American Journal of Pathology, Vol. 151, No. 4, October 1997

Short Communication

Bile Ductular Damage Induced By Methylene Dianiline Inhibits Oval Cell Activation

Bryon E. Petersen, Valerie F. Zajac, and George K. Michalopoulos

From the Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania

Administration of 2-acetylaminofluorene (2-AAF) given before a two-thirds partial hepatectomy (PHx), results in suppression of hepatocyte proliferation and stimulation of oval cell proliferation. Our objective in this study was to examine the oval cell response and associated α-fetoprotein (AFP) gene expression by combining 2-AAF with selective hepatic damage caused by either carbon tetrachloride (CCl_4) exposure or by PHx. We also examined oval cell response with the above two protocols (2-AAF/CCl_4 and 2-AAF/PHx) as affected by previous bile ductular damage caused by 4,4′-methylene dianiline (4,4′-diaminodiphenylmethane, DAPM) exposure. DAPM is an aromatic diamine, known to cause bile ductular damage in both humans and animals. Using the protocols of 2-AAF/CCl_4 and 2-AAF/PHx, when DAPM was given 24 hours before the hepatic injury, no oval cell proliferation was seen (histological) and AFP expression was not detected by Northern blot analysis. These results provide direct evidence that oval cells are closely associated with the biliary epithelial cells and supports the theory that hepatic oval cells may originate from cells derived from either intraportal or periportal ductules. *(Am J Pathol 1997, 151:905–909)*

The origin of precursors of oval cells in the adult liver of humans and animals has been the subject of controversy over the past several decades. Activation of the hepatic stem cell compartment is histologically detectable only when hepatocyte proliferation is suppressed or delayed. 2-Acetylaminofluorene (2-AAF) given before two-thirds partial hepatectomy (PHx) results in suppression of hepatocyte proliferation, thus allowing for sustained proliferation of oval cells.[1] Oval cells arise in the periportal region of the liver. Morphologically, these oval cells are small in size (approximately 10 μm) and have a large nucleus to cytoplasmic ratio, with an oval-shaped nucleus (hence their name).[2] They have similarities to bile ductular cells with distinct isoenzyme profiles, expressing certain keratin markers (eg, CK-19) and γ-glutamyl transpeptidase (GGT) and expressing hepatic markers such as α-fetoprotein (AFP).[3–7] Monoclonal antibodies exist, such as OV6, OC2, and BD1, which also aid in their characterization.[8,9] Oval cells also express high levels of certain mRNAs such as AFP and stem cell factor.[10]

Several previous studies have documented the emergence of oval cells after chemical injury, such as with ethionine[11] and, more recently, with galactosamine.[12,13] The injury caused by the above chemicals is diffuse throughout the entire lobule, as is the regenerative response induced by PHx. Other chemicals, however, such as CCl_4, are associated with induction of histological damage that is limited to a specific lobular zone. We performed the studies mentioned in this report to examine the relationship between oval cells and biliary epithelium. Previous studies have also described the phenotypic and histochemical similarities between oval cells and biliary epithelium and have provided strong indirect evidence for the relationship between the oval cells and the epithelium of small intraportal ducts or canals of Herring.[7] On the other hand, oval cells also express histochemical markers associated with hepatocytes, such as AFP,[9,10] and expression of hepatitis B surface antigen.[14] To directly examine the relationship between bile ductular epithelium and the origin of the oval cells, we used the toxin 4,4′-methylene dianiline (4,4′-diaminodiphenylmethane, DAPM). This toxin is known to induce selective damage limited to the destruction of the biliary epithelium[15] by mechanisms not yet fully understood (see Discussion). We treated rats with DAPM before induction of oval cell proliferation to investigate the effect of destruction of biliary epithelial cells associated with portal and extraportal (canal of Herring) structures in the genesis of oval cells.

Supported in part by NIH grants CA35373 and CA30241.

Accepted for publication June 26, 1997.

Address reprint requests to Dr. George Michalopoulos, Professor and Chair, Department of Pathology, University of Pittsburgh, Pittsburgh PA 15261.

Materials and Methods

CCl_4, 99% pure HPLC grade, DAPM, and 2-AAF were purchased from Aldrich Chemical Co. (St. Louis, MO). 2-AAF crystals were inserted into time-released pellets (70 mg/pellet over a 28-day release (2.5 mg/day); Innovative Research, Toledo, OH). Fischer male 344 rats were obtained from Fredricks Laboratories (Frederick, MD). X-ray film was obtained from Eastman Kodak Co. (Rochester, NY). GeneScreen Plus nylon membrane was purchased from NEN Research Products (Boston, MA). Microscope Superfrost Plus slides, buffered Formalin-Fresh, and dextran sulfate were obtained from Fisher Scientific (Pittsburgh, PA). Qiagen Maxi-Prep plasmid purifying kit, and QiagenGel extraction kit were bought from Qiagen (Chatsworth, CA). RNAzol B was purchased from Cinna-Biotecx Laboratories (Houston, TX). [^{32}P]dCTP and Multiprime labeling kit were obtained from Amersham (Arlington Heights, IL). Agarose Ultrapure and all restriction enzymes were purchased from Gibco/BRL (Gaithersburg, MD). Hematoxylin was purchased from Anatech (Battle Creek, MI). Eosin and all other chemicals used were obtained from Sigma Chemical Co. (St. Louis, MO). Rat AFP cDNA probe was obtained from the laboratory of Dr. Joseph Locker (University of Pittsburgh).

Administration of Chemicals

A LD_{50} of CCl_4, was administered on the basis of the LD_{50} dose as determined by Lewis.[16] A single CCl_4 dose of 1.9 ml/kg (1500 mg/kg) of body weight in a 1:1 v/v dilution in corn oil was administered by intraperitoneal injection. In the case of DAPM, the dose administered was on the basis of the LD_{50} dose as determined by Lewis.[16] DAPM was dissolved in 100% ethanol and then diluted to 50 mg/ml in 35% ethanol with distilled water as described by Kanz et al.[15] Animals were given DAPM via intraperitoneal injection. The total dose of DAPM used for this study was 50 mg/kg, approximately one-fourth of an actual LD_{50} dose.

Animal Euthanasia

All procedures regarding animals were conducted according to institutionally approved protocols. Rats were anesthetized by injection with sodium pentobarbital (0.1 ml/100 g body weight) before being sacrificed.

Northern Analysis of Total RNA

Total RNA was extracted from 0.25 g of liver tissue per time point using 10.0 ml of RNAzol B according to the manufacturer's guidelines. RNA concentration and purity was determined by routine spectrophotometry. Twenty micrograms of total RNA for each sample was electrophoresed in a 1% agarose/formaldehyde denaturing gel. Once adequate separation of the 28 S and 18 S bands was obtained, the RNA was transferred to a synthetic membrane GeneScreen Plus via capillary action overnight. After cross-linking membranes using a Stratagene Cross-Linker (La Jolla, CA), membranes were hybridized overnight using a specific rat AFP cDNA probe.[17] The glyceraldehyde phosphate dehydrogenase cDNA probe was produced in our laboratory and was used for loading quantification purposes. The cDNA probes were labeled with [α-^{32}P]dCTP using an Amersham random primer labeling kit. The membranes were washed under high-stringency conditions and then exposed to x-ray film and kept at −80°C until developed (2 to 12 hours).

Immunohistochemistry

Tissue obtained was fixed in 10% buffered formalin for at least 24 hours before being processed into paraffin. All staining procedures for light microscopy of paraffin-embedded tissue were carried out on 4-μm-thick sections. Routine histological examinations were performed on all liver tissue samples on sections stained with hematoxylin and eosin.

Protocols for Stimulation of Proliferation of Oval Cells

2-AAF pellets (70 mg/28-day release, 2.5 mg/day) as previously described by Hixson et al[18] and Novikoff et al[13] were inserted 7 days before hepatic injury following a similar protocol described by Evarts et al.[1] The time points obtained were counted from when the hepatic injury (CCl_4 or PHx) was induced. For the PHx, rats were hepatectomized under anesthesia according to the methods described by Higgins and Anderson.[19] The dose and delivery for CCl_4 was discussed earlier in the compound delivery section and performed in the same manner.

Results

DAPM was used as a selective biliary toxin[15] to examine the relationship between biliary epithelial cells (including epithelium of intraportal bile ductules and canals of Herring) and oval cell proliferation. DAPM was administered in the presence of 2-AAF and before PHx or CCL_4. The effects of DAPM (and 2-AAF) alone, without accompanying administration of CCL_4 or PHx are shown in Figure 1. Liver sections were examined from rats at 24 hours (Figure 1A), 72 hours (Figure 1B), and 10 days (Figure 1C) after exposure to DAPM. Maximal destruction of the biliary tree is seen at 24 hours after administration of DAPM. There is fibrinoid necrosis of the wall of the ductules and disappearance of the biliary epithelium. This was seen in more than 90% of the total triads. By 72 hours, most triads contained ducts with incomplete lining by new biliary epithelium. Many bile infarcts, classically described in cholangiolar injury in human liver pathology,[20,21] were also noted. These are areas in which bile seepage causes necrosis of circumscribed areas of hepatic parenchyma adjacent to portal triads. By 10 days, most of the triads had normal lining of the biliary ductules. The above findings are consistent with studies previously per-

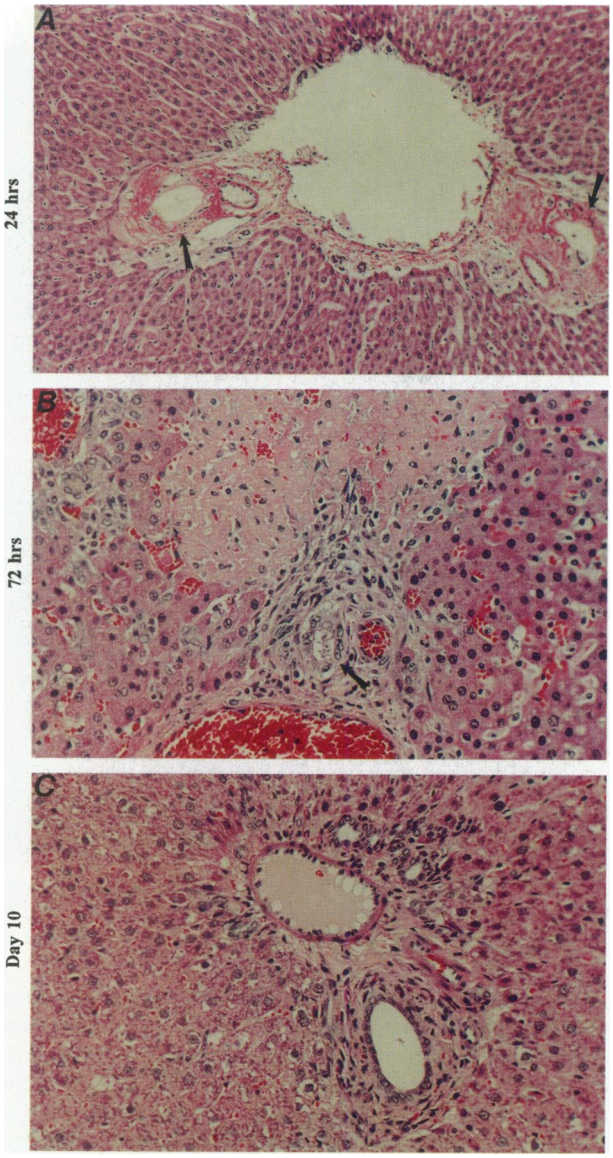

Figure 1. Histological changes of rat livers exposed to DAPM (50 mg/kg) and 2-AAF for 24 hours (**A**), 72 hours (**B**), and 10 days (**C**) after exposure to DAPM. In **A** and **B**, there is marked destruction of the epithelium and the wall of small portal ductules. A bile infarct (death of hepatocytes in a strictly circumscribed area adjacent to the portal triad) due to biliary leakage is also shown with **arrows** in **B**. Repair of the damaged sites and restoration of the biliary ductules is shown in **C**. No oval cells can be seen in all three photomicrographs. Original magnification, ×100 (**A**) and ×200 (**B** and **C**). **Arrows** indicate destroyed bile ducts (**A**) and the beginnings of ductular repair (**B**).

formed in which the effect of DAPM on the biliary ductules was first described.[15]

In view of the above findings in which the maximal destruction of the biliary tree by DAPM is seen at 24 hours, we administered DAPM 24 hours before the administration of CCL_4 or performing a PHx. Figure 2 is a collage of photomicrographs of sections of liver from rats exposed to 2-AAF for 7 days, followed by CCl_4 (Figure 2A) or PHx (Figure 2B), and sacrificed 9 days after surgery or hepatic injury. In both models, oval cell proliferation was induced. Figure 2, C and D, shows the histological changes in the livers when DAPM is administered to the rats 24 hours before the hepatic injury by CCl_4 (Figure 2C) and PHx (Figure 2D). Oval cell proliferation was dramatically suppressed. These findings are also corroborated by comparison of the expression of AFP with and without DAPM administration. Whereas AFP was expressed after 2-AAF/CCL_4 or 2-AAF/PHx as described above (Figure 3A), no expression of AFP was detected after pretreatment with DAPM (Figure 3B).

Discussion

The nature, site, and cell of origin of the oval cells that emerge in liver after different types of injury has been a subject of analysis of several studies.[1–10] The appearance of both biliary and hepatocytic markers in these cells has reinforced the concept of the hepatic stem cell, and many workers in the field consider these cells as the immediate progeny of a putative hepatic stem cell type.[9] Chemical injury to the liver induced by a variety of agents such as galactosamine[12] and ethionine[11] causes an increase in numbers of oval cells. A more standard protocol applied in recent studies is that established by Thorgeirsson and co-workers[9] as an adaptation of a carcinogenesis protocol originally established by Solt and Farber.[22] The purpose of this study was the analysis of the lobular site of liver injury (centrilobular *versus* ductular) as a determining parameter of the oval cell response. Previous studies that had shown the induction of oval cells as a result of chemical injury had used agents (ethionine and galactosamine) that cause a diffuse injury affecting all lobular areas of the liver. Very little oval cell response was seen after CCl_4 in a study that directly compared it with that induced by galactosamine.[23] For the purposes of this study, we used protocols in which the injury was zonally applied and that could be easily timed in terms of initiation of the injury leading to the proliferative response. This was especially critical for the part of our study involving DAPM. In view of this, we used the chemical CCl_4, known to cause injury of the hepatic centrilobular area in combination with the chemical 2-AAF, which has been shown by Solt and Farber[22] and Evarts and Thorgeirsson[1,3,9] to block proliferation of hepatocytes, thus allowing oval cells to continue to proliferate in large numbers. In essence, our protocol (2-AAF/CCl_4) in this regard is the same as the one used by Thorgeirsson et al, except that we used an agent to induce hepatic damage instead of PHx.

The phenotypic similarities of the oval cells with biliary epithelium[24,25] as well as the organization in ductular structures have always been cited as strong evidence that the oval cells are derived by or related to biliary epithelial cells of the small ductules or canals of Herring. In a most convincing evidence, Sarraf et al[26] injected the biliary tree with pigmented gelatin and demonstrated that most of the cells in the oval cell population were part of a system of tortuous channels connected to the biliary tree. Similar conclusions were reached by combined morphological and biochemical studies by Lenzi et al.[27] Our studies with the chemical DAPM provide further support to this relationship. The long-term injury of biliary cells

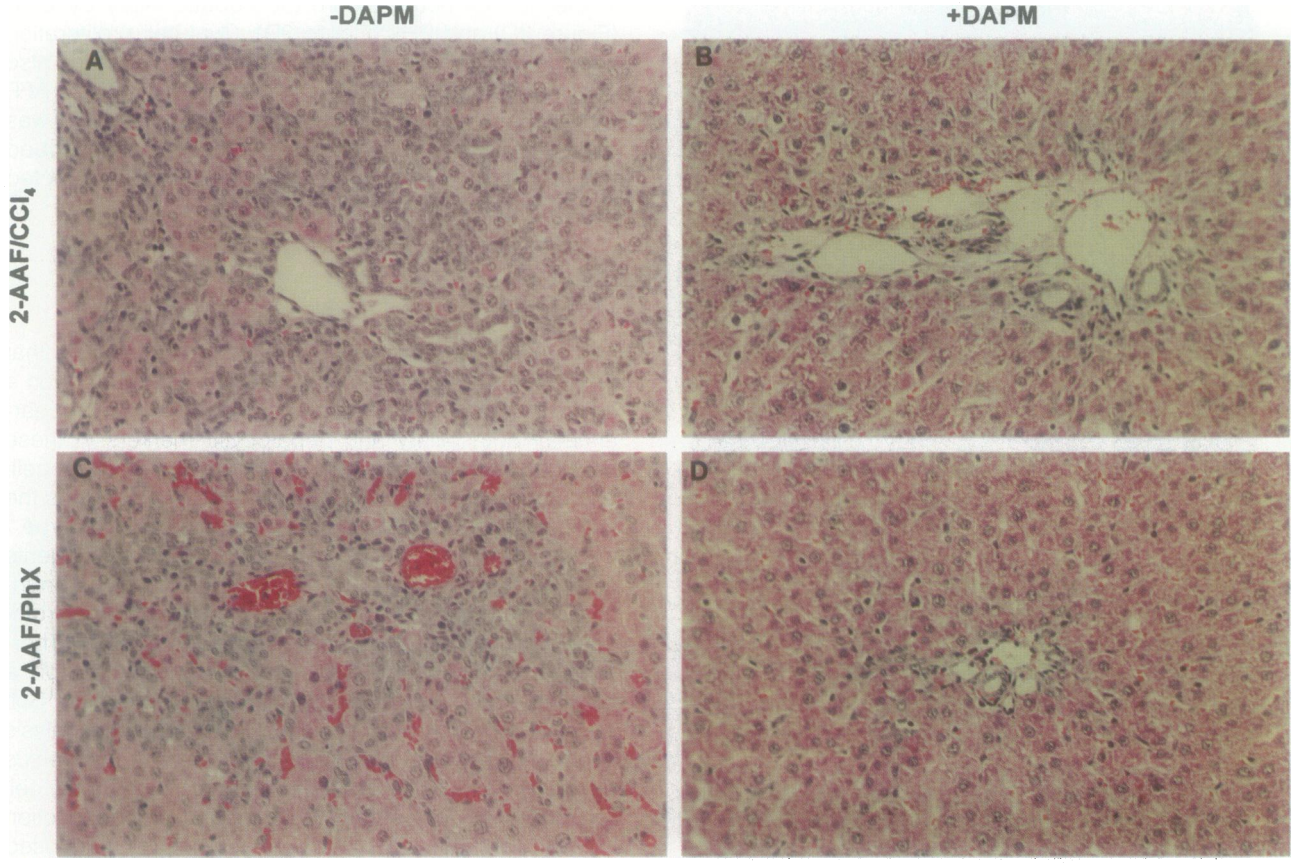

Figure 2. Effect of DAPM on histological changes induced by either 2-AAF/CCl_4 (**A**) or 2-AAF/PHx (**C**) on day 9 after hepatic injury. Oval cells are easily noted with both 2-AAF/CCl_4 and 2-AAF/PHx protocols (**A** and **C**) as described above. Oval cell expansion is abolished when DAPM is administered (**B** and **D**) before the above protocols. Original magnification, ×200.

induced by DAPM was first studied by Fukushima et al.[28] These authors noticed that long-term administration of DAPM led to destruction of the biliary tree, proliferation of biliary ductular structures and oval cells, and scarring. A more recent study addressing the mechanisms of hepatic injury induced by DAPM was that of Kanz et al.[15] The authors concluded that the biliary epithelium is the direct target of DAPM and the reason for the associated hepatotoxicity. The authors presented evidence that any damage to hepatocytes was secondary to that of the biliary cells and that hepatocytes are not a primary target of DAPM. We also noticed (Figure 2B) that bile infarcts were the only sign of hepatocyte damage. This type of damage is known to be associated with leakage of bile from damaged cholangioles[20,21] and causes indiscriminate destruction of all hepatocytes exposed to the bile, leading to a circumferential site of hepatocyte destruction in close proximity to the portal triads. The authors concluded that the toxic metabolites of DAPM are excreted in the bile at high concentration, thus leading to the direct biliary toxicity. As we show, DAPM given 24 hours before the initiation almost entirely abolished the oval cell response, as evidenced by both morphology as well as measurement of AFP mRNA. The findings of Figure 2A show the striking degree of destruction of the biliary epithelium seen at 24 hours after DAPM. The elimination of the oval cell response under these circumstances most strongly argues that the cells from which oval cells originate are likely to be targets of DAPM. This would be most compatible with a cell of biliary type and topologically exposed to the bile stream containing DAPM[15,28] as the forerunner of the oval cells. We should also point that DAPM did not interfere with the effectiveness of CCl_4. The same pattern and extent of damage was induced by CCl_4 with or without DAPM (data not shown). These results provide direct evidence that oval cells are closely associated with the biliary epithelium. Clearly, additional investigation needs to be performed to fully understand the mechanisms involved with DAPM toxicity and oval cell activation.

Acknowledgments

We thank Linda Shab, Jeff Levis, and Mike Chasky, the members of the Department of Pathology photography lab, for their excellent work in preparing the figures for this manuscript. We also thank the members of the Department of Pathology Research Histology Lab, Eni Sonmez-Alpan Ph.D., Karen Mulé, and Kim Stanis. We express appreciation to Drs. Doug Hixson, Benito Lombardi, and Joseph Locker for their generous gifts of antibodies and cDNA, which was crucial in characterization of the oval cells described in this study, Dr. Mary

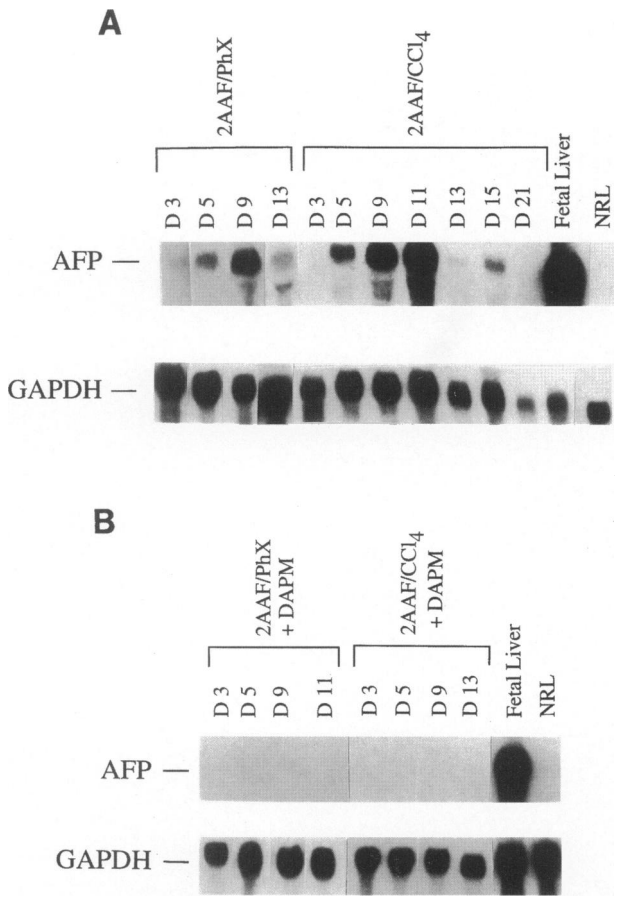

Figure 3. Expression of AFP mRNA from rats exposed to 2-AAF/PHx or 2-AAF/CCl_4 protocol (**A**). **B:** Expression of AFP mRNA from rats under the same protocols except that DAPM exposure occurred 24 hours before hepatic injury. Total RNA (pooled 20 μg) was loaded per lane per time point. Fetal rat liver RNA was used as a positive control for AFP expression. Normal rat liver (NRL) was used as a negative control. The glyceraldehyde phosphate dehydrogenase gene was used as internal control of RNA amount loaded per lane. This figure represents a collage of four autoradiograms, all hybridized at the same time with the same radiolabeled cDNA probe. All autoradiograms are of an overnight exposure to radiolabeled filters.

Kanz for her advice in DAPM dosing and critical reference materials. We also thank Dr. Donna Beer-Stolz for her critical review of this manuscript.

References

1. Evarts RP, Nagy P, Nakatsukasa H, Marsden E, Thorgeirsson SS: In vivo differentiation of rat oval cells into hepatocytes. Cancer Res 1989, 49:1541–1547
2. Farber E: Similarities in the sequence of early histological changes induced in the liver of the rat by ethionine, 2-acetylaminofluorene, and 3′-methyl-4-dimethylaminoazobenzene. Cancer Res 1956, 16:142–148
3. Evarts RP, Nagy P, Marsden E, Thorgeirsson SS: A precursor-product relationship exists between oval cells and hepatocytes in rat liver. Carcinogenesis 1987, 8:1737–1740
4. Germain L, Noel M, Gourdeau H, Marceau N: Promotion of growth and differentiation of rat ductular oval cells in primary culture. Cancer Res 1988, 48:368–378
5. Shiojiri N, Lemire JM, Fausto N: Cell lineage and oval cell progenitors in rat liver development. Cancer Res 1991, 51:2611–2620
6. Dabeva MD, Shafritz DA: Activation, proliferation, differentiation of progenitor cells into hepatocytes in the D-galactosamine model of liver regeneration. Am J Pathol 1993, 143:1606–1620
7. Fausto N: Liver stem cells. The Liver Biology and Pathobiology, ed 3. Edited by Arias IM, Boyer JL, Fausto N, Jakoby WB, Schachter DA, Shafritz DA. New York, Raven Press, 1994, pp 1501–1518
8. Faris RA, Monfils BA, Dunsford HA, Hixson DC: Antigenic relationship between oval cells and subpopulation of hepatic foci, nodules, and carcinomas induced by the "resistant hepatocyte" model system. Cancer Res 1991, 51:1308–1317
9. Thorgeirsson SS, Evarts RP, Bisgaard HC, Fujio K, Hu Z: Hepatic stem cell compartment: activation and lineage commitment. Proc Soc Exp Biol Med 1993, 204:253–260
10. Fujio K, Evarts RP, Hu Z, Marsden ER, Thorgeirsson SS: Expression of stem cell factor and its receptor, c-kit, during liver regeneration from putative stem cells in the adult rat. Lab Invest 1994, 70:511–516
11. Lemire JM, Shiojiri N, Fausto N: Oval cell proliferation and the origin of small hepatocytes in liver injury induced by D-galactosamine. Am J Pathol 1991, 139:535–552
12. Shinozuka H, Lombardi B, Sell S, Iammarino RM: Early histological and functional alterations of ethionine liver carcinogenesis in rats fed a choline-deficient diet. Cancer Res 1978, 38:1092–1098
13. Novikoff P, Yam A, Oikawa I: Blast-like cell compartment in carcinogen-induced proliferating bile ductules. Am J Pathol 1996, 148:1473–1492
14. Vandersteenhoven AM, Burchette J, Michalopoulos GK: Characterization of ductular hepatocytes in end-stage cirrhosis. Arch Path Lab Med 1990, 114:403–406
15. Kanz MF, Kaphalia L, Kaphalia BS, Romagnoli E, Ansari GAS: Methylene dianiline: acute toxicity and effects on biliary function. Toxicol Appl Pharmacol 1992, 117:88–97
16. Lewis RJ (editor): SAX Dangerous Properties of Industrial Material, ed 8, vol 3. New York, Van Nostrand Reinhold, 1993, pp 52 and 1149
17. Muglia L, Locker J: Developmental regulation of albumin and α-fetoprotein gene expression in the rat. Nucleic Acids Res 1984, 12(17):33–48
18. Hixson DC, Faris RA, Thompson NL: An antigenic portrait of liver during carcinogenesis. Pathobiology 1990, 58:65–77
19. Higgins GM, Anderson RM: Experimental pathology of the liver. I. Restoration of the liver of the white rat following partial surgical removal. Arch Pathol 1931, 12:186–202
20. Alpini G, Phillips JO, LaRusso NF: The Liver Biology and Pathobiology, ed 3. Edited by Arias IM, Boyer JL, Fausto N, Jakoby WB, Schachter DA, Shafritz DA. New York, Raven Press, 1994, pp 1501–1518
21. Desmet VJ: Cholestasis: extrahepatic obstruction and secondary biliary cirrhosis. Pathology of the Liver. Edited by Macsween RN, Anthony PP, Scheuer PJ. New York, Churchill Livingstone, 1979, pp 272–305
22. Solt DB, Farber E: New principle for the analysis of chemical carcinogenesis. Nature 1976, 263:701–703
23. Dabeva MD, Alpini G, Hurston E, Shafritz DA: Models for hepatic progenitor cell activation. Proc Soc Exp Biol Med 1993, 204:242–252
24. Bisgaard HC, Nagy P, Santoni-Rugiu E, Thorgeirsson SS: Proliferation, apoptosis, and induction of hepatic transcription factors are characteristics of early response of biliary epithelial (oval) cells to chemical carcinogens. Hepatology 1996, 23:62–70
25. Yang L, Faris RA, Hixson DC: Phenotypic heterogeneity with clonogenic ductal cell populations isolated from normal adult rat liver. Proc Soc Exp Biol Med 1993, 204:280–288
26. Sarraf C, Lalani E-N, Golding M, Anikurmar TV, Poulsom R, Alison M: Cell behavior in the acetylaminofluorene-treated regenerating rat liver: light and electron microscopic observations. Am J Pathol 1994, 145:1114–1126
27. Lenzi R, Liu MH, Tarestti F, Slott PA, Alpini G, Zhai W-R, Paronetto F, Lenzen R, Tavoloni N: Histogenesis of bile duct-like cells proliferating during ethionine hepatocarcinogenesis: evidence for biliary epithelial nature of oval cells. Lab Invest 1992, 66:390–402
28. Fukushima S, Shibata M, Hibino T, Yoshimura T, Hirose M, Ito N: Intrahepatic bile duct proliferation induced by 4,4′-diaminodiphenylmethane in rats. Toxicol Appl Pharmacol 1978, 48:145–155