Liver sinusoidal endothelial cells (LSECs) constitute the sinusoidal wall, also called the endothelium, or endothelial lining (see website chapter W-26). The liver sinusoids can be regarded as unique capillaries which differ from other capillaries in the body, because of the presence of fenestrae lacking a diaphragm and a basal lamina underneath the endothelium. The first electron microscopic observation of LSEC fenestrae was accomplished in 1970 by Wisse (1), who applied perfusion fixation to the rat liver and demonstrated groups of fenestrae arranged in sieve plates. In subsequent reports, Widmann (2) and Ogawa (3) confirmed the existence of fenestrae in LSECs by using transmission electron microscopy (TEM). In general, endothelial fenestrae measure 150 to 175 nm in TEM, occur at a frequency of 9 to 13 per \( \mu \text{m}^2 \), and occupy 6% to 8% of the endothelial surface in scanning electron microscopy (SEM) (4). In addition, differences in fenestrae diameter and frequency in periportal and centrilobular zones were demonstrated; in SEM the diameter decreases slightly from 110.7 \( \pm \) 0.2 nm to 104.8 \( \pm \) 0.2 nm, whereas the frequency increases from 9 to 13 per \( \mu \text{m}^2 \), resulting in an increase in porosity from 6% to 8% from periportal to centrilobular (5). Recent atomic force microscopic observations on glutaraldehyde-fixed and living LSECs in culture revealed that the fenestrae diameter is about 240 to 280 nm (6,7).

Other ultrastructural characteristics of LSECs are the presence of numerous bristle-coated micropinocytotic vesicles and many lysosome-like vacuoles in the perikaryon, indicating a well developed endocytotic activity. The nucleus sometimes contains a peculiar body, the sphaeridium (8).

On the basis of morphological and physiological evidence, it was reported that the grouped fenestrae act as a dynamic filter (4,9). Fenestrae filter fluids, solutes and particles that are exchanged between the sinusoidal lumen and the space of Disse, allowing only particles smaller than the fenestrae to reach the parenchymal cells or to leave the space of Disse (Fig. 30.1). At present, it has become clear that alteration of the endothelial filter affects the bidirectional macromolecular exchange, and therefore may determine the balance between health and disease. Although the majority of the research has been descriptive, the role of the liver sieve has been demonstrated in various diseases such as hyperlipoproteinemia, cirrhosis and cancer (Reviewed in ref. 10). Another functional
characteristic of LSECs is their high endocytotic capacity. This function is reflected by the presence of numerous endocytotic vesicles and by the effective uptake of a wide variety of substances from the blood by receptor-mediated endocytosis [(11), reviewed in ref. 12]. This endocytotic capacity, together with the presence of fenestrae and the absence of a regular basal lamina, makes these cells different and unique from any other type of endothelial cell in the body (13).

**Preparation of Endothelial Cells**

LSECs seem to be vulnerable cells, a fact that becomes obvious during the isolation, purification and cultivation procedures for *in vitro* studies. LSEC suspensions from rats and mice can be obtained through a variety of isolation and purification techniques, all including perfusion of the liver with one or more tissue-dissociating enzymes. Specific methods can be chosen to satisfy particular requirements in terms of cell yield, purity, intact morphology, responsiveness, and ability to survive in culture (Reviewed in ref. 14).

We found that the use of a low-rate nonrecirculating perfusion of rat liver with collagenase plus fetal calf serum, followed by purification using isopycnic sedimentation in a two-step Percoll gradient and removal of contaminating cells by selective adherence, gave a vital and morphologic intact LSEC population of high purity, enabling the study

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**FIGURE 30.1.** Scanning electron micrographs of the sinusoidal endothelium from rat liver. A: Low magnification showing the fenestrated wall, the space of Disse (SD) and the bordering parenchymal cells (Pc). Notice also the clustering of fenestrae in sieve plates (arrow). Scale bar, 1 μm. B: Higher-power micrograph depicting fenestrae (arrow). Scale bar, 250 nm. C: Low magnification showing the sinusoidal endothelium of a corn oil-fed rat. After administration of a dose of corn oil, chylomicrons (arrow) with diameters in the range of 250 to 600 nm were present in the liver sinusoids. Scale bar, 1 μm. D: Higher magnification shows lipid particles passing the fenestrae (arrow), illustrating the sieving effect of fenestrae. Scale bar, 250 nm. (Courtesy of Dr. R. De Zanger.)
of structure and function of these cells *in vitro* (Fig. 30.2) (15). Comparable isolation and purification methods have been described to obtain LSEC cultures from pig (16) and humans as well (17).

**Identification of Endothelial Cells**

To discriminate LSECs from other liver cells (pit cells, stellate cells, Kupffer cells (KC), parenchymal cells, and bile duct cells), immunocytochemical, receptor ligand, and ultrastructural studies can be used. RECA-1 and SE-1 have been reported as suitable antibodies to stain rat LSECs *in vitro* and *in situ* (18,19). However, RECA-1 antibodies do in fact label other microvascular endothelia as well (18). A commercial LSEC-specific human monoclonal antibody named HM 15/3 (BMA Biomedical AG, Switzerland) is available as well. Staining with von Willebrand factor or factor VIII-related antigen is commonly used as a cytochemical marker for vascular endothelium and has been used in attempts to identify LSECs (16,20–21). However, this marker should be used with caution, because the presence of von Willebrand factor in LSECs is controversial. Variations in activity and presence among various species make it inadvisable to use this marker. Until this controversy has been settled, markers other than von Willebrand should be used to identify LSECs.

LSECs possess cell membrane receptors, which enable them to clear rapidly from the blood specific substances such as hyaluronan and other extracellular matrix components, which can be used to specifically label LSECs (12). Besides hyaluronan, Smedsrod et al. (22) conjugated fluorescein isothiocyanate to chondroitin sulphate proteoglycan, collagen alpha chains, and N-terminal propeptide of procollagen type I. All these substances are endocytosed exclusively and with a remarkable efficiency by LSECs. Therefore, in systems with viable cells, this way of distinguishing LSECs from other types of cells is probably the most reliable and specific method available at present.

LSECs in culture display their normal morphologic and functional characteristics, such as fenestrae and pinocytotic vesicles (1), and can also be identified easily from other liver cells by their ultrastructural morphology (Fig. 30.2) (13,15).

**Biology of Endothelial Cells**

At present, studies are focused on the molecular biology and the clinical aspects of this intriguing class of cells (23–24). LSECs can be regarded as outlined earlier: (a) as a "selective sieve" for substances passing from the blood to parenchymal and stellate cells, and *vice versa* (10), and (b) as a "scavenger system" which clears the blood from many different macromolecular waste products, which originate from turnover processes in different tissues (22). In addition, LSECs have (c) a variety of adhesion molecules (25–26), (d) a capacity to secrete cytokines (26–27), and (e) an important role in various diseases such as liver cancer (28), posttransplantation rejection (29), alcoholic liver disease (30), lipoprotein metabolism (31), fibrosis (32), and viral infection (23).
Interactions between leukocytes or cancer cells and LSECs have been known to be involved in the pathogenesis of liver injury (26). In these cell-to-cell interactions, a wide spectrum of adhesion molecules play a key role, and their expression is mainly regulated by inflammatory cytokines such as interleukin-1, tumor necrosis factor-α and interferon-γ (27). LSECs in normal liver express intercellular adhesion molecule-1, intercellular adhesion molecule-2, leukocyte function-associated-3, very late antigen-5, and CD44. In patients with acute or chronic liver disease, intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression are markedly enhanced in the inflamed liver tissue (27,33–34). Selectins are not present in normal conditions, but are induced after lipopolysaccharide administration (35).

Colorectal tumors often metastasize in the liver. In general, it has been supposed that single tumor cells get stuck in the sinusoids when entering the liver, because their size largely exceeds the diameter of a sinusoid. After plugging, their adhesion molecules might react with the surface molecules of the LSECs, enabling them to extravasate and enter the liver parenchyma. Therefore, the adherence of tumor cells to LSECs is a crucial step in early stages of hepatic metastasis [(36), reviewed in ref. 37].

The success of liver transplantation depends at least partially on the functioning of LSECs in the transplanted liver. Preparing the liver for transplantation includes perfusion of preserving fluid, lowering the temperature, stagnant flow during preservation, and reperfusion with warm oxygenated blood when connecting the liver to the donor circulation (29). Many studies point to the LSECs as being very sensitive in this procedure, possibly leading to essential and lethal deficiency during the posttransplantation period (23,38).

Experimental data have accumulated suggesting that alcohol-induced pathological changes of LSECs precede the pathological changes of the hepatocytes. Moreover, due to its strategic position in the liver sinusoid, LSEC dysfunction and structural alterations have far-reaching repercussions for the whole liver (39). There is evidence suggesting that alcohol-induced LSEC alterations are mostly due to KC activation induced by alcohol rather than to a direct action of alcohol on LSEC. In alcoholemia, the activated KC secretes a spectrum of mediators that affect both the structure and function of LSECs. Alcohol-induced LSEC dysfunction comprises a dramatic decrease in hyaluronan uptake by the scavenger receptors of LSECs, a decreased porosity of the liver sieve, and an increment in the secretion of the vasoconstrictor endothelin-1 (40).

LSECs also have important functions in the handling of lipoproteins and the regulation of lipoprotein metabolism (31). They possess several cell membrane receptors for the various types of apoproteins. Moreover, these scavenger receptors have been implicated in adhesion, clearance of dying cells, and host defense against foreign organisms [(41), reviewed in ref. 42].

The role of LSECs in liver fibrosis seems to be quite passive; that is, the cells might become involved in the process of capillarization, but active participation in the synthesis of extracellular matrix products seems to be the task of activated stellate cells (reviewed in ref. 32). However, in cirrhotic liver, compared to normal liver, LSECs exhibit as much as five times the collagen type I mRNA, whereas mRNA for type IV collagen and laminin is decreased by up to 50% (43). Moreover, transforming growth factor-β1 stimulates the synthesis of basement membrane proteins laminin, collagen type IV and entactin in rat LSEC cultures (44).

LSECs are permissive for mouse hepatitis virus 3, at the same time showing a decrease in the number of fenestrae in vivo and in vitro (45). Therefore, it has been hypothesized that viral infection of LSECs may cause hyperlipoproteinemia. However, feline immunodeficiency virus, as a model for human immunodeficiency virus type 1, did not change the number of fenestrae upon infection. Moreover, the infection of LSECs with the feline immunodeficiency virus contributed to the progression of the infection (46). When human LSECs were infected with human immunodeficiency virus type 1 in vitro, they showed the budding of new viral particles, indicating the production of new viruses. This infection was probably facilitated by CD4 surface antigens on LSECs, as were shown to be present by immunogold-EM (47). Therefore, it is concluded that LSECs might be a target and a reservoir for human immunodeficiency viruses.

**Biology of Fenestrae**

To date, one of the widely accepted hypotheses maintains that drugs which dilate fenestrae, such as pantethine, acetylcholine and ethanol improve the extraction of dietary cholesterol from the circulation while drugs such as nicotine, long-term ethanol abuse, adrenalin, noradrenalin and serotin, which decrease the endothelial porosity, play a role in the development of drug- and stress-related atherogenesis (48). As a consequence, alterations in the number or diameter of fenestrae by drugs, hormones, toxins, and diseases can produce serious perturbations in liver function (10).

Current interest focuses on the role of the actin cytoskeleton in regulating the diameter and number of fenestrae. Immunoelectron microscopic studies of LSECs in the early 1980s revealed the first information regarding the structural basis of the contraction and dilatation machinery of fenestrae. Oda et al. (49) described in 1983 the presence of actin filaments in the neighborhood of fenestrae, indicating that the cytoskeleton of LSECs plays an important role in the modulation of fenestrae. At present, it is widely accepted that contractile bundles of actin and myosin around fenestrae regulate fenestrae diameter under the control of intracellular calcium levels (Fig. 30.3) (50–53).

In 1986, Steffan et al. (54–55) provided the first evidence that LSEC fenestrae are inducible structures. Treatment of LSECs in situ and in vitro with the microfilament-
inhibiting drug cytochalasin B resulted in an increased number of fenestrae. SEM observations of detergent-extracted LSECs revealed that the increase in the number of fenestrae was related to an alteration of the cytoskeleton. Moreover, the effect of cytochalasin B on the number of fenestrae and cytoskeleton could be reversed after removal of the drug. However, when LSECs were treated with various microtubule-altering drugs, there was no effect on the number of fenestrae, thereby demonstrating that microtubules are not involved in the formation of the endothelial pores (55–56). These observations indicate that fenestrae are dynamic structures which may undergo changes in number in response to local external stimuli and that the actin-cytoskeleton has a major role in this process. Later, Bingen et al. (57) noted, in freeze-fracture replicas of cytochalasin B-treated LSECs, areas which were more or less devoid of intramembrane particles having the size of fenestrae. Those authors proposed that fenestrae are formed by fusion between opposite sheets of plasma membrane which are depleted of intramembrane particles. In addition, Taira (58) elaborated the study of Bingen et al. (57) and found some new evidence about the formation of sieve plates or clustered fenestrae. The study of the luminal cell membrane of freeze-fractured LSECs revealed the presence of trabecular meshworks which were attached to the E- and P-face of the cell membrane of both the cell body and the attenuated cell processes. The author demonstrated that these meshworks give rise to a stepwise formation of sieve plates by ballooning, fusion and flattening of the cell membrane and cytosol among these plasmalemmal invaginations.

The recent availability of a battery of new actin binding drugs that affect the polymerization of actin by different mechanisms greatly enhances the precision with which the dynamics and functions of the actin cytoskeleton in various cell types can be dissected (59). The study of the numerical dynamics of LSEC fenestrae is an example of such a cellular process, in which the use of several microfilament-disrupting drugs was necessary to identify one that could selectively reveal the process of fenestrae formation. In the past we demonstrated that treatment of LSECs with cytochalasin B, latrunculin A, swinholide A, misakinolide A or jaspilkinolide induces an increased number of fenestrae (59–61). However, only by treating LSECs with misakinolide were we able to visualize the process of fenestrae formation and to identify a new structure involved in the process of fenestrae formation, as we describe below (61).

Actin filament staining of untreated LSECs displays intense circular bundles lining the cell periphery and few straight bundles oriented parallel to the long axis of the cell (Fig. 30.4A). The maximal effect of misakinolide on F-actin was obtained as soon as 10 to 15 minutes after treatment and caused loss of F-actin bundles. Further incubation did

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**FIGURE 30.3.** Scheme of the serotonin signal pathway showing the steps in fenestral contraction and relaxation, as postulated by Arias and co-workers (50–53): (I) serotonin binds to a ketanserin-inhibitable receptor, coupled to a pertussis-toxin sensitive G-protein; (II) a calcium channel opens, causing an influx of calcium ions; (III) the intracellular calcium level increases rapidly, and (IV) calcium binds to calmodulin, (V) the calcium-calmodulin complex activates myosin light chain kinase, and (VI) as a result phosphorylation of the 20-kd light chain of myosin occurs, resulting in (VII) an increased actin-activated myosin ATPase activity, which finally initiates contraction of fenestrae. The mechanism for the relaxation of liver sinusoidal endothelial cell fenestrae is presently unclear and probably involves dephosphorylation of myosin light chains (MLC) as represented by dashed lines: a decrease in the cytosolic free calcium concentration leads to dissociation of calcium and calmodulin from the kinase, thereby inactivating myosin light chain kinase (MLCK); under these conditions myosin light chain phosphatase, which is not dependent on calcium for activity, dephosphorylates myosin light chain and finally causes relaxation of fenestrae. ADP, adenosine diphosphate; ATP, adenosine triphosphate.
not result in additional alterations in actin organization or in adverse effects on cell shape and viability (Fig. 30.4B). Remarkably, within one hour of misakinolide treatment, we could observe with the aid of SEM small cytoplasmic unfenestrated areas, surrounded by rows of very small fenestrae within the area of fenestrated cytoplasm (Fig. 30.4C). To study these areas in more detail we applied whole-mount TEM to LSECs cultured on collagen-coated grids, after slight prefixation and extraction with detergent, as this technique allows the visualization of the cytoskeleton with minimal disruption of the cells (62) (Fig. 30.4D–F). Examination of control LSECs at low magnification showed the existence of an extensive network of cytoskeletal elements that fills and structurally organizes the cytoplasm (Fig. 30.4D). Treatment with misakinolide for 10 to 30 minutes resulted in the disappearance of microfilaments, and in the appearance of small cytoplasmic unfenestrated areas of intermediate electron density (gray centers) within the cytoplasm of all treated cells. In several of these unfenestrated areas, a singular structure could be observed, consisting of rows of fenestrae with increasing diameter, emanating from the gray centers and fanning out into the surrounding cytoplasm (Fig. 30.4E). These structures are suggestive of de novo fenestrae formation, and we therefore named them “fenestrae-forming center” (FFC). By two hours of treatment, the burst of fenestrae formation has subsided and the small unfenestrated areas (gray centers) did not show the presence of rows of fenestrae (Fig. 30.4F). Thorough investigation of swinholide A-, jasplakinolide-, cytochalasin B-, and latrunculin A-treated LSECs revealed only small unfenestrated areas, but no sign of connected fenestrae rows. The disassembly of actin filaments in LSECs by these compounds, together with the increase in the number of fenestrae and the presence of inactive FFCs, suggests nevertheless a common mechanism of fenestrae formation for all actin binding agents. Apparently, specific alterations in actin organization at particular locations and at particular times are required to bring to light nascent fenestrae emerging from the FFCs.

When the short period of time necessary to form new fenestrae is taken into account, it is most likely that this process does not involve de novo protein synthesis. We have performed experiments with cycloheximide, an inhibitor of protein synthesis, to check this hypothesis. In this set-up, the use of cycloheximide in combination with misakinolide resulted also in the appearance of active FFCs and in an increment in the number of fenestrae. Therefore, it is obvious that the process of fenestrae formation is not driven via protein synthesis. Instead, we consider a reorganization of preexisting FFCs. By using dry-cleaving of LSECs, we recently observed a sponge-like framework of three-dimensional organized fenestrae grouped along the nucleus, as early as 5 minutes after microfilament-disruption (59). We suppose therefore that FFCs are normally anchored to the actin cytoskeleton in the perinuclear area of LSECs, where they cannot be resolved in whole-mount TEM or TEM sections due to the mass thickness and/or the complex three-dimensional organization of the cytoskeletal proteins in this area (59). Disruption of the actin cytoskeleton can, therefore, free the preexisting FFCs from their anchor in the perinuclear region, resulting in their translocation into the 300- to 400-nm-thick peripheral cytoplasm, thereby giving rise to flattened FFCs as depicted in Figure 30.4E.

The fusion of two opposing cell membranes to form fenestrae in LSECs requires the presence of unique compositional membrane microdomains (57,63) and a cell membrane-associated cytoskeletal structure (61). Several theories have been used to model the possible mechanisms of membrane fusion and pore formation. In general, the process leading to membrane fusion is subdivided into the following: adhesion-dehydration; disappearance of the hydration bar-

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**FIGURE 30.4.** A: Actin distribution in control liver sinusoidal endothelial cells (LSECs) showing the presence of stress fibers (arrow), mainly oriented parallel to the long axis of the cells, and peripheral bands (arrowhead) of actin bundles that line the cell margin. Scale bar, 5 μm. B: LSECs treated with 25 nM misakinolide A for 10 minutes show a loss of actin bundles and the appearance of curly actin aggregates (arrow). Peripheral actin bands (arrowhead) are less dense and interrupted. Scale bar, 5 μm. C: High-power scanning electron micrograph of the fenestrated cytoplasm obtained after 1 hour of exposure to 25 nM misakinolide. Note a typical cytoplasmic unfenestrated area (asterisk), surrounded by rows of very small fenestrae (arrow). Scale bar, 250 nm. D–F: Transmission electron micrographs of whole-mount, formaldehyde-prefixed, cytoskeleton buffer-extracted LSEC of control (D) and misakinolide-treated LSECs. (E–F) D: Low magnification showing the cell nucleus (N) and extracted cytoplasm. Note that the sieve plates are well defined by a dark border (arrowheads). Inside the sieve plates, fenestrae can be observed (arrow). Scale bar, 2 μm. E: Treatment with 25 nM misakinolide A for 30 to 60 minutes resulted in the appearance of small cytoplasmatic unfenestrated areas of intermediate density (asterisk) within the fenestrated cytoplasm and showing the initial step of fenestrae formation (arrow). Scale bar, 200 nm. F: Low magnification showing the cell nucleus (N) and the highly fenestrated cytoplasm (small arrow) after 120 minutes of 25 nM misakinolide treatment. Compare with Fig. 4D for the difference from control. Note the thin cytoplasmic arms (arrowheads) which run from the nucleus into the cytoplasm. In the cytoplasm, inactive FFCs (large arrow) can be observed. Scale bar, 5 μm.
rier; contact between phospholipid bilayers, and molecular rearrangement, resulting in pore formation (64–66). As for LSECs, the first step corresponds to the formation of intramembrane protein-free zones (57), while the appearance of peristomal rings of sterols around fenestrae probably corresponds to the final step (63). It seems reasonable to consider that these events take place in the rim of FFCs. However, although nothing realistic can be said about the molecular composition of FFCs, we speculate that the presence of fusion proteins (64), which pull the bilayers of the cell membranes together on the rim of FFCs, may contribute as well to the fusion-fission process. In addition, based on our gallery of electron micrographs taken from misakinolide-treated LSECs, it became clear that the nascent fenestrae emanating from an FFC are already decorated with the earlier described fenestrae-associated cytoskeleton ring (FACR) (Fig. 30.4E) (62). This probably indicates that FFCs already contain the necessary cytoskeletal proteins for assembling the FACR.

In conclusion, despite the multidisciplinary approach taken to study the structure, origin, dynamics and formation of fenestrae, there are still important gaps in information. Our knowledge needs considerable consolidation and expansion at the structural and biochemical level to reveal how the different proteins form a contractile unit, and which signal transduction pathways are involved in fenestral dilatation and relaxation. Moreover, further investigation is needed to determine the molecular composition of the FACR, the FFC, and the exact mechanism of fenestrae formation in the FFCs.

For the near future, we expect that forthcoming research will focus on therapeutic strategies by altering the sieve's porosity. For example, the discovery of new drugs that increase the porosity of the liver sieve may be of great benefit. Such agents may not only improve the extraction of atherogenic lipoproteins from the circulation; they could also be used for enhancing the efficiency of liposome-mediated gene or drug delivery to parenchymal cells.

**PIT CELLS, THE LIVER-SPECIFIC NATURAL KILLER CELLS**

Natural killer (NK) cells are functionally defined by their ability to kill certain tumor cells and virus-infected cells without prior sensitization (67). NK cells comprise about 10% to 15% of lymphocytes in the peripheral blood, and most of these cells in human and rat have the morphology of large granular lymphocytes (LGL) (68). However, it has been demonstrated that small agranular lymphocytes, lacking CD3 expression, have cytolytic activity comparable to that of NK cells (69). These variations may be related to the stage of NK cell differentiation or heterogeneity (70). Moreover, some cytotoxic T lymphocytes (CTL) also display LGL characteristics (70). Besides NK cells in peripheral blood, NK cells are also found in tissue compartments such as the spleen, lung, intestine, lymph nodes, bone marrow and liver (70). NK cells in the liver, also called pit cells (71), constitute a unique resident population in the liver sinusoids. Their immunophenotypical, morphological and functional characteristics differ from those of blood NK cells (72). Morphologic and cytotoxic differences between pit cells of different species have been observed (73).

**Identification, Structure, and Tissue Distribution of Pit Cells**

Pit cells were first described in 1976 by Wisse (71). The name pit cell was introduced because of the characteristic cytoplasmic granules, which in Dutch language are called pit, resembling the pits in a grape (71). The hypothesis that pit cells might possess NK activity was formulated by Kaneda et al. (74), and was based on their morphologic resemblance to LGL. The isolation and purification of pit cells from rat liver and the evidence of spontaneous cytotoxicity against YAC-1 cells confirmed these cells to be hepatic NK cells (75–76).

Pit cells inhabit the liver sinusoids and often adhere to LSECs, although they incidentally contact KCs (Fig. 30.5).
They face the blood directly. Pseudopodia of pit cells can penetrate the fenestrae of the LSECs and enter the space of Disse, and can directly contact the microvilli of hepatocytes (4,71). Their appearance in the space of Disse is not a common feature (77). By morphological investigation, the frequency of pit cells in rat liver tissue was found to be about an average of 1 pit cell per 10 KCs. The number of pit cells, in untreated rats, is therefore estimated to be 1.4 to 2 × 10⁶ cells per gram liver weight (75). By immunohistochemistry, using mAb 3.2.3 against NKR-P1A (a specific marker of NK cells), the number of pit cells in frozen sections of rat liver was counted as 13.7 per mm² (78). After intravenous injection of biological response modifiers (BRM), the number of pit cells increases 4- to 6-fold in rat liver treated with zymosan (79), and 43-fold with interleukin-2 (IL-2) (80). The surplus of pit cells is considered to originate from local proliferation and from the bone marrow (79–80). Pit cells were found to be more numerous in the periportal than in the pericentral region of the liver lobule (74,78).

Pit cells have essentially the same morphology as NK cells from blood and other organs, that is, LGL morphology (Fig. 30.5). The cells are characterized by a relatively large size compared to other lymphocytes, the presence of granules in the cytoplasm, a pronounced asymmetry of the cell and an indented or kidney-shaped nucleus of high density (81). Pit cells in the rat are about 7 μm in diameter and vary in shape, possessing well-developed pseudopodia. They show a pronounced polarity with an eccentric nucleus and most organelles lying at one side of the nucleus.

The most conspicuous organelles are the electron-dense granules. These granules have several characteristics. They are azurophilic and can be seen after Giemsa staining of a cell smear or cytospin preparation with light microscopic examination. As measured by electron microscopy, the granules differ in size between different pit cell subpopulations [low-density (LD) and high-density (HD) pit cells], but within one cell type the granules are rather homogeneous with respect to size, shape and electron density (82). The granules are membrane-bound and range in size between 0.2 μm in LD pit cells and 0.5 μm in lymphokine-activated killer (LAK) cells. The granules contain lysosomal enzymes, such as acid phosphatase (75,83). Although perforin and granzymes, which have been isolated from NK cell granules (84–85), have not yet been identified in pit cell granules, it is believed by analogy that these molecules are present in the granules of pit cells.

Rod-cored vesicles are small inclusions, ranging in diameter from 0.17 to 0.2 μm, and are exclusively found in LGL (74,83). They contain a straight rod structure that is 30 to 50 nm in length, which bridges the entire diameter of the vesicle (74,83). Rod-cored vesicles derive from and distribute preferentially around the Golgi apparatus. Possibly rod-cored vesicles may also contain cytotoxic factors functioning in natural cytotoxicity (77).

Pit cells also exist in human and mouse liver, but their identification is difficult because they contain a low number of small granules and only a very few rod-cored vesicles (86–88). On the other hand, 5% to 25% of human pit cells contain “parallel tubular arrays” (PTA), which were also reported in human blood NK cells and are considered as a characteristic of these cells (72,87).

### Surface Phenotype of Pit Cells

Most surface antigens found on rat pit cells are similar to those found on spleen or blood NK cells (Table 30.1) (76,78,89,90). Pit cells were found to express NKR-P1 (78) (Fig. 30.6). NKR-P1 was first identified in the rat (91) and has now been shown to be expressed by mouse and human

| TABLE 30.1. CHARACTERISTICS OF RESTING NK CELLS, LD, HD PIT CELLS, AND IL-2-ACTIVATED-NK CELLS |
|---------------------------------------------------|-------------------------------------------------|----------------------------------|---------------------------------|--------------------------|
| **Morphology** | Resting NK Cells | HD Pit Cells | LD Pit Cells | IL-2-Activated-NK Cells |
| Size of the cell (μm) | 6–7 | 6–7 | 6–7 | 8–13 |
| Number of rod-cord vesicles per cell | 0.5 | 0.8 | 1.0 | n.d.³ |
| Size of specific granules (μm) | Large (0.3–0.5) | Intermediate (±0.2) | Small (±0.17) | Very Large (0.5–0.9) |
| Number of granules per cell | Low (n = 10) | Intermediate (n = 20) | High (n = 50) | n.d. |
| **Surface antigens** | | | | |
| CD2 | 80 | 80 | 80 | 100 |
| CD8 | 40 | 100 | 100 | n.d. |
| CD11a | 54 | 90 | 90 | 90–100 |
| CD18 | 90 | 90 | 90 | 90–100 |
| CD54 | 35 | 35 | 35 | 97 |
| Asialo-GM1 | 100 | 70 | 36 | n.d. |
| NKR-P1 | 94 | 95 | 95 | 95 |
| **Cytotoxicity** | | | | |
| NK activity | + | ++ | +++ | ++++ |
| P815 cell killing | – | – | + | + |

Data summarized from references 68, 76, 78, 82, 83, 89, and 91; ³n.d., no data; ³Approximate percentage of cells that express antigen.
NK cells (92–93). NKR-P1 is present on 94% of rat LGL and serves as a molecule triggering cytotoxic action (91). The anti-NKR-P1 monoclonal antibody (mAb) 3.2.3 is considered to be useful for NK cell identification (91). However, a subset of T lymphocytes and polymorphonuclear leukocytes (PMN) also expresses NKR-P1 (91,93). CD11a is present on 90% of rat pit cells, which is different from rat peripheral blood NK cells (54%) (89). Approximately 90% of rat pit cells express CD18, 35% express CD54 and 80% express CD2 (89). Asialo-GM1, which is expressed by all rat blood NK cells (82), is present on 36% of LD pit cells and 70% of HD pit cells (82). CD8, a marker of NK cells and cytotoxic T lymphocytes (68), is present on all rat pit cells (76). However, the composition of CD8 in NK cells and T lymphocytes is different. Most CD8+ NK cells express CD8α/CD8α homodimers rather than the CD8α/CD8β heterodimers prevalent on cytotoxic T cells (94). In addition, rat pit cells do not express T cell receptor and CD5 antigen (a pan T cell marker) (76–77).

**Isolation and Purification of Pit Cells**

The isolation method for rat pit cells is based on a nonenzymatic, high-pressure washout technique (75) followed by purification, based on the magnetic negative selection of cells using mAbs against surface antigens found on T and B cells (72,95). Since pit cells are apparently not heavily anchored in the liver sinusoids, the cells can be washed out by a nonenzymatic, high-pressure (50 cm water) perfusion of the liver via the portal vein with phosphate-buffered saline supplemented with 0.1% EDTA (75). The washout is collected from the vena cava. The erythrocytes, granulocytes and cell debris in the washout are removed by Ficoll-Paque gradient centrifugation. The mononuclear cells recovered from the interface of Ficoll-Paque gradient are composed of T cells, pit cells, B cells, monocytes, and a few LSECs. Adherent monocytes and B cells in this population can be selectively removed in a nylon wool column (75). Pit cells are further purified by magnetic cell sorting (95). With this system, a highly purified population of pit cells can be obtained by negative selection, that is, by elimination of remaining monocytes, T and B cells using specific antibodies and immunomagnetic beads. By this method, pit cells with a purity of more than 90% and a viability of more than 95% can be obtained (Fig. 30.7). Moreover, this nonenzymatic method does not destroy cell surface molecules.

**Heterogeneity and Origin of Pit Cells**

A considerable set of data indicates that rat pit cells constitute a heterogeneous population. Based on the cell density, pit cells can be separated into LD and HD cells by apply-
ing 45% iso-osmotic Percoll gradient centrifugation (82). These two cell populations have been shown to differ immunophenotypically, morphologically and functionally from each other and from blood LGL (Table 30.1) (82,89,90,96). LD pit cells (Fig. 30.8A) contain more rod-cored vesicles and more but smaller granules than blood NK cells (Fig. 30.8B) (82). The number of rod-cored vesicles and granule composition (number and size) of HD pit cells are intermediate between LD pit cells and blood NK cells (82). Immunophenotypically, almost all blood NK cells are asialo-GM1 positive, and 70% of HD pit cells are strongly positive, whereas only 36% of LD pit cells are weakly positive (82). Furthermore, functional differences have been observed among these three populations. The LD pit cells are more cytotoxic against YAC-1 cells and colon carcinoma (CC531s) cells than blood NK cells (96). The HD pit cells have intermediate cytotoxic activity between LD pit cells and blood NK cells (96). In addition, LD pit cells are able to lyse LAK-sensitive P815 mastocytoma targets, which are resistant to normal blood NK cells and hepatic HD pit cells (96).

Pit cells are considered to originate from blood NK cells (97). Several types of evidence support the concept that blood NK cells immigrate into the hepatic sinusoids to become HD pit cells, which further differentiate into LD pit cells. Importantly, the characteristics and functions of HD pit cells are intermediate between blood NK cells and LD pit cells (97). Kinetic experiments with sublethal total body irradiation (700 cGy) showed that blood NK cells and HD pit cells were depleted about one week after irradiation, whereas LD pit cells had totally disappeared at two weeks after irradiation. Shielding of the liver gave similar results, and splenectomy did not affect pit cell number (97). With the use of intravenous anti-asialo-GM1 antiserum injection, blood NK and HD pit cells totally disappeared within one week of treatment, whereas LD pit cells disappeared from the liver one week later (97). The direct evidence for LD pit cells originating from asialo-GM1-positive precursors (blood NK and HD pit cells) was given by the adoptive transfer of fluorescent-labeled HD pit cells into syngeneic rats. After three days, 5% of labeled cells were recovered in the LD fraction, and these cells displayed typical LD pit cell morphology (97). These observations also indicate that the lifespan of pit cells in the liver is about two weeks (72,97).

The mechanism behind the migration of blood NK cells to the liver sinusoids is not fully understood. Several adhesion molecules were found to be involved in the process (89). Rat blood NK and pit cells express LFA-1 (CD11a/CD18) and CD2 (LFA-2) adhesion molecules (89). Their ligands, CD54 (ICAM-1) and CD58 (LFA-3) were found to be present on liver LSECs (98). After intravenous injection of antibodies against CD2, CD11a and CD18 into rats, the number of pit cells in the liver decreased significantly, indicating that the interactions of LFA-1/CD54 and CD2/CD58 are involved in the recruitment of pit cells to the liver (89).

Once marginated in the liver sinusoids, blood NK precursors first differentiate into HD pit cells, then into LD pit cells. The microenvironment of the liver sinusoid is believed to be responsible for this differentiation process (99). Van-
derkerken et al. (99) found that KCs were selectively eliminated three days after intravenous injection of liposomes containing the cytotoxic drug dichloromethylene diphosphonate. This treatment kills the KCs, whereas other cells remain unharmed. The number of HD pit cells declined three days after the injection. At that time, the LD pit cell population showed no change, but a decline of about 80% was seen seven days after the injection (99). These data indicate that pit cells are KC-dependent, and therefore it is supposed that KCs play a role in the differentiation of pit cells in the liver. However, it remains unclear what factor(s) derived from KC are responsible for this differentiation. In addition, LSECs may work synergically with KCs and may contribute to pit cell differentiation, since co-culture of HD pit cells with KCs failed to induce the full differentiation of HD into LD pit cells (99).

Functions of Pit Cells

As a member of the NK cell family, pit cells have demonstrated cytotoxic activity against various tumor cells. The production of cytokines and participation in the resistance to microbial pathogens is less well known (67). Rat pit cells have a high spontaneous cytotoxic activity against various tumor cell lines, such as YAC-1, P815, CC531s, DHD-K12, L929, 3LL, and 3LL-R (81). Compared with blood NK cells, pit cells are four to eight times more cytotoxic against YAC-1 and CC531s cells, and are able to kill the NK-resistant but LAK-sensitive P815 cells (82,96,100). This evidence supports the fact that pit cells become activated by the hepatic microenvironment. Furthermore, NK activity in the liver could be augmented by BRM (79,88). Interestingly, an increase in function seems to coincide with a large increase in the number of LGL (79,88).

Mechanisms in Pit Cell-Mediated Cytotoxicity

It is believed that, like NK cells (101), pit cell-mediated target killing is a multistep process, including recognition of target cells, binding of effector to target cells (conjugation), activation of effector cells, delivery of the lethal signal to target cells, and effector cell detachment and recycling (68,70,101).

The prerequisite of pit cell killing is the binding of one or more effector cells to a target cell (72). Adhesion molecules are considered to be responsible for this process. It has been found that the interaction between β2 integrins (CD11a/CD18) and ICAMs (intercellular adhesion molecules) is the most important mechanism of binding of NK cells to their targets (102–103). In addition, LFA-1 (CD11a/CD18) also participates in the signal transduction in NK cells required for NK cell activation (104). Crosslinking of LFA-1 on NK cells with its antibody is known to induce a calcium influx, phosphoinositide turnover, and tumor necrosis factor-α (TNF-α) production (104), and to inhibit the target cell killing by NK cells (105). LFA-1 was also found to be involved in pit cell-mediated cytotoxicity. The antibody against LFA-1 inhibits not only the binding of pit cells to target cells, but also the killing of target cells by pit cells (106). Taken together, this information suggests that LFA-1 on effector cells may have a dual function of binding to target cells and triggering cytolysis.

CD2 is an adhesion molecule involved in T cell (107) and NK cell cytotoxicity (108–109). Approximately 80% of rat pit cells express CD2 (89). Anti-CD2 mAb had no effect on the binding of pit cells to CC531s, or on the cytotoxicity against CC531s cells (106). However, the anti-CD2 mAb enhanced the cytolytic activity of rat pit cells against FcγR+ P815 target cells (106).

NKR-P1 is a well-known triggering receptor on NK cells (102,110). mAbs against mouse and rat NKR-P1 were found to trigger NK cell-mediated lysis of FcγR+ target cells, termed re-directed antibody-dependent cellular cytotoxicity (ADCC) (91). This action also involves a rise in intracellular Ca++ levels (111) and cytokine production (112). Furthermore, mAbs to NKR-P1 stimulate phosphoinositide turnover (111), arachidonic acid generation (113) and granule exocytosis (91). NKR-P1 has been found to be involved in pit cell-mediated cytotoxicity against FcγR+ P815 target, but not in FcγR+ CC531s target killing (114). These data indicate that NKR-P1 and CD2 depend on subclass specificity of target cell IgG-FcR (109), and may serve as activation structures on pit cells.

NK cytotoxicity was originally thought to be spontaneous and major histocompatibility complex (MHC) class I-unrestricted. However, increasing evidence indicates that NK cells preferentially kill cells lacking MHC class I (115–119). Masking of MHC class I by an mAb enhances pit cell-mediated cytotoxicity against CC531s cells, indicating that MHC class I on CC531s cells protects these cells from being killed by rat pit cells (114). An explanation of this observation is that the cytotoxic activity of NK cells is regulated by positive and negative signals from triggering and inhibitory membrane receptors. The final outcome, that is, triggering of cytotoxic activity or inhibition of cytotoxicity, appears to depend on the balance between the positive and negative signals (102,120). Inhibitory receptors on effector cells recognize MHC class I, and this recognition generally inhibits the lysis of MHC class I+ cells (110,120–123). However, inhibitory receptors like Ly49 have not been directly identified on pit cells yet.

Studies have demonstrated that NK cell-mediated cytotoxicity can mainly be implemented by two pathways, the perforin/granzyme (granule exocytosis) pathway and the Fas/FasL pathway (124,125). The perforin/granzyme pathway is a Ca++-dependent pathway and is mediated by the pore-forming protein perforin and granzymes, especially granzyme B, both of which are stored in NK cell granules (125), of which pit cells have plenty (82). After contact...
between effector and target cells, perforin and granzymes are released in a directed manner into the intercellular space between these cells. Perforin alone induces lysis without inducing apoptosis, that is, fragmentation of target cell DNA. Granzymes play a critical role in the rapid induction of DNA fragmentation by CTLs, NK cells and pit cells (126,127). It has been shown that pit cells induce apoptosis in CC531s tumor cells (Fig. 30.9) by the perforin/granzyme pathway (126).

The Fas pathway of apoptosis is mediated by the interaction of Fas ligand (FasL, CD95L) with the apoptosis-inducer Fas (CD95/APO-1) molecule expressed on target cells (124,128,129). Fas is widely expressed on lymphoid and nonlymphoid cells, and some tumor cells (126,129). FasL is expressed by activated T cells, NK cells and pit cells (126,129). It has been demonstrated that Fas/FasL plays an important role in the killing of virus-infected cells and tumor cells by CTLs and NK cells (130). Although Fas is expressed on CC531s cells and FasL is expressed on rat pit cells, pit cell-mediated CC531s apoptosis was found to be exclusively implemented by the perforin/granzyme exocytosis pathway (126).

In conclusion, there is growing evidence that pit cells are highly active, liver-specific NK cells. Pit cells are located in the liver sinusoids and can be separated into LD and HD fractions by 45% iso-osmotic Percoll gradient centrifugation. These two subpopulations differ morphologically, phenotypically and functionally from each other and from blood NK cells. LD pit cells contain a higher number of small granules, have a higher expression of LFA-1, are more cytotoxic against several tumor cell lines as compared to blood NK cells, and are able to kill NK-resistant but LAK-sensitive P815 cells. The characteristics of HD cells are intermediate between those of LD pit cells and blood NK cells. Pit cells most probably originate from blood NK cells, and the recruitment of pit cells in the liver is mediated by adhesion molecules. A major challenge is to achieve a better understanding of the mechanisms of pit cell cytotoxicity and the cooperation between pit cells and other cells in the liver (i.e., KCs, LSECs, T-, and NK-T cells). Moreover, since pit cells are located in a strategic position in the hepatic sinusoids, they represent a first line of cellular defense against metastasizing colon cancer cells. The role of pit cells in a number of liver pathologies, such as viral hepatitis, deserves more attention.

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