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Isolation of Oval Cells by Centrifugal Elutriation and Comparison with Other Cell Types Purified from Normal and Preneoplastic Livers[1]

Paul Yaswen,[2] Nancy T. Hayner,[2] and Nelson Fausto[3]

Department of Pathology, Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912

ABSTRACT

Oval cells and biliary epithelial cells were isolated from livers of rats fed a choline-deficient diet containing 0.1% ethionine and from normal rat livers, respectively. Nonparenchymal cell suspensions prepared from these livers by collagenase perfusion followed by digestion of undissociated tissue with 0.1% collagenase, 0.1% Pronase, and 0.004% DNase I were separated into six fractions by centrifugal elutriation. Cells in each fraction were characterized histochemically for γ-glutamyl transpeptidase, peroxidase, alkaline phosphatase, and glucose-6-phosphatase activities, and for albumin and α-fetoprotein by immunocytochemical methods. Cells from Fraction 5 of the elutriation procedure had various features predicted for oval cells and were selected for further studies. The cell yield in this fraction, from each preneoplastic liver, was 5.7×10^7 cells, 93 ± 2% of which were γ-glutamyl transpeptidase positive, 6 ± 1% peroxidase positive, 61% albumin positive, and 29% α-fetoprotein positive. Cells in this fraction have a median diameter of 13.1 μm and are diploid and cycling. The majority of these cells has morphological features characteristic of biliary epithelial cells, although some cells display features intermediate between duct cells and hepatocytes. Nucleic acid hybridization using specific probes revealed that these cells contain albumin and α-fetoprotein messenger RNAs, while hepatocytes from normal and preneoplastic liver contain only albumin messenger RNA. Biliary cells obtained from normal livers do not contain albumin messenger RNA. The large-scale purification and characterization of cell populations from preneoplastic livers is an important step in elucidating the cellular derivation of liver tumors.

INTRODUCTION

Although liver carcinogenesis is a common model for the study of tumor induction in animals, its essential molecular and cellular events are poorly understood. Current theories of hepatocarcinogenesis suggest that neoplastic transformation derives from "dedifferentiation" of mature hepatocytes or from the abnormal differentiation of precursor cells (27, 43). It is not yet possible, however, to identify, at the early stages of liver carcinogenesis, the cells which will give rise to tumors or to distinguish the steps which intervene between cell initiation and neoplasia (6, 35).

One of the first changes induced by most chemical carcinogens is the emergence in the liver of cells, distinct from hepatocytes, which resemble biliary epithelial cells (5, 9, 22, 38). This population of cells proliferates to such an extent that it may constitute a majority of the liver cellular elements at the early stages of hepatocarcinogenesis, particularly in animals given carcinogens in conjunction with diets deficient in lipotropic factors (30). Some of these cells form typical and atypical bile ducts and, although it is assumed that the population is derived from biliary cells, its lineage and function are unclear. Because of these uncertainties, ductular-like cells which proliferate during carcinogenesis are generally referred to as oval cells, a name which reflects their morphology but is noncommital as to origin or biological role (5).

A central issue regarding oval and ductular cells derives from observations which suggest that these cells are capable of differentiating into hepatocytes. In some systems, such as azo dye carcinogenesis, it is possible that oval cells constitute the progenitor population of neoplastic hepatocytes (15, 25). This hypothesis is strengthened by the demonstration that, at the early stages of carcinogenesis induced by some chemical agents, oval cells, rather than hepatocytes, contain oncodevelopmental proteins commonly associated with liver neoplasia (12, 19, 38). The observations of Grisham *et al.* (10), that hepatic epithelial cells may derive from nonparenchymal cell precursors in tissue culture, have led them to suggest that "terminal biliary ductular cells are facultative stem cells for hepatocytes."

A direct analysis of the developmental potential and the biochemical properties of oval cells requires the isolation of large numbers of these cells free or nearly free from contamination by other cellular types, especially hepatocytes. In this paper, we describe the isolation, by centrifugal elutriation, of oval cells from preneoplastic livers of rats fed for 4 to 6 weeks a CDE[4] diet. Since oval cells might be derived from and share morphological features with bile duct cells, we have, for comparative purposes, isolated biliary epithelial cells from normal rat liver. Both cell populations, as well as hepatocytes purified from normal and preneoplastic livers, have been analyzed for: (*a*) the activity of several enzymes (GGT, G6P, ALKP, and peroxidase) with histochemical methods; (*b*) the presence of serum proteins (AFP and ALB) by immunocytochemistry; (*c*) DNA content (ploidy) using microspectrophotometry; and (*d*) the presence of AFP and ALB mRNAs by nucleic acid hybridization with specific probes. A study of the isozyme composition of oval cells isolated from preneoplastic livers and biliary epithelial cells obtained from normal livers, and of parenchymal cells from normal and carcinogen-treated animals, is presented in an accompanying paper (13).

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass.) weighing 130 to 150 g were fed either a standard

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[3] To whom requests for reprints should be addressed.

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[4] The abbreviations used are: CDE, choline-deficient diet containing 0.1% DL-ethionine; GGT, γ-glutamyl transpeptidase; G6P, glucose-6-phosphatase; ALKP, alkaline phosphatase; AFP, α-fetoprotein; ALB, albumin; MEM, minimum essential medium.

laboratory chow or a CDE diet (38) prepared by Teklad Test Diets (Madison, Wis.). Rats receiving the carcinogenic diet were killed 4 to 6 weeks after the start of the feeding. Histological examination showed that oval cells were abundant in the livers of the animals receiving the carcinogenic diet, as described previously (1, 32, 38).

Cell Suspension

Rats were anesthetized with a mixture of ether and oxygen. Each liver was perfused *in situ* via the portal vein with oxygenated calcium-free Hanks' balanced salt solution [with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, without bicarbonate, pH 7.4] for 10 min at 22 ml/min at 37°. The liver with the cannula in place was then excised, and a recirculating perfusion was established using 50 ml of 0.10% collagenase (type I; Sigma Chemical Co., St. Louis, Mo.) in oxygenated calcium-supplemented buffer [3.9 g NaCl, 0.5 g KCl, 0.7 g $CaCl_2 \cdot 2H_2O$, and 24.0 g 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid in 1000 ml; pH 7.6, at 37°] (33). After 15 min of recirculating perfusion, the liver was removed to a large Petri dish containing 50 ml of calcium-free Hanks' solution. The liver capsule was cut, and the tissue was dissociated by shaking and combing.

Nonparenchymal Cells. After decanting the released cells (which were saved for the purification of parenchymal cells), the remaining undissociated tissue was minced in 25 ml of Joklik-modified MEM (Grand Island Biological Co., Grand Island, N. Y.) with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, containing 0.1% collagenase, 0.1% Pronase (Calbiochem-Behring Corp., La Jolla, Calif.), and 0.004% DNase I (DN-25; Sigma), pH 7.4. The minced tissue was incubated 3 times with the enzyme solution in a trypsinizing flask. For each incubation, the flask was kept at 37° for 20 min in a shaking water bath at 200 rpm. After each incubation, the supernatant was decanted and filtered through 45-μm nylon mesh (TETKO, Inc., Elmsford, N. Y.). Cold MEM with 10% calf serum (Grand Island Biological Co.) was added, bringing the volume to 50 ml. Tissue fragments retained on the filters after the first and second digestion were reincubated by including these filters in the final enzyme incubation. The filtered cell suspensions were centrifuged at 300 × *g* for 5 min at 4°, resuspended in MEM with 10% calf serum, and recentrifuged. The pellets obtained from each incubation were combined and resuspended in a final volume of 10 ml of MEM containing 10% calf serum.

Parenchymal Cells. Cell suspensions obtained after collagenase perfusion of normal livers were filtered through 220 μm mesh (TETKO) and then centrifuged twice at 50 × *g* for 2.5 min at 4°. Cell pellets were pooled and resuspended in appropriate volumes of Hanks' Solution or MEM with 10% calf serum. To prepare cell suspensions from preneoplastic livers, perfused livers were minced in 25 ml MEM with 0.1% collagenase and 0.004% DNase I, and the minced tissue incubated in a trypsinizing flask at 37° at 125 rpm for 15 min. The cell suspension was decanted, filtered, and centrifuged as described for normal parenchymal cells. The tissue which remained undissociated was treated once more with enzyme solution. The cell pellets were pooled and suspended in MEM with 10% calf serum.

Samples of all cell suspensions were taken for cell counts, determination of cell viability by trypan blue exclusion, and light and electron microscopic examination.

Cell Separation

Nonparenchymal Cells. Centrifugal elutriation of the cell suspension obtained by collagenase/Pronase digestion was performed in a Beckman JE-6 elutriator rotor equipped with a standard Beckman separation chamber (Beckman Instruments, Palo Alto, Calif.) run at 2500 rpm and kept at 10°. MEM with 10% calf serum and 0.004% DNase I was used as the elutriation medium. Approximately 9 ml of cell suspension were injected into the mixing chamber. Five 100-ml fractions were collected at increasing pump flow rates of 18, 24, 26, 28, and 40 ml/min. A sixth "blowout" fraction was collected with the rotor stopped and maximal pump flow rate. The elutriated cells from each fraction were centrifuged at 500 × *g* for 10 min, and the pellets were resuspended in MEM containing 10% calf serum.

Parenchymal Cells from Preneoplastic Livers. Parenchymal cells from preneoplastic livers were purified by centrifugal elutriation. Nine ml of cell suspension, containing 0.5 to 2.0 × 10^8 parenchymal cells, were loaded into the JE-6 elutriator rotor with the rotor speed set at 1500 rpm. Five 100-ml fractions were collected at pump flow rates of 20, 25, 30, 35, and 40 ml/min. An additional blowout fraction was also collected as described above. The cells in each fraction were centrifuged at 50 × *g* for 2.5 min, and the pellets were resuspended in MEM containing 10% calf serum.

Histochemistry

Cell smears were prepared without prior fixation and used immediately or frozen at −20°. Separate slide sets were stained for GGT (31), peroxidase (7, 41), ALKP (14), and G6P (45). In each preparation, 400 cells were surveyed, and the percentage of positive cells was determined.

Immunohistochemistry

ALB and AFP were localized by the peroxidase-antiperoxidase method (40) in smears of isolated cells which were washed in buffer and fixed in periodate/lysine/*p*-formaldehyde fixative (23). Rabbit antibody to rat ALB (Cappel Laboratories, West Chester, Pa.) and the IgG fraction of rabbit antibody to mouse AFP (Miles Laboratories, Elkhart, Ind.) were pretested and titrated on known positive control tissue sections and were checked for specificity and cross-reactivity against purified rat ALB (Pel Freez, Rogers, Ariz.), purified mouse AFP (a gift of T. Tamaoki), calf serum (Grand Island Biological Co.), and goat serum (Grand Island Biological Co.). Nonimmune rabbit serum (KC Biologicals, Lenexa, Kans.) or the IgG fraction from this serum was used, at the same titer as the antibody preparations, to assess the degree of background staining on control slides. Cell smears stained by the peroxidase-antiperoxidase method for either ALB or AFP were counterstained with Giemsa; 400 cells on each slide were scored to determine the percentage of AFP- or ALB-positive cells.

Electron Microscopy

Samples of the isolated cell suspensions were fixed at 4° for 45 min in 1.6% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, containing 0.1 M sucrose. Fixed cells were centrifuged at 15,000 × *g*, and the pellets were postfixed for 20 min with 0.1% OsO_4 in 0.1 M sodium cacodylate buffer, dehydrated in graded ethanol and propylene oxide, and embedded in Spurr resin. Thin sections were stained with uranyl acetate and Reynolds lead citrate.

Cell Sizing

Cells in suspension were sized using a Coulter Model ZM particle counter with a 140-μm orifice (Coulter Electronics, Edison, N. J.), in conjunction with a Coulter size distribution analyzer (Coulter Channelyzer). Median cell sizes were calibrated using microspheres of known diameter as standards (Coulter Electronics, Hialeah, Fla.).

DNA Microspectrophotometry

Cell smears were fixed in methanol/acetic acid (3/1). Staining with 0.1% 4′-6-diamidino-2-phenylindole, and microspectrophotometric determination of fluorescence of individual nuclei were performed according to the method described by Coleman *et al.* (3).

ALB and AFP mRNAs in Isolated Cell Fractions

Cytoplasmic extracts were prepared from isolated cell fractions as described by White and Bancroft (47), and amounts equivalent to 10 to 80 μg of protein were spotted on nitrocellulose sheets (BA85, 0.45 μm;

Schleicher and Schuell, Keene, N. H.). The cloned DNA probes, pAF6 for AFP mRNA and pmalb2 for albumin mRNA (17, 20), were gifts from Dr. T. Tamaoki and were labeled by nick translation (29) with [^{32}P]dCTP (3200 Ci/mmol; New England Nuclear, Boston, Mass.). Dot-blot hybridizations were performed for 72 hr at 42° using 1 × 10^6 cpm/ml hybridization buffer (42). After hybridization, the filters were washed, placed in contact with Kodak XAR-5 film, and kept at −70°. To determine sensitivity of the procedure and possible cross-reactivity of the probes, from 3 to 100 pg of unlabeled, heat-denatured pAF6 and pmalb2 DNA were spotted on the filters and hybridized with labeled DNA. Cytoplasmic extracts treated with 10 μg of RNase A (Boehringer Mannheim, Indianapolis, Ind.) for 1 hr at 37° prior to denaturation were used as controls for the hybridization procedure.

RESULTS

Oval Cell Isolation from Preneoplastic Livers. On the basis of the published data from this and other laboratories, we adopted the following operational criteria for the recognition of oval cells: (*a*) cell diameter, ranging between 10 and 15 μm, about one-half of that of hepatocytes; (*b*) presence of GGT activity, demonstrable by histochemical staining; and (*c*) absence of histochemically demonstrable peroxidase activity. Initially, we attempted to isolate GGT-positive nonparenchymal cells from preneoplastic livers by dissociating the livers by collagenase perfusion and separating parenchymal from nonparenchymal cells by differential centrifugation. Nonparenchymal cell fractions obtained with this method contained no more than 2% GGT-positive cells and proved unsuitable for further purification. Histological examination of the liver tissue which remained undissociated after collagenase treatment revealed that the tissue was very highly enriched in GGT-positive cells and in structures resembling bile ducts or ductules, enveloped in a connective tissue matrix. After experimenting with a number of proteolytic enzymes alone or in combination, we found that a collagenase/Pronase/DNase digestion step was most satisfactory for the preparation of monodispersed nonparenchymal cell suspensions enriched in GGT-positive cells. Digestion of the undissociated tissue with the collagenase/Pronase/DNase mixture yielded preparations which contained 62 ± 3% (S.E.) GGT-positive cells and 13 ± 1% peroxidase-positive cells. Parenchymal cells constituted 2 ± 1% of the population. Approximately 5.9 ± 1.2 × 10^8 nonparenchymal cells with 95 ± 2% viability by trypan blue exclusion were obtained from each liver of rats kept on the CDE diet for 4 to 6 weeks.

The cells obtained by collagenase/Pronase/DNase digestion were separated according to size and density using the centrifugal elutriation procedure. In the initial experiments, 10 different fractions were collected at increasing pump speeds to determine the optimal conditions for cell separation. The procedure which was finally adopted involved the collection of 6 fractions as indicated in "Materials and Methods" and shown in Chart 1. Fractions 1 and 2 contained small cells with the characteristics of endothelial cells, lymphoid cells, and erythrocytes in addition to some cellular debris. In Fractions 3 and 4, nearly 90% of the cells were GGT positive but Fraction 5, which contained 93 ± 2% GGT-positive cells and 6 ± 1% peroxidase-positive cells with a 93 ± 1% viability, was the most homogenous. In this fraction, approximately 5.7 × 10^7 viable cells were collected from one preneoplastic liver (Chart 1). No viable parenchymal cells were detected in this fraction, although nonviable hepatocytes were

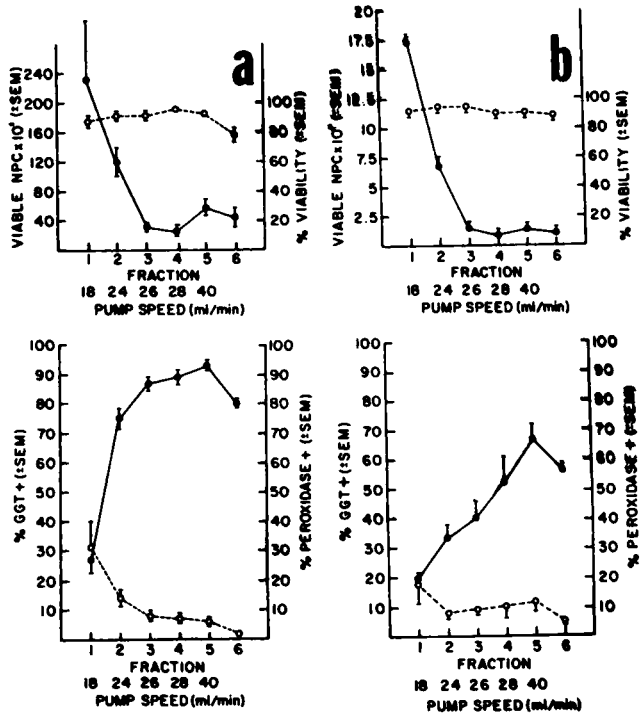

Chart 1. Nonparenchymal cells (*NPC*) from the livers of 6 rats maintained for 4 to 6 weeks on a CDE diet (*a*) and from the livers of 3 rats maintained on a normal diet (*b*) were separated into 6 fractions by centrifugal elutriation. *Top*, number of cells in each elutriated fraction was counted in a hemocytometer (●), and viability was determined by 0.2% trypan blue exclusion (○). *Bottom*, duplicate cell smears were made of cells from each elutriated fraction. Smears were stained histochemically for GGT (●) or peroxidase (○); 400 cells were counted in each smear.

occasionally found. Fraction 6, a "blowout" fraction obtained after the rotor had stopped, contained clumped cells and some viable hepatocytes. The average overall recovery of viable nonparenchymal cells, after centrifugal elutriation (Fractions 1 to 6) followed by centrifugation and resuspension of the cells, was approximately 86%.

Size and Ploidy of Isolated Oval Cells. The size profile of cells in Fraction 5 was determined with a Coulter Channelyzer (Chart 2). The median diameter of the cells was 13.1 μm, a value which conforms to the expectation for oval cells, although the coefficient of variation was relatively large.

Microspectrophotometric analysis of the cells present in Fraction 5 showed that, overall, they have a diploid DNA content and constitute a cycling population (Chart 3), an observation which agrees with data on thymidine labeling of oval cells in preneoplastic livers (9, 19, 36).

Morphological Characterization and Enzyme Histochemistry. The majority of the cells of Fraction 5 have the following features: (*a*) irregularly shaped nuclei with variable amounts of condensed chromatin; (*b*) high nuclear/cytoplasmic ratios; (*c*) little rough endoplasmic reticulum; and (*d*) mitochondria of small size and number (Fig. 1). Occasional lipid droplets as well as other unidentified cytoplasmic inclusions are also apparent, and the surfaces of some of the cells appear to have protruding microvilli. These features have been noted in previous descriptions of oval and biliary cells in liver and in isolated cell fractions (4, 9, 15, 25, 37, 39). These cells are easily distinguishable from the few Kupffer cells present which have abundant lysosomal structures and cytoplasmic projections. Some of the cells in

Fraction 5 are slightly larger and have more abundant cytoplasm than do typical biliary epithelial cells (Fig. 2). These larger cells have more and bigger mitochondria than do biliary cells, and may be "transitional" cells in various stages of maturation to hepatocytes.

Only about 1% of the Fraction 5 cells stained for G6P, a marker for mature hepatocytes, while approximately 46% were ALKP positive (Table 1). Since histochemical demonstration of these enzymes was done only by light microscopy, it is not clear whether the cells which displayed demonstrable G6P activity were transitional cells. The same histochemical method detected G6P activity in all hepatocytes collected in the "blowout" fraction of the elutriation procedure and in parenchymal cells isolated from normal or preneoplastic livers (see below).

Isolation of Biliary Epithelial Cells from Normal Livers. The method described for oval cell isolation from preneoplastic livers also proved to be the most satisfactory for the preparation from normal livers of monodispersed nonparenchymal cell suspensions enriched in GGT-positive cells. Histological examination of the tissue remaining after collagenase dissociation, but before the collagenase/Pronase/DNase step, showed a variety of duct structures surrounded by connective tissue. After treatment of the undissociated tissue with collagenase/Pronase/DNase, the cell suspensions were elutriated using same pump speeds as for oval cell isolation. The proportion of GGT-positive cells progressively increased from Fractions 1 to 5 (Chart 1). Cells isolated in Fraction 5 stained for: GGT, 67 ± 9%; peroxidase, 12 ± 7%, G6P, less than 1%; and ALKP, 11% (Chart 1; Table 1). Approx-

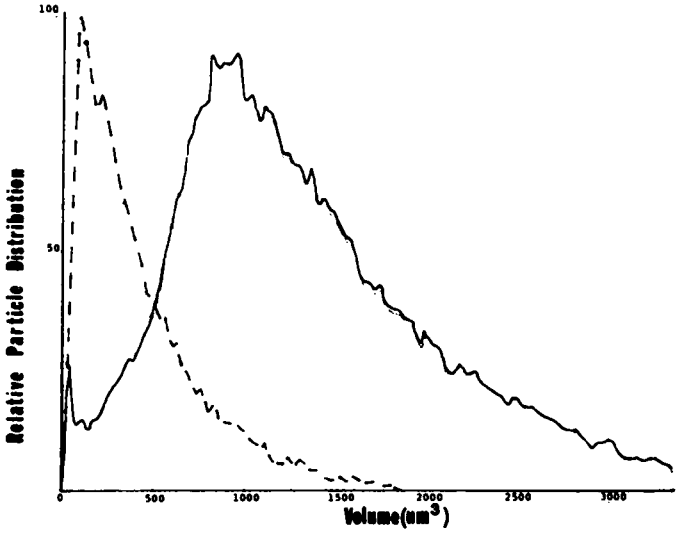

Chart 2. Size distributions (Coulter Channelyzer) of cells from an unfractionated nonparenchymal cell suspension obtained after collagenase/Pronase/DNase digestion of a CDE liver (– – – –), and of cells from Fraction 5 obtained after centrifugal elutriation of the cell suspension (——). The median volume of the cells in Fraction 5 was 1172.5 cu μm, and the coefficient of variation was 49%.

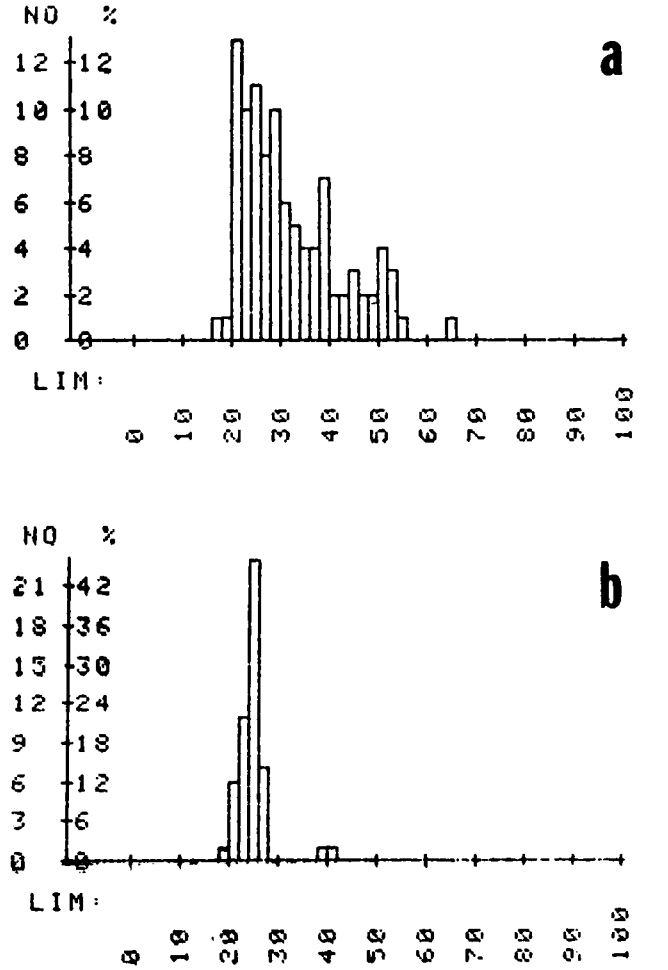

Chart 3. Relative fluorescence measurements using 4′-6-diamidino-2-phenylindole (0.1 μg/ml) of cells from elutriated Fraction 5 of: (*a*) CDE nonparenchymal cells; and (*b*) normal nonparenchymal cells. In *a*: the number of nuclei counted was 100; the mean fluorescence value was 32.13; and the coefficient of variation was 31.64%. In *b*: the number of nuclei counted was 50; the mean fluorescence value was 24.34; and the coefficient of variation was 14.89%. In both cases, the background fluorescence was less than 1.00.

Table 1

Histochemical and immunohistochemical characteristics of cells from elutriated nonparenchymal cell fractions

Each vertical column represents one experiment; 400 cells were counted in each slide preparation, and the percentages which stained positively are shown.

Fraction	ALKP				G6P				AFP		ALB			
	CDE[a]		Normal[b]		CDE		Normal		CDE		CDE		Normal	
1	25	18	11	13	0	1	0	0	0	6	12	1	1	9
2	42	28	17	18	1	1	0	0	9	12	56	28	64	7	6
3	58	35	11	14	4	1	0	0	16	12	66	53	73	8	1
4	53	30	13	13	3	2	1	0	23	17	60	51	63	8	3
5	46	45	13	9	1	1	1	1	22	35	68	60	54	6	2
6	37	45	13	13	18	9	17	11	19	13	45	34	26	31	18

[a] These cells were obtained from livers of rats maintained on a choline-deficient diet with 0.1% ethionine for 4 to 6 weeks.

[b] These cells were obtained from livers of rats fed a normal choline-supplemented diet without carcinogen.

imately 1.7 ± 0.3 × 10^6 GGT-positive nonparenchymal cells with 91 ± 3% viability were collected in Fraction 5 from one rat liver. These cells had similar size and general morphological characteristics as oval cells isolated from preneoplastic livers, although "transitional" forms were not observed. Microspectrophotometric analysis (Chart 3) of the DNA content of individual cells indicated that they constituted a diploid, noncycling cell population.

Isolation of Parenchymal Cells from Normal and Preneoplastic Livers. Parenchymal cells were isolated from normal livers by the collagenase digestion procedure described by Seglen (33). Because the purity of these preparations is very high, elutriation proved to be unnecessary. However, collagenase digestion of preneoplastic livers yielded preparations of parenchymal cells which were heavily contaminated by other cell types and had to be further purified by elutriation (see "Materials and Methods"). The fraction obtained at a pump speed of 35 ml/min was the purest, containing 87 ± 5% parenchymal cells. All of the parenchymal cells of this fraction were positive for G6P; and approximately 49 ± 8% were GGT positive. The median cell diameter was 25 μm, and the population contained diploid, tetra-, and a few octoploid cells with typical hepatocyte morphology characterized by abundant rough endoplasmic reticulum, large mitochondria, and round nuclei.

AFP and ALB mRNAs in Isolated Oval Cells, Biliary Epithelial Cells, and Parenchymal Cells. RNA contained in cytoplasmic extracts of parenchymal and oval cells isolated from the livers of preneoplastic rats was hybridized with ^{32}P-labeled, cloned AFP cDNA using a dot-blot procedure. Cytoplasmic extracts of oval cells pretreated with RNase served as controls for the hybridization procedure. Fig. 3 shows that oval cell extracts contain AFP mRNA while, in parenchymal cells isolated from preneoplastic livers, this mRNA is barely detectable. Hybridization of RNA in cytoplasmic extracts with [^{32}P]ALB cDNA (Fig. 3) demonstrated that: (*a*) parenchymal cells isolated from normal and preneoplastic livers contain large amounts of ALB messenger, as expected; (*b*) biliary epithelial cells from normal liver do not have ALB mRNA; and (*c*) oval cells isolated from preneoplastic livers contain ALB mRNA. Extracts of oval or parenchymal cells pretreated with RNase had no detectable levels of ALB mRNA.

Histochemical Distribution of AFP and ALB in Oval and Biliary Epithelial Cells. Using specific immunocytochemical procedures, we found that 29% of oval cells elutriated in Fraction 5 stained positively for AFP, while 61% were positive for ALB (Table 1). Most parenchymal cells isolated from normal and preneoplastic livers and approximately 2% of cells in biliary epithelial cell isolates from normal livers stained for ALB. ALB-positive cells in these latter preparations might be Kupffer cells containing phagocytized cellular debris.

DISCUSSION

Understanding the biological significance of oval cell proliferation in the liver depends on knowledge of the origin, characteristics, fate, and developmental potential of these cells. We approached the problem by isolating oval and transitional cells from preneoplastic livers in large enough quantities to permit biochemical analysis of isolated cell fractions and comparisons between these cells, hepatocytes, and cells from normal biliary epithelium.

Conventional collagenase perfusion techniques proved inadequate for the preparation of monodispersed cell suspensions from preneoplastic livers. A method which uses collagenase perfusion followed by treatment of the undissociated tissue with a mixture containing collagenase, Pronase, and DNase was devised for the isolation of nonparenchymal cells from preneoplastic livers. Pronase, a mixture of bacterial proteases, has been widely used for the purification of liver nonparenchymal cells due to the selective sensitivity of hepatocytes toward these proteases (18, 33). Although Pronase may adversely affect cell surface proteins and receptors and decrease the ability of cells to form colonies in culture, the cells we have isolated have high viability as measured by Trypan blue exclusion, well-preserved morphological structure, as well as intact mRNAs for ALB, AFP, and some oncogene products.[5] When Pronase is omitted from the isolation procedures, the oval cell fractions recovered after elutriation are contaminated by a small proportion of hepatocytes. In addition, the number of oval cells obtained is much reduced, because the large number of hepatocytes present in the initial cell suspension limits the amount of nonparenchymal cells loaded in the elutriation rotor.

Centrifugal elutriation separates cells according to size and density (28) and permits the isolation of discrete cell types or subpopulations in relatively large amounts. This method has been used for the separation of subpopulations of hepatocytes from normal and preneoplastic livers (2, 46), and for the isolation of Kupffer and endothelial cells (20, 48) but, so far, it has not been used for the separation of oval cells. Methods used to isolate oval cells from preneoplastic livers and ductular cells from normal livers have included isokinetic centrifugation in Ficoll gradients, free-flow electrophoresis, and isopyknic centrifugation in metrizamide gradients (8, 16, 24, 37). In the most homogeneous elutriated oval cell fraction (Fraction 5), we routinely obtained approximately 57 million cells from each liver of rats fed the carcinogenic diet for 4 to 6 weeks. The cell yields and the percentage of GGT-positive cells in this fraction are significantly greater than those reported with other isolation techniques (16, 37). The total number of GGT-positive cells in the combined oval cell Fractions 3, 4, and 5 isolated from preneoplastic livers was approximately 1.0 × 10^8. With the same methodology, the total yield of GGT-positive biliary epithelial cells isolated from normal livers was approximately 2.2 × 10^6.

In normal liver, AFP mRNA constitutes approximately 0.006% of the polysomal polyadenylated liver RNA but, in preneoplastic livers at 4 weeks after the start of the diet, AFP mRNA corresponds to approximately 0.21% of the liver polyadenylated RNA (26). In contrast, AFP mRNA proportions are no higher than 0.012% of the polysomal polyadenylated RNA during liver regeneration after partial hepatectomy (26), during which more than 90% of hepatocytes proliferate. Thus, the increase of AFP mRNA during carcinogenesis induced by the CDE diet could be a consequence of: (*a*) a change in the expression of the AFP gene in hepatocytes brought about by the diet but unrelated to hepatocyte proliferation; or (*b*) the presence of a new population of cells which are capable of synthesizing the protein. To distinguish between these 2 possibilities, we hybridized a cloned AFP complementary DNA probe to cytoplasmic extracts of isolated hepatocytes and oval cells obtained by elutriation from rats receiving the CDE diet for 4 weeks. With this procedure, we

[5] P. Yaswen, N. T. Hayner, and N. Fausto, unpublished observations.

detected AFP mRNA primarily in oval cells but not in hepatocytes. Using an ALB DNA probe, we found that both hepatocytes and oval cells isolated from livers of rats fed the CDE diet contained ALB mRNA (although the intensity of the signal was higher in hepatocytes), but that cytoplasmic extracts from elutriated normal biliary epithelial cells did not contain the messenger. Although dot-blot hybridization procedures are not strictly quantitative, the results of these assays indicate that the oval cell fraction isolated from preneoplastic livers contains both AFP and ALB mRNA and, thus, is capable of synthesizing proteins which are characteristic of fetal and adult hepatocytes, respectively. In contrast, hepatocytes isolated from preneoplastic livers of rats fed the CDE diet for 4 weeks contain ALB but very little AFP mRNA, suggesting that dedifferentiation, at least in relationship to these markers, is not taking place in parenchymal cells at this stage of carcinogenesis.

Measurements of AFP and ALB mRNA in isolated cell fractions do not address the question of the expression of these genes in individual cells, *i.e.*, whether all or only a fraction of the oval cells express ALB and/or the AFP gene. The only available way, at this time, to answer this question is to determine the proportion of oval cells which stain for ALB or AFP with immunochemical methods. Immunoperoxidase staining of the isolated oval cells (Fraction 5) showed that over 60% of the cells stained for ALB, and 29% of the cells stained strongly for AFP. Although we do not know if ALB is synthesized in transitional rather than oval cells, it is clear that GGT-positive nonparenchymal cells can have typical hepatocyte markers. Interestingly, only a very small proportion of these cells, no more than 5%, were positive for G6P, another marker of mature hepatocytes. Several laboratories have reported that, in tissue sections of preneoplastic livers, oval or transitional cells may stain for ALB and/or AFP (4, 11, 19, 34) and that hepatocytes stain for ALB but not AFP (38). Cells forming ducts do not stain for either of these markers, while oval cells stain for AFP, ALB, or both (38).

There was a much larger number of ALKP-positive cells in the oval cell fractions (46% positive in Fraction 5) than in the corresponding fractions obtained from normal livers (11%). In sections of normal and preneoplastic livers, ALKP stain was located in canalicular surfaces of hepatocytes and in some cells surrounding small bile ducts but not in cells lining the larger ducts. It has been reported that "connective tissue" cells located near bile ducts and cells which line cholangioles may be positive for ALKP (21, 44). Since more than 90% of cells in the elutriated oval cell fractions are GGT positive; most cells in these fractions which are ALKP$^+$ must also be GGT positive. The ALKP- and GGT-positive cells isolated from preneoplastic livers may be transitional cells which have acquired some plasma membrane specialization to form bile canaliculi, or they may be cholangiole-like biliary cells.

In this paper, we show that oval cell fractions isolated from preneoplastic livers are comprised of cycling, diploid GGT-positive cells with a median diameter of approximately 13 μm. It is likely that the GGT-positive cells in these fractions may belong to various subpopulations with differing characteristics. Morphologically, the isolated cells resemble biliary epithelial cells or small, transitional hepatocytes. They share biochemical features with biliary cells (isozymes and GGT), with normal hepatocytes (ALB), and with cells of neoplastic livers (AFP and isozymes) (14). To date, most of the knowledge about the biology of oval cells has been derived from the study of liver sections. The isolation of large numbers of viable oval cells with identifiable markers (14) will facilitate the detailed biochemical analysis of these cells, their separation into subpopulations, and the study of their developmental potential *in vivo* and *in vitro*.

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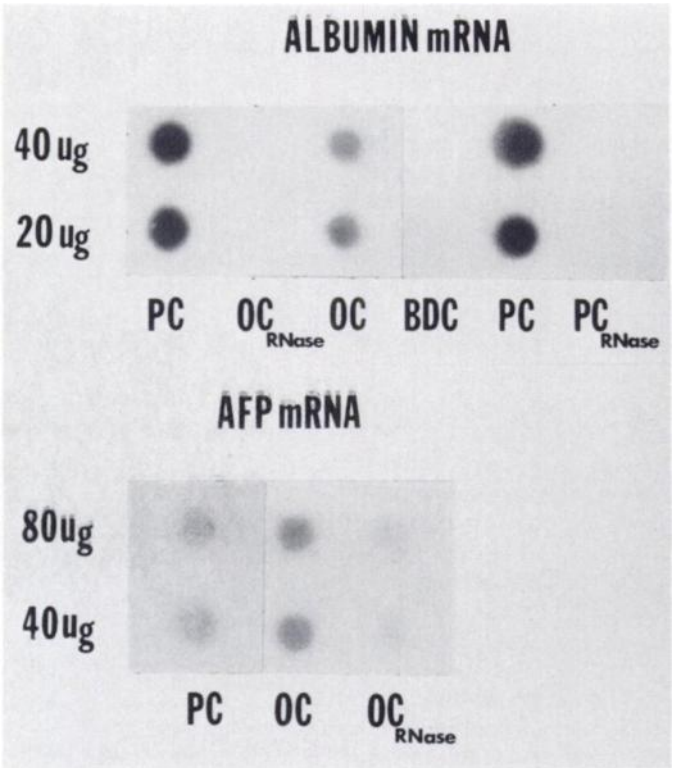
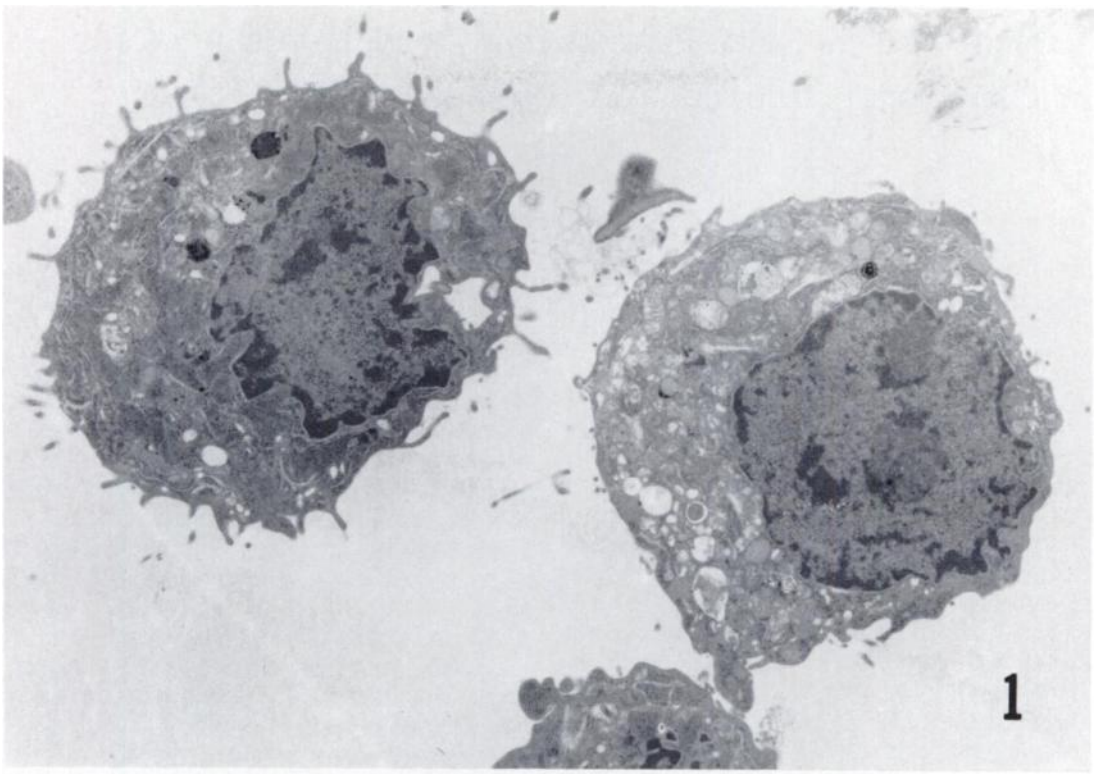


Fig. 3. ALB and AFP mRNAs in isolated cell fractions. Cytoplasmic extracts were prepared from elutriated nonparenchymal cell Fraction 5 from normal liver (*BDC*) and from the liver of a rat fed the CDE diet for 4 weeks (*OC*). Parenchymal cell (*PC*) cytoplasmic extracts were prepared from unelutriated hepatocytes from normal liver (*ALB*) and elutriated hepatocytes from the liver of a rat fed the CDE diet for 4 weeks (*AFP*). Cytoplasmic extracts treated with RNase were used as negative controls. Extracts containing 20, 40, or 80 μg of protein were spotted on nitrocellulose filters and hybridized with ^{32}P-labeled, cloned DNA probes.



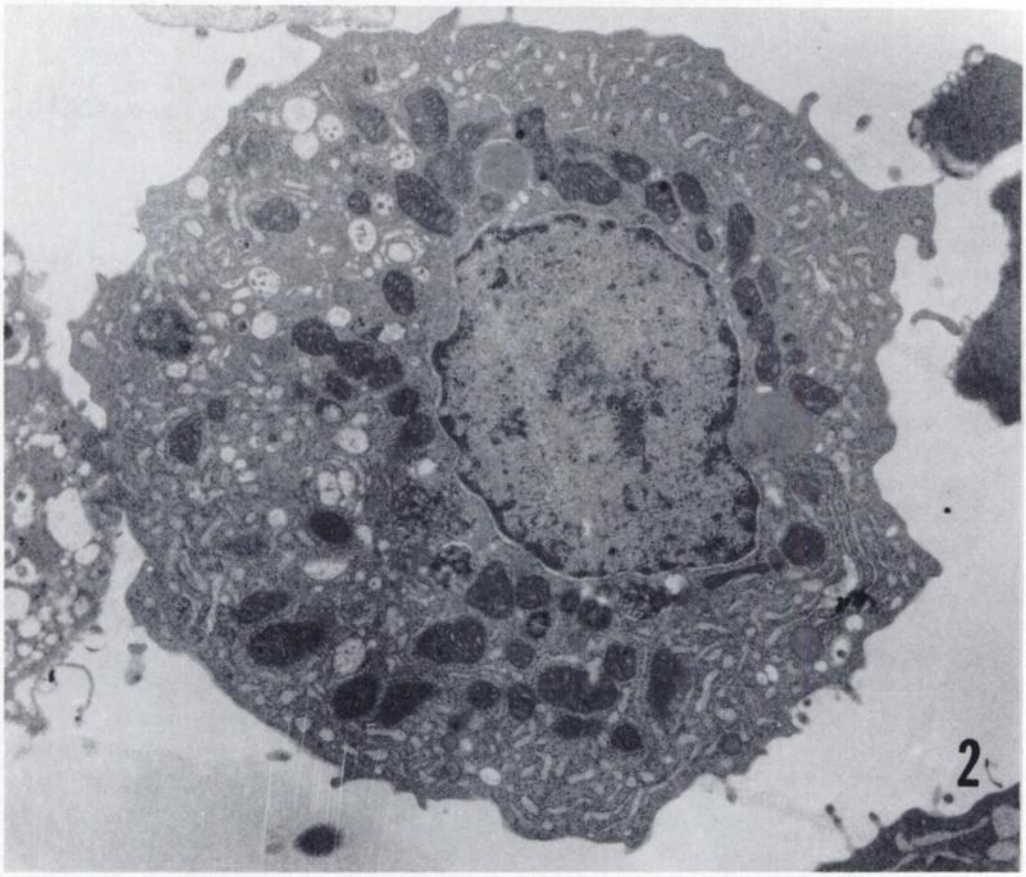


Fig. 1. Electron micrograph of typical cells from elutriated Fraction 5 from CDE liver. Note the high ratio of nucleus to cytoplasm, the irregular shapes of the nuclei, the sparsity of mitochondria, and the presence of microvilli. Uranyl acetate and lead citrate, × 7576.

Fig. 2. Electron micrograph of a cell from elutriated Fraction 5 from CDE liver which displays "transitional" characteristics. Note the greater area of the cytoplasm in relation to the nucleus, and the the more abundant mitochondria of this cell in relation to the cells of Fig. 1. Uranyl acetate and lead citrate, × 11,669.