Studies on the Proliferation and Fate of Oval Cells in the Liver of Rats Treated With 2-Acetylaminofluorene and Partial Hepatectomy

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The kinetics of oval cell proliferation in the liver and their fate were studied by combined autoradiography and immunohistochemical staining for epidermal prekeratin and epoxide hydrolase (EH). The oval cell proliferation was induced in rats by exposure to dietary 2-acetylaminofluorene (2-AAF) for 2 weeks with the midway performance of partial hepatectomy (PH). The labeling with ^{3}H-thymidine [^{3}H-TdR] was done in different groups of rats by two procedures: continuous exposure for 1 week with the aid of a minipump and brief exposure by the administration of a single dose. The livers of groups of animals were examined from 1 to 10 weeks after PH. Oval cells and duct epithelium showed positive staining for prekeratin and negative for EH, whereas hepatocytes showed the reverse pattern of staining. A critical finding was the observation that the exposure to the 2-AAF inhibited virtually completely the labeling of hepatocytes with [^{3}H]-TdR in the caudate lobe and incompletely in the right lobe without interfering with the labeling of the oval cells in either lobe. This made it possible to study the fate of the oval cells vis-à-vis hepatocytes. This qualitative–quantitative study of oval cells and hepatocytes clearly indicates that oval cells under these experimental conditions do not become hepatocytes within 10 weeks. Over 80% of oval cells disappear within this period, and the remainder persist as such. These results indicate that under one set of experimental conditions related to hepatocarcinogenesis in the rat, no evidence for the conversion of oval cells to hepatocytes was obtained. (Am J Pathol 1984, 114:418–430)

FUNDAMENTAL to our understanding of any malignant neoplasm is the identification of the possible sequences of biological, biochemical, and structural changes that initiated cells undergo during the pathogenesis of cancer. For hepatocellular carcinoma development in the rat, one overall sequence has been established by observation of a direct cellular continuity between carcinogen-induced resistant hepatocytes, hepatocyte ("hyperplastic") nodules, a small subset of these nodules (persistent nodules), and nodules within such nodules,[1] including metastasizing cancer.[2-6] This sequence was seen after initiation with diethylnitrosamine and promotion by dietary 2-acetylaminofluorene (2-AAF) plus partial hepatectomy (PH). Findings consistent with this conclusion have been reported with aramite[1], ethionine[7,8], 4-dimethylaminoazobenzene or related carcinogens,[9-11] and 2-AAF.[12]

A second possible sequence has been suggested to arise from a subset of oval cells.[13-22] These are cells that appear in and about the portal areas early during liver carcinogenesis with many chemical carcinogens[5,22,23] and penetrate the liver acinus as they proliferate. These "oval cells" are especially prominent in models of carcinogenesis involving long exposure to

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carcinogens. Although there was a general impression among several investigators that oval cells did not seem to be major sites of origin for hepatocellular carcinoma with most carcinogens,[2,5,9-11,24] this has been questioned more recently. The finding of several phenotypic markers in common between oval cells or subsets of oval cells and some hepatocellular carcinomas,[2,12,16-18,22,25-33] together with other observations indicating an observable transition between oval cells and hepatocytes in livers of rats fed azo dyes,[10,13,15,17,34,35] has led to a reevaluation of the possible role of oval cells in the genesis of liver cancer. The recent markers used include α-fetoprotein (AFP),[16,18,19,22,26,28,31-33] γ-glutamyltransferase (γ-GT),[2,12,17,25,27,29,30] and fetal aldolases.[26]

The biology of the oval cells, including their origin or origins, life history, and fate or fates, remain poorly understood. Origins from ductular epithelial cells, including the canals of Hering,[7,13,14,16,36-40] from hepatocytes,[41-43] or from hypothetical "stem" cells presumed to be located in the portal zone[16,18,21,22] have been suggested. With respect to their fate, some reports propose their differentiation to hepatocytes,[10,13,14,19,35,36,39,42,44,45] while others suggest atrophy and disappearance.[38,46-49]

Grisham and Hartoft,[37] using electron microscopy in rats fed either choline deficient or ethionine-supplemented diets, could find no ultrastructural changes in the oval cells that suggested anything other than a ductular identification. In a subsequent study, Grisham and Porta[38] used both electron microscopy and labeling with ^{3}H-thymidine (^{3}H-TdR) and could find no evidence for transformation of oval cells ("ductular cells") into hepatocytes and, vice versa, under a variety of experimental conditions. Rubin and colleagues,[47,49] using prelabeling with thymidine under both similar and different experimental conditions, found oval cells ("ductular cells") to be short-lived and could observe no transformation to hepatocytes.

A source of difficulty in these studies was the inability to mark or label selectively the oval cells without concomitantly labeling variable numbers, often a considerable proportion, of hepatocytes. This difficulty has been eliminated in the present study by finding conditions to selectively label proliferating oval cells with radioactive thymidine without labeling the surrounding hepatocytes. During an ongoing investigation of mechanisms underlying the resistant hepatocyte model of liver carcinogenesis,[2,5,50] it was found that the post-PH hepatocyte proliferation was virtually completely inhibited by dietary 2-AAF in the caudate lobe of the liver. The oval cell proliferation associated with this experimental condition[51] allows the study of some important aspects of oval cell biology and is the major subject of this communication. An observation of interest made during this study was the intense staining of the vast majority, if not all, of the oval cells by an immunoperoxidase method for prekeratin.[54] This finding provides a staining method that allows the ready distinction between the proliferating oval cells and the surrounding hepatocytes.

Materials and Methods

Animals

Male Fischer 344 rats (Charles River Breeding Laboratories, Wilmington, Mass, and Detroit, Mich) weighing from 130 to 160 g were maintained on a semisynthetic diet containing 26% protein (#101) (Bio-Serv, Frenchtown, NJ). The animals were given food and water *ad libitum* with a 12-hour light and dark daily cycle and were acclimatized to their environment for 1 week before the start of each experiment.

Experimental Regimens

One group of 3 rats were used as controls. They were fed the basal diet without added 2-AAF and were subjected to a standard two-thirds PH. All the remaining animals were part of 3 different experimental groups. Animals in each experimental group were placed on the basal diet containing 0.02% 2-AAF for 1 week, then subjected to a standard two-thirds PH and continued for an additional week on the 2-AAF-containing diet.[2,3] The rats were returned to the basal diet until the termination of the experiment. The dietary 2-AAF was used to inhibit proliferation of hepatocytes,[2,52] and the PH provided a strong stimulus for liver regeneration. Under these experimental conditions, the liver shows vigorous oval cell proliferation without initiation of carcinogenesis or the development of foci or nodules of altered resistant hepatocytes.[2,3,51]

Experiment 1

Thirty-two animals each received 6 intraperitoneal injections of 50 μCi ^{3}H-Tdr (New England Nuclear, Boston, Mass, 71.8 Ci/mmol) per day at 4-hour intervals for 4 days, beginning 48 hours after the time of PH. Groups of 3 or 4 rats were sacrificed at intervals by cervical dislocation after an 18-hour period of fasting, beginning the day of termination of the 2-AAF-containing diet, ie, 7 days after PH, and

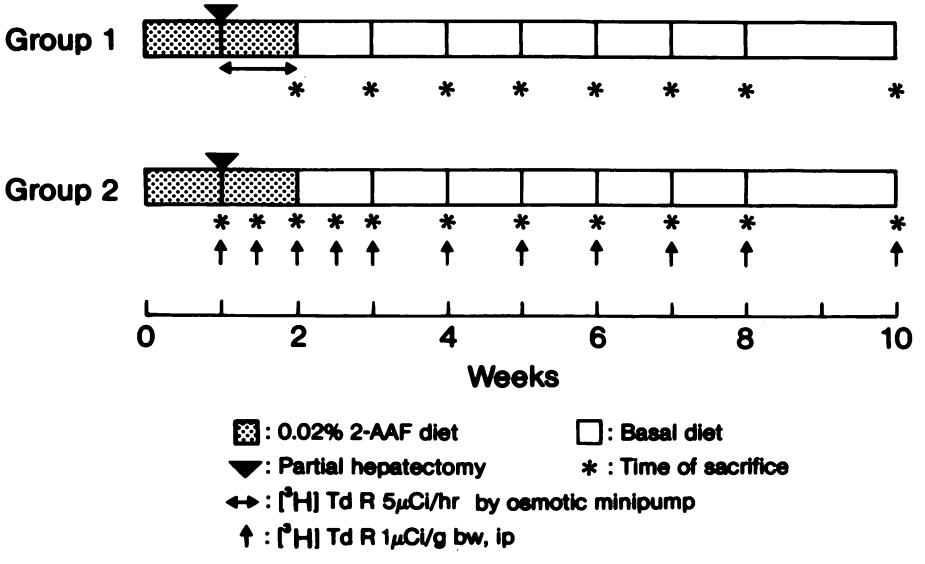


Figure 1—Scheme of experimental design for Experiment 2. In Group 2, the thymidine (^{3}H-TdR) was given 1 hour before the time of sacrifice.

ending on Day 49 after PH. Aliquots from each lobe of the liver were fixed in cold acetone for staining for γ-GT[29,53] and in formalin for hematoxylin and eosin (H&E).

Experiment 2 (Figure 1)

In *Group 1*, 33 rats were each given ^{3}HTdR (New England Nuclear, Boston, Mass, 73.6 Ci/mmol) continuously for 7 days, beginning immediately after the PH. Alzet osmotic minipumps, model 2001 (Alza, Palo Alto, Calif), delivering 5 μCi/hr, were implanted subcutaneously in the back. After the 7-day exposure, the minipump was removed. Four or 5 animals were sacrificed at 2, 3, 4, 5, 6, 7, 8, and 10 weeks after the beginning of the experiment. The 3 control rats received ^{3}H-TdR beginning immediately after the PH and were sacrificed at 7 days after PH.

In *Group 2*, 28 rats were each given ^{3}H-TdR intraperitoneally as a single pulse dose of 0.5 μCi/g body weight 1 hr prior to sacrifice. Three or four animals were sacrificed at 7, 10, and 14 days and 3, 4, 6, 8, and 10 weeks after the beginning of the experiment.

In all animals in experiment 2 (Groups 1 and 2), very careful attention to each lobe remaining after PH was performed. The post-PH liver lobes were separated into the larger right lobe (the right anterior and the right posterior) and the smaller caudate lobe. Some liver slices from each lobe were fixed in cold acetone for 24 hours and embedded in low-melting paraffin.[29] Three contiguous paraffin sections were used, one stained for perkeratin, one for epoxide hydrolase, and one for H&E. In addition, other slices, again from each lobe, were fixed in 10% formalin and stained for routine H&E.

Combined Immunohistochemical Studies for Prekeratin and Epoxide Hydrolase (EH) and Autoradiography

The following antibodies were used: 1) rabbit antiserum against human prekeratin from stratum corneum from the human foot[54]—the prekeratin antiserum reacted positively with prekeratin from stratum corneum of rat leg on Ouchterlony double-immunodiffusion test (kindly provided by Dr. Reuben Baumal[54])—and 2) rabbit antiserum against rat epoxide hydrolase[55] (EC. 3.3.2.2.).

Prekeratin and epoxide hydrolase were localized by means of the peroxidase–antiperoxidase (PAP) method according to Sternberger et al.[56] The paraffin sections were deparaffinized in benzene for 10 minutes and dried quickly. They were then sequentially treated with normal swine serum, rabbit anti-human epidermal prekeratin (1:1000 dilution) or rabbit anti-rat epoxide hydrolase (1:400 dilution), swine anti-rabbit IgG (1:10 dilution), and rabbit peroxidase complex (1:50 dilution). The site of peroxidase binding was revealed by the diaminobenzidine method of Graham and Karnovsky[57]. The peroxidase-stained sections were coated with Kodak NTB 3 emulsion and kept in a sealed dessicator for 6 weeks at 4 C. After development, the slides were counterstained with hematoxylin for microscopic examination.

As a positive control for specificity of anti-prekeratin antibody, sections of normal rat skin were stained with the prekeratin antiserum. As a negative control for specificity of anti-prekeratin or anti-EH antibody preimmune rabbit serum was used instead of the prekeratin or EH antiserum.

Quantitation of Oval Cells and Labeled Cells

The lobules were divided into the three zones of Rappaport, ie, periportal, mizonal, and central portions, by visually dividing the lobule into three equal widths designated—Zone 1, Zone 2, Zone 3, respectively. The labeling index (LI) for hepatocytes and oval cells were calculated by counting more than 5000 cells of each type in randomly selected areas, with an equal representation of each zone.

For measuring the number of oval cells and hepatocytes, use was made of a 10-mm ocular grid divided into 100 squares (American Optical #1408A) at × 100 magnification. All oval cells staining for prekeratin and all hepatocytes staining for EH were counted in 10 randomly selected fields, each containing 100 squares. For adjustment for possible variations in the number of oval cells or hepatocytes per unit sectional area, the relation of the weight of the caudate lobe and maximal horizontal sectional area of the caudate lobe was analyzed by a bivariate curve-fitting program. The image of the caudate lobe, projected orthographically, as the maximum horizontal sectional area, was measured by a Kontron image analyzer. We did this to compensate for the fact that even if the absolute number of oval cells did not change, their number would show an apparent decrease if the number of hepatocytes increased.

Results

Experiment 1

The results of the first experiment indicated the complete labeling with ^{3}H-TdR of all the proliferating oval cells in Zones 1 and 2 at 7 days after PH and the progressive loss of the majority of these cells by 49 days after PH. At the latter time, 18% of the oval cells were still present and labeled. The nearby hepatocytes, mainly in Zone 1, showed a small percentage, about 6%, to be labeled with ^{3}H-TdR on Day 14 after PH. In this experiment, no separate analysis was made of the right and caudate lobes remaining after PH, and this made a definitive and conclusive analysis of the possible fates of the oval cells impossible.

However, at that time, it was observed that the labeling patterns of hepatocytes were quantitatively different between the caudate and right lobes. This discovery, together with the use of staining for prekeratin and for EH, made it possible to carry out the following definitive analysis of the biologic features of the oval cells in the caudate and right lobes.

Experiment 2

Changes in Liver Weight

The changes in liver weight as a function of time in Groups 1 and 2 were not significantly different from each other (Table 1), thus suggesting that the exposure to the radioactive TdR for 1 week in the animals in Group 1 did not influence adversely the overall response following PH, as compared with the animals in Group 2 which received only a single dose of TdR just before sacrifice. The weights of the right lobe increased significantly faster than those of the caudate lobe ($P < 0.01$ or 0.001) after release from the 2-AAF diet, such that the weight of the caudate lobe relative to the total liver weight decreased from 30% at 1 week to about 18% at 6–8 weeks.

Histologic Changes

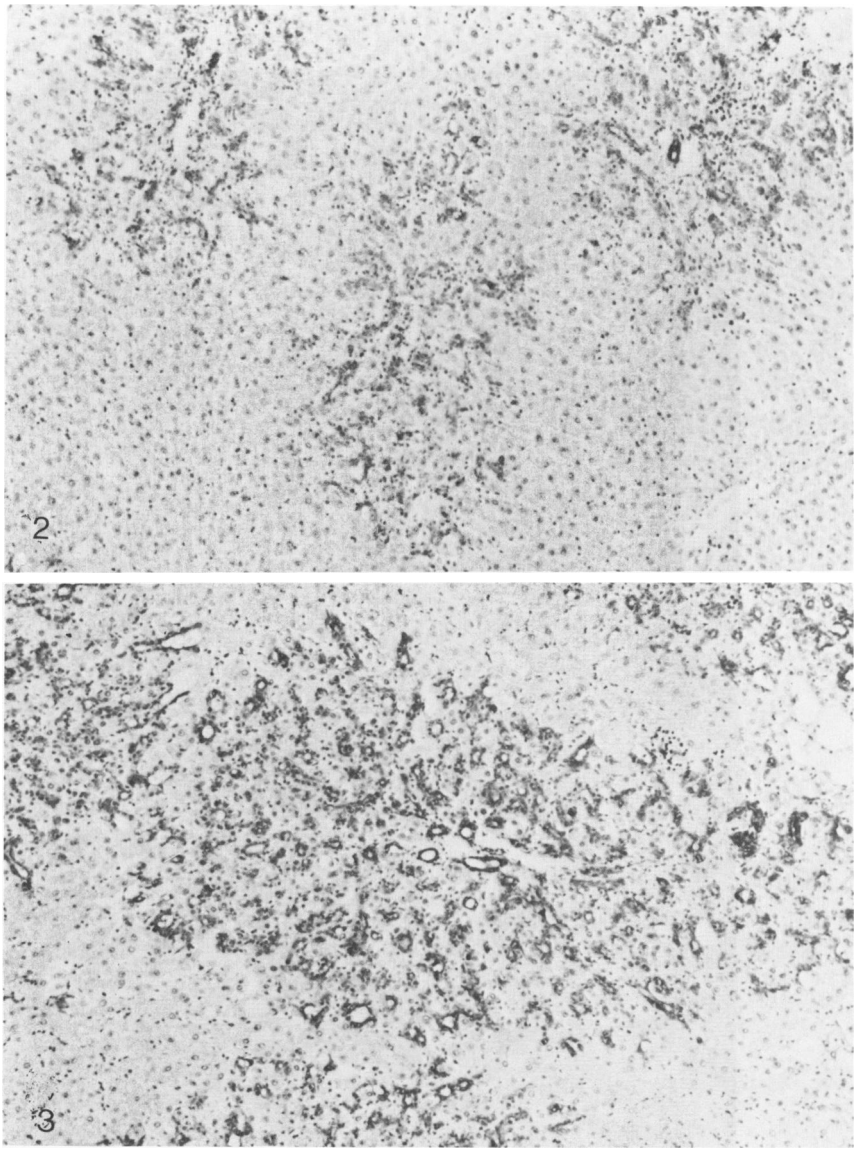
In agreement with previous results[51], the livers of animals fed the basal diet containing 0.02% 2-AAF for 1 week were not visibly different from the livers of control rats. No necrosis, cell hypertrophy, or other histologic or cytologic changes were evident by light microscopy.

Following the performance of PH after 1 week's exposure to the dietary 2-AAF and with the exposure continued for a second week as in this study, the liver began to show obvious proliferation of "oval cells" in the portal tracts along with proliferation of bile duct epithelium (Table 1, Figures 2–5). These oval cells had scanty lightly basophilic cytoplasm and pale-blue-staining nuclei. The majority of the nuclei contained a fine chromatin network and prominent nucleoli. The nuclear membrane was sharply outlined in contrast to the cell boundary, which was indistinct. The nuclei in most of the bile duct epithelial cells were paler and more vesicular than in normal liver. An occasional mitotic figure was seen. A scattering of polymorphonuclear leukocytes was seen in some areas of greatest oval cell proliferation. At 3 weeks the oval cells were much more abundant and had extended well into Zone 2 of the liver acinus. They were present between the hepatocytes and the sinusoids. Isolated

Table 1—Changes in Liver Weight and Number of Oval Cells in Groups 1 and 2 (Experiment 2)

	Weeks after beginning of experiment									
	1	1.5 (10 days)*	2	3	4	5	6	7	8	10
Group 1										
Number of rats	–	–	5	4	4	4	4	4	4	4
Liver weight (mg)										
Right lobe	–	–	1.87 ± 0.12†	3.02 ± 0.21	4.99 ± 0.30	6.70 ± 0.45	7.30 ± 0.63	7.45 ± 0.60	7.55 ± 0.52	7.82 ± 0.61
Caudate lobe	–	–	0.77 ± 0.06†	0.88 ± 0.07	1.10 ± 0.10	1.41 ± 0.10	1.59 ± 0.12	1.65 ± 0.14	1.76 ± 0.10	1.95 ± 0.14
			(29.20 ± 0.80)‡	(22.60 ± 1.10)§	(18.10 ± 0.80)‖	(18.10 ± 0.80)‖	(17.40 ± 0.80)‖	(17.90 ± 0.80)‖	(18.90 ± 1.10)‖	(20.00 ± 1.20)
Number of oval cells per unit sectional area (sq mm)										
Right lobe	–	–	490 ± 46¶	1082 ± 120	616 ± 72**	462 ± 53**	258 ± 32¶	142 ± 17¶	104 ± 17¶	80 ± 12¶
Caudate lobe	–	–	475 ± 42¶	1191 ± 137	798 ± 84**	598 ± 49**	350 ± 37¶	223 ± 20¶	162 ± 14¶	124 ± 10¶
Group 2										
Number of rats	3	4	4	4	4		3		3	3
Liver weight (mg)										
Right lobe	1.52 ± 0.09	1.63 ± 0.11	1.88 ± 0.10	3.12 ± 0.26	4.87 ± 0.34		7.27 ± 0.70		7.62 ± 0.58	7.90 ± 0.70
Caudate lobe	0.65 ± 0.03	0.70 ± 0.02	0.78 ± 0.06	0.90 ± 0.09	1.12 ± 0.11		1.62 ± 0.10		1.74 ± 0.13	1.92 ± 0.12
	(30.00 ± 0.70)	(30.00 ± 0.60)	(29.30 ± 1.00)	(22.40 ± 1.50)§	18.7 ± 0.70‖		(18.20 ± 0.70)‖		(18.60 ± 0.80)‖	(19.60 ± 1.00)
Number of oval cells per unit sectional area (sq mm)										
Right lobe	9 ± 3¶	110 ± 21¶	480 ± 41¶	1052 ± 101	625 ± 64**		241 ± 29¶		100 ± 18¶	85 ± 15¶
Caudate lobe	8 ± 4¶	102 ± 18¶	460 ± 52¶	1180 ± 115	810 ± 81**		352 ± 34¶		158 ± 17¶	120 ± 16¶

* Ten days after the beginning of the experiment.
† Mean ± SD.
‡ Number in parentheses indicates the percent weight of the caudate lobe per total liver weight after partial hepatectomy.
§ Significantly different from the value at Week 2 ($P < 0.01$.)
‖ Significantly different from the value at Week 2 ($P < 0.001$).
¶ Significantly different from the value at Week 3 ($P < 0.001$).
** Significantly different from the value at Week 3 ($P < 0.01$).



Figures 2-6 – Immunoperoxidase staining of oval cells using antibody to prekeratin. All the slides were counterstained with hemotoxylin. Prekeratin staining was strong in all oval cells and negative in hepatocytes. **Figure 2** – Section of the caudate lobe from an animal in Group 1 at Week 2. Notice the proliferation of prekeratin-positive oval cells around portal triads. (×40) **Figure 3** – Section of the caudate lobe from an animal in Group 1 at Week 3. Notice the further extension of the proliferation of oval cells and the arrangement of many as small ducts. (×40)

degenerating liver cells with some polymorphonuclear leukocytic infiltration were present between the oval cells in some areas. A few of the liver cells in regions of oval cell proliferation showed hyperchromatic nuclei with large nucleoli, while neighboring parenchymal cells were atrophic. In many instances, the oval cell extensions in Zones 1 and 2 consisted of two parallel rows of oval cells with a small space between the cells. This space often enlarged to form ductlike spaces (Figures 3-5).

During the period of oval cell "disappearance" between weeks 4 and 10, the liver showed a progressive return toward the control appearance. By Week 10, only minimal numbers of oval cells could be seen immediately surrounding portal areas (Figure 6). Unlike what is seen with exposure to azo dyes such as butter yellow and derivatives,[9,10,13,14,34,35] in which the oval cells undergo an obvious cytologic change with enlargement of nuclei and rounding of the cell border, the oval cells, except those that lined ducts, in this study did not change their appearance at any time. Unlike the hepatocytes in the control animals, the hepatocytes, under these conditions, showed only very minimal proliferation, as noted previously.[2-6]

Patterns of Staining for Prekeratin and EH

Normal rat liver consistently showed strong positive staining of bile duct epithelium for prekeratin but very little staining in hepatocytes and an absence of staining in other cells. At 1 week after PH in the 2-AAF-PH regimen, proliferating "oval cells" were observed in the portal triads and were found between

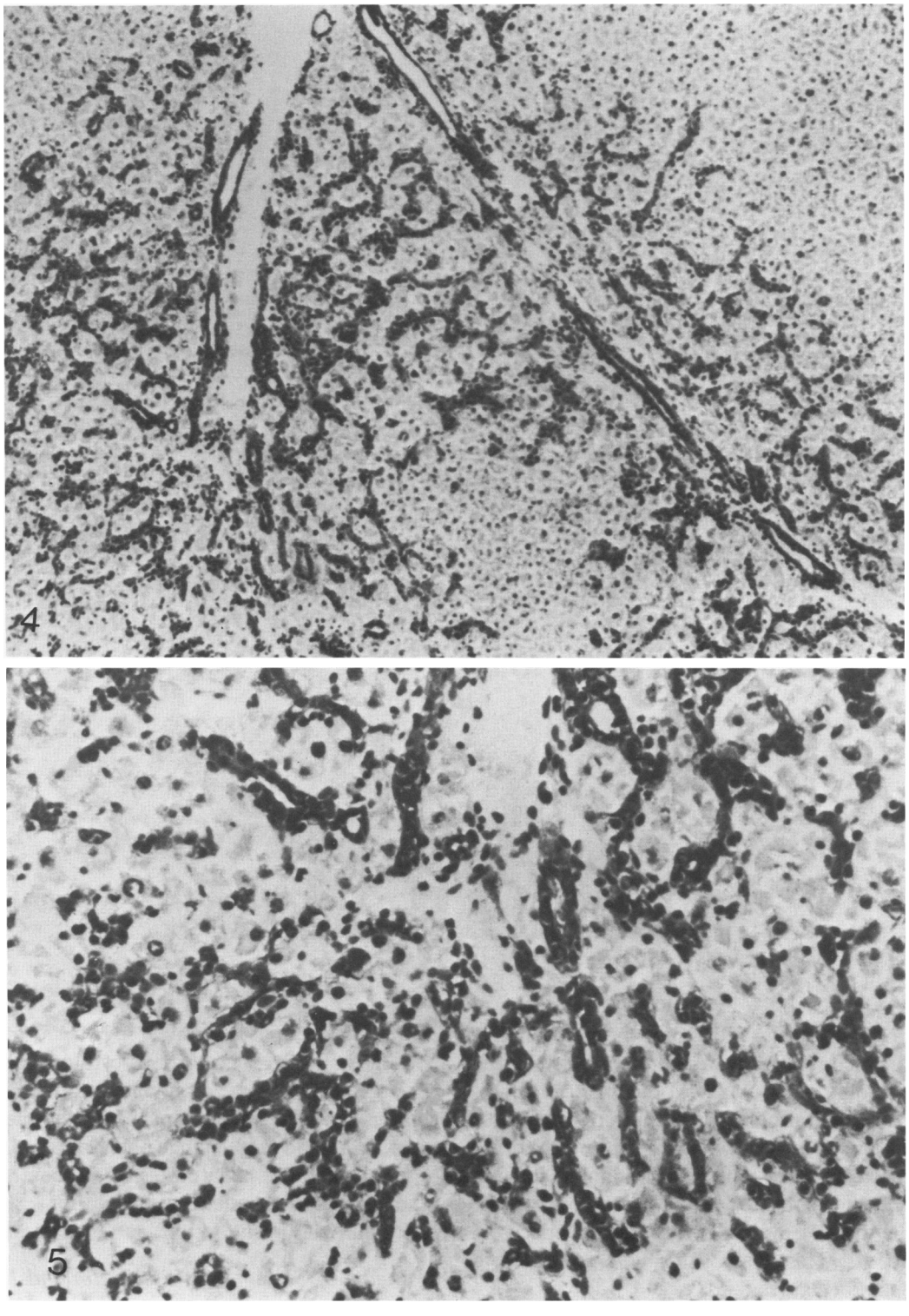


Figure 4—Section of the caudate lobe from an animal in Group 1 at Week 2 (same animal as in Figure 3) at higher magnification. Notice the positively stained "oval cell proliferation" and the unstained hepatocytes. (×80) **Figure 5**—Section of the caudate lobe from an animal in Group 1 at Week 2. Higher magnification of Figure 4. Notice the frequent arrangement of oval cells about lumens (×120)

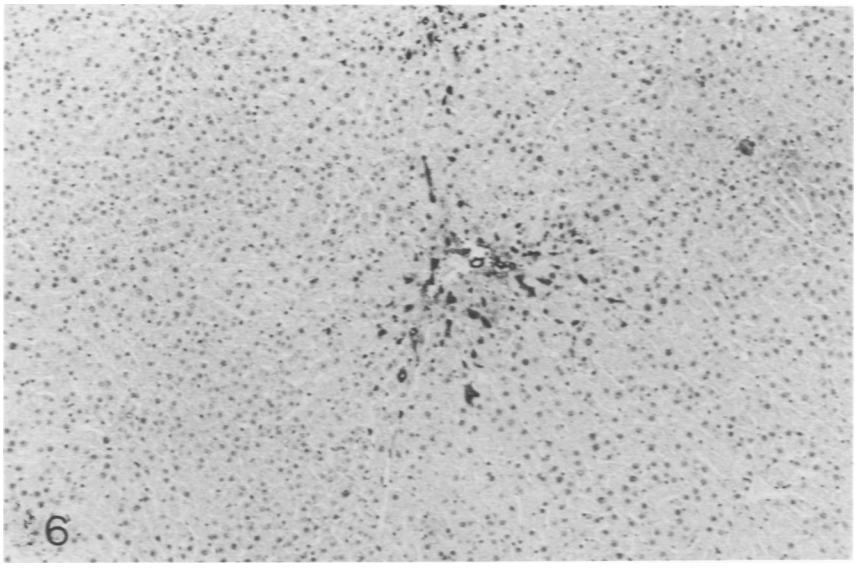


Figure 6—Section of the caudate lobe from an animal in Group 1 at Week 10. Notice the marked decrease in the number of oval cells (compare with Figures 2 and 3) and their presence only around portal triads. (×40)

parenchymal cells in Zone 1 and Zone 2 of virtually all liver acini (Figures 2–5). These cells showed uniformly heavy staining for prekeratin but with slightly lesser intensity than in bile duct epithelium. The surrounding hepatocytes were uniformly negative for prekeratin staining (Figures 2–5). In the sections of rat skin used for positive controls, prekeratin was detected in all layers of the epidermis and in some skin adnexa, including hair follicles, sweat gland ducts, and ducts of sebaceous glands. The prekeratin-positive areas in liver and skin were negative when preimmune rabbit serum was used in place of the immune serum.

The localization of EH activity in the normal rat liver showed a characteristic zonal distribution[55,58] with more intense staining in Zone 3 than in Zone 1. The staining was confined to the cytoplasm of the hepatocytes and was absent from hepatocyte nuclei, bile duct epithelium, and vascular endothelium. On exposure to dietary 2-AAF for 2 weeks, the intensity of staining increased in all three zones of the liver lobules as previously described.[55,58] Ductular cells and proliferating oval cells were evident in the periportal area (Zone 1), but these cells were uniformly negative. After release from the 2-AAF diet, hepatocytes showed an obvious decrease in staining with a return to the control zonal pattern. However, liver cells located between oval cells or around periportal areas remained strongly positive until 4 weeks in the right lobe and 6 weeks in the caudate lobe. Epoxide hydrolase is an excellent marker for distinguishing hepatocytes from oval cells or bile duct epithelium.

Table 2—Changes in Labeling Indexes of Oval Cells and Hepatocytes in Rats in Group 1 After Cumulative Labeling of Osmotic Minipump for 7 Days

	Weeks after beginning of experiment							
	2	3	4	5	6	7	8	10
Number of animals	4	3	3	3	3	3	3	3
Oval cells								
Right lobe	99.4 ± 0.5*	99.5 ± 0.7	98.6 ± 1.2	97.5 ± 1.4	98.2 ± 1.9	97.0 ± 1.7	96.3 ± 3.0	96.0 ± 2.9
Caudate lobe	99.5 ± 0.6	98.7 ± 1.6	99.2 ± 1.0	98.6 ± 1.3	97.3 ± 2.0	98.1 ± 1.5	95.4 ± 2.8	96.2 ± 2.6
Hepatocytes								
Right lobe	7.6 ± 1.2	20.4 ± 4.9†	>30‡	>30‡	>30‡	>30‡	>30‡	>30‡
Caudate lobe	0.2 ± 0.1§	0.4 ± 0.2§	0.2 ± 0.1§	0.2 ± 0.2§	0.1 ± 0.1§	0.1 ± 0.1§	<0.1§	<0.1§

* Mean ± SD.
† Significantly different from the value at Week 2 ($P < 0.01$).
‡ Significantly different from the value at Week 2 ($P < 0.001$).
§ Significantly different from the value of the right lobe at the same experimental period ($P < 0.001$).

Table 3—Changes in Labeling Indexes of Oval Cells and Hepatocytes in Rats in Group 2 After a Single Dose of Thymidine

	Weeks after beginning of experiment							
	1	1.5 (10 days)	2	3	4	6	8	10
Number of animals	3	4	4	4	4	3	3	3
Oval cells								
Right lobe	ND*	35.2 ± 5.2	32.8 ± 5.4	5.7 ± 2.0†	0.4 ± 0.2†	0.1 ± 0.1†	<0.1†	<0.1†
Caudate lobe	ND	33.6 ± 6.1	31.4 ± 4.3	6.0 ± 1.3†	0.5 ± 0.2†	0.1 ± 0.1†	<0.1†	<0.1†
Hepatocytes								
Right lobe	0.0	<0.1	0.2 ± 0.1	5.2 ± 0.7‡§	1.9 ± 0.4‡	0.4 ± 0.1	0.1 ± 0.1	<0.1
Caudate lobe	0.0	0.0	<0.1	1.8 ± 0.5‡	1.4 ± 0.3‡	0.8 ± 0.2‖	0.2 ± 0.1	0.1 ± 0.1

* Number of oval cells is insufficient to calculate the LI of oval cells.
† Significantly different from the values at Weeks 1, 1.5, and 2 ($P < 0.001$).
‡ Significantly different from the values at Weeks 1, 1.5, and 2 ($P < 0.01$).
§ Significantly different from the value of the caudate lobe at the same period ($P < 0.05$)
‖ Significantly different from the values at Weeks 1, 1.5, and 2 ($P < 0.05$).

Control liver sections from each experimental period incubated with preimmune rabbit serum were consistently negative for staining.

Cell Dynamics

Group 1

The sequential changes in LI of oval cells and hepatocytes in each liver lobe are presented in Table 2. Almost 100% of oval cells in each liver lobe were labeled at Week 2, and the LI did not decrease below the 95% level during the course of the experiment (Table 2). Although the grains over the nuclei of oval cells decreased because of cell division, the average number of grains did not fall below 12.4 ± 3.1 (± SD, n = 4) at Week 10. The LI of EH-positive hepatocytes in the right lobe at Week 2 was 7.6% ± 1.2%, in contrast to that in the caudate lobe, where the LI was very low, 0.2 ± 0.1% (± SD, n = 5) ($P < 0.001$). Some of the hepatocytes in the right lobe showed mitotic activity after partial hepatectomy during the 2-AAF feeding period, but proliferation of hepatocytes in the caudate lobe was completely inhibited by the dietary 2-AAF during the week when the ^{3}H-TdR was administered by the osmotic minipump. After release from the 2-AAF diet, labeled hepatocytes in the right lobe quickly increased through their cell division, and the grains over their nuclei decreased. The grains over the very few labeled hepatocytes in the caudate lobe also decreased through mitosis, but the LI of hepatocytes in this lobe did not increase significantly.

In addition to the oval cells and hepatocytes, cells lining the sinusoids become labeled with thymidine. They slowly become less labeled over the next 8 weeks. Otherwise, they did not appear to change in appearance.

Group 2

During the period of 2-AAF feeding, the oval cells in both liver lobes showed a high LI (Table 3). This decreased rapidly from over 30% at 10 days and 2 weeks to about 6% at 3 weeks ($P < 0.001$). The labeling of oval cells by a single dose of thymidine was virtually absent after 4 weeks. Hepatocytes showed no labeling in the caudate lobe and only a rare labeled hepatocyte in the right lobe during the 2-AAF feeding period. After release from the 2-AAF diet, hepatocytes in both liver lobes began to proliferate. At week 3, the LI of hepatocytes in the right lobe was significantly higher ($P < 0.05$) than that in the caudate lobe. These labeled hepatocytes usually were found away from oval cells. Changes in labeling indices of oval Cells and hepatocytes in the animals in Group 2 are presented in Table 3.

Some of the cells lining the sinusoids became labeled. This did not seem to vary in an obvious way at any time during the study.

Quantitation of Oval Cells and Hepatocytes

The number of oval cells per unit sectional area in both right and caudate lobes was maximum at Week 3 and decreased with time as indicated Figures 2, 3, and 6 and Table 1. Because no significant differences in the number of oval cells between groups 1 and 2 were found, the differences in the amounts of the ^{3}H-TdR given apparently did not influence the induction and fate of oval cells.

Because the cumulative LI of hepatocytes in caudate lobe at Week 2 was significantly lower ($P < 0.001$) than that in right lobe in Group 1, further

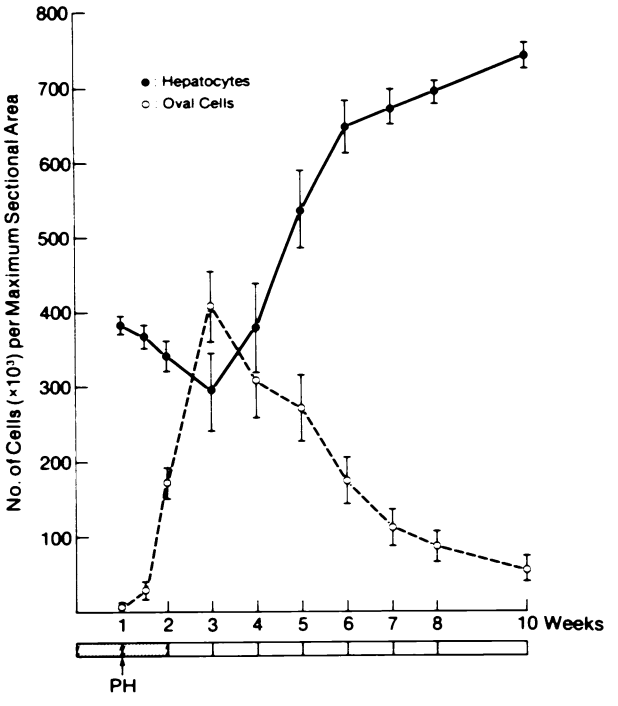


Figure 7 – Sequential changes in the number of oval cells and hepatocytes per maximum horizontal sectional area of caudate lobe. The points and bars are means and standard deviations of values calculated in animals from Groups 1 and 2. *PH*, partial hepatectomy. ▨, 0.02% 2-AAF. □, basal diet.

analysis of quantitation of oval cells and hepatocytes was focused on the caudate lobe. The relation between weight (W, mg) of the caudate lobe and maximum horizontal sectional area (A, sq mm) as analyzed by a bivariate curve-fitting program is:

$$A = 5.49\ W^{0.61}\ (r = 0.995)$$

Changes of the maximum horizontal sectional area thus showed a significant relation to changes in liver weight. Accordingly, changes in the number of oval cells and hepatocytes per maximum horizontal sectional area approximate closely the relative changes in numbers of oval cells and hepatocytes in the caudate lobe, although the numbers do not indicate the absolute number of oval cells and hepatocytes per liver. Sequential changes in the number of oval cells and hepatocytes per maximum horizontal sectional area of the caudate lobe are summarized in Figure 7. The adjusted number of oval cells increased until Week 3 and then decreased. In contrast, the number of hepatocytes decreased until Week 3 and then increased.

Discussion

It is clearly evident from this study that the proliferating oval cells induced by the dietary 2-AAF–PH in all remaining liver lobes label intensely with ^{3}HTdR and stain well for prekeratin but not for EH. A novel finding in this regimen is that hepatocytes in the caudate lobe label only at a very low level. This has made it possible to pose an important question in liver pathology and carcinogenesis: do oval cells, as a group or as a significant subgroup, become hepatocytes? Under the conditions of this study no hepatocytes became labeled over the 10-week period during which the oval cells showed a maximum proliferation and regression. Whatever the mechanism of "disappearance" or "regression" of the oval cells, differentiated hepatocytes are not an obvious "product" of the process in the caudate lobe.

For example, the number of oval cells per horizontal sectional area of the caudate lobe was maximal at Week 3 ([408 ± 48] × 10^3) and continuously decreased until Week 10 ([56 ± 15] × 10^3). During this period, about 352 × 10^3 oval cells per maximum horizontal sectional area disappeared. If all the oval cells that disappeared differentiated into hepatocytes, at least 45% of the hepatocytes would have become labeled. No labeled hepatocytes were seen in the caudate lobe at any time during the experiments. With over 80% of the oval cells disappearing by 10 weeks without any evident residuum, it is probable that the majority of, if not all, such cells have quite a short half-life. Rubin et al[47] reported that the half-life of ductular or oval cell DNA in ethionine-treated rats was 7.7 days and that removal of the stimulus for their proliferation led to their disappearance without altering their life span. Our results are not inconsistent with a reasonably short half-life for the oval cells as a group.

The conclusion concerning lack of oval cell conversion to hepatocytes would seem to be valid for the 10-week period of study, providing the oval cells did not lose their label before their presumed conversion to hepatocytes. If the conversion involved a preliminary further period of cell proliferation during the differentiation process, it might theoretically be possible for all the label that was incorporated during the post-PH week to have been lost before the conversion became evident. This possibility is virtually eliminated by the observation (Table 3) that cell proliferation of oval cells, as indicated by pulse labeling with thymidine before sacrifice, was very low at 4 weeks and thereafter. Also, if any significant number of oval cells began to change their appearance at some time prior to the end of the experiment at 10 weeks, this would have become evident. For example, in oval cell proliferation induced in rats exposed to butter yellow

or derivatives,[9,10,35] a change in the appearance of the oval cells is readily obvious on even a casual histologic examination.

The conclusions made from this study are in good agreement with those of Grisham and associates[37,38] and of Rubin and colleagues,[47,49] using quite different models with different patterns of labeling and different chemicals. Their evidence also failed to suggest any observable degree of conversion of oval cells ("ductular cells") to hepatocytes.

However, an ever present difficulty concerns the possible heterogeneity of the "oval cells." Are they one type of cell or several types? If the latter, perhaps only a small number have the potential to become tranformed into hepatocytes. Again, judging by the findings in animals exposed to azo dyes, the change in appearance of the oval cells involves the majority of cells, not a small number.

Also, although oval cells do not appear to give rise to hepatocytes in the present regimen, the possibility remains that oval cells might give rise to altered hepatocytes in livers initiated for carcinogenesis. The 2-AAF–PH regimen used to generate oval cells in this study is not associated with the initiation of hepatocarcinogenesis, but only with the selection of preexisting initiated cells for promotion.[3,4,52,59] Conceivably, during initiation with chemicals, some biochemical or molecular change could occur in the unidentified precursors of proliferating oval cells which might then be biologically different from that in uninitiated liver. Under such theoretical circumstances, some oval cells might then be able to differentiate to hepatocytes and act as one precursor cell for the altered hepatocytes that constitute the foci and nodules early in hepatocarcinogenesis. The present regimen, with initiation added, could be appropriate to test this hypothesis.

The histologic appearance of the oval cell proliferation in the caudate lobe, including their periods of appearance and disappearance, resembles closely that seen in other models with several different hepatocarcinogens[2,7,10,17,19,21,23,24,44,47] *with the exception of the azo dyes.*[9,10,35] It is likely, therefore, that the oval cells in the caudate lobe in this study are not fundamentally different from oval cells in the many other situations relating to hepatocarcinogenesis in the rat.

The major basis for the suggestions to date concerning the possible role of a subset of oval cells in the genesis of hepatocellular carcinoma is the finding of phenotypic histochemical markers common to oval cells and to hepatocellular carcinoma. As indicated in a recent summary of all of these studies,[60] any selection of common markers is quite arbitrary. It is possible to "prove" any suggested histogenetic sequence by selecting the histochemical markers that fit the particular hypothesis and ignoring all other findings.

One of the important aspects of this study is the regional quantitative difference in the response of the liver to a chemical. The basis for this regional difference in the inhibition of hepatocyte proliferation by 2-AAF is not understood. Although the dietary 2-AAF inhibited to a major extent the proliferation of hepatocytes in all remaining liver, there was a small proportion of uninhibited hepatocytes in the right lobe. Variations in vascular supply, in nervous innervation, in temperature, or in the activities of enzymes concerned with the metabolism and activation of carcinogens, such as 2-AAF, could play some part in this difference. Regional differences in the growth of normal and neoplastic cells in the skin have been related to temperature differences, as one important variable.[61]

In this discussion we have purposely avoided the issue of the origin or origins of the oval cells. Although the common pattern of staining for prekeratin could be interpreted as evidence that virtually all the oval cells are derived from duct or ductular epithelium, this is not justified by a comparison of all the various published phenotypic histochemical markers in liver.[60] Some more sharply defined model would probably be necessary for us to pose this question in a conclusive manner.

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