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Chronic Hepatitis and Primary Liver Cancer in Woodchucks (*Marmota Monax*) Associated with Chronic Woodchuck Hepatitis Virus (WHV) Infection (13-Nov-2004)

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Naturally Occurring WHV Infection and Other Mammalian and Avian Hepadnaviruses

The hepatitis B virus (HBV) of humans and great apes is the prototype of the family Hepadnaviridae, a group hepatotropic DNA viruses that infect several mammalian (Genus: Orthohepadnavirus) and avian (Genus: Avihepadnavirus) species. The second member of the hepadnavirus family, WHV, was identified in the Eastern woodchuck. The virus was described by Summers and his colleagues in a colony of woodchucks that originated in the native habitat but were maintained by Snyder in a conventional laboratory animal environment. For some years, the colony experienced high rates of chronic hepatitis and hepatocellular carcinoma (HCC), hepatic diseases known to be associated in humans with chronic HBV infection. WHV in the serum of many of the woodchucks was, on the basis of morphological and molecular analyses, classified as a member of the HBV virus family.

The natural habitat of the woodchuck 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, the Yukon Territory, and a region in southeastern Alaska. A comprehensive, seroepidemiologic study of WHV infection has not yet been performed and the prevalence of WHV infection throughout most of this range remains unknown. WHV infection, however, is hyperendemic in the Mid-Atlantic States, and the woodchucks originally studied by Summers et al. were from Pennsylvania. In one study, 23 percent of woodchucks from Pennsylvania, New Jersey and Maryland were shown to be test positive for the woodchuck hepatitis surface antigen (WHsAg) and an additional 36 percent were positive for anti-WHs antibody for an overall infection rate of 59 percent. In some parts of Maryland, the rate of chronic WHs antigenemia was 33 percent and similar high rates of infection in the Mid-Atlantic States have been confirmed by others. In contrast, the rate of WHV infection in central New York State has been estimated to be approximately 1 - 2 percent based on the presence of anti-WHs and anti-WHc antibodies, and the rate of persistent WHs antigenemia is approximately 0.2 percent. A single woodchuck from Quebec with HCC has been shown to be infected with WHV. Although the number of woodchucks tested is small, we have found no serologic evidence of WHV infection in Vermont, Massachusetts, or in Iowa.

Since description of WHV, several closely-related hepadnaviruses have been described in other mammalian and avian species. The genetic organization, morphology, and replication strategies are similar. The ground squirrel hepatitis virus (GSHV) has been described in California ground squirrels (*Spermophilus beecheyi*). Persistent GSHV infection is associated with chronic hepatitis and HCC, although the incidence of HCC is lower than that associated with chronic WHV infection and hepatic tumor development occurs at an older age in California ground squirrels. A virus closely related to WHV and GSHV has been described in the arctic ground squirrel (*Spermophilus parryi*) and named the arctic ground squirrel hepatitis virus (AGSHV). The prevalence of AGSHV infection appears to be high in certain Alaskan populations and persistent infection is associated with a high rate of HCC. Evidence for a hepadnavirus infecting Eastern gray squirrels (*Spermophilus carolinensis*) has been reported from Pennsylvania. Although lesions of hepatitis were described, there was no evidence of HCC. There is evidence for a hepadnavirus infecting Richardson's ground squirrels (*Spermophilus richardsonii*) originating in Alberta, Canada. The hepatic lesions of Richardson's ground squirrels including HCC were remarkably similar to those of WHV infected woodchucks. The duck hepatitis B virus (DHBV) was first described in domestic Pekin ducks (*Anas*

domesticus) and apparently has a worldwide distribution. Closely related avian hepadnaviruses have been reported in grey herons (*Ardea cinerea*, the heron hepatitis B virus, HHBV) and in snow geese (*Anser caemlescens*, snow goose hepatitis virus, SGHVB) from Germany. DHBV infection has not been shown to cause HCC and hepatic neoplasms have not been associated with HHBV or SGHBV infection.

Experimental WHV Infection

HBV is among the most important human pathogens world-wide and the etiologic role of HBV in chronic hepatitis and in hepatocarcinogenesis is well recognized. Hepatocellular neoplasms were described early in the twentieth century in woodchucks from the Philadelphia Zoological Garden. Hepatic tumors later were described in woodchucks from the Washington Zoological Park and from Bethesda, Maryland. A high rate of HCC was reported in laboratory-maintained woodchucks trapped in Pennsylvania, New York, Maryland, and Delaware.

From the beginning, the limitations of the use of wild woodchucks for experimental studies of WHV pathogenesis and hepatocarcinogenesis were recognized. It was impossible, for example, to know at what age and for how long trapped woodchucks had been infected with WHV or to be certain about nutritional or environmental factors that could influence the outcome of WHV infection. Importantly, hepatic lesions caused by *Ackertia marmotae* and *Capillaria* sp. are common in wild woodchucks and complicated the interpretation of experimental investigations. To utilize the woodchuck as an experimental animal model, the National Institute of Allergy and Infectious Diseases and the National Cancer Institute jointly supported the establishment of a breeding colony of woodchucks at Cornell University in 1980. This allows the genetic background of experimental woodchucks to be known, their diet to be defined, and it is possible to eliminate natural exposure to WHV infection and to other diseases that are endemic in wild woodchuck populations. The breeding colony now serves as a source of specific pathogen free woodchucks for studies of the pathogenesis of experimental WHV infection, for studies of viral hepatocarcinogenesis, and for antiviral drug development.

Woodchucks born in the laboratory are inoculated at birth with serum from standardized infectious pools derived from chronic, WHV carrier woodchucks. Following inoculation, woodchucks are monitored using specific serologic markers of WHV infection (WHV DNA, WHsAg, anti-WH core and anti-WH surface antibodies). Chronic WHV infection develops in 60 to 65 of woodchucks experimentally infected at birth. Kaplan-Meier survival analysis has been used to compare the survival of chronic WHV carriers and woodchucks in which WHV infection was resolved with control woodchucks not infected with WHV but born and raised under similar laboratory conditions. All WHV carriers were dead by 56 months of age, and the lifetime risk of HCC was 100 percent. In contrast, 42 percent of the woodchucks with resolved WHV infection and 62 percent of uninfected controls were alive at 56 months of age. Although the rate of HCC in WHV carriers was significantly higher, 17 percent of the woodchucks in which neonatal WHV infection was resolved developed HCC. HCC was not observed in uninfected, laboratory-reared, control woodchucks. 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 providing direct experimental evidence for the carcinogenicity of WHV and, by analogy, for other hepadnaviruses including HBV.

Histogenesis of Viral HCC

Hepatocarcinogenesis is a multistage process. In chemically induced rodent models, development of hepatic neoplasms is preceded by the appearance of microscopic foci of altered hepatocytes (FAH) that are similar to those observed in chronic WHV infection. Dysplastic hepatocytic changes occur in HBV infection and in some cases have been considered precancerous lesions. Recently, FAH have been described in the livers of patients undergoing liver transplantation for chronic, end stage viral hepatitis with or without HCC and are considered to be identical to those caused by chemical hepatocarcinogens in rats and mice and that are observed in chronic WHV infection.

We have investigated the temporal development of FAH in woodchucks with experimentally induced chronic WHV infection. The earliest detection of FAH in chronic WHV carriers was at six months of age. By 9 - 10 months, more than 30% of WHV carriers had such lesions and thereafter, almost all the livers of chronic WHV carriers examined contained FAH. In experimentally induced, chronic WHV infection, small HCCs have been observed as early as nine months of age. The median time to tumor detection by ultrasonography in the model was 24 months and the median time to tumor death 30 months of age.

Molecular Genetic Alterations in HCC Associated with WHV Infection - Integrated hepadnaviral nucleic acid sequences have been demonstrated in the cellular DNA of most hepatic tumors of woodchucks chronically infected with WHV and, as

in HBV infection, a direct molecular role of hepadnaviruses in hepatocarcinogenesis has been hypothesized. Integration of hepadnaviral nucleic acid sequences is believed to be a critical mutagenic event that alters the expression of cellular regulatory genes (protooncogenes, tumor suppressor genes) that ultimately, results in the neoplastic transformation of hepatocytes.

Buendia and her colleagues have shown that *N-myc* mRNA was over expressed in 60% of woodchuck HCCs examined, and this transcript was not detectable in normal woodchuck liver. Woodchucks were found to have two *N-myc* loci. One *N-myc* locus was homologous to other mammalian *N-myc* genes. The other was an intronless gene with the characteristic structure of a retrotransposon and was called *N-myc2*. The expression of *N-myc2* which has been mapped to the X chromosome, is highly restricted, and the brain is the only normal woodchuck tissue in which *N-myc2* RNA has been detected. The functional significance of *N-myc2* remains unknown, but current evidence indicates that a distinctive feature of hepatocarcinogenesis in woodchucks with chronic WHV infection is viral integration into or near the *myc* family of proto-oncogenes.

In both naturally acquired and experimental WHV infection, viral integrations seem to be preferentially associated with the *N-myc2* locus and are clustered either within a 3-kb region upstream of *N-myc2* or in the 3' non-coding region of the gene. Insertion of WHV enhancer sequences either upstream or downstream of the *N-myc2* coding domain results in increased production of either normal *N-myc2* RNA or of a hybrid *N-myc2*/WHV transcript that is initiated at the normal *N-myc2* start site. Transcriptional activation of *myc* family proto-oncogenes by enhancer insertion seems to be a common mechanism and a recently identified liver-specific regulatory element in the WHV genome appears to control cis activation *N-myc2*. Distant, downstream integration of WHV DNA on the X chromosome at two separate sites also has been associated with *N-myc2* activation.

Buendia and her colleagues have reported transgenic mice carrying the *N-myc2* gene under the control of WHV regulatory sequences. These mice are highly predisposed to liver cancer and 70 % develop hepatocellular adenomas or HCC. A transgenic founder carrying the unmethylated WHV/*N-myc2* transgene sequence died at 2 months of age with a large hepatic tumor, demonstrating the high oncogenic capacity of the woodchuck *N-myc* retroposon. Mutations or deletions of the beta-catenin gene were present in 25% of the hepatic tumors of the *N-myc2* transgenic mice and were similar to those reported in HCCs of humans. 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 experimental evidence for synergy between activation of the *N-myc2* gene and decreased expression of p53 in hepatocarcinogenesis.

Like the woodchuck, the California ground squirrel possesses an *N-myc2* locus that is transcriptionally active in the brain. Increased *N-myc2* expression is unusual in the HCCs of California ground squirrels infected with GSHV. Amplification of *c-myc* expression, however, is more frequent in ground squirrels than in woodchucks. Marion et al. have shown that HCC characteristically develops less frequently in GSHV infected ground squirrels than in WHV carrier woodchucks and in ground squirrels, HCC characteristically develops at an older age.

Seeger et al. demonstrated that woodchucks were susceptible to GSHV infection. He successfully infected neonatal woodchucks with both WHV and GSHV and compared the oncogenic potential of the two viruses in the same host species. HCC developed at a significantly earlier age in WHV carrier woodchucks than in woodchucks chronically infected with GSHV. Hepatocellular carcinomas from these woodchucks have been analyzed by Hansen et al. They confirmed the propensity for WHV genomic DNA to integrate in or near the *N-myc2* locus in HCCs from WHV carriers. Seven of 17 WHV-induced tumors (41%) had demonstrable rearrangements of the *N-myc2* allele while only 1 of 16 woodchuck tumors induced by GSHV tumors (6%) had such an *N-myc2* rearrangement. Comparable results were obtained when *N-myc2* gene expression was determined. Based on these observations, it was concluded that the differences in hepadnavirus insertion and *N-myc2* activation between woodchucks and California ground squirrels were related primarily to genetic differences in the respective viruses rather than to differences in the animal host.

Relationship of Size and Histologic Grade of Hepatic Neoplasms to WHV Integration and *N-myc* Rearrangement

Jacob, et al., have investigated the relationship between the size and histologic grade of hepatic neoplasms of woodchucks and the presence of WHV DNA integrations and *N-myc* 2 rearrangements. The 13 chronic WHV-carrier woodchucks of their study ranged in age from 24 to 39 months (median age, 30 months) and had been born, reared, and maintained in a laboratory environment. The clinical diagnosis of HCC was based initially on increasing serum activity of gammaglutamyltransferase (GGT) and on hepatic ultrasound examinations. Fifty-five hepatocellular neoplasms and matched non-tumorous hepatic tissue were obtained postmortem and the frequency of WHV DNA integrations and of *N-myc* rearrangements compared in

tumors of different size and histologic grade.

Four small tumor nodules were classified histologically as adenomas. Integrated sequences of WHV DNA were detected in two of the four and in one of the two there was evidence of *N-myc* rearrangement. Fifty-one of the neoplasms were classified as HCC. Seven were grade 1 HCCs and WHV DNA integrations were demonstrated in 43% but none had *N-myc* rearrangements. In 20 grade 2 HCCs, 80% had WHV DNA integrations and in 38%, *N-myc* rearrangements were detected. Twenty-four, grade 3 HCCs had integrations of WHV DNA in 79% and *N-myc* rearrangements were present in 74%. In two other grade 3 HCCs rearrangements of *N-myc* were detected in the absence of WHV DNA integrations. The 12 largest tumors in the series all were grade 2 or 3 HCCs, and in 83%, both WHV DNA integrations and *N-myc* rearrangements were demonstrated. The molecular changes observed in this study suggest a progression in genetic alterations that provide proliferative stimulus and/or growth advantage and that have an etiologic role in hepatocarcinogenesis of woodchucks with chronic WHV infection.

Chemoprevention of Hepatocellular Carcinoma in Woodchucks by Long-Term Nucleoside Analog Treatment

In a study reported by Colonna, et al., the guanosine nucleoside, entecavir, which has potent antiviral activity against WHV and HBV, was used to determine the influence of long term suppression of viral replication on hepatocarcinogenesis in woodchucks. Beginning at 8 months of age, WHV carriers were given entecavir orally at a dose of 0.5 mg/kg/ day for 8 weeks then weekly at a dose of 0.5 mg/kg/week. In 6 woodchucks, treatment was stopped after a total of 14 months and in 5, treatment was continued for a total of 36 months. Hepatic expression of viral antigens and covalently closed circular WHV DNA were significantly reduced by long term entecavir treatment of woodchucks. Three of the 6 woodchucks treated for 14 months had sustained antiviral responses and developed no evidence of HCC during the next 2 years. In woodchucks treated for 36 months, there was no evidence of HCC in 4 of the 5 (80% HCC free survival). Compared to historical controls in which 4 year HCC survival was 4%, entecavir treatment significantly delayed development of HCC and prolonged survival.

In a second study of long term antiviral therapy reported by Menne, et al., one and two year-old woodchucks were treated for 32 weeks with the highly potent nucleoside, clevudine (10 mg/kg day) and controls received placebo. Half of clevudine treated woodchucks and half of the placebo recipients then received 4 doses of alum adsorbed, WHsAg vaccine during the next 16 weeks. Vaccination alone elicited low-level antibody responses to WHsAg in most carriers but did not affect serum WHV DNA, serum WHsAg or liver enzyme responses. Carriers treated first with clevudine to reduce serum WHV DNA and WHsAg and then vaccinated developed more robust anti-WHs response and normalized liver enzymes. Following vaccination, WHsAg-specific cell-mediated immunity (CMI) was demonstrated in both vaccinated groups, but was significantly enhanced in carriers treated initially with clevudine, and was broadened to include responses to WHV core antigen (WHcAg) and to selected peptide epitopes of WHcAg and WHsAg. It was concluded that vaccination with WHsAg following treatment with L-FMAU disrupted virus-specific humoral and cell-mediated immune tolerance in chronic WHV infection and enhanced the immune response profiles beyond those seen with either clevudine or with vaccine monotherapy. Therapy with the clevudine-vaccine combination resulted in immune response profiles that resembled those observed during resolution of experimental WHV infection.

In 10 of the woodchucks reported by Menne, et al., clevudine treatment (with or without vaccine) was initiated at one year of age. Marked and sustained reductions in serum WHV DNA and WHs antigenemia were observed during treatment and for a period of 18 months following drug withdrawal. Liver biopsies were obtained at the time treatment was initiated, at the end of treatment (20 months of age) and six and 12 months following drug withdrawal (24 and 32 months of age). At every time point after clevudine treatment was begun, hepatic WHV nucleic acids and WHcAg expression were reduced compared to placebo treated controls. The percentage of biopsies with FAH was significantly lower in the clevudine treated group than in controls. The development of HCC also was delayed and the survival at three years of age in the 10 clevudine treated woodchucks was 50% compared to 25% in controls. After four years, the survival in the clevudine group was 25% compared to 6% in controls.

In a third, long-term study of chemoprevention, 20 eight month old, WHV carriers were treated for life with lamivudine (5 mg then 15 mg/kg/day) and 20 WHV carrier controls received placebo. Serum WHV DNA decreased by 4 to 5 logs in lamivudine treated woodchucks and the antiviral effect was sustained for more than one year. Thereafter, recrudescence of viral replication was detected that was associated with mutations of the WHV polymerase B domain gene. There was a significant delay in the development of HCC in lamivudine treated woodchucks and a corresponding increase in survival. The median time to death in placebo treated controls was 32 months and in lamivudine treated woodchucks was 44 months. A similar beneficial effect on HCC development was described recently in a controlled clinical trial in chronic HBV carriers treated for a median period of 32 months with lamivudine. During the study, HCC developed in 16 of 215 (7%) placebo

treated control patients and in 17 of 436 (4%) patients treated with lamivudine.

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