.

Ito T, Nemoto M. 1952. Uber die Kupfferschen Sternzellen und die "Fettspeicherungszellen" ("fatstoring cells") in der Blunkapillarenwand der menschlichen Leber. Folia Anat Jpn 24:243–258.

Johnson RJ, Floege J, Yoshimura A, Iida H, Couser WG, Alpers CE. 1992. The activated mesangial cell: a glomerular "myofibroblast"? J Am Soc Nephrol 2:S190.

Jones EA, Summerfield JA. 1982. Kupffer cells. In: Arias I, Popper H, Schachter D, Shafritz DA, editors. The liver, biology and pathobiology. New York: Raven Press. p 507–523.

Kanai M. 1989. Ultrastructural and biochemical studies of lipolysis by lipolysosomes in chick hepatocytes. Cell Tissue Res 255:559–565.

Kanai M, Watari N, Soji T, Sugawara E. 1994. Formation and accumulation of lipolysosomes in developing chick hepatocytes. Cell Tissue Res 275:125–132.

Kanai M, Murata Y, Mabuchi Y, Kawahashi N, Tanaka M, Ogawa T, Doi M, Soji T, Herbert DC. 1996. In vivo uptake of lecithin-coated polystyrene beads by rat hepatocytes and sinusoidal endothelial cells. Anat Rec 244:175–181.

Kanai M, Soji T, Herbert DC. 1997. Biogenesis and function of lipolysosomes in developing chick hepatocytes. Microsc Res Tech 39:444–452.

Kato M, Kato K, Goodman DS. 1985. Immunochemical studies on the localization and on the concentration of cellular retinal-binding protein in rat liver during perinatal development. Lab Invest 52:475–484.

Kiassov AP, Eyken PV, van Pelt JF, Depla E, Fevery J, Desmet VJ, Yap PSH. 1995. Desmin expressing nonhematopoietic liver cells during rat liver development: an immunohistochemical and morphometric study. Differentiation 59:253–258.

Kirn A, Steffan AM, Anton M, Gendrault JL, Bingen A. 1978. Phagocytic properties displayed by mouse hepatocytes after virus induced damage of the sinusoidal lining. Biomedicine 29:25–28.

Kirn A, Gut JP, Gendrault JL. 1982. Interaction of viruses with sinusoidal cells. In: Popper H, Schaffner F, editors. Progress in liver diseases. Vol. VII. New York: Grune and Stratton. p 377–392.

Luft JH. 1961. Improvements in epoxy resin embedding methods. J Biophys Biochem Cytol 9:409–414.

Matsumoto E, Hirosawa K, Abe K, Naka S. 1984. Development of the vitamin A-storing cell in mouse liver during late fetal and neonatal periods. Anat Embryol (Berl) 169:249–259.

McVicker BL, Tuma DJ, Kubik JA, Hindemith AM, Baldwin CR, Casey CA. 2002. The effect of ethanol on asialoglycoprotein receptor-mediated phagocytosis of apoptotic cells by rat hepatocytes. Hepatology 36:1478–1487.

Millonig G. 1962. Advantages of a phosphate buffer for OsO4 solutions in fixation. J Appl Physiol 32:1637.

Moghimi SM, Hunter AC. 2001. Recognition by macrophages and liver cells of opsonized phospholipid vesicles and phospholipid headgroups. Pharm Res 18:1–8.

Moghimi SM, Patel HM. 2002. Modulation of murine liver macrophage clearance of liposomes by diethylstilbestrol. The effect of vesicle surface charge and a role for the complement receptor Mac-1 (CD11b/CD18) of newly recruited macrophages in liposome recognition. J Control Release 78:55–65.

Naito M, Wisse E. 1977. Observations on the fine structure and cytochemistry of sinusoidal cells in fetal and neonatal rat liver. In: Wisse E, Knook DL, editors. Kupffer cells and other liver sinusoidal cells. Amsterdam: Elsevier/North-Holland Biomedical Press. p 497–505.

Naito M, Wisse E. 1978. Filtration effect of endothelial fenestrations on chylomicron transport in neonatal rat liver sinusoids. Cell Tissue Res 190:371–382.

Niki T, Pekny M, Hellemans K, Bleser PD, Berg KV, Vaeyens F, Quartier E, et al. 1999. Class VI intermediate filament protein nestin is induced during activation of rat hepatic stellate cells. Hepatology 29:520–527.

Nitou M, Ishikawa K, Shiojiri N. 2000. Immunohistochemical analysis of development of desmin-positive hepatic stellate cells in mouse liver. J Anat 197:647–657.

Ohata M, Ito T. 1986. Experimental study on the fine structure of chicken liver parenchyma with special references to extrasinusoidal cells and macrophages in the normal and India ink-perfused livers. Arch Histol Jpn 49:83–103.

Ohata M, Tanuma Y, Ito T. 1982. Electron microscopic study on avian livers with special remarks on the fine structure of sinusoidal cells. Okajimas Folia Anat Jpn 58:325–368.

Pinzani M. 1995. Hepatic stellate (ITO) cells: expanding roles for a liver-specific pericyte. J Hepatol 22:700–706.

Rabinovitch M. 1995. Professional and non-professional phagocytes: an introduction. Trends Cell Biol 5:85–87.

Romero EL, Morilla MJ, Regts J, Koning GA, Scherphof GL. 1999. On the mechanism of hepatic transendothelial passage of large liposomes. FEBS Lett 448:193–196.

Sasaki K, Iwatsuki H. 1997. Origin and fate of the central macrophages of erythroblastic islands in the fetal and neonatal mouse liver. Microsc Res Tech 39:398–405.

Sasaki K, Iwatsuki H, Suda M, Itano C. 1993. Scavenger macrophages and central macrophages of erythroblastic islands in liver hemopoiesis of the fetal and early postnatal mouse: a semithin light- and electron-microscopic study. Acta Anat 147:75–82.

Sato T. 1968. A modified method for lead staining of thin sections. J Electron Microsc (Tokyo) 17:158–159.

Savill J, Smith J, Sarraf C, Ren Y, Abbott F, Rees A. 1992. Glomerular mesangial cells and inflammatory macrophages ingest neutrophils undergoing apoptosis. Kidney Int 42:924.

Scherphof GL, Kamps JAAM. 2001. The role of hepatocytes in the clearance of liposomes from the blood circulation. Prog Lipid Res 40:149–166.

Soji T, Murata Y, Ohira A, Nishizono H, Tanaka M, Herbert DC. 1992. Evidence that hepatocytes can phagocytize exogenous substances. Anat Rec 233:543–546.

Steffan AM, Gendrault JL, Kirn A. 1978. Synthesis of vaccinia specified antigens in mouse hepatocytes after Frog Virus 3-induced damage to the sinusoidal cells. FEMS Microbiol Lett 3:5–7.

Steffan AM, Gendrault JL, McCuskey RS, McCusky PA, Krin A. 1986. Phagocytosis, an unrecognized property of murine endothelial liver cells. Hepatology 6:830–836.

Tatsumi H, Fujita H. 1983. Fine structural aspects of the development of Ito cells (vitamin A uptake cells) in chick embryo livers. Arch Histol Jpn 46:691–700.

Tsutsumi M, Takada A, Takase S. 1987. Characterization of desmin-positive rat liver sinusoidal cells. Hepatology 7:277–284.

Vassy J, Rigaut JP, Briane D, Kraemer M. 1993. Confocal microscopy immunofluorescence localization of desmin and other intermediate filament proteins in fetal rat livers. Hepatology 17:293–300.

Viñas O, Bataller R, Sancho-Bru P, Gines P, Berenguer C, Enrich C, Nicolas JM, Ercilla G, Gallart T, Vives J, Arroyo V Rodes J. 2003. Human hepatic stellate cells show features of antigen-presenting cells and stimulate lymphocyte proliferation. Hepatology 38:919–929.

Vinores SA, Herman MM. 1993. Phagocytosis of myelin by astrocytes in explant of adult rabbit cerebral white matter maintained on Gelfoam matrix. J Neuroimmunol 43:169–176.

Wake K. 1971. Sternzellen in the liver: perisinusoidal cells with special reference to the storage of vitamin A. Am J Anat 132:429–461.

Watanabe K, Osborne D, Kim SU. 1989. Phagocytic activity of human adult astrocytes and oligodendrocytes in culture. J Neuropathol Exp Neurol 48:499–506.

Watson ML. 1958. Staining of tissue sections for electron microscopy with heavy metals. J Biophys Biochem Cytol 4:475–478.

Widmann JJ, Cotran SR, Fahimi HD. 1972. Mononucleal phagocytes (Kupffer cells) and endothelial cells. Identification of two functional cell types in rat liver sinusoids by endogenous peroxidase activity. J Cell Biol 52:159–170.

Wisse E. 1970. An electron microscopic study of the fenestrated endothelial lining of rat liver sinusoids. J Ultrastruct Res 31:125–150.

Yamamoto M. 1975. Ultrastructure and function of Ito cell (fat-storing cell) in the liver. Med J Hiroshima Univ 23:245–275.

Yokoi Y, Namihisa T, Kuroda H, Komatsu I, Miyazaki A, Watanabe S, Usui K. 1984. Immunocytochemical detection of desmin in fat-storing cells (Ito cells). Hepatology 4:709–714.