Insulinlike Growth Factor II Expression and Oval Cell Proliferation Associated with Hepatocarcinogenesis in Woodchuck Hepatitis Virus Carriers

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Insulinlike growth factor II (IGF-II) is a highly mitogenic fetal growth factor suspected of regulating the growth of a wide spectrum of tissues via an autocrine or paracrine mode of action or both. High steady-state levels of IGF-II RNA were detected in 45% of hepatocellular carcinomas (HCCs) arising from woodchuck livers with persistent woodchuck hepatitis virus (WHV) infection. Analysis of WHV RNA in the same HCCs revealed that HCCs with high levels of IGF-II RNA contained low or undetectable levels of WHV RNA and HCCs with low levels of IGF-II RNA contained high levels of WHV RNA. Integrated WHV DNA was present in HCCs from both groups, but viral DNA replicating forms were present, predominantly in HCCs with low levels of IGF-II. Several IGF-II RNAs, the most prominent of which were poly(A) species of approximately 3.75 and 1.1 to 1.3 kilobases, were detected only in precancerous nodules and HCCs. Levels of IGF-II were elevated two- to three-fold in the serum of woodchucks with chronic active hepatitis preceding the occurrence of HCC. Proliferation of a population of oval cells, which arise from portal tract regions in the liver, preceded the development of HCC and was a prominent feature of livers from which tumors with high levels of IGF-II occurred. The HCCs tended to have distinct histological features according to their growth factor status. Tumors with low levels of IGF-II were generally highly differentiated acinar-trabecular HCCs, whereas tumors with high levels of IGF-II were more anaplastic, with regions of fibrosis and fatty accumulation. A model to relate the pathology of WHV infection to oval cell proliferation and IGF-II expression in the development of these heterogeneous HCCs is presented.

The isolation of woodchuck hepatitis virus (WHV) by Snyder and Summers established a viral etiology of hepatitis in woodchucks (37, 41; R. L. Snyder, A. J. Pathol. 52:32, 1968). The association of hepatocellular carcinoma (HCC) with persistent WHV infection was also reported at the time of discovery of the virus (37, 41), and subsequent studies have shown a nearly 100% lifetime risk of HCC among WHV carrier woodchucks (24, 32; G. V. Tyler, Ph.D. dissertation, the University of Pennsylvania, Philadelphia, 1984). While primary HCCs are the predominant tumor which arise in WHV carriers after long-term infections (2 years or longer), rare cholangiocarcinomas have also been reported (41). Proliferation of anaplastic spindle-shaped cells, resembling sarcoma cells, in whorls around proliferations of blood vessels has also been reported (41).

Considerable controversy exists over the question of which cells in the precancerous liver are the precursors of HCC (7, 9, 34). Classical interpretation of rat hepatocarcinogenesis models has led many investigators to conclude that hepatocytes are the precursor cells of HCC (9). Experiments tracing the expression of two fetal genes, alpha fetoprotein and gamma glutamyltranspeptidase, have raised questions as to the cellular origin of HCCs (9, 34). Alpha fetoprotein and gamma glutamyltranspeptidase are expressed primarily in a population of cells called oval cells, which originate in the portal tracts and proliferate exten-

sively in the early stages of hepatocarcinogenesis (9, 15, 34). The differentiation of oval cells into basophilic, albumin-producing hepatocytes has been demonstrated by using pulse-chase labeling of cellular DNA (7). The oncogenic potential of oval cells has also been demonstrated with several rat oval cell lines (21, 44). Treatment of one oval cell line with carcinogens, followed by cloning and injection of the clones into nude mice led to the production of a spectrum of tumors, including differentiated HCC, cholangiocarcinoma, and anaplastic carcinomas (21; H. Dunsford and J. McMahon, Fed. Proc. 46:428, 1987). Using another approach, Braun et al. (4) transformed oval cells with a c-Ha-ras gene and demonstrated that the transformed clones produced HCCs with various phenotypes when injected in nude mice. In light of these observations, the possible role of oval cells in WHV-mediated hepatocarcinogenesis is of considerable interest.

In addition to controversy over the cellular origin of HCC, very little information is available regarding endogenous growth factors or oncogenes which might participate in the initiation, promotion, and progression stages of primary HCC. Since the reexpression of fetal genes is a prominent feature of many cancers, including HCC (8, 34), we investigated the transcription of insulinlike growth factor II (IGF-II) in WHV-infected woodchuck livers and HCCs. IGF-II was of particular interest because it is a fetal growth factor expressed in high levels in fetal liver (10, 16, 26, 38). IGF-II is also highly mitogenic in cell culture assays (6, 42) and is

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produced by the BRL-3A liver epithelial cell line (H. Coon, J. Cell Biol. 39:29, 1968).

IGF-II is believed to function by autocrine or paracrine mechanisms to affect the growth and development of fetal liver and many other organs (10, 26, 33, 39). We have undertaken a study of the transcription of the IGF-II gene in woodchuck HCCs and liver tissues to determine whether it might have a function in the pathophysiology of persistent WHV infection leading to HCC. Analysis of IGF-II and WHV RNAs revealed two classes of biochemically distinct HCCs. One class of HCCs contained high levels of IGF-II RNA and low or undetectable levels of WHV RNA, and a second class exhibited low levels of IGF-II RNA and high levels of WHV RNA. The two classes of tumors had distinctive histological features similar to those of tumors generated in nude mice by oval cell lines. This has led us to speculate that IGF-II expression may affect the phenotype of HCCs which arise in woodchucks. Proliferating oval cells were also observed in liver and preneoplastic nodules of WHV carriers, raising the possibility that their role in hepatocarcinogenesis affects the phenotype of HCCs. Our results are discussed in relation to models of hepatocarcinogenesis in rats.

MATERIALS AND METHODS

Animals and tissues. Captive woodchucks were housed at the Penrose Research Laboratory of the Philadelphia Zoological Society and were maintained on the previously reported diet (36). Woodchucks with persistent WHV infection developed multifocal HCCs between 3 and 5 years of age, and uninfected woodchucks did not develop HCCs. When multiple tumors were present, each was excised separately. Tumor and normal tissues were snap frozen in liquid N₂ immediately after surgical removal and were stored in a liquid N₂ freezer. Liver samples from control woodchucks were obtained by wedge biopsy and were snap frozen. Tissue samples of each tumor and the surrounding nontumorous region were fixed in buffered formaldehyde and were paraffin embedded for histological analysis. Tissue sections were stained with hematoxylin and eosin, periodic acid-Schiff with or without diastase digestion or trichrome.

DNA hybridization probes and Northern (RNA) and Southern blot analysis. A cDNA clone encoding human preproinsulinlike growth factor II (prepro-IGF-II) plasmid phigf2, was used in these studies (2). The phigf2 cDNA contains a coding region of 543 base pairs plus a 5' untranslated region of 250 base pairs and a 3' untranslated region of 253 base pairs. The phigf2 cDNA does not contain a polyadenylation signal or a poly(A) tract. The coding sequences span the pre-B, -C, -A, -D, and -E domains of human prepro-IGF-II which are highly homologous in amino acid and nucleotide sequence to the corresponding domains in rat prepro-IGF-II (39).

WHV RNA and DNA was detected by using a 3.2-kilobase (kb) WHV DNA fragment comprising the entire WHV genome. The fragment was purified from plasmid pW8 after *EcoRI* digestion (23). The phigf2 cDNA insert, for use as a hybridization probe, was excised from the plasmid vector with *PstI* and was gel purified as was the WHV *EcoRI* insert. Probe DNAs were radiolabeled with ³²P to a specific activity of approximately 2 × 10⁸ to 4 × 10⁸ cpm/μg, using the random primer extension method (40).

Total cell RNA was prepared by the guanidine thiocyanate-cesium chloride procedure (5). Poly(A)⁺ RNA was further purified by chromatography on oligo(dt)-cellulose

(1). For Northern blot analysis, 5 μg of total cell RNA or 1 μg of poly(A)⁺ RNA was electrophoresed on formaldehyde-1% agarose gels in the presence of 0.5 μg of ethidium bromide per ml and then transferred to nylon membranes (GeneScreen) by standard methods. The RNAs were checked for intactness (18S and 28S rRNA) and equal loading by UV illumination of the nylon membranes. Hybridization with a β-actin probe was carried out as a further check for equal RNA loading (data not shown).

Genomic DNAs were isolated from tumor and liver as previously described (23), and 10 μg of each sample, digested with the appropriate restriction endonuclease, was used for Southern blot analysis (23) using nitrocellulose (Schleicher & Schuell, Inc.) or nylon (Zeta bind; AmF Cuno) membranes. Northern and Southern blots were hybridized (no prehybridization) with 40 × 10⁶ to 80 × 10⁶ cpm of the appropriate hybridization probe at high stringency (5× SSC, [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 50% formamide, 2.5× Denhardt solution, 42°C) for 16 to 24 h and washed at high stringency (23). Autoradiographic exposure of blots hybridized with phigf2 was for 16 to 48 h at -70°C and 4 to 24 h at -70°C for blots hybridized with WHV by using intensifying screens. Autoradiograms were analyzed by scanning densitometry with a densitometer (Ultrascan XL; LKB).

Measurements of IGF-II in serum. A radioreceptor assay was used to measure IGF-II in woodchuck serum (17). Rat placental membranes prepared as previously reported for BRL-3A membranes (17) were used as a source of IGF-II receptors. The IGF-II level was determined by competition between woodchuck serum and synthetic ¹²⁵I-labeled IGF-II for receptor binding. Synthetic human IGF-II (18) was used for standard competition curves for each membrane preparation, duplicate samples were measured, and nonspecific counts were determined in the presence of excess unlabeled IGF-II.

RESULTS

Analysis of IGF-II RNA in woodchuck livers and tumors. Analysis of IGF-II RNA in cellular RNA from woodchuck tumors and liver was carried out by hybridizing a human cDNA clone encoding prepro-IGF-II (2) to Northern blots of total cellular RNAs. Our initial hybridization analysis of tumors showed that some contained very high levels of IGF-II RNA, whereas others contained low levels of IGF-II RNA, comparable to the level in normal adult liver (Fig. 1, upper panel). The proportion of tumors containing high levels of IGF-II RNA from any given animal varied from 0 to 100%. In one animal with five tumors, all five contained very high levels of IGF-II RNA (Fig. 1C, upper panel). The most predominant IGF-II RNAs were a large species of approximately 3.75 kb, plus a heterogeneous population of smaller fragments ranging from approximately 0.7 to 2.4 kb. The most abundant poly(A)⁺ woodchuck IGF-II RNAs migrated exactly the same as those of the rat and were approximately 3.75 and 1.1 to 1.3 kb (Fig. 1C, lanes 8 and 9).

We next determined levels of IGF-II RNA in tissues at various stages of hepatocarcinogenesis, including normal uninfected liver, liver recovered from acute infection, persistently infected liver, and precancerous nodules. Normal uninfected adult woodchuck liver contained a single IGF-II species of approximately 4.2 kb (Fig. 2a). This RNA was also present in tissue which had recovered from acute infection, and some recovered livers also contained a heterogeneous group of smaller transcripts similar in size to those

FIG. 1. Northern blot analysis of IGF-II (upper panel) and WHV (lower panel) RNA present in total cellular RNA from WHV-infected woodchuck livers and HCCs. Northern blots were hybridized with purified ^{32}P -labeled cDNA inserts from plasmid phigf2 (2). After exposure, the blots were eluted and rehybridized with ^{32}P labeled WHV DNA. Lanes: A1, uninfected adult woodchuck liver RNA; A2, B1, and C7, WHV-infected liver RNA; A3 to A7, B2 to B4, C1 to C5, independent HCCs isolated from three animals (panel A, woodchuck CW428; panel B, woodchuck WC156; and panel C, woodchuck J9); C6, spleen RNA; C8 and C9, poly(A)⁺ RNA from woodchuck HCCs. Moderate degradation of RNA in lanes C7 was observed, resulting in a smear of WHV hybridization rather than specific WHV RNA bands.

in tumors but in much lower abundance (Fig. 2b). The 4.2-kb IGF-II RNA was also the predominant species in persistently infected livers (Fig. 2c). The level of heterogeneous smaller fragments varied considerably in persistently infected tissues and this could be due to the severity of chronic

infection. Three small whitish nodules were isolated from the liver of woodchuck J9 (which had HCC) and were divided in half for histological and RNA analysis. Northern blot analysis of RNA from half of each nodule, with the IGF-II probe, revealed a 3.75-kb transcript plus a series of

FIG. 2. Northern blot analysis of IGF-II RNA present in total cellular RNA samples from (a) uninfected adult livers; (b) liver which had recovered from acute WHV infection; (c) persistently WHV-infected livers before HCC developed; (d) precancerous nodules isolated from woodchuck J9 liver. (e) Small whitish nodular tissue fragments isolated from J9 liver. Lanes: 1, nodule 1; 2, nodule 2; 3, nodule 3; 4, nodule 4.

smaller RNAs which were present in varying abundance (Fig. 2d). Histological analysis revealed each nodule to be precancerous, with unique features (see below).

In addition to the above-described nodules, four small (2 to 4 mm) fragments of tissue which appeared as whitish nodular regions within the nontumorous portion of the liver of animal J9 were isolated and total RNA was extracted. Northern blot analysis of these RNAs revealed both the 4.2-kb IGF-II RNA observed in normal liver plus the 3.75-kb IGF-II RNA (Fig. 2e). Thus, small nodules which are either precancerous growths or early tumors contain a 3.75-kb IGF-II RNA which is not detectable in normal liver and becomes highly abundant in tumors.

A comparison of woodchuck and rat IGF-II RNAs (from BRL-3A cells) on the same Northern blot revealed closely related profiles with many common size transcripts (data not shown). The two most abundant common transcripts were 3.75 and 1.1 to 1.3 kb and were the major poly(A) species in both woodchuck and rat. A complex transcription and posttranscriptional processing mechanism involving two transcription initiation sites and more than one transcription termination site is involved in the generation of the sets of RNAs observed in the rat BRL-3A cell line (10, 39), and the multiple IGF-II RNAs in the woodchuck are probably generated in a similar manner. Since the IGF-II RNA profiles are very similar, the woodchuck and rat IGF-II genes are probably closely related in structure.

Analysis of WHV RNA in woodchuck liver and tumors. The same Northern blots were eluted and rehybridized with a WHV DNA probe. These experiments revealed the unexpected finding that those tumors which contained high levels of IGF-II RNA contained very low or undetectable levels of WHV RNA (Fig. 1, lower panel). In contrast, those tumors which contained low levels of IGF-II RNA usually contained high levels of WHV RNA, comparable to levels observed in the surrounding nontumorous liver. These results also demonstrated that the lack of hybridization to the IGF-II probe in the samples with low levels of IGF-II was not due to degradation of the RNA sample. Three predominant WHV RNAs of approximately 3.4, 2.4, and 2.1 kb were identified. These WHV RNAs migrate in positions expected of the RNAs coding for the WHV pregenome, pre-S1 protein and surface antigen of WHV (11). In our samples, the 3.4-kb RNA was in high abundance compared with the smaller surface antigen RNAs. In some cases, a heterogeneous mixture of variable-sized WHV RNAs of between 3.2 and less than 0.5 kb partially obscured the specific transcripts (Fig. 1A, lanes 2, 4, and 6; Fig. 1B, lane 1, and Fig. 1C, lane 1, lower panel). These heterogeneously sized RNAs were probably extracted from replicative intermediates present in the tissue. Interestingly, two of the precancerous nodules (Fig. 2d, and lanes 2 and 3) and one tumor (Fig. 1C, lane 1) exhibited both high levels of IGF-II RNA and WHV RNA, indicating that either some cells are permissive for both IGF-II and WHV transcription or a heterogeneous population of permissive and nonpermissive cells exists within some nodules and tumors.

A survey of 30 tumors from 13 woodchucks supported our initial observations of two predominant tumor types with regard to IGF-II and WHV transcription. There were 45% of the HCCs with medium to high levels of IGF-II RNA and low to undetectable levels of WHV RNA, and 37% contained low to undetectable levels of IGF-II RNA and high levels of WHV RNA. The 15% incidence of tumors with both low levels of IGF-II and WHV RNAs could be due to RNA degradation in a few samples. Nine persistently in-

fectured livers exhibited highly variable levels of IGF-II RNA, ranging from undetectable to medium, and 83% contained medium to high levels of WHV RNA.

Analysis of WHV DNA in HCCs. The occurrence of multiple tumors within a single liver could be due to several independent transformation events or to transformation followed by metastases. Tumors which arise by separate transformation events would be expected to contain different WHV DNA integrations, whereas tumors which arise by metastases within the liver would be expected to contain identical WHV DNA integrations. Cellular DNA was subjected to Southern blot analysis by using a WHV DNA hybridization probe, and autoradiograms of Southern blots from animals CW428 and J9 (five tumors each) are shown in Fig. 3. Tumor DNAs (Fig. 3) and RNAs (Fig. 1) were from the same set of tumors. Unique WHV DNA integrations identified as bands greater than 3.2 kb were present in all the tumor DNAs, and the integration patterns were different for all the tumors from an individual animal, except for tumors three and five from animal CW428. These tumors contained identical WHV DNA integration patterns when analyzed with several restriction endonucleases (Figure 3B, lanes 3 and 5). Therefore, tumors 3 and 5 (woodchuck CW428) fit the criteria for a metastasis. Interestingly, both were the high-IGF-II, low-WHV RNA type. The presence of abundant free viral DNA in tumor 5 could be due to the presence of some normal tissue in the tumor sample.

Southern blot analysis of many tumors revealed that high-WHV RNA tumors, in general, contained abundant WHV DNA replicating forms. In contrast, the tumors with high levels of IGF-II RNA, in general, were nonpermissive or very weakly permissive for viral transcription.

IGF-II in woodchuck serum. We next determined whether the level of IGF-II in the serum of woodchucks with multiple HCCs was elevated in comparison to the level of IGF-II in the serum of uninfected woodchucks, woodchucks with acute or persistent WHV infection without HCC, and woodchucks with acute or persistent WHV infection with HCC (Table 1). Uninfected woodchucks, approximately 15 months old, exhibited a mean of 470 ng of IGF-II per ml (6.2×10^{-8} M IGF-II) and acutely infected woodchucks exhibited a mean of 474 ng/ml. In contrast, woodchucks of the same approximate age (14 to 16 months) with chronic active hepatitis (CAH) and no HCC (infection at birth) averaged 762 ng of IGF-II per ml in serum, with a high degree of variability among animals. The data show that IGF-II elevation in serum can precede the development of HCC (1½ years later these animals had not developed HCC). Woodchucks with HCC exhibited high levels of IGF-II in the serum, averaging 1,308 ng of IGF-II per ml. The data support the conclusion that translation of the IGF-II RNAs in the liver and tumors results in increased secretion of IGF-II into the blood.

Histological analysis of precancerous and HCC woodchuck tissues. We next undertook a detailed histologic analysis of precancerous and HCC tissues to identify any structural characteristics might distinguish the biochemically heterogeneous tissues. We observed extensive proliferation of oval cells in the livers of WHV carrier woodchucks. The proliferation was particularly pronounced in the nontumorous regions of the livers immediately adjacent to HCCs. The phenotypes of the proliferating oval cells were virtually identical to those of oval cells previously reported in rats (8).

Oval cell proliferation extended from the portal regions up the sinusoids in woodchucks with CAH. This resulted in focal accumulations of oval cells in the centrilobular regions

FIG. 3. Southern blot analysis of WHV DNA present in cellular DNAs from liver and HCCs of woodchucks CW428 (A and B) and J9 (C). Southern blots were hybridized with 32 P-labeled WHV DNA. (A) DNA were digested with *EcoRI* before Southern blot analysis. Lanes: L, liver DNA, 1 to 5, DNA from woodchuck CW428 tumors 1 to 5, respectively. Analysis of RNA from these same tumors is presented in Fig. 1A, lanes 3 to 7, respectively. (B) Southern blot analysis of DNA from woodchuck WC428, tumors 3 and 5 (lanes 3 and 5), with restriction endonucleases *EcoRV* and *HpaI*, respectively (*EcoRV* and *HpaI* do not cut WHV DNA). (C) Lane L, DNA from J9 liver. Note WHV hybridization to high molecular mass cellular DNA, indicating WHV integration in nontumorous liver. Lanes 1 to 5, DNA from J9 tumors 1 to 5, respectively. Analysis of RNA from these tumors is presented in Fig. 1C, lanes 1 to 5, respectively. J9 DNAs were digested with *HpaI* before Southern blot analysis. Note lack of WHV replication forms in tumor DNAs and different WHV DNA integration patterns in each tumor. Lanes λ , Molecular size markers.

of the liver (Fig. 4A). Oval cell hyperplasia appears to be continuous during CAH in woodchucks and was extensive at the time of HCC development in the livers of the animals we studied (Fig. 4B). In nontumorous regions of livers from

which HCCs have arisen, oval cell proliferation surrounding or intermingling with hepatocytes was common (Fig. 4B, insert). Three of the dysplastic and presumptively precancerous nodules which were high in IGF-II RNA (Fig. 2d) were analyzed histologically. Each nodule contained numerous oval cells, and the hepatocytes were arranged in abnormal architecture.

Histological analysis of woodchuck HCCs revealed several pathological characteristics which were predominant in high-IGF-II tumors. These included fatty degeneration (Fig. 5A) of both the macro- and microvesicular type and a more anaplastic cellular organization (Fig. 5B). High-IGF-II tumors also contained large regions of fibrosis and many megakaryocytes (Fig. 5B), features reported earlier (24, 25, 37, 41). Low-IGF-II tumors were usually highly differentiated (trabecular-acinar) or mixed (trabecular and acinar) HCCs with minimal fibrosis or fatty degeneration (Fig. 5C).

DISCUSSION

A series of RNAs were shown to arise from either the -2 promoter (exon -2) or the -1 promoter (exon -1) of the rat IGF-II gene in BRL-3A cells (10, 16, 39). Woodchuck IGF-II RNAs comprise a series of transcripts which are nearly identical in size to rat IGF-II RNAs in BRL-3A cells. This leads us to conclude that the structure and transcription of the woodchuck IGF-II gene is probably very closely related to the rat IGF-II gene. This is not surprising since the human and rat IGF-II genes have been shown to be highly conserved in DNA sequence, exon-intron organization, and multiple RNA species produced (10, 39).

The 3.75- and 1.1- to 1.3-kb woodchuck IGF-II RNAs were only detected in precancerous nodules and HCCs of woodchucks. The 4.2-kb woodchuck IGF-II RNA which is present in low levels in normal liver is also present in low

TABLE 1. Summary of IGF-II levels in woodchuck sera

Description and animal no.	IGF-II (ng/ml)	Avg (ng/ml)
Uninfected woodchuck (no WHV)		
CW525	637	470
CW527	353	
CW528	456	
J38	435	
Woodchuck with acute WHV infection (WHsAg ⁺) ^a		
J36	368	474
J37	256	
J39	583	
J40	608	
J41	556	
Woodchuck with persistent infection, no tumor (WHsAg ⁺ , anti-WHcAg)		
CW521	885	762
CW522	1,134	
CW523	633	
CS524	398	
Woodchuck with persistent infection and tumors (HCC)		
J9	1,478	1,308
CW428	1,160	
CW490	1,718	
CW745	876	

^a WHsAg⁺, WHV surface antigen positive; WHcAg, WHV core antigen.

FIG. 4. Histological features of WHV-infected woodchuck liver with CAH and liver isolated from woodchuck J9. (A) Centrilobular proliferation of oval cells in woodchuck with persistent WHV infection and no HCC. (Insert) Higher magnification of woodchuck oval cells. (B) Section from nontumorous liver of woodchuck J9 at the time of HCC, demonstrating massive proliferation of oval cells extending from portal tracts into centrilobular regions, disruption of lobular architecture, accumulation of plasma cells and lymphocytes, and scattered necrosis of hepatocytes. (Insert) Higher magnification of one area of same J9 nontumorous liver, illustrating oval cell proliferation infiltrating and disrupting the normal arrangement of hepatocytes, some of which appear entrapped.

levels in tumors. If the woodchuck IGF-II gene has two promoters like the rat gene, as is strongly suspected, then the 3.75- and 1.2-kb IGF-II RNAs probably initiate in exon -1 and the 4.2-kb IGF-II RNA in the -2 exon (relative to the rat gene). If this is the case, the high level of the 3.75-kb and 1.1- to 1.3-kb IGF-II RNAs we have observed in tumors is probably due to activation of the -1 promoter, 5' to exon -1.

Our observations that IGF-II RNAs accumulate in only about 50% of the primary woodchuck HCCs is consistent with other studies of rat and human hepatoma cell lines. Soares et al. (39) reported that rat hepatoma H4 contained a 1.1-kb species specific to the -1 exon, whereas another hepatoma cell line (McA-RH777) did not contain any detectable IGF-II RNA. In our studies of human HCC cell lines, we have observed highly elevated IGF-II transcription in HepG2, Hep3B, and Huh1 cell lines and no detectable IGF-II RNA in the PLC/PRF/5, Malavhu, and Huh2 cell lines. These data are consistent with two categories of human tumors which may have properties similar to those of primary woodchuck HCCs. One model to explain the lack of IGF-II RNA in some HCCs is that IGF-II functions primarily in the initiation stages of HCC and that continued expression is not required for tumor progression.

In the case of Wilms tumors and Wilms tumor cell lines, IGF-II expression is elevated to levels equivalent to that of expression in embryonal tissues, including liver and kidney (27, 33). Other tumors of embryonal origin, including rhabdomyosarcoma and hepatoblastoma, also exhibited elevated IGF-II transcription (33). In these cases, it was proposed that loss of regulatory genes (suppressor genes) at one locus may allow the overexpression of IGF-II located at a second locus (27, 33). A contrasting view, which places less importance on IGF-II in tumorigenesis, would be that increased IGF-II expression was simply an epiphenomenon involving the reestablishment of the fetal phenotype as a result of transformation (19). Our data demonstrating IGF-II expression in precancerous livers before HCC makes it more likely that IGF-II plays an active role in tumor initiation.

The presence of WHV DNA integrations in all the tumors is of interest for several reasons. First it shows that all the tumors arose from cells which were infected by WHV. Second, since viral DNA integration is a rare event in normal hepatocytes (31), tumors may arise preferentially from cells which contain integrations. Third, integrations were present in tumors which were both permissive or nonpermissive for viral transcription. In the case of nonpermissive cells, one possibility is that infecting viral DNA is shunted into an integration pathway. Although a specific role for integrations in the mechanism of HCC is unclear, integrations serve as focal points of chromosome rearrangements in many tumors 14, 22, 28, 29, 30; H. P. Wang and C. E. Rogler, *Cytogenet. Cell Genet.*, in press). Factors which promote chromosome instability are clearly important factors in oncogenesis.

A model to explain the possible relationship of reparative cellular proliferation during CAH and the eventual occurrence of HCCs with specific biochemical and histological phenotypes is presented in Fig. 6. The model proposes that once WHV persistent infection is established, portal hepatitis ensues and several pathological reactions occur on a self-perpetuating basis. These include death and regeneration of hepatocytes, an inflammatory reaction primarily limited to the portal tracts, and mild proliferation of bile ducts (Fig. 6, line 2). As the disease progresses to CAH, involvement of the rest of the hepatic lobule becomes evident (lobular hepatitis) (Fig. 6, line 3). Oval cells either isolated or in clusters can be found along the sinusoids from portal tracts to the midzone and centrilobular areas of the hepatic lobule. Woodchucks displaying lesions of this severity showed two- to threefold elevations of IGF-II in the serum.

Tumor phenotypes arising in woodchuck livers (HCCs and rare cholangiocarcinomas) may reflect the cellular origin of each particular tumor or the state of differentiation of a stem cell at the time of malignant transformation. The predominant view has been that HCCs arise from fully differentiated hepatocytes. Thus, transformation of fully differentiated hepatocytes could result in high-WHV, low-IGF-II tumors

FIG. 6. Diagram illustrating the proposed progressive stages of WHV infection leading to the generation of biochemically and histologically heterogeneous primary hepatocellular carcinomas in woodchucks. Structures illustrated are: O, oval cell; H, hepatocyte; BD, bile duct; CV, central vein; PV, portal vein; HA, hepatic artery; MN, mononuclear cell; F, fibroblast; MK, megakaryocyte; FD, fatty degeneration. Line 1 schematic illustration of normal liver architecture comprising portal tracks (left) connected by single cell plates of hepatocytes and central hepatic veins (right). Line 2, after establishment of WHV persistent infection, portal hepatitis develops. Line 3, portal hepatitis progresses to CAH. Oval cell proliferation is prominent. Line 4, sustained by the dynamics of protracted infection, inflammation, cell necrosis, and growth factor production, heterogeneous types of tumors arise. Illustrated are (a) low-IGF-II, high-WHV tumors, (b) high-IGF-II, low-WHV tumors with more dysplastic features, or (c) very rare instances of cholangiocarcinoma (1).

without altering the transcriptional program of WHV and IGF-II genes in those hepatocytes. Alternatively, if a population of stem cells within the liver are the precursors of HCC, the final phenotype of the tumor may reflect the state of differentiation of the stem cell at the time transformation occurs. In such a case, transformation of a less-differentiated stem cell would more likely generate a tumor closer to the fetal phenotype, which could be nonpermissive for WHV transcription and should express high levels of IGF-II RNA. Oval cells which proliferate in woodchuck livers with CAH represent a potential source of cells for this differentiation pathway.

The preceding model is consistent with general mechanisms of tumorigenesis involving the activation of growth factor genes at an early stage of carcinogenesis. In the case of adult T-cell leukemias, the interleukin 2 and interleukin 2 receptor genes are transcriptionally activated by the p40

gene of human T-cell leukemia virus type I, resulting in the early polyclonal proliferation of T cells preceding malignant transformation (12, 20, 35). This could be analogous to the proliferation of oval cells in chronically infected woodchuck livers, although the direct role of WHV in this cellular proliferation has not yet been demonstrated. Interleukin 2 continues to be expressed in only a fraction of adult T-cell leukemias, demonstrating that its continued presence is not required during clonal expansion of malignantly transformed T cells (43). This is also consistent with our observation that roughly half of the HCCs arising in woodchucks continue to express high levels of IGF-II. Clearly, secondary events are required before malignant transformation occurs in both systems. Many other analogies can be drawn between these and other systems, involving growth factor activation during the initiation stages of tumorigenesis (3, 13).

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FIG. 5. Distinctive histological features of HCCs from WHV carriers. (A) Woodchuck CW428, tumor 1 (high-IGF-II RNA; Fig. 1A, lane 3) exhibiting acinar arrangement of cells with regions of macrovesicular fatty cell degeneration. (B) Woodchuck J9, tumor 5 (high-IGF-II RNA; Fig. 1C, lane 5). Area of extramedullary hematopoiesis (megakaryocytes) intermingling with tumor cells with acinotrabeular arrangement. Lymphoplasmatic infiltration is prominent, with moderate dysplasia and marked architectural disorganization, plasma cell infiltration, and mild fibrosis. (C) Woodchuck WC156, tumor 2 (low-IGF-II RNA; Fig. 1B, lane 3), illustrating a highly differentiated HCC displaying trabecular-acinar arrangement with no fibrosis.

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