ABSTRACT

During hepatocarcinogenesis, α-fetoprotein (AFP) synthesis may be dramatically increased while albumin synthesis is frequently decreased. Therefore, a reciprocal modulation between both gene expressions has been hypothesized. In this work, we combined in situ hybridization and immunoperoxidase on parallel liver tissue sections in order to analyze at the cellular level, AFP gene expression and its relation to ALB gene expression in both early and neoplastic lesions induced by 3MeDAB in the rat. In early lesions, cell populations were heterogeneous as regards AFP expression. High levels of AFP transcripts were detected both in oval type cells and in a subset of basophilic hepatocytes within preneoplastic lesions. In these two highly AFP-expressing cell populations, significant levels of ALB transcripts were concomitantly detected. In the majority of altered hepatocytes, no AFP expression was detected while the level of ALB expression was decreased. In neoplastic lesions, AFP expression was strikingly heterogeneous and independent from the degree of morphological differentiation. No evidence of reciprocal modulation with ALB gene expression could be assessed. In both preneoplastic and neoplastic lesions, a few altered hepatocytes displayed significant levels of AFP transcripts while no corresponding protein could be detected; such a discrepancy was not observed for ALB. This work shows that during 3MeDAB hepatocarcinogenesis, AFP gene activation occurs in heterogeneous cell populations and according to different cellular patterns. Our observations lend no support to the hypothesis of a reciprocal modulation between AFP and ALB gene expressions during rat azo-dye hepatocarcinogenesis.

INTRODUCTION

Hepatocarcinogenesis is one of the few situations in which AFP gene, normally repressed, may be reexpressed in adult life. Indeed, AFP synthesis is dramatically induced in certain hepatocarcinomas and hepatoma cell lines and during the course of chemical hepatocarcinogenesis in the rat (1-4). Since concomitantly, albumin synthesis is frequently decreased (1, 2, 3), the hypothesis that AFP and albumin gene expressions are reciprocally modulated during hepatocarcinogenesis has been raised. Furthermore, since the pattern of protein synthesis achieved during hepatocarcinogenesis simulates that observed in fetal liver, such a gene modulation has been assigned to a process of retrodifferentiation, i.e., the reexpression of fetal characters by dedifferentiated mature hepatocytes (5). Such hypotheses have been subsequently challenged by the demonstration that, at least in the perinatal period (6) and in regenerating rat liver (7), AFP and albumin gene expressions are independently regulated. That this may also be the case in hepatocarcinogenesis, is suggested by the results of molecular hybridization studies sequentially performed in liver homogenates obtained from carcinogen-treated rats (7-9). These studies have brought no evidence for a reciprocal modulation between AFP and albumin gene expressions in vivo. An increase in AFP mRNAs, paralleling the variations in AFP plasma concentration, has been actually demonstrated, but was not found to be accompanied by a reciprocal decrease in the amount of ALB mRNAs (7-9). However, investigation of gene expression at the tissue level does not take into account the striking cellular heterogeneity observed in the liver during chemical hepatocarcinogenesis and therefore, cannot rule out the possibility that AFP and albumin are expressed by distinct cell populations. In situ hybridization has proven to be an accurate and sensitive method for investigating gene expression at the cellular level, making it possible to perform a morphological analysis of cells containing specific mRNAs. This approach has been recently used to analyze albumin expression in preneoplastic lesions induced either by diethylnitrosamine (10) or according to the so-called resistant hepatocyte protocol (11), in an attempt to correlate albumin gene expression with various other phenotypic markers indicative of the state of differentiation. However, these works have not addressed the possible relations between albumin and AFP gene expression. Furthermore, AFP expression has been not detected in any cell population (10) or has been observed only in oval cells (11). These studies have therefore not confirmed the results of previous immunohistochemical investigations of AFP expression at the early stage of rat hepatocarcinogenesis. In order to provide a comprehensive analysis of AFP and albumin gene expression during rat liver carcinogenesis, we selected as a model azo-dye hepatocarcinogenesis in the rat, which is known to be accompanied by a dramatic increase in AFP synthesis (9, 12). In situ hybridization has been used for detection of gene transcripts and immunoperoxidase for detection of the corresponding proteins on parallel liver tissue sections. The whole spectrum of early and neoplastic lesions has been investigated. The aims of the present work are: (a) to reevaluate the cellular patterns of AFP gene expression in rat hepatocarcinogenesis, and (b) to reassess the possibility of a reciprocal modulation between AFP and albumin gene expression at the cellular level.

MATERIALS AND METHODS

Study Design

Male Fischer 344 rats (Animalerie de l’IRSC, Villejuif, France) were used throughout the study. Animals were housed in an air-conditioned room at controlled temperature and humidity, with a 12-h light-dark cycle. They received tap water ad libitum. 24 rats, constituting the study group, were fed a diet containing 0.06% 3MeDAB (Koch Light Laboratories, London, England) (13) from the age of 4 weeks to their sacrifice. Eight rats, used as controls, were fed a standard commercial diet (UAR, Villacoublay, France). Animals were sacrificed at the following time points after initiation of DAB administration: 4, 6, 10, 12, 14, 16, 18, and 22 weeks. At each time point, at least two animals from the study group and one from the control group were sacrificed.

Tissue Preparation

Rats were anesthetized with sodium pentobarbital (Clin-Midy, Saint-Jean, France). Through the portal vein, livers were washed for 4 min.
with 0.1 M PBS bubbled with O2 95% and CO2 5%, then fixed for 10 min with cold, freshly prepared 4% paraformaldehyde (Merck, Darmstadt, RFA) diluted in phosphate buffer pH 7.4. Liver slices, 1 to 2 mm thick, were then postfixed by immersion in the same fixative for 2 to 4 h at 4°C.

Embedding and sectioning were performed as previously described (14). Briefly, after dehydration in graded ethanol, blocks were immersed in three baths of 1-butanol (Prolabo, Paris, France), then in three paraffin baths (Medite, Burgdorf, FGR) before embedding. 4-μm sections were collected on slides pretreated as previously described (14) and dried for 24 h at 37°C.

Adjacent sections were used for in situ hybridization and immunoperoxidase. An additional section was stained with hematoxylin & eosin for routine histological observation.

**In Situ Hybridization**

**In situ** hybridization for ALB and for AFP mRNAs was simultaneously performed during the same experiment on parallel sections from the same block.

**Molecular Probes.** The probes used in this study were the ALB and AFP cDNA inserts respectively excised from the recombinant plasmids pRSA 8 (15) and pRAFP 65 (16), as previously described (17). Probes were then labeled by nick-translation (18) using 35S-labeled dATP and dCTP (Amersham, Little Chalfont, UK), to a specific activity of 1–2 × 109 cpm/μg.

**In situ Hybridization Procedure.** After pretreatment of tissue sections as previously described (14), 0.5 to 5.105 cpm of the labeled probe resuspended in hybridization buffer (14, 17) were applied to each section. Hybridization was performed overnight at 37°C. Sections were washed by immersion in two successive baths of 50% formamide, 10 mm Tris-HCl, pH 7.5, 600 mm NaCl, 1 mm EDTA, at 37°C for 1 h. Sections were then rinsed in the following solutions: 2x SSC for 2 h at 37°C, 0.1x SSC for 90 min at 52°C, 0.1x SSC for 30 min at room temperature. Sections were finally dehydrated in graduated ethanol containing 0.3 M ammonium acetate (Merck, Darmstadt, FRG), and dipped in NTB2 emulsion (Eastman Kodak, Rochester, NY). After 15–20 day exposure, slides were developed in Dektol (Kodak, Rochester, NY, USA), fixed in HYPAM (Ilford, Mobberley, UK), and stained with 2% Giemsa (Prolabo, Paris, France).

**Control Reactions.** RNase controls were performed after slide pretreatment by incubation for 1 h at 37°C with a 1 mg/ml RNase A solution (Sigma, Saint-Louis, MO, USA). Slides were rinsed in PBS, dehydrated and air-dried. They were subsequently hybridized in the same way as the others. pBR controls were also performed by hybridizing tissue sections with 35S-labeled pBR 325 DNA.

**Immunoperoxidase Technique**

An indirect immunoperoxidase technique was applied to sections adjacent to those used for in situ hybridization. After deparaffinization, sections were immersed for 30 min in methanol (Prolabo, Paris, France) containing 0.5% hydrogen peroxide (Merck, Darmstadt, FRG) for inhibition of endogenous peroxidase activity and then treated with a solution containing 2 μg/ml of proteinase K (Sigma, St. Louis, MO) as previously described (17). Sections were then incubated for 90 min at room temperature in (a) rabbit anti-rat AFP polyclonal antibody (kindly provided by J Uriel, Villejuif, France) diluted 1:50 in PBS or (b) rabbit anti-ALB antiserum (Nordic, Tilburg, Netherlands) diluted 1:50 in PBS or (c) rabbit anti-AFP antiserum (Nordic, Tilburg, Netherlands) diluted 1:50 in PBS. Sections were then incubated for 90 min at room temperature in peroxidase-labeled anti-rabbit immunoglobulin diluted 1:400 in PBS (Dakopatts, Copenhagen, Denmark). Peroxidase activity was demonstrated according to Graham and Karnovsky (19). Sections were finally lightly counterstained with Mayer’s hematoxylin (Prolabo, Paris, France), dehydrated and mounted. For control reactions, primary polyclonal antibody was omitted and replaced by PBS.

**RESULTS**

**Control Rat Liver**

**AFP Expression.** AFP mRNAs were present at very low levels in all hepatocytes, without evidence of zonal difference in signal intensity. No AFP-positive cell could be detected by immunoperoxidase. No expression was detected in bile duct cells.

**Albumin Expression.** All hepatocytes contained ALB mRNAs. As previously shown (20), ALB hybridization signal was more intense in periportal and mediolobular zones than in perivenous zones. By immunoperoxidase, the corresponding protein displayed a similar heterogeneous staining pattern. No expression was detected in bile duct cells.

**Early Lesions (4–16 Weeks)**

Early lesions induced by 3MeDAB were characterized (a) by the marked proliferation of so-called oval cells accompanied by an initial hepatocellular injury and (b) by the subsequent development of typical preneoplastic foci and nodules constituted by heterogenous cell populations (20–25).

**Oval Cell Proliferation.** Oval cell proliferation was marked at 4 weeks and maximal at 6 weeks. Subsequently, oval cells decreased in number but remained present in significant amounts until 16 weeks. They constituted tracts expanding from the portal triads and dividing the liver lobules into nodules of variable size (Fig. 1a). A number of duct-like structures were interspersed among typical oval cells and radiated inside hepaticoytic areas (Fig. 1a). Cells lining these structures, or “ductal cells,” are usually regarded as cells deriving from oval cells. The marked oval cell proliferation observed at Weeks 4 and 6 was accompanied by the disruption of hepatic lobular architecture and by signs of hepatocellular injury.

**AFP Expression.** AFP expression was detected in both oval and ductal cells. While AFP mRNAs were present in both cell

---

**Fig. 1.** Histological appearance of early and neoplastic lesions in 3MeDAB-induced hepatocarcinogenesis in the rat. a (×180, hematoxylin & eosin staining), corresponding to an early stage of 3MeDAB-induced lesions, shows a tract of oval cells (arrows) expanding from a portal triad (P) and dividing the hepatic lobule into residual hepatocytic islands (*). b (×180, hematoxylin & eosin staining) and c (×230, hematoxylin & eosin staining), feature two aspects of preneoplastic nodules. In b, the pleiomorphism of the cell population is evident, with a mixture of acidophils and clear cells. c shows a focus of basophilic hepatocytes. d (×180, hematoxylin & eosin staining), shows a neoplastic lesion characterized by the presence of marked nuclear atypias and several mitotic figures. Bar, 40 μm.
0-FETOPROTEIN EXPRESSION IN HEPATOCARCINOGENESIS

types (Fig. 2a), hybridization signal was higher in ductal cells than in typical oval cells. By immunoperoxidase (Fig. 2b), typical oval cells were inconstantly reactive for AFP. In contrast, duct-like structures were constantly labeled. Reactivity pattern was different for the two cell types (Fig. 2b). Typical oval cells displayed a diffuse cytoplasmic staining. In ductal cells, staining was restricted to the apical region.

In residual hepatocytic islands, a few cells displayed a moderate increase in AFP mRNAs. These AFP-expressing cells were larger than typical oval cells, did not form pseudo-ductular structures, and possessed a fairly abundant basophilic cytoplasm which displayed no immunoperoxidase labeling for the corresponding protein.

Albumin Expression. Albumin expression was also detected in both oval and ductal cells. The hybridization signal (Fig. 2c) was comparable in the two populations. By immunoperoxidase, a faint cytoplasmic labeling was observed for ALB in both oval and ductal cells.

ALB expression was decreased in the altered hepatocytes intermingled with oval cells. These zones of low ALB-expressing hepatocytes surrounded residual hepatocytic islands displaying normal level of ALB transcripts.

Preneoplastic Foci and Nodules. Preneoplastic foci and nodules observed in every animal from Weeks 10 to 16, consisted of a mixture of morphologically altered hepatocytes, which, according to accepted cytological classifications (23), could be classified as acidophilic, clear, or basophilic (Fig. 1, b and c). Acidophilic hepatocytes were in majority (Fig. 1b). Clear cells were large hepatocytes with an abundant cytoplasm faintly stained by conventional histological techniques. Basophilic hepatocytes constituted a minority of cells organized in small foci (Fig. 1c).

AFP Expression. The majority of morphologically altered hepatocytes contained AFP mRNAs at low levels, comparable to those present in normal adult hepatocytes. Increased amounts of AFP mRNAs were present in a minority of cells. Two distinct phenotypes were observed. Certain AFP-expressing cells (Fig. 3, a and b) were scattered, contained increased amounts of cytoplasmic and nuclear AFP mRNAs (Fig. 3a), whereas they displayed no labeling for the corresponding protein by immunoperoxidase (Fig. 3b). Such a phenotype was observed in acidophilic and clear cells. The other AFP-expressing cells (Fig. 3, c and d) constituted cellular foci, expressed high amounts of AFP mRNAs (Fig. 3c) and stained for the corresponding protein by immunoperoxidase (Fig. 3d). These cells presented the cytologic characters of the so-called basophilic hepatocytes (23, 26).

Albumin Expression. All hepatocytes contained albumin mRNAs. The level of transcript detection was variable. In the majority of morphologically altered hepatocytes, the amount of transcripts was decreased as compared to normal hepatocytes (Fig. 3e). By immunoperoxidase, the corresponding protein remained detectable but the staining intensity was highly variable. In contrast, a high level of transcript detection, comparable to or higher than that of normal hepatocytes, was present in
foci of altered hepatocytes presenting the cytological features of basophilic hepatocytes (Fig. 3). Those cells displayed an immunoperoxidase labeling for the corresponding protein.

Neoplastic Lesions (16–22 Weeks)

Hepatocarcinomas with varying degrees of morphological differentiation (Fig. 1d) were observed in seven animals from the 16th to the 22nd weeks of administration (23).

AFP Expression. AFP mRNAs were inconstantly detected in the tumor samples investigated. Some tumors were entirely negative. In most cases, a mosaic pattern was observed: positive areas coexisted with negative ones within the same tumor (Fig. 4a). In some instances, cells containing high amounts of AFP mRNAs were found adjacent to AFP-negative cells. The presence of AFP mRNAs in neoplastic cells could not be correlated with their degree of morphological differentiation. Comparison of serial sections showed that, in differentiated areas, significant amounts of AFP mRNAs could be coexpressed with significant amounts of ALB mRNAs (Fig. 4c and d). By immunoperoxidase, the corresponding protein was usually detected in cells containing AFP mRNAs (Fig. 4b). However, in some instances, no AFP protein could be detected by immunoperoxidase despite the presence of high amounts of AFP mRNAs in corresponding cells from adjacent sections (Fig. 4e and f).

Albumin Expression. ALB mRNAs were detected in all tumor samples investigated. The level of transcript detection was highly variable. In poorly differentiated areas, hybridization signal was usually markedly decreased as compared to surrounding nonneoplastic tissue. In differentiated areas, the amount of transcripts detected was comparable to that of surrounding hepatocytes or only moderately decreased. By immunoperoxidase, the majority of differentiated neoplastic cells displayed a cytoplasmic staining for the corresponding protein. Poorly differentiated cells were usually negative.

Control Reactions

No significant hybridization was obtained after RNase treatment and with radioactive pBR fragment. No immunoperoxidase labeling was obtained in the controls.
DISCUSSION

This work, combining in situ hybridization and immunoperoxidase techniques, shows that AFP gene expression occurs in heterogenous cell populations during rat azo-dye hepatocarcinogenesis, and that several patterns of gene activation are identifiable. Our observations suggest an absence of reciprocal modulation between AFP and albumin expression at the cellular level, in both early and neoplastic lesions.

In the early lesions induced by 3MeDAB in the rat, we identified two cell subsets characterized by a high level of AFP gene expression. One is the oval cell subset, in which large amounts of AFP mRNAs are present not only in typical oval cells, as shown by earlier studies (10, 11, 27), but also in the so-called ductal cells. The other highly AFP expressing cells belong to a cell subset of altered hepatocytes with distinctive morphological features, corresponding to the so-called basophilic hepatocytes (23, 26). In situ hybridization therefore confirms previous immunohistochemical studies according to which oval cell population is the main, but not the unique cellular source of AFP at the early stage of azo-dye hepatocarcinogenesis (28–32). It is of interest that the two cell populations associated with high AFP expression, i.e., oval cells and basophilic hepatocytes, comprise a majority of cycling cells, as indicated by previous histoautoradiographic studies (28, 33). These observations concur with those performed during normal liver regeneration (34) to suggest that AFP expression is related, at least in part, to cell entry into the proliferative cycle (35, 36).

The sensitive in situ hybridization technique makes it possible to identify a pattern of moderate AFP gene activation which has been overlooked by previous immunohistochemical studies at the early stage of rat azo-dye hepatocarcinogenesis (27–29, 31). This pattern is characterized by a mild increase in nuclear...
and cytoplasmic AFP mRNAs, without immunohistochemically detectable levels of the corresponding protein. Two previous in situ hybridization studies performed in rats treated with various carcinogens have also failed to identify such cells (10, 11). While the use of different technical procedures may turn into differences in the sensitivity of in situ hybridization technique, differences in the carcinogenic regimens may also account for these apparently conflicting data. A characteristic effect of 3MeDAB is to induce a limited restitutive proliferation (10, 11). While the use of different technical procedures may with various carcinogens have also failed to identify such cells and cytoplasmic AFP mRNAs, without immunohistochemistry, this cannot explain our morphological observations. Indeed, in a model of human hepatomas transplanted into athymic mice (41), AFP expression was found to be related to cell proliferation.

In situ hybridization results bring no support to the hypothesis of a reciprocal modulation between AFP and albumin gene expression at the cellular level during rat azo-dye hepatocarcinogenesis. In both preneoplastic and neoplastic lesions, serial sections showed that cells displaying high levels of AFP transcripts may also contain high amounts of albumin mRNAs. The decrease in albumin expression that, in accordance with previous studies (10, 11), we have observed in most preneoplastic hepatocytes, was not associated with an increase in AFP expression.

Our cellular analysis of albumin and AFP-expressing cells, in both preneoplastic and neoplastic lesions, further supports the hypothesis that the control of AFP and albumin expression during hepatocarcinogenesis is mainly at the transcriptional level (42). Indeed, in most cases, comparison between in situ hybridization and immunoperoxidase results suggests that the level of mRNA and protein expression are roughly parallel. However, that in certain cells, within both preneoplastic and neoplastic lesions, AFP protein could not be detected despite the presence of significant levels of AFP mRNAs, raises the possibility of an additional posttranscriptional control for AFP. Interestingly, comparable observations have been performed in related conditions, such as human cirrhosis associated with hepatocellular carcinoma (43), and teratocarcinomas (44). However, alternative possibilities may account for the discrepancies observed between mRNA and protein expression. While the sensitivity of immunoperoxidase may be lower than that of in situ hybridization, this cannot explain our morphological findings. Indeed, within the same section, for a comparable level of AFP mRNAs, certain cells contained detectable levels of AFP protein, while others did not. A toxic effect of 3MeDAB on RNA processing (45) or the presence of several AFP-mRNAs species, as shown at the early stage of rat hepatocarcinogenesis (27), may be hypothesized but cannot be tested with the probe used in this study. Another hypothesis is that an abnormal posttransductional processing of AFP results in loss of the normal immunoreactivity of the corresponding protein; however, the use in our study of a polyclonal antibody makes this possibility unlikely.

**REFERENCES**


25. Ma, M. H., and Webber, A. J. Fine structure of liver tumors induced in the...


Cellular Expression of α-Fetoprotein Gene and Its Relation to Albumin Gene Expression during Rat Azo-Dye Hepatocarcinogenesis


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/49/7/1790

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/49/7/1790. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.