The Woodchuck Model of Hepatitis B Virus Infection

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Abstract

The woodchuck hepatitis virus (WHV) was the first of the mammalian and avian hepadnaviruses described after discovery of the virus of hepatitis B (HBV). Woodchucks chronically infected with WHV develop progressively severe hepatitis and hepatocellular carcinoma, which present as lesions that are remarkably similar to those associated with HBV infection in humans. The initial virological studies and studies of pathogenesis utilized woodchucks that had been trapped in the wild and had acquired WHV infection naturally. Research with wild woodchucks was complicated by lack of knowledge of their backgrounds (e.g., dietary history, exposure to parasites or environmental toxins, and source and duration of WHV infection). Breeding colonies of woodchucks have been established and maintained in laboratory animal facilities, and laboratory-reared woodchucks are superior for experimental studies of pathogenesis or hepatocarcinogenesis. It is possible to infect neonatal woodchucks born in the laboratory with standardized inocula and produce a high rate of chronic WHV carriers that are useful for controlled investigations. WHV has been shown experimentally to cause hepatocellular carcinoma, supporting conclusions based on epidemiological and molecular virological studies that HBV is an important etiological factor in human hepatocarcinogenesis. Chronic WHV carrier woodchucks have become a valuable animal model for the preclinical evaluation of antiviral therapy for HBV infection, providing useful pharmacokinetic and pharmacodynamic results in a relevant animal disease model. It also has been shown that the pattern of toxicity and hepatic injury observed in woodchucks treated with certain fluorinated pyrimidines is remarkably similar to that observed in humans that were treated with the same drugs, suggesting the woodchuck has significant potential for the preclinical assessment of antiviral drug toxicity.

Key Words: antiviral drug; hepatitis B virus; hepatocellular carcinoma; nucleoside analogs; woodchuck hepatitis virus

Introduction

Blumberg and colleagues’ (1965, 1967) identification of the Australia antigen and the subsequent establishment of its relation to hepatitis B virus (HBV) (Dane et al. 1970; Okuchi and Murakami 1968; Prince 1968) are among the most important medical discoveries of modern times and have resulted in major advances in understanding the natural history and control of viral infections of the liver. As a result of HBV infection, a small percentage of adults and a much larger proportion of infants and young children develop persistent infection, becoming chronic carriers and potential sources of HBV. Characteristically chronic HBV carriers develop chronic hepatitis that often progresses to cirrhosis (Alberti et al. 1978, 1979; Dudley et al. 1972; Prince et al. 1970), and there is convincing epidemiological evidence that HBV is an etiological factor in the pathogenesis of primary hepatocellular carcinoma (HCC) of humans (Blumberg and London 1982; Blumberg et al. 1975; Hadziyannis 1981; Macnab et al. 1976; Maupas et al. 1975, 1980; Popper et al. 1982; Prince et al. 1975; Sherlock et al. 1970; Szmuness 1978).

In at least four members of the family Sciuridae, naturally occurring infection with HBV-like viruses (hepadnaviruses) has been described (Marion et al. 1980, 1983, 1986; Minuk et al. 1986; Snyder 1968; Snyder and Ratcliffe 1969; Summers et al. 1978; Tennant et al. 1991b). The objective of this article is to summarize the experimental studies that have been conducted with the Eastern woodchuck (Marmota monax, groundhog), with the woodchuck hepatitis virus (WHV) and the use of this species as an animal model for HBV research.

Natural History of WHV Infection

Summers and his colleagues (1978) described WHV infection in a colony of woodchucks in which high rates of chronic hepatitis and HCC had been observed (Summers et al. 1978). The serum from 15% of the woodchucks contained viral
DNA polymerase activity and viral particles that morphologically resembled HBV. It was concluded that WHV was a member of the same family of viruses to which HBV belonged (Summers et al. 1978).

WHV is classified as a member of the genus Orthohepadnavirus, family Hepadnaviridae (Gust et al. 1986; Melnick 1982; Robinson et al. 1982). The genetic organization of WHV is similar to HBV and to other mammalian hepadnaviruses. Numerous 22-nm filaments and spherical particles are found in the serum of infected woodchucks and are composed of the envelope protein of the virus. Complete virions are 42 to 45 nm in diameter and are composed of the exterior envelope (WHsAg1), the nucleocapsid or core protein (WHcAg1), and, within the nucleocapsid, the DNA genome (Ganem and Varmus 1987; Tiollais et al. 1985). The replicative cycle of WHV appears to be identical to that of HBV (Ganem and Varmus 1987; Summers 1987; Tiollais et al. 1985). Transition of viral DNA to RNA during the life cycle of hepadnaviruses has similarities to that of retroviruses (Ganem and Varmus 1987; Summers 1987). Integration of viral DNA into the host genome is not, however, essential for replication of hepadnaviruses as is the case with retroviruses. The integration of WHV DNA sequences into host cell genomic DNA may have an important role in hepatocarcinogenesis (described below).

The woodchuck habitat extends from northern Georgia, Alabama, and Mississippi in the southern United States, west to Oklahoma, Kansas, Nebraska, and North and South Dakota, north to Quebec and Labrador, and across Canada to British Columbia and the Yukon Territory, including a region in southeastern Alaska. A comprehensive, seroepidemiological study of WHV infection has not been performed, and the prevalence of WHV infection throughout most of this range remains unknown. WHV infection is hyperendemic in the mid-Atlantic states, and the woodchucks originally studied by Summers and colleagues (1978) were from Pennsylvania. Of the woodchucks from Pennsylvania, New Jersey, and Maryland, 23% were test positive for WHsAg, and an additional 36% were positive for anti-WHs antibody for 59% prevalence rate of infection (Tyler et al. 1981). Others have confirmed high rates of WHV infection in the mid-Atlantic states (Wong et al. 1982). In contrast, the rate of WHV infection in central New York State has been estimated to be approximately 2% based on the presence of antibody to the WHV surface antigen antibody (Wong et al. 1982), and the rate of persistent WH surface antigenemia is less than 0.2%. Although the number of woodchucks tested is small, no serological evidence of WHV infection has been found in Vermont, Massachusetts, or Iowa (Lutwick et al. 1982; Summers 1981; Young and Sims 1979).

Hepatocellular neoplasms were described in woodchucks from the Philadelphia Zoological Garden many years ago in the form of multiple hepatic “adenomas” that ranged from 0.3 to 1.0 cm in diameter (Fox 1912; Ratcliffe 1933). Two woodchucks were later described with primary hepatic neoplasms, one from the Washington Zoological Park and one trapped in Bethesda, Maryland (Habermann et al. 1954). In addition to woodchucks from Pennsylvania (Snyder 1968; Snyder and Ratcliffe 1969), other cases of hepatic neoplasia have been reported in laboratory-maintained woodchucks originally trapped in New York and Maryland (Bond 1970; Long et al. 1975).

The hepatic neoplasms associated with naturally acquired WHV infection characteristically were well-differentiated HCCs with hyperchromatic nuclei and prominent nucleoli (Snyder and Summers 1980). In most cases, chronic, active hepatitis was present in the non-neoplastic liver, with abundant portal mononuclear cell infiltration extending beyond the limiting plate. There also was scattered parenchymal hepatocellular necrosis, bile duct proliferation, and in some there was evidence of early fibrosis (Popper et al. 1981; Roth et al. 1985; Snyder and Summers 1980; Snyder et al. 1982). Progression of neoplasia from foci of altered hepatocytes, to small neoplastic nodules and to frank HCC, was described, and some HCCs contained significant numbers of infiltrating hematopoietic cells (Popper et al. 1981). Trabecular, pseudo-glandular, and pelioid histological patterns have been observed in HCCs of woodchucks, similar to that of humans (Roth et al. 1985). Metastasis of HCC outside the liver, which occurs in humans and experimental animal models with some frequency, has not been reported in woodchucks by most investigators, although pulmonary metastases have been observed (Roth et al. 1985).

**Other Naturally Occurring Hepadnavirus Infections of Animals**

Since the description of WHV, closely related hepadnaviruses have been described in several other mammalian and avian species (Table 1). The morphology and genetic organization of these hepadnaviruses are similar (Ganem and Varmus 1987; Tiollais et al. 1985). The ground squirrel hepatitis virus (GSHV1) was described in California ground squirrels (Spermophilus beecheyi) (Kodama et al. 1985; Marion et al. 1980, 1986; Weiser et al. 1983). Persistent GSHV infection is associated with chronic hepatitis and with HCC, although the frequency of HCC is believed to be less than that associated with chronic WHV infection and develops at an older age (Cullen and Marion 1996; Marion et al. 1986).

A similar hepadnavirus has been described in the arctic ground squirrel (Spermophilus parryi) and named the arctic ground squirrel hepatitis virus (AGSHV1). Infection with AGSHV was associated with a remarkably high rate of HCC (Testut et al. 1996). Infection with a putative hepadnavirus has been described in Eastern gray squirrels (Spermophilus carolinensis) in Pennsylvania (Feitelson et al. 1986). Lesions of hepatitis were reported, but hepatic tumors were not observed. Evidence also has been reported for hepadnavirus infection in Richardson’s ground squirrels (Spermophilus richardsonii) originating in Alberta (Minuk et al. 1986; Tennant et al. 1991b). The hepatic lesions including HCC were remarkably similar to those described in woodchucks, California ground squirrels, and Arctic ground squirrels.
Table 1 Hepatitis B viruses (hepadnaviruses) of animals

<table>
<thead>
<tr>
<th>Virus Scientific Name</th>
<th>Host</th>
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<tbody>
<tr>
<td><strong>Genus: Orthohepadnavirus</strong></td>
<td></td>
</tr>
<tr>
<td>Hepatitis B virus (HBV)*</td>
<td>Human</td>
</tr>
<tr>
<td>Woodchuck hepatitis virus (WHV)</td>
<td>Woodchuck, groundhog</td>
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<tr>
<td>California ground squirrel hepatitis virus (GSHV)</td>
<td>California ground squirrel</td>
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<tr>
<td>Arctic ground squirrel hepatitis virus (AGSHV)</td>
<td>Arctic ground squirrel</td>
</tr>
<tr>
<td>Woolly monkey hepatitis B virus (WMHBV)</td>
<td>Woolly monkey</td>
</tr>
<tr>
<td><strong>Genus: Avihepadnavirus</strong></td>
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<tr>
<td>Duck hepatitis B virus (DHBV)</td>
<td>Domestic duck, Pekin duck</td>
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<tr>
<td>Heron hepatitis B virus (HHBV)</td>
<td>Grey heron</td>
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<tr>
<td>Snow goose hepatitis B virus (SGHBV)</td>
<td>Snow goose</td>
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* Naturally acquired HBV infection also has been demonstrated in the chimpanzee, gorilla, gibbon, and orangutan.

Infection with the duck hepatitis B virus (DHBV) has been reported in domestic Pekin ducks (Anas domesticus) (Mason et al. 1980; Omata et al. 1983). DHBV has a worldwide distribution. From Germany, avian hepadnaviruses also have been described in grey herons (Ardea cinerea) (Sprengel et al. 1988) (heron hepatitis B virus) and in snow geese (Anser caerulescens) (Chang et al. 1999). Much of the current understanding of hepadnavirus replication is based on research using DHBV in vivo and in vitro (Marion et al. 1987). HCC has been described infrequently in association with DHBV infection (Omata et al. 1983), and the hepatocarcinogenicity of DHBV in ducks remains questionable. Hepatic neoplasms have not been associated with heron hepatitis B virus infection (Sprengel et al. 1988).

### Experimental Hepadnavirus Infection of Woodchucks

The original observations of WHV infection were made with woodchucks trapped in the native habitat and subsequently maintained in the laboratory. Such woodchucks, in which WHV infection was naturally acquired, proved to be valuable sources of virus and hepatic tissue for histological and molecular analyses; however, their limitations for experimental purposes were soon recognized. It was impossible to know when and for how long trapped woodchucks had been infected with WHV or to be certain about their nutritional history and/or exposure to environmental factors, which might have influenced the course of WHV infection. Importantly, hepatic lesions caused by nematodes such as *Ackeria marmotae* and *Capillaria* sp. were common in wild woodchucks (Cohn et al. 1986) and could complicate the interpretation of experimental results.

Summers and colleagues (1980) described the experimental infection of 4- to 8-mo-old woodchucks with serum from chronic WHV carriers. They described successful productive infection, but infection was self-limited, and no woodchucks became chronic carriers. Attempts by others to infect juvenile or adult woodchucks experimentally resulted in acute WHV infection (Millman et al. 1984; Tyler et al. 1986; Wong et al. 1982) but, with one exception, did not cause chronic WHV infection. Morphological and molecular virological studies of the liver have shown that virtually 100% of hepatocytes become infected after experimental WHV infection (Kajino et al. 1994). Although replicative forms were cleared rapidly during recovery, covalently closed circular WHV DNA persisted in three of 10 woodchucks after evidence of WHV replication had ceased (Kajino et al. 1994). Clearance of experimental WHV infection in adult woodchucks is associated with robust humoral and cell-mediated immune responses (Guo et al. 2000; Menne et al. 1997; Shanmuganathan et al. 1997). Treatment with immune suppressive doses of cyclosporine A significantly increases the rate of chronic WHV infection (Cote et al. 1991, 1992).

When adult humans are infected with HBV, less than 5% become chronic carriers (Seeff et al. 1987; Tassopoulos et al. 1987). HBV infection early in life, however, results in high rates of chronic infection (Blumberg and London 1982; Ganem and Varmus 1987). The high rates of chronic WHV infection observed in woodchucks from hyperendemic areas (Popper et al. 1981; Snyder and Summers 1980; Tyler et al. 1981) suggested that, as in humans, infection in woodchucks early in life (or possibly vertical transmission) must be necessary to account for the high WHV carrier rates.

To utilize the advantages of the woodchuck as an experimental animal model, it became necessary to breed and rear...
woodchucks in laboratory animal facilities. The result allowed knowledge of the experimental woodchucks’ genetic background, definition of their diet, and, possibly, prevention of their natural exposure to WHV infection and other diseases that are endemic in wild woodchuck populations. To meet this requirement, a breeding colony of WHV-negative woodchucks was established at Cornell University in 1979. The colony now serves as the source of woodchucks for experimental studies of WHV infection and hepatocarcinogenesis. Woodchucks born in the laboratory animal setting are inoculated at birth with diluted serum from standardized infectious pools obtained from chronic WHV carrier woodchucks (Cote et al. 2000a,b; Gerin et al. 1989; Popper et al. 1987). After inoculation, woodchucks are monitored using specific serological markers of WHV infection (WHV DNA, WHsAg, anti-WH core antibody, and anti-WH surface antibody) (Cote et al. 1993; Wong et al. 1982).

The rate of chronic WHV infection after neonatal inoculation is 60% or greater (Cote et al. 2000a,b; Gerin et al. 1989; Popper et al. 1987; Tennant et al. 1988). Survival analyses have been made of chronic WHV carriers experimentally infected at birth with WHV, of woodchucks that recovered from neonatal WHV infection by clearing WH viremia and developing anti-WHs antibody, and of control woodchucks not infected with WHV but born and raised under similar laboratory conditions. All WHV carriers were dead by 56 mo of age, and the lifetime risk of HCC was 100% (Gerin et al. 1989; Popper et al. 1987). The median time to death from HCC in WHV carriers was 29 mo. In contrast, 42% of the woodchucks with resolved WHV infection and 62% of uninfected controls were alive at 56 mo of age. Although the rate of HCC in WHV carriers was significantly higher than that of woodchucks in which WHV infection was resolved, 17% of the woodchucks that recovered from neonatal WHV infection developed HCC (Gerin et al. 1989). HCC was not observed in the uninfected, laboratory-reared, control woodchucks. These results provide compelling direct experimental evidence for the carcinogenicity of WHV and, by analogy, for other mammalian hepadnaviruses (HBV, GSHV, and AGHSV) in which naturally acquired hepadnavirus infection has been associated with HCC. The rate of HCC in woodchucks with experimentally induced chronic WHV infection was similar to that observed in woodchucks with naturally acquired chronic WHV infection (Popper et al. 1981; Roth et al. 1985; Snyder and Summers 1980), and the presence of preneoplastic foci of altered hepatocytes with progressive aneuploid change (Cullen et al. 1994; Mi et al. 1994) also was similar (Abe et al. 1988; Toshkov et al. 1990).

In contrast to humans and experimental rats, the sex of experimental woodchucks does not influence their rate of infection with HCC. This result was unexpected because both in humans infected with HBV and in laboratory rodents in which HCC is induced by chemical hepatocarcinogenesis, males tend to have higher rates of HCC than females. The explanation may relate to the unusual circannual reproductive cycle of the woodchuck. For at least 8 mo of the year, the testicles of male woodchucks are abdominal and produce little or no testosterone, resulting effectively in functional castration (Baldwin et al. 1985).

With one exception, the diseases caused by WHV infection are confined to the liver. Immune-mediated glomerulonephritis has been reported with increased frequency in woodchucks chronically infected with WHV, and it appears to be similar etiologically to the glomerulonephritis caused by WHV (Peters et al. 1992).

Investigation of hepadnavirus replication has been impeded by the limited availability of tissue culture systems. However, full-length clones of the genomes of HBV (Will et al. 1982), WHV (Miller et al. 1990), DHBV (Sprengel et al. 1984), and GSHV (Seeger et al. 1984, 1987) have been shown to be infectious; and injection directly into the hepatic parenchyma of the homologous host species results in productive hepadnavirus infection. During transfection experiments to determine the viral gene or genes responsible for host range restriction, it was found that woodchucks could be infected with GSHV (Seeger et al. 1987, 1991). The chipmunk (Eutamias species) also has been shown to be susceptible to GSHV infection (Trueba et al. 1985).

HCC in woodchucks chronically infected with WHV infection has been reported to occur more frequently (Gerin et al. 1989; Popper et al. 1981, 1987) than in California ground squirrels chronically infected with GSHV; and in GSHV infection, HCC develops at an older age (Marion et al. 1986). Because woodchucks could be infected with both hepadnaviruses, it could be determined whether the apparent differences in oncogenicity of GSHV and WHV in their respective natural hosts were the result of differences in host response or of viral genetic differences. When neonatal woodchucks were experimentally infected with WHV or GSHV, the rates of chronic infection for each virus were similar, and at 2 yr of age, no difference was noted in the severity of chronic hepatitis (Seeger et al. 1991). By 2 yr, hepatic neoplasms had developed in 13 of 16 chronic WHV carriers. In sharp contrast, only one of 16 chronic GSHV carriers at that age had developed a single, grossly identifiable hepatic tumor nodule (5 mm diameter), which was classified as a hepatic adenoma. On the basis of these observations, it was concluded that the difference between GSHV and WHV in oncogenic capacity that had been observed in their respective natural hosts must be due to genetic differences between the viruses, although additional differences in host factors could not be excluded.

It would be useful to infect California ground squirrels with WHV, but this may not be possible. Attempts to transfect California ground squirrels with WHV DNA have not yet been successful (Seeger et al. 1987). It should, however, be possible to assess differences in the viral genetic determinants of oncogenicity using WHV/GSHV chimeric hepadnavirus constructs, and such investigations are under way.
Experimental Infection of Ducks with DHBV

DHBV was identified originally in Pekin ducks in China (Omata et al. 1983, 1984), where it caused persistent infection. Subsequently, DHBV infection was demonstrated in domestic duck populations in the United States (Mason et al. 1980, 1983), and it probably has a worldwide distribution. In contrast to mammalian hepadnaviruses, chronic DHBV infection is characteristically not associated with significant histological changes in the liver (Cullen et al. 1989; Marion et al. 1984) or with mild changes. As mentioned previously, DHBV appears not to be a primary cause of HCC in ducks.

Experimental studies of DHBV infection (Fukuda et al. 1987; Jilbert et al. 1988, 1996; Marion et al. 1987; Mason et al. 1983; Vickery and Cossary 1996) have shown age to be a critical determinant of the outcome of hepadnavirus infection as it is in humans and other mammals. Congenitally infected ducklings or ducklings infected at an early age develop persistent DHBV infection. Infection of older ducks is followed typically by resolution of infection, often with inflammatory changes in the liver. As in the woodchuck, adult DHBV infection results in the infection of almost all hepatocytes with the expression of DHBV DNA and of viral proteins. Immune clearance of DHBV infection occurs rapidly and apparently without massive hepatocellular necrosis (Jilbert et al. 1992).

Much of what is known about the replication of hepadnaviruses has been learned using the DHBV system both in vitro and in vivo (Summers and Mason 1982). The role of covalently closed circular DNA as the template for viral transcription was first demonstrated in the duck system (Summers 1987). The molecular structure (Newbold et al. 1995), the mechanism of replenishment of the cccDNA pool, and the control of this pathway by surface antigen molecule (Summers et al. 1990; 1991) all have been investigated primarily using DHBV. The duck also has been valuable in antiviral drug development (Tsiquaye et al. 1996).

Mechanisms of Hepatocarcinogenesis Associated with Hepadnavirus Infection

There are three lines of evidence that indicate HBV is an important etiological factor in human hepatocarcinogenesis. The first is the epidemiological evidence demonstrating the close relation between the prevalence of HBV infection and that of HCC (Maupas et al. 1980; Szmuness 1978). Significantly higher rates of HCC are observed in individuals with chronic HBV infection than in case controls both in the hyperendemic regions of Africa (Blumberg et al. 1975; Maupas et al. 1980; Prince et al. 1975) and Asia (Asano et al. 1982; Chien et al. 1981; Hann et al. 1982; Okuda et al. 1982) and in the United States and Great Britain, where the rates of HBV infection and HCC were relatively low (Omata et al. 1979; Tabor et al. 1977; Viola et al. 1981). Compelling evidence of the etiological role of HBV infection in hepatocarcinogenesis comes from prospective epidemiological studies in Taiwan. These now-classical investigations demonstrated that the relative risk of HCC in chronic HBV carriers was 100 times higher than in noncarrier individuals (Beasley 1982, 1988; Beasley and Lin 1978; Beasley et al. 1981). A similar high relative risk of HCC in HBV carriers also has been reported in the United States (Prince and Alcabes 1982).

A second line of molecular evidence comes from the demonstration of covalent integration of truncated HBV DNA sequences in the cellular DNA of hepatic tumors of most HBV carriers (Brechot et al. 1981; Chen et al. 1982; Shafritz 1982). These observations suggest that HBV has a direct oncogenic role similar to that of other tumor-producing viruses in which integrated viral DNA or, in the case of retroviruses, proviral DNA causes malignant transformation by insertional mutagenesis (Chen et al. 1982; Shafritz 1982).

Finally, the comparative medical evidence strongly supports an etiological role of hepadnaviruses in hepatocarcinogenesis. At least four members of the family Sciuridae have been described in which persistent hepadnavirus infection has been closely associated with development of HCC. How hepadnaviruses actually cause HCC, however, is not completely understood.

Two general mechanisms have been proposed to explain hepatavirus-associated hepatocarcinogenesis (Tennant 1992). One hypothesis is that hepatavirus have a direct molecular role (Buendia 1994; Buendia and Pineu 1995). In this model, integration of hepadnaviral nucleic acid sequences into host cell DNA is considered a critical mutagenic event, which causes an alteration in expression of genes that regulate the cell cycle (protooncogenes and tumor suppressor genes). Ultimately, these changes result in neoplastic transformation of hepatocytes. Hepadnavirus integration is thought to initiate cell transformation in a manner comparable with that caused by chemical hepatocarcinogens (Farber and Sarma 1987; Pitot and Dragan 1991).

Support for the molecular hypothesis comes from detection of integrated hepadnaviral sequences in the cellular DNA of most primary hepatic tumors from HBV carriers (Brechot et al. 1981; Chen et al. 1982; Shafritz 1982). Similar integrations of viral nucleic acids have been observed in other mammalian cancers caused by DNA tumor viruses (Johnson and Williams 1982). Integrations in apparently non-neoplastic hepatic tissue of HBV carriers suggest integration precedes formation of hepatic neoplasms. Although occasional integrations have been identified in or near a potential oncogene, the large majority of HBV integrations in human HCC appear to be random.

Integrated WHV sequences also have been found in most HCCs from WHV-infected woodchucks (Korba et al. 1989). Characteristically, only portions of the viral genome are integrated, and the sequences are often rearranged (Ganem and Varmus 1987; Schödel et al. 1980). Molecular cloning and analyses of integrated WHV DNA and associated flanking sequences of cellular DNA initially demonstrated that integrations occurred at multiple sites within the woodchuck genome (Fuchs et al. 1989; Kaneko et al. 1986; Korba et al. 1989; Mitamura et al. 1982; Ogston et al. 1982; Rogler 1989).
N-myc and c-myc in 20% of the tumors studied. Woodchucks have two N-myc transcripts identified as N-myc2. Activation was the result of insertional mutagenesis (Hsu et al. 1988). In a large series of woodchuck HCCs, activation of c-myc was observed in 10% of the tumors. Insertions of WHV DNA have been reported in apparently nontumorous liver of woodchucks (Fuchs et al. 1989; Korba et al. 1989; Mitamura et al. 1982; Ogston et al. 1982; Rogler and Summers 1984; Rogler et al. 1987). These reports, combined with the unique integration patterns of individual hepatic tumors, suggest that integration occurs early in hepatocarcinogenesis and before clonal expansion. Evidence of integrations in early neoplastic nodules has been described before development of frank HCC (Rogler 1991; Yang et al. 1993, 1996).

Mammalian hepadnaviruses do not contain oncogenes similar to those found in transforming retroviruses (Ganem and Varmus 1987; Tiollais et al. 1985). Up-regulation of most of the well-characterized protooncogenes has not been demonstrated (Moroy et al. 1986). Increased expression (5- to 50-fold) of c-myc was observed, however, in three of a series of nine woodchuck HCCs (Hsu et al. 1988; Moroy et al. 1986, 1989), and truncation and rearrangement of the gene were demonstrated. In one tumor, no direct linkage between WHV DNA integration and c-myc activation was demonstrated. In that case, rearrangement and activation of c-myc appeared to be similar to that observed in Burkitt’s lymphoma and in acute B- and T-cell leukemias in which chromosomal translocations are present (Moroy et al. 1986).

In the other two integrations, c-myc activation was the result of insertional mutagenesis (Hsu et al. 1988), and WHV DNA insertions interrupted different regions of the c-myc locus. The position and orientation of the WHV and c-myc sequences and the use of the c-myc promoter excluded involvement of the hepadnaviral promoter in c-myc activation (i.e., promoter insertion). In both cases, WHV sequences analogous to one of the HBV enhancers were present, suggesting an enhancer insertion mechanism (Hsu et al. 1988). In a large series of woodchuck HCCs, activation of c-myc was observed in 10% of the tumors (Dejean and deThe 1990).

Buendia and her colleagues now have demonstrated that N-myc messenger RNA was overexpressed in 60% of woodchuck HCCs examined, and this transcript was not detected in normal woodchuck liver. Insertions of WHV nucleic acid sequences were located adjacent to cellular N-myc sequences in 20% of the tumors studied. Woodchucks have two N-myc loci. One N-myc locus was homologous to the N-myc gene of other mammalian species; the other was an intronless gene with the characteristic structure of a retrotransposon and was identified as N-myc2 (Fourel et al. 1990). N-myc2 was mapped to the X chromosome. Expression of N-myc2 is highly restricted, and the brain is the only normal tissue of the woodchuck in which N-myc2 RNA was detected (Fourel et al. 1992).

The physiological function, if any, of N-myc2 remains unknown (Fourel et al. 1992). A distinctive feature of hepatocarcinogenesis in woodchucks with chronic WHV infection appears to be the coupling of viral integration into the myc family of protooncogenes. Viral integrations appear to be preferentially associated with the N-myc2 locus (Flajolet et al. 1997; Wei et al. 1998). Insertion of WHV enhancer sequences either upstream or downstream of the N-myc2 coding domain result in increased transcription either of normal N-myc2 RNA or of a hybrid N-myc2/WHV transcript that is initiated at the normal N-myc2 start site. Transcriptional activation by enhancer insertion appears to be a common mechanism (Wei et al. 1992). A liver-specific regulatory element in the WHV genome has been identified that appears to control cis activation of N-myc2 (Fourel et al. 1996). Downstream integration of WHV DNA also has been associated with activation of N-myc2 (Fourel et al. 1994).

Recently, Buendia and her colleagues have reported that transgenic mice carrying the N-myc2 gene under the control of WHV regulatory sequences are highly predisposed to cancer of the liver (Renard et al. 2000). Seventy percent developed either HCC or hepatocellular adenomas. A transgenic founder that carried the unmethylated WHV/N-myc2 transgene sequence died at the age of 2 mo with a large liver tumor, demonstrating the high oncogenic capacity of the woodchuck N-myc retroposon. Mutations or deletions of the β-catenin gene similar to those of HCCs from humans and mice (De La Coste et al. 1998) were present in 25% of the hepatic tumors of the N-myc2 transgenic animals, and tumor latency (time to tumor) was significantly reduced (Renard et al. 2000). When N-myc2 transgenic mice were crossed with p53 null mice, the absence of one p53 allele markedly accelerated the onset of liver cancer, providing direct experimental evidence for synergy in multistage hepatocarcinogenesis between activation of N-myc2 and diminished expression of p53 (Renard et al. 2000).

Like the woodchuck, the California ground squirrel possesses a functional, transcriptionally active N-myc2 locus in the brain (Quignon et al. 1996). However, increased N-myc2 expression is unusual in HCCs from California ground squirrels infected with GSHV. Amplification of C-myc expression, however, is more frequent in ground squirrel hepatocellular neoplasms than in those of woodchucks (Transy et al. 1992). HCCs from woodchucks experimentally infected with WHV or with GSHV have been analyzed (Hansen et al. 1993). The propensity for WHV genomic DNA to integrate in or near the N-myc2 locus in HCCs from chronic WHV carriers was confirmed, whereas in woodchuck HCCs associated with GSHV infection, such integrations were exceptional. Seven of 17 (41%) WHV-induced tumors had rearrangements of the N-myc2 allele. Only one of 16 GSHV-associated tumors (6%), however, had such N-myc2 rearrangements. Based on the observations in GSHV-induced HCCs from ground squirrels and from woodchucks, it was concluded that the differences in hepadnavirus insertion and in N-myc activation between woodchucks and ground squirrels are due primarily to viral genetic differences and not to differences...
Transfection of a nonmalignant, SV-40 T antigen-transformed mouse hepatic cell line with HBV DNA produced cells with a malignant phenotype that grew in soft agar and were tumorigenic in nude mice. The hepatitis B virus X gene (HBX) transcript was expressed at a much higher level than that observed in vivo (Hohne et al. 1990). In subsequent studies, it was concluded that overexpression of the HBX was required for malignant transformation of the cell line (Seifer et al. 1991). HBX is known to promote hepatocarcinogenesis in c-myc transgenic mice (Terradillos et al. 1997) and may promote hepadnavirus-induced hepatocarcinogenesis (Seeger and Mason 2000).

The X gene appears to be essential for normal replication of hepadnaviruses in vivo (Chen et al. 1993). The woodchuck hepatitis X (WHX) protein is coexpressed with WHCag in the liver of chronic WHV carrier woodchucks (Feitelson et al. 1993; Jacob et al. 1997). Feitelson and colleagues demonstrated that when WHX was cotranslated in vitro with p53, WHX/p53 complexes developed, and similar WHX/p53 complexes were demonstrated in the livers of chronic WHV carriers (Feitelson et al. 1997). Combined with observations in HBV transgenic mice (Ghebranious and Sell 1998; Ueda et al. 1995), the observations of Feitelson et al. suggest that binding of WHX to p53 may prevent entry of p53 into the nucleus, diminish tumor suppressor activity, and represent an important mechanism by which the hepadnavirus X-gene product could promote hepatocarcinogenesis (Feitelson et al. 1997). Mutations of p53 also may alter its tumor suppressor activity, but such mutations were found primarily in the less differentiated hepatic tumors, suggesting the mutations altered tumor progression at a later stage of development (Hsia et al. 2000; Ueda et al. 1995).

The second hypothesis regarding the role of hepadnavirus in hepatocarcinogenesis is that hepatic injury caused by viral infection and related hepatocellular regeneration provide an environment that enhances fixation of spontaneous mutations, rearrangements, or chromosomal translocations that are responsible for malignant transformation of hepatocytes. Such a role for hepadnaviruses in hepatocarcinogenesis would be comparable to the processes of promotion and/or progression recognized in multistage chemical hepatocarcinogenesis (Farber and Sarma 1987; Hanahan and Weinberg 2000; Pitot and Dragan 1991; Tennant 1992).

Support for this model comes from the observation that chronic liver injury associated with cirrhosis frequently precedes the development of HCC in HBV-infected people (Kalayci et al. 1991; Zhou et al. 1999). Chronic hepatitis C virus infection results in progressively severe chronic hepatitis and cirrhosis and is frequently associated with development of HCC. A significant increase in the rate of HCC has been observed in inherited forms of chronic liver disease in humans including hemochromatosis and alpha-1-antitrypsin deficiency (Schafer and Sorrell 1999). These inherited diseases also are associated with development of progressive hepatocellular injury, regeneration, and cirrhosis that precede development of HCC (Johnson and Williams 1987).

Experimental evidence supporting the role of hepatic inflammation and hepatocellular injury as a key event in hepadnavirus-associated HCC comes from the work of Chisari and his colleagues with transgenic mice described above (Chisari 2000; Chisari et al. 1989; Dunsford et al. 1990; Moriyama et al. 1990). In the lines of transgenic mice they have studied, a direct relation was found between the expression and retention of HBsAg in hepatocytes and the severity of hepatitis. A close correlation also was observed between the severity of hepatitis in transgenic mice and the rate of development of HCC (Chisari et al. 1989; Dunsford et al. 1990; Sell et al. 1991). Increased free radical production within the liver was associated with oxidative DNA damage in this model, similar to that suggested in woodchucks (Bannasch et al. 1995) and that observed in other mice (Faux et al. 1992).

**Interaction of Hepadnaviruses and Chemical Carcinogens in Hepatocarcinogenesis**

Aflatoxin is among the most potent hepatocarcinogens known and is a frequent contaminant of the diets of populations with high incidence rates of HBV infection and HCC. The possible interaction between hepadnavirus infection and aflatoxin has been investigated using the woodchuck model (Bannasch et al. 1995; Rivkina et al. 1996; Tennant et al. 1991a). When administered early in life to woodchucks experimentally infected at birth with WHV, aflatoxin B1 did not appear to increase the rate of chronic WHV infection or the rate of development of HCC (Tennant et al. 1991a). When administered beginning at 1 yr of age to established chronic WHV carriers, the time to tumor was moderately but significantly reduced by aflatoxin B1 treatment (Bannasch et al. 1995). A similar synergistic interaction between HBV and aflatoxin has been reported in the tree shrew (Yan et al. 1996).

It has been shown that HBV transgenic mice are more susceptible to chemical hepatocarcinogenesis than controls (Dragani et al. 1990). When transgenic mice that expressed HBsAg were exposed to nitrosodiethylamine (NDMA) or aflatoxin, they developed hepatic adenomas and HCC more rapidly and more extensively than unexposed transgenic controls or normal mice receiving either of these hepatocarcinogens. These results demonstrated a significant synergistic interaction between chemical hepatocarcinogens and the chronic hepatocellular injury induced by overexpression of HBsAg in transgenic mice (Sell et al. 1991). A similar synergistic effect of HBX expression in transgenic mice and NDMA in hepatocarcinogenesis has also been reported (Slagle et al. 1996).

Another possible hepatocarcinogenic mechanism related to hepatic inflammation has been suggested by studies of nitric oxide (NO) production in chronic viral hepatitis. In the
liver, NO biosynthesis from arginine is catalyzed by inducible NO synthetase. Endotoxin, γ-interferon, and other cytokines (Billiar et al. 1992a,b; Cullen et al. 1989; Curran et al. 1990, 1991; Geller et al. 1993; Hortellano et al. 1992; Knowles et al. 1990; Wood et al. 1993) can increase the activity of this enzyme significantly. Under certain circumstances, NO may have a protective effect against experimental hepatic injury (Curran et al. 1991). Under other conditions, hepatic production of NO may contribute to development of hypotension in septic shock (Kilbourn et al. 1990).

Woodchucks chronically infected with WHV excrete more nitrate in the urine and more NDMA than uninfected control woodchucks (Liu et al. 1991). Similar increased NO production has been observed in HBV transgenic mice (Chisari 2000). Nitrate and NDMA are derived from NO produced from L-arginine. Urinary nitrate excretion also is increased in a human patient with chronic HBV infection (Nguyen et al. 1992).

In primary hepatocyte cultures from normal woodchucks, NO synthesis can be induced by endotoxin, and hepatocytes cultured from chronic WHV carriers produce significantly more NO and nitrosamine than hepatocytes from uninfected controls (Liu et al. 1992). SV-40 T antigen-transformed woodchuck hepatocyte cell lines have the capacity to produce NO in response to endotoxin (Liu et al. 1993) utilizing the L-arginine-NO pathway and to produce NDMA. WHsAg purified from woodchuck serum can induce NO production in woodchuck hepatocyte cultures (Liu et al. 1994). NO reacts with O₂ to produce NO₂, which exists in equilibrium with N₂O₃ and N₂O₄. N₂O₃ and N₂O₄ are potent nitrosating agents capable of reacting with water to form nitrite and nitrate (Marletta et al. 1988) or, in neutral solution, of nitrosating dialkylamines to form nitrosamines. NO could act as a carcinogen indirectly by causing formation of hepatocarcinogenic nitrosamines such as NDMA, which is a strong alkylating agent. Point mutations can be induced by NDMA by methylation of guanine to 7-methyl and to O⁶-methylguanine, or by methylation of adenine to 3-methyladename (Archer 1989; Magee 1989).

NO also has the capacity to act as a direct mutagen (Wink et al. 1991). In vitro, NO can deaminate deoxynucleosides, deoxynucleotides, or intact DNA and contribute to depurination-related mutations. It has been predicted based on studies of the rate of deamination of guanine to xanthine in vitro that guanine/cytosine → adenine/thymine transitions could be a frequent form of mutation induced by NO. Adenosine deamination to hypoxanthine would be expected to result in adenine/thymine → guanine/cytosine transitions, and removal of the xanthine formed by guanine deamination would result in depurination and transversions (Nguyen et al. 1992). The demonstration of increased NO formation in the hepadnavirus-infected liver suggests that NO could have an important role in hepatocarcinogenesis.

**Use of the Woodchuck Model in Development of Antiviral Therapy**

Woodchucks with experimentally induced chronic WHV infection are now frequently used in the preclinical assessment of antiviral drugs being developed for treatment of chronic HBV infection. Nucleoside analogs (Chu et al. 1998; Hostetler et al. 2000; Korba et al. 2000a,b, c) and immune response modifiers have been tested (Gangemi et al. 1996; Tennant et al. 1996b). Before testing in woodchucks, the drugs are screened for antiviral activity against HBV in the 2.2.15 cell system, a HepG2 cell line engineered to produce HBV constitutively (Korba and Gerin 1992). Acyclovir and azidothymidine, which had no selectivity in 2.2.15 cells, had no antiviral effect in the woodchuck model of HBV infection. Adenine-5′-arabinoside monophosphate, which had moderate antiviral activity in vitro, had significant antiviral activity in woodchucks in vivo, and activity was increased by specific liver targeting (Enriquez et al. 1995). Most nucleoside analogs with intermediate antiviral activity in vitro against HBV had intermediate antiviral activity in vivo. One exception was 1′,2′-deoxy-2′-fluoro-1-β-D arabinofuranosyl-5-ido-uracil, which had modest in vitro activity. In woodchucks, however, 1′,2′-deoxy-2′-fluoro-1-β-D arabinofuranosyl-5-ido-uracil had potent antiviral activity but was highly toxic, as had been observed in humans (Tennant et al. 1998). The in vitro activity of 1′-(2-fluoro-5-methyl-β-L-arabinofuranosyl) uracil (L-FMAU, clevudine) was only moderate but was highly potent in vivo in WHV carrier woodchucks (Chu et al. 1998; Peak et al. 2000). Lobucavir also had intermediate in vitro activity in the 2.2.15 cell system but, at the dosage studied, had potent antiviral activity against WHV (Tennant et al. 1996a).

Lamivudine, which had a very high selective index in vitro, was a potent antiviral drug in woodchucks with favorable pharmacokinetics (Rajagopalan et al. 1996) and was without toxicity at doses of 5 or 15 mg/kg/day for 28 days (Korba et al. 2000b; Peak et al. 1997). The absence of effect of lamivudine on hepatic cccDNA was postulated to be due to the absence of cell division (Moraleda et al. 1997).

Lamivudine has been shown in the woodchuck model to act synergistically both with alpha-interferon (Korba et al. 2000a) and with famciclovir (Korba et al. 2000c). Similarly, high antiviral potency of the closely related emtricitabine has been shown in WHV carrier woodchucks (Korba et al., 2000d). Extended lamivudine treatment of woodchucks with chronic WHV infections delayed the development of HCC and significantly extended survival in one study (Peak et al. 1997). In another study, no effect of lamivudine was observed on hepatocarcinogenesis. Although treatment was begun at an older age, the duration of treatment was not as long and the apparent effect of treatment on viral load was not as great (Mason et al. 1998). A very high rate of polymerase gene mutations was detected in both humans and woodchucks.
after long-term lamivudine therapy (Baumert et al. 1998; Brechot et al. 1981; Peek et al. 1997; Zhou et al. 1999).

Summary

Animal models of hepatitis B virus infection have been valuable in determining the mechanisms of hepadnavirus replication, for studies of pathogenesis, and for investigation of viral hepatocarcinogenesis. The woodchuck also appears to be useful in the discovery and development of antiviral drugs to treat HBV infection and for testing new forms of immunotherapy. In particular, the woodchuck is ideal for studying the impact of antiviral treatment and immunotherapy on the outcome of hepadnavirus infection and on survival. The median life expectancy of experimentally infected chronic WHV carriers is approximately 29 mo, and almost all develop HCC. New types of prophylaxis or therapy, therefore, can be evaluated under controlled experimental conditions in a relevant animal model within a reasonable time frame.

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