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Small Epithelial Cells and the Histogenesis of Hepatoblastoma

Electron Microscopic, Immunoelectron Microscopic, and Immunohistochemical Findings

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The wide range of epithelial and mesenchymal lines of differentiation seen in hepatoblastoma suggests that this tumor derives from a pluripotent stem cell. To test this hypothesis, seven hepatoblastomas of various subtypes were investigated for the presence of cells with the features of the oval cells found during hepatocarcinogenesis in rodents that are thought to be closely related to hepatic stem cells. Because similar cells, referred to as "small cells," have been described in human liver disease with chronic ductular reaction, five liver biopsies from infants with biliary atresia were also investigated. The specimens were investigated by electron microscopy, immunoelectron microscopy, and immunostaining for cytokeratins 7, 8, 18, and 19. Small epithelial cells (SEC) corresponding to the oval cells of the rat and the "small cells" in humans were found in both biliary atresia and hepatoblastoma. These cells were oval and exhibited intercellular junctions, tonofilament bundles, and a biliary epithelium-type cytokeratin profile. SEC were found in small numbers in fetal hepatoblastoma and in moderate numbers in embryonal hepatoblastoma. In small cell hepatoblastoma, nearly all the tumor cells exhibited SEC-like ultrastructural features and a corresponding cytokeratin profile. Thus, cells exhibiting morphological and immunophenotypic features of hepatic stem cells are detectable in hepatoblastoma. Their numbers vary according to the subtype, reflecting the differing degrees of differentiation of the various subtypes, consistent with the theory propounded in the literature that embryonal and, with further differentiation, fetal tumor cells derive from precursor small cells. The findings support the hypothesis that hepatoblastoma derives from a pluripotent, probably entodermal or even less committed, stem cell. (Am J Pathol 1996, 148:321–329)

Hepatoblastoma, the most common malignant tumor of the liver in childhood, exhibits a wide range of epithelial and mesenchymal lines of differentiation,[1] on which the subclassification of this tumor is based. The classification system of Ishak and Glunz[2] distinguishes between pure epithelial and mixed hepatoblastoma, the latter containing both epithelial and mesenchymal components, eg, osteoid or spindle cell areas. The epithelial hepatoblastomas include the fetal and embryonal subtypes, so named on the basis of histological similarities with these stages of liver development. The least differentiated epithelial form is the small cell or anaplastic subtype.[3] Endocrine, melanocytic, or neural differentiation may also be seen.[4–7] The histogenesis of this interesting tumor has not yet been established. However, its multidirectional spectrum of differentiation has led both us and other authors to advance the hypothesis that hepatoblastoma derives from a pluripotent stem cell.[7–10]

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In humans, unlike in rodents, the number and significance of hepatic stem cells, from which both hepatocytes and biliary epithelial cells are thought to be derived, have been investigated in relatively few studies.[11–14] It has recently been shown that so-called "small cells" appear in the human liver in hepatic diseases accompanied by chronic ductular reaction.[12] These cells exhibit morphological similarities with the oval cells found in the liver of rodents after exposure to hepatocarcinogens. The oval cells exhibit a cytokeratin (CK) profile similar to that of biliary epithelial cells and are thought to represent the stem cells of the liver, or at least be closely related to these cells (see review[15]). This study was undertaken to investigate whether such cells are also found in hepatoblastoma and, if so, what their significance could be in the histogenesis of this tumor. The material investigated consisted of hepatoblastomas of various subtypes and liver biopsy specimens from cases of extrahepatic biliary atresia (BA), a disease of childhood in which there is a pronounced chronic ductular reaction. Small epithelial cells (SEC) similar to the oval cells were found in both conditions. These cells were studied by electron microscopy and their cytokeratin profile investigated by immunohistochemistry and immunoelectron microscopy.

Materials and Methods

Seven hepatoblastomas (from patients aged 7 months to 5 years, 4 months) and five liver wedge biopsy specimens from patients with BA (age range 2 weeks to 4 months) were investigated. Three hepatoblastomas contained a mesenchymal component and were thus classified as mixed hepatoblastomas. Of the four pure epithelial tumors, two contained both fetal and embryonal areas, and two were pure small cell hepatoblastomas. The specimens were all obtained at operation except in the case of one small cell hepatoblastoma, from which only tissue obtained by needle biopsy was available. Adjacent normal liver tissue was also investigated in four cases. Tissue from each specimen was fixed in 4% formalin and embedded in paraffin. Sections were cut at 5 μm and stained with hematoxylin and eosin or dewaxed and immunostained by the avidin-biotin-peroxidase complex (ABC) method[16] with the antibodies listed in Table 1. 3,3′-diaminobenzidine (Sigma Chemical Co., Munich, Germany) was used as the chromogen.

For electron microscopy, tissue from four hepatoblastomas and all the BA cases was fixed in 4% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.3) for 4 hours immediately after resection, post-fixed in 2% osmium tetroxide in cacodylate buffer (pH 7.4), and embedded in Araldite (Serva, Heidelberg, Germany). Tissue from one mixed hepatoblastoma and one small cell hepatoblastoma that had initially been embedded in paraffin was re-embedded in Araldite. Ultrathin sections were contrasted with 5% uranyl acetate and lead citrate.

Table 1. *Antibodies Used in the Study*

Antibody* against	Dilution
CK-7†	1:25
CK-8‡	1:25
CK-18‡	1:20
CK-19†	1:50

*All antibodies listed were obtained from DAKO (Glostrup, Denmark).
†Pretreatment of sections with 0.1% pronase (Sigma, Munich, Germany) for 7 minutes at 37°C.
‡Microwave pretreatment of sections in citrate buffer (pH 6.0) for 3 × 5 minutes.

Tissue from all the BA cases and one mixed hepatoblastoma (with both fetal and embryonal areas and calcified osteoid) was also available for investigation by immunoelectron microscopy. This was undertaken to investigate whether the SEC, which in most cases occurred in only very small numbers and could not be identified with certainty at the light microscopic level, expressed CK-7, an intermediate filament typically expressed by biliary epithelium and hepatic stem cells. The tissue was fixed in 3% paraformaldehyde/0.1% glutaraldehyde in phosphate-buffered saline (PBS, pH 7.4) for 2 hours at room temperature and embedded in Lowicryl K4M (Bal-Tec, Walluf, Germany). Ultrathin sections were collected on nickel grids coated with Formvar (Merck, Darmstadt, Germany). They were then incubated with 0.5% bovine serum albumin and 0.1% gelatin in PBS (PBS-BSA), followed by 10% goat serum diluted in PBS-BSA. They were next incubated with the primary antibodies for 90 minutes, washed with PBS-BSA, and incubated with gold-labeled goat anti-mouse immunoglobulin G (IgG) (Amersham International, Little Chalfont, UK) diluted 1:20 in PBS-BSA. The sections were contrasted with uranyl acetate and lead citrate. For negative controls, the primary antibody was replaced by PBS-BSA.

Results

Conventional Histology

The BA biopsy specimens exhibited the histological picture typical of this disease, with portal fibrosis,

cholestasis, and proliferation of bile ductules. Cirrhosis was seen in one case.

The histological features of the various different epithelial and mesenchymal components of the hepatoblastomas also corresponded largely to the typical histological picture described in the literature.[1–3,17] The fetal areas contained cords and clusters of large tumor cells with distinct borders, abundant, usually pale, cytoplasm, and only slightly pleomorphic nuclei. The tumor cells in the embryonal areas were smaller, had basophilic cytoplasm, a higher nuclear/cytoplasmic ratio, markedly pleomorphic nuclei, and greater mitotic activity. Tubular structures were often seen here. The small cell hepatoblastomas contained sheets of small, anaplastic tumor cells with high mitotic activity. A few small tubular structures were also seen in one case. The mesenchymal component of the mixed hepatoblastomas was represented by osteoid and/or spindle cell areas. The epithelial component consisted of fetal and embryonal areas in two cases and embryonal areas in one case. One of the mixed hepatoblastomas, which contained both melanin-producing cells and cells with endocrine differentiation, is described in detail elsewhere.[7] In another of the mixed hepatoblastomas, areas with tubular differentiation that resembled proliferating bile ductules were seen both at the periphery of embryonal and fetal areas and in connective tissue septa.

Immunohistochemistry

In the normal liver, the biliary epithelium was immunoreactive for CK-7, CK-8, CK-18, and CK-19, and the hepatocytes for CK-8 and CK-18. In addition, a few cells in the liver parenchyma, especially near the portal tracts, were immunoreactive for CK-7 and CK-19. These cells were smaller than hepatocytes and were often clustered in small groups of up to five cells.

The same staining pattern was seen in the BA cases. The bile duct epithelium was found to exhibit all the CKs investigated, and the hepatocytes CK-8 and CK-18. The number of small cells in the parenchyma that stained for CK-7 and CK-19 was slightly greater than in the normal liver.

In the hepatoblastomas, the great majority of tumor cells in the fetal and embryonal areas and in the small cell tumors was reactive for CK-18. The number of cells reactive for CK-8 was generally small in the fetal areas, small to moderate in the embryonal areas, and moderate in the small cell hepatoblastomas.

Cells immunoreactive for CK-19 were generally very few in number in fetal areas. However, in embryonal areas they were found in moderate numbers (Figure 1a), cells in the tubular structures being particularly strongly stained. CK-19-immunoreactive cells were often located predominantly in the periphery of the tumor nodules, and in the tumor with ductular differentiation they were also seen in large numbers at the periphery of fetal areas. In small cell hepatoblastoma the overwhelming majority of the tumor cells was immunoreactive for CK-19 (Figure 1b). Immunoreactivity for CK-7 was generally absent in fetal areas, but a few stained cells were seen in the embryonal areas, again mainly in the tubular structures. The tumor with ductular differentiation was again an exception in which numerous stained cells were found at the periphery of fetal and embryonal areas. In the small cell hepatoblastomas, only occasional stained cells were seen in the solid areas, but the cells in the tubular structures found in one tumor were, almost without exception, all stained (Figure 1c).

A few cells immunoreactive for CK-7 (Figure 1d), CK-18, and CK-19 were found in osteoid areas, but no definite immunoreactivity for any of the CKs was found in the spindle cell areas.

Electron Microscopy

The ultrastructural appearances of the biliary epithelial cells and hepatocytes in BA were the same as those described by other authors.[18,19] Unlike in the normal liver, a very small number of cells that have not previously been described in BA were found in the peripheral parts of the portal tracts and adjacent parts of the lobules. These represent the cells referred to as SEC. They were characterized by their oval shape; small size (7 to 18 μm maximum diameter); scanty electron-dense cytoplasm; an oval, electron-dense nucleus in which the heterochromatin was seen as small clumps dispersed in the nucleoplasm with peripheral condensation; tonofilament bundles; and tight junctions or desmosome-like junctions (Figure 2). Such junctions were found both between SEC and hepatocytes and between SEC and the epithelial cells of small bile ductules. The number of organelles in the SEC varied. Some contained numerous free ribosomes, and others contained free ribosomes and rough endoplasmic reticulum. A few SEC exhibited small surface microvilli and formed bile canaliculi with neighboring hepatocytes. Some SEC were partly surrounded by a basement membrane. A more detailed classification of the SEC in BA into different morphological subtypes

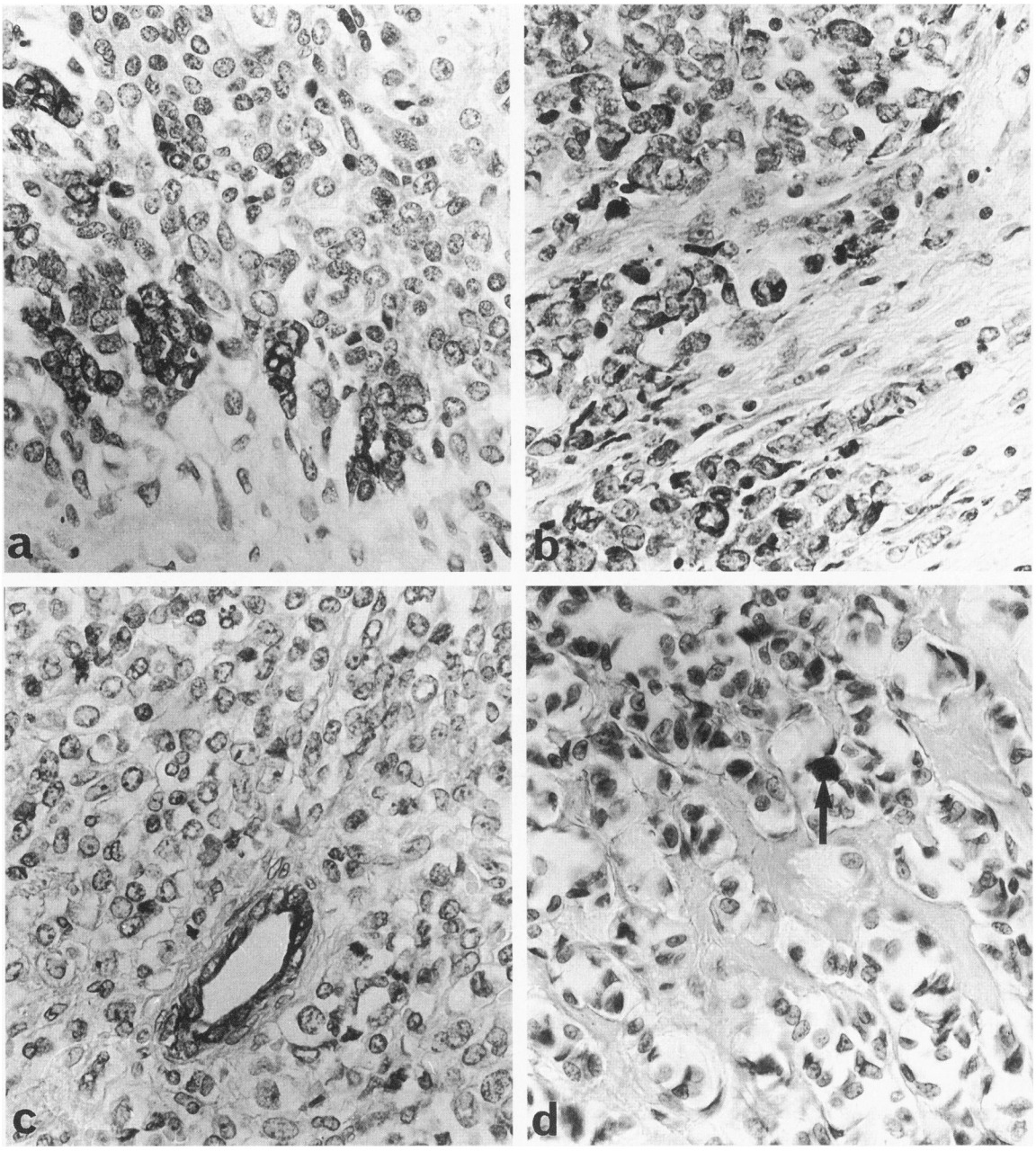

Figure 1. (a) *Embryonal hepatoblastoma. A moderate number of tumor cells, mainly at the periphery of the tumor, are reactive for CK-19.* (b) *Small cell hepatoblastoma. The great majority of the tumor cells is reactive for CK-19.* (c) *Small cell hepatoblastoma. The tumor cells in a tubular structure are reactive for CK-7, but other tumor cells remain unstained.* (d) *Osteoid from a mixed hepatoblastoma. A single cell with reactivity for CK-7 is seen* (arrow). a–d: ×*360.*

will be described elsewhere (J-C Xiao, P. Ruck, and E. Kaiserling, manuscript in preparation). The SEC differed from hepatocytes in their shape, their higher nuclear/cytoplasmic ratio, their more electron-dense nucleus, and the presence of tonofilament bundles. They differed from biliary epithelial cells in their more electron-dense cytoplasm and the fact that most exhibited no basement membrane. They could be distinguished from Ito cells, which also have an electron-dense cytoplasm, by their tonofilaments, tight junctions, and lack of dense bodies and fat droplets.

Immunoelectron microscopy revealed immunoreactivity of the SEC for CK-7. The immunogold marking was located in relation to the tonofilament bundles.

The electron microscopic findings in the fetal and embryonal areas of the hepatoblastomas also largely corresponded to those reported in the literature.[17,20]

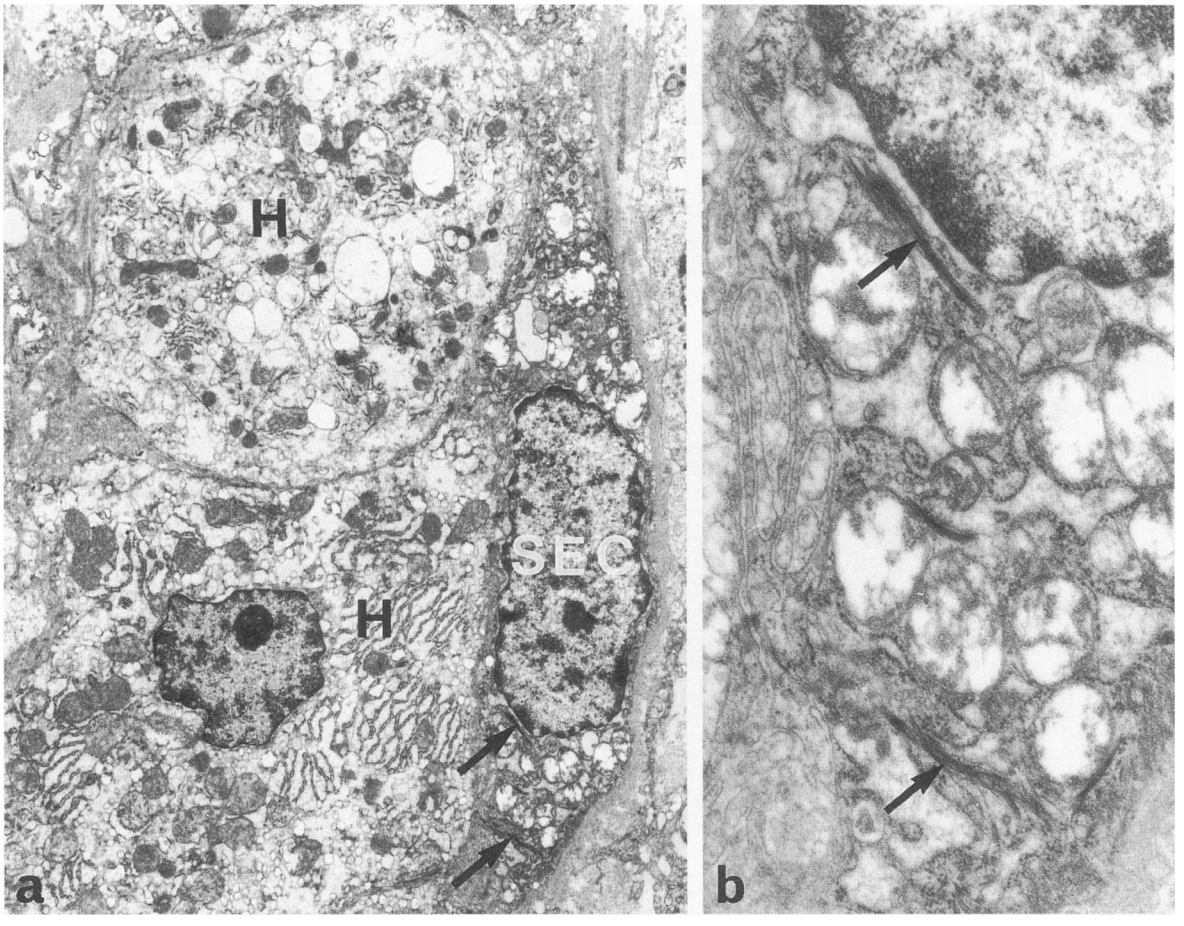

Figure 2. *BA.* (a) *An SEC can be seen at the periphery of a lobule (H = hepatocytes). The SEC is oval in shape, has an electron-dense cytoplasm, and contains an oval nucleus with peripherally condensed heterochromatin. The cytoplasm contains tonofilament bundles* (arrows), *as seen in the area from the lower part of* a *shown at higher magnification in* b. a: ×5000; b: ×25,000.

No mesenchymal areas were present in the tissue available for electron microscopy. Cells with the same morphology as the SEC noted in BA were found in small numbers in fetal areas (Figure 3) and moderate numbers in embryonal areas, and were found mainly at the periphery of the tumor nodules. Tight junctions were often seen between SEC and the tumor cells. In small cell hepatoblastoma, the overwhelming majority of the tumor cells exhibited an ultrastructure very similar to that of the SEC, except that these cells and their nuclei were often more round than oval (Figure 4). The diameter of these tumor cells was about 7 to 14 μm, the cytoplasm and nucleus were relatively electron dense, and the heterochromatin was seen as small clumps dispersed in the nucleoplasm with peripheral condensation. These tumor cells also contained numerous tonofilaments, were joined by intercellular junctions, and occasionally exhibited microvilli.

The tonofilaments of the SEC in hepatoblastoma, as in BA, were found on immunoelectron microscopic investigation to be marked by the antibody against CK-7 (Figure 5).

Discussion

Most of the information relating to stem cells in the liver has been obtained in investigations of experimental hepatocarcinogenesis in rodents, in which the administration of hepatocarcinogenic substances leads to proliferation of oval cells with distinct morphological features, including a narrow rim of cytoplasm and an oval cell nucleus.[9,21–26] It is believed that these oval cells are closely related to hepatic stem cells. The actual stem cell compartment in the liver is probably located in the transitional bile ductules, which link the bile canaliculi to the canals of Hering, and/or in a distinct population of periductular cells.[9,26–28] Few studies have investigated stem cells in the human liver. Cells with morphological and immunophenotypic features of rat

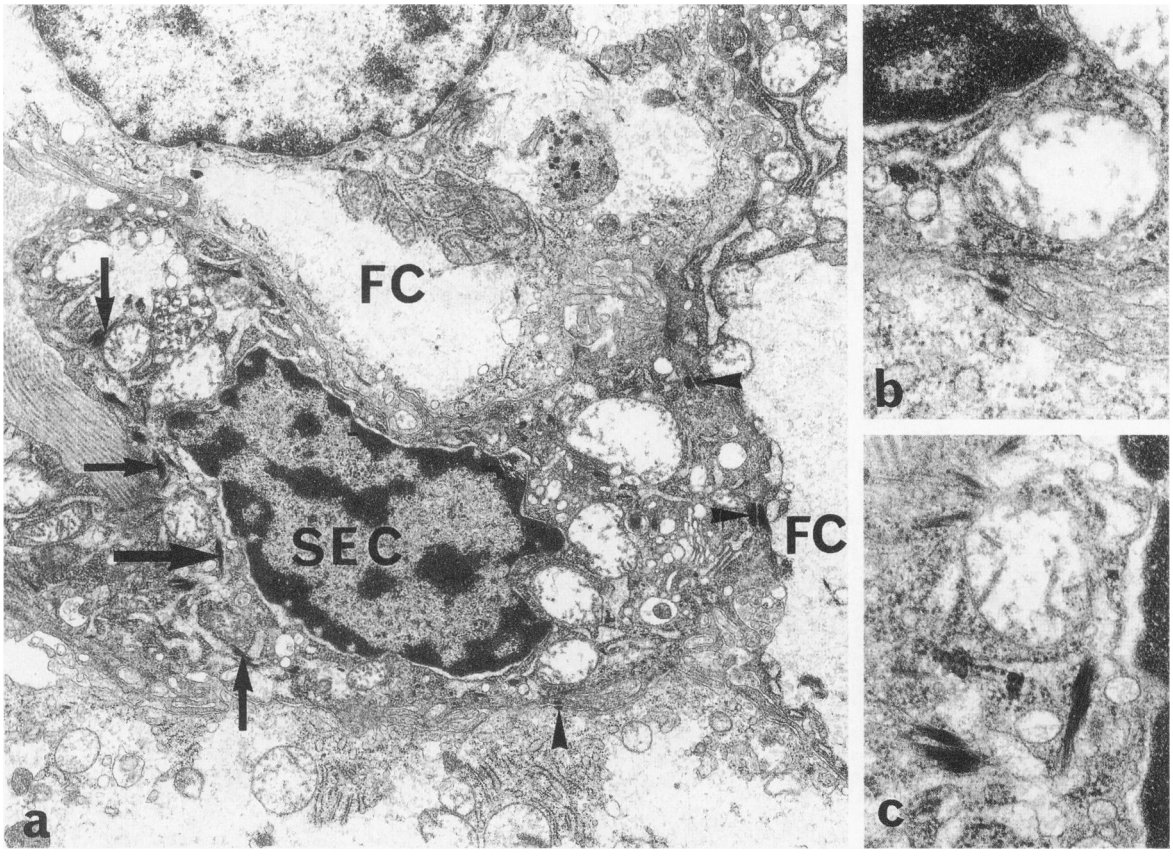

Figure 3. *Fetal hepatoblastoma.* (a) *An SEC is seen lying between the fetal tumor cells (FC), which have an electron-lucent cytoplasm rich in glycogen. The SEC and FC are connected by desmosome-like junctions* (arrowheads), *the lower one of which is seen at higher magnification in* b. *The cytoplasm of the SEC contains numerous tonofilament bundles* (arrows), *of which those in the area indicated by the large arrow are seen at higher magnification in* c. a: ×*10,000;* b *and* c: ×*31,000.*

oval cells have been described in fetal liver,[29] in alcoholic hepatitis,[30] and in massive hepatic necrosis.[31] Hsia et al[13] found oval-type cells at the margins of hepatocellular carcinoma in humans that were found to express CK-7 and CK-19 and were also reactive with an antibody directed against the oval cells of the rat. A detailed electron microscopic investigation by De Vos and Desmet[12] demonstrated "small cells," similar to the oval cells and thought to correspond to or be closely related to hepatic stem cells, in the human liver in diseases with chronic ductular reaction. However, the CK profile of these cells was not investigated.

To define the ultrastructure and CK profile of the corresponding cells in the liver in childhood, we first investigated liver biopsy specimens from cases of BA, which all exhibited chronic ductular reaction, by electron microscopy and immunoelectron microscopy. SEC, characterized by their size and shape, electron-dense cytoplasm, oval electron-dense nucleus, and tonofilament bundles, were found in all the cases of BA investigated. The ultrastructure of these cells corresponded to that of the oval cells of the rat and the "small cells" described by De Vos and Desmet.[12] Immunoelectron microscopic investigation of the CK profile of the SEC revealed immunoreactivity located in the tonofilament bundles for CK-7, which is expressed by biliary epithelial cells but not by hepatocytes.[32,33] The antibody against CK-19 was found to be unsuitable for use in immunoelectron microscopic investigations, but was found by light microscopy to stain both biliary epithelium and cells that on the basis of their size and location were probably SEC. Thus, SEC correspond to the oval cells of the rat not only in their ultrastructural appearance but also in their CK profile.[34]

Cells with the same morphology and immunoelectron microscopic CK profile as the SEC in BA were found in hepatoblastoma. Their number varied according to the subtype, being small in fetal areas and moderate in embryonal areas. In small cell hepatoblastoma nearly all the tumor cells exhibited SEC-like ultrastructural features.

The SEC could not be identified with certainty at the light microscopic level. However, in line with the electron microscopic findings, there was an increase

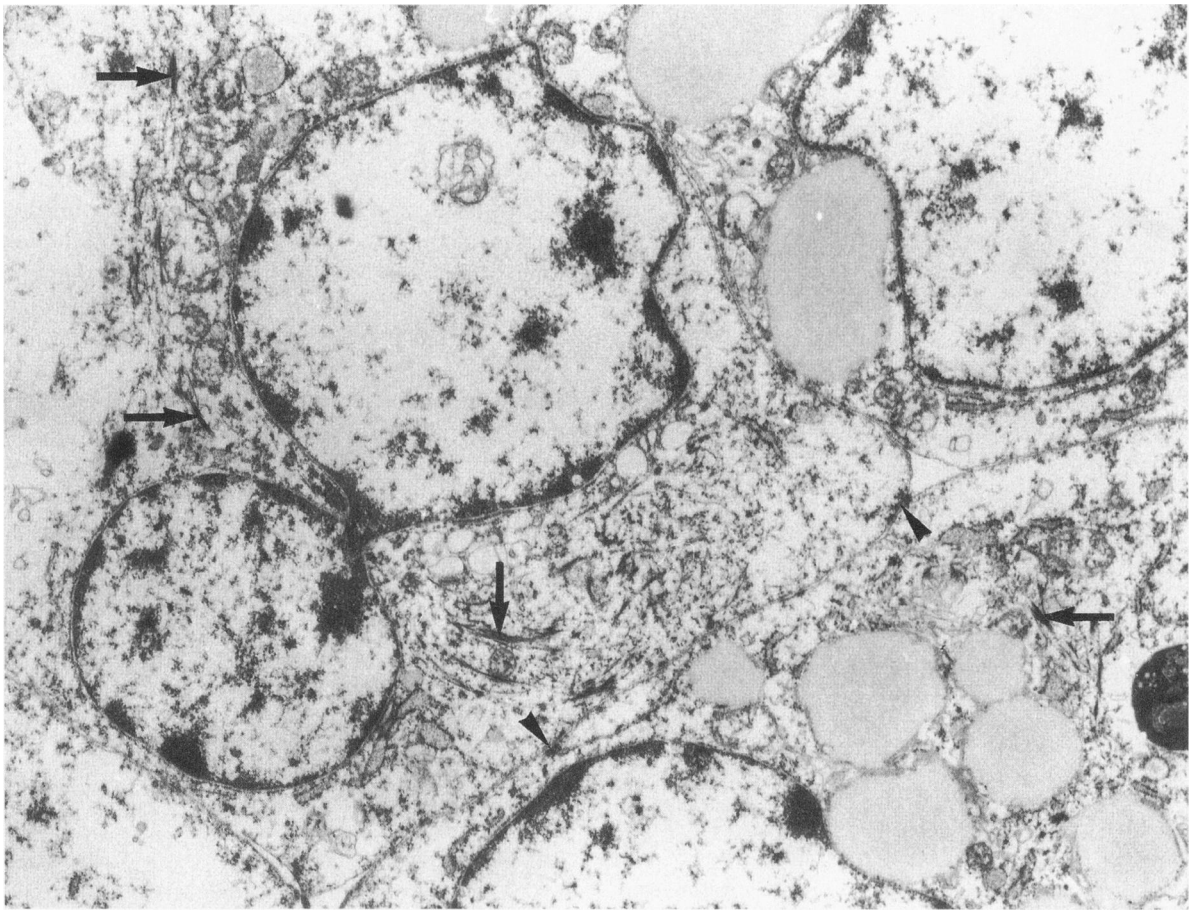

Figure 4. *Small cell hepatoblastoma. The nuclei of the tumor cells exhibit peripheral condensation of the heterochromatin, one nucleus being bilobular. The cytoplasm contains numerous tonofilament bundles* (arrows). *Intercellular junctions can also be seen* (arrowheads). ×*10,000; material originally embedded in paraffin.*

from the fetal, through the embryonal, to the small cell subtype in the number of cells expressing the CKs typical for biliary epithelium (CK-7 and CK-19, especially the latter), that is, CKs that are also expressed by the oval cells of the rat.[34] The findings in the small cell hepatoblastomas were particularly interesting here, because the overwhelming majority of the tumor cells expressed CK-19.

These findings are consistent with the notion based on conventional tumor histology that the variously differentiated epithelial hepatoblastoma subtypes correspond to certain stages in the embryological development of the liver. The decrease in numbers of SEC from the embryonal to the fetal subtype also finds a parallel in liver development, during which the number of bipotent stem cells, which express CKs typical for biliary epithelium, decreases with increasing gestational age.[11,29,35] Small cell hepatoblastoma, which consists almost exclusively of tumor cells with SEC-like ultrastructural features, occupies a special position. Our findings are therefore consistent with the hypothesis put forward by Abenoza et al[17] concerning the histogenesis and differentiation of the various hepatoblastoma subtypes, according to which precursor small cells give rise to embryonal hepatoblastoma cells and, after further maturation, fetal hepatoblastoma cells. The tumor cells of small cell hepatoblastoma exhibit no differentiation or, at most, abortive differentiation as represented by the formation of occasional tubular structures seen in one case, which we have described in a previous publication.[36] The undifferentiated phenotype of this tumor is reflected in the fact that it may coexpress keratin and vimentin[17] and we have recently demonstrated that it may express CD34, an antigen associated with hemopoietic stem cells.[37] Our findings show that the tumor cells of small cell hepatoblastoma closely resemble the putative rodent and human hepatic stem cells in their morphology and immunophenotype. It could therefore be postulated that small cell hepatoblastoma arises when further differentiation of the

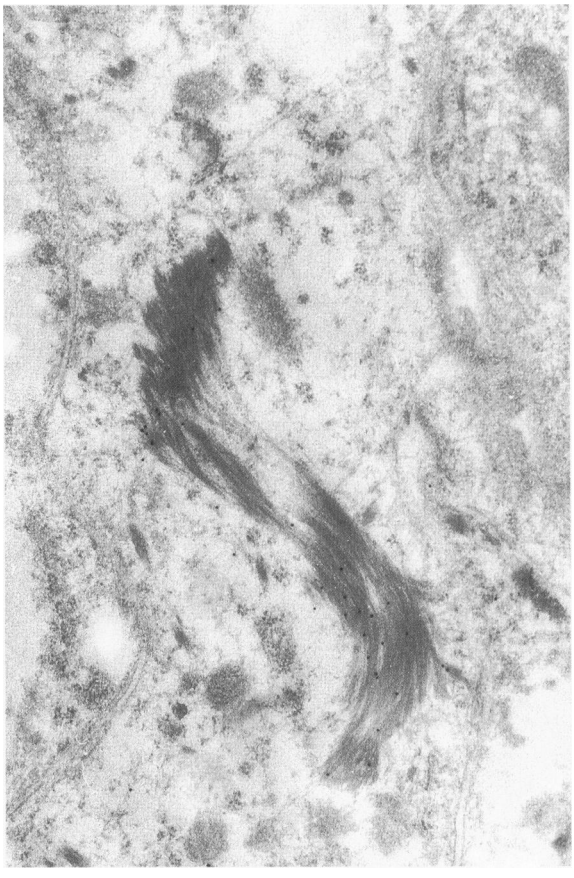

Figure 5. *Embryonal hepatoblastoma. The tonofilament bundles of an SEC exhibit immunogold marking for CK-7. ×25,000.*

stem cell is almost completely blocked by malignant transformation. If further differentiation is not completely blocked, embryonal or fetal hepatoblastoma develop, depending on the stage of differentiation at which the block is situated.

The histogenesis of the mesenchymal components of mixed hepatoblastoma also fits into this concept, although osteoid requires special consideration. As both we and other authors have found,[17,38] a few cells in the osteoid exhibit immunoreactivity for CKs, which is a characteristic of epithelial cells. This finding led Abenoza et al[17] to postulate a derivation from embryonal tumor cells by osteoid metaplasia. By contrast, no CK-immunoreactive cells are found in the spindle cell areas. Some indication of the histogenesis of these and other mesenchymal elements is offered by the findings of investigations into the biological behavior of malignant oval cells. Tsao and Grisham[8] showed that the transplantation into isogeneic rats of chemically transformed cultured liver epithelial cells, which are nearly identical to oval cells, could result in numerous different tumors, among them sarcoma, mixed hepatoblastoma, and tumors exhibiting ductular differentiation, which was also seen in one of our cases. The authors interpreted this as indicating that the mesenchymal components of mixed hepatoblastoma could develop by metaplastic sarcomatous transformation of a primitive stem cell.

As the SEC described are defined by morphological features and CK expression alone, no definite phenotypic marker for hepatic stem cells having yet been developed,[28] their exact relationship to hepatic stem cells cannot be established with certainty from the findings of this study. However, the SEC exhibit morphological features and a CK profile similar to that of the putative human and rodent hepatic stem cells, so that their presence in hepatoblastoma can be considered further evidence that this tumor derives from a stem cell that is able to differentiate in various different directions. The property of multidirectional differentiation suggests that hepatoblastoma, at least the mixed type, does not derive from the bipotent hepatic stem cells that give rise to hepatocytes and biliary epithelial cells, but rather from pluripotent entodermal, or even less committed, stem cells.

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