Hepatocellular carcinoma in ground squirrels persistently infected with ground squirrel hepatitis virus (hepatitis B virus/animal model/liver neoplasms)

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ABSTRACT Although persistent infection with hepatitis B virus and woodchuck hepatitis virus has been associated with development of hepatocellular carcinoma in the host, little has been known of such an association with ground squirrel hepatitis virus (GSHV), which is closely related to the woodchuck virus. Colonies of GSHV-infected and -uninfected Beechey ground squirrels were observed for tumors for a period of 5 years. Tumors developed in seven squirrels after a minimum of 2.4 years of observation per animal; each of the seven animals was over 4 years old when the tumor was detected. The predominant type of tumor was hepatocellular carcinoma, which appeared in 1 of 28 GSHV-bearing animals studied and in 1 of 23 squirrels with antibody to the virus. No hepatocellular carcinoma appeared in 24 GSHV marker-free squirrels. Integrated GSHV DNA was found in the hepatocellular carcinoma tissue of the one carrier animal examined, paralleling the frequent findings of integrated hepatitis B and woodchuck hepatitis viral DNA in human and woodchuck hepatocellular carcinoma. Although the incidence of liver carcinoma reported here in carrier ground squirrels is neither as great as that in carrier woodchucks nor statistically different from the incidence in noncarrier squirrels, the data presented suggest that persistent infection with GSHV may also be associated with hepatocellular carcinoma.

In recent years an association between persistent hepatitis B virus (HBV) infection and hepatocellular carcinoma (HCC) has been strongly indicated (1, 2) and repeatedly reviewed (e.g., ref. 3). Viruses similar in characteristics to HBV (designated here as hepatitisnaviruses) and infecting lower animals have been discovered (4–6). As with HBV, infection with woodchuck hepatitis virus (WHV), ground squirrel hepatitis virus (GSHV), and duck hepatitis B virus (DHBV) can cause persistent infection. Persistent WHV infection is associated with a high incidence of HCC in captive woodchucks (4). It is not clear whether the association of HCC with persistent infection is a characteristic of just HBV and WHV or whether it is a feature of all hepatitisnaviruses.

HCC usually develops in human carriers after decades of persistent infection, with an incidence of less than 1% per year. Viral DNA is frequently but not always found to be integrated in the host DNA of HCC (reviewed in ref. 7). In woodchucks, HCC development occurs after a shorter period of infection, several months or years, and at a much higher rate, with approximately one-third of carrier animals developing tumors per year (8, 9). Most tumors contain integrated viral DNA (10). The association of DHBV with HCC is not yet clear. DHBV is found in some domestic ducks in China that have developed HCC (11, 12). However, HCC is also present in ducks without detectable virus or viral DNA. Although most HCC from ducks from China have not had demonstrable integrated viral DNA, evidence for integration has been found in one such tumor (13). HCC has not yet been reported in persistently infected domestic ducks in the United States.

The fourth hepatitisnavirus, GSHV, is very closely related to WHV. Both viruses infect members of the squirrel family. There is considerable antigenic crossreactivity between the two viruses (14), and the DNA homology has been calculated to be 82% (15). We have been observing colonies of virusinfected and uninfected Beechey ground squirrels for disease since 1980. In 1983 we had detected no liver tumors in infected or uninfected ground squirrels (16). Liver tumors were also not noted in another large colony of Beechey ground squirrels (17). The failure to observe HCC in GSHV-infected ground squirrels has contrasted sharply with the observations in WHV-infected woodchucks and has raised the question of whether such tumors ever arise in GSHVinfected animals. Here we report HCC in three ground squirrels over 4 years of age during the fifth year of observation of our colonies, two in GSHV-bearing animals and one in an animal with antibody to the virus.

MATERIALS AND METHODS

Ground Squirrel Colonies. Beechey ground squirrels were trapped live at various locations on the San Francisco Peninsula from 1980 through 1984. The ages were estimated as described (16). While the animals were individually held in quarantine for 1 month, serum from each animal was tested for ground squirrel hepatitis surface antigen (GSHsAg) by radioimmunoassay, for antibody to GSHsAg (anti-GSHs) by radioimmunoassay, and for virion-associated DNA polymerase activity (5). Animals with serum GSHsAg and serum DNA polymerase activity were housed in a separate room from GSHsAg-negative animals. Animals were fed an ad libitum diet of rat chow and water, supplemented once a week with sunflower seeds and with apples or carrots.

Histology. Tissue specimens taken at necropsy were fixed in 10% formalin and embedded in paraffin. Stained sections were histologically evaluated without knowledge of experimental data.

DNA Analysis. DNA from liver was extracted and purified, treated with restriction enzymes, and analyzed by agarose gel electrophoresis as described (18). As radioactive DNA probes for molecular hybridization, GSHV DNA, either with

Abbreviations: HCC, hepatocellular carcinoma(s); HBV, hepatitis B virus; DHBV, duck hepatitis B virus; WHV, woodchuck hepatitis virus; GSHV, ground squirrel hepatitis virus; GSHsAg, ground squirrel hepatitis surface antigen; anti-GSHs, antibody to GSHsAg.

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plasmid vector pBR322 or separated from the plasmid DNA by gel electrophoresis, was radiolabeled with $^{32}$P by nick-translation as described (18, 19). Southern blot and slot blot hybridization on nitrocellulose filters were also carried out as previously described (12, 18).

RESULTS

Occurrence and Histologic Characteristics of Tumors in Beechey Ground Squirrels. In necropsies followed by histologic study of 75 ground squirrels, 3 animals had HCC (Table 1). All were 4.5 years of age or older, with none of 58 younger animals developing any tumors. Of other old animals, one had a thymoma, one a renal endocrine carcinoma, and one each a pulmonary adenocarcinoma and adenoma. In 28 squirrels (2 with HCC and 1 with thymoma) the serum was GSHsAg- and viral DNA polymerase-positive (see ref. 5); 23 were GSHsAg-negative and anti-GSHs-positive, and 24 were GSHV marker-free. All tumors were observed in squirrels held at least 2.4 years in captivity, with totals of 10 GSHV-positive, 5 anti-GSHs-positive, and 6 GSHV marker-free animals observed for 2.4 years or longer. Tumors occurred in both males and females.

The HCC of GSHV carrier TrO8 was a spherical outgrowth 1.3 cm in diameter, located near the gallbladder. TrO8 had been treated with the antiviral compound 2-(1,3-dihydroxy-2-propoxy)methylguanine (20) 22 months before detection of the newly well-differentiated HCC. The small cells were arranged in cords, or trabeculae (Fig. 1). Their nuclei varied significantly in size but were barely enlarged, although because of relatively little cytoplasm the nuclear/cytoplasmic ratio was high. In places the cytoplasm was eosinophilic and had small fat droplets. The cells occasionally surrounded small cavities containing protein but no bile. Littoral cells were sometimes increased. Focal and larger necroses were noted, seemingly ischemic because segmented leukocytes surrounded cell fragments.

The HCC of GSHV carrier Tr02 was in the left lobe of the liver, 1.4 cm in diameter, and softer and darker in color than the uninvolved liver. The tumor cells were similar in appearance and trabecular arrangement to TrO8 except that autolysis was present.

The HCC of the GSHV-negative, anti-GSHs-positive animal GL21 was in the right lobe and 2.5 cm in diameter. The large nodule was a moderately anaplastic medullary carcinoma; large, rather anaplastic, eosinophilic tumor cells were arranged in sheets (Fig. 2). There were great nuclear variations and few mitoses. The acidophilic alteration of the cytoplasm progressed to acidophilic bodies. Some tumor cells had small fat droplets. Bile canaliculi were not observed. Transition of normal liver—without significant change—to cancer was seen in the same microscopic field. The littoral cells were slightly increased in number.

Detection of High Molecular Weight Viral DNA in Tumor DNA. DNA was purified from both the HCC and uninvolved liver of animal TrO8. DNA of both tissues was digested with various restriction enzymes and compared to undigested DNA by Southern blot analysis with a GSHV DNA probe.

![Fig. 1. Trabecular type of HCC in squirrel Tr08. (Hematoxylin/eosin stain.) (Upper) The tumor cells form irregularly arranged trabeculae that differ from the hepatic parenchyma in left lower corner. (×100.) (Lower) In places, narrow lumina filled with proteinaceous material are in the center of the cords (straight arrows). Littoral cells are increased in number. Segmented leukocytes (curved arrows) replace necrotic cells. (×240.)](image-url)

Tumor DNA untreated with any restriction enzyme contained viral DNA predominantly of the sizes expected for virion DNA or viral replicating forms [migrating in positions corresponding to linear double-stranded DNAs of length 4.4 kilobases (kb) or less]; higher molecular weight viral DNA was not detected (Fig. 3A, lane 1). Tumor DNA digested with HindIII produced a strong band migrating at 14.5 kb which was not observed in undigested tumor DNA (Fig. 3A, lane 2 and Fig. 3B, lane 1). The failure to detect the 14.5-kb band in HindIII-digested DNA from nontumorous liver (lanes 5 and 5') suggests that this viral DNA-containing HindIII fragment was from HCC cells and not from nontumorous liver tissue contaminating the HCC tissue. The amount of viral DNA in the 14.5-kb band present in HindIII-cut tumor DNA was estimated to be equivalent to 3.4 viral genomes per haploid cell genome. Two enzymes, Kpn I and Xba I, each cleaved

Table 1. GSHV markers associated with tumors in Beechey ground squirrels

<table>
<thead>
<tr>
<th>Animal</th>
<th>Sex</th>
<th>Age, years</th>
<th>Years in captivity</th>
<th>Tumor site</th>
<th>Histological classification</th>
<th>GSHV</th>
<th>Anti-GSHs</th>
<th>High Mf, GSHV DNA in tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tr02</td>
<td>F</td>
<td>≥6.0</td>
<td>4.5</td>
<td>Liver</td>
<td>Trabecular HCC</td>
<td>+</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Tr08</td>
<td>M</td>
<td>≥4.5</td>
<td>2.4</td>
<td>Liver</td>
<td>Trabecular HCC</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>GL21</td>
<td>M</td>
<td>=5.0</td>
<td>4.0</td>
<td>Liver</td>
<td>Medullary HCC</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>RV23</td>
<td>F</td>
<td>=5.5</td>
<td>4.0</td>
<td>Thymus</td>
<td>Thymoma</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

ND, not done.
animals with GSHV markers. In the period, three hepatocellular carcinomas were observed in animals with GSHV markers. Two of these were trabecular

the viral DNA at one site (data not shown), and digestion of tumor DNA with these enzymes produced distinct high molecular weight viral DNA containing bands of 15.7 and 4.3 kb, respectively, as shown in Fig. 3B, lanes 2 and 3. When tumor DNA was digested with EcoRI, which cleaved the viral genome at two sites (data not shown), no high molecular weight fragment containing viral DNA was detected. Several faint high molecular weight bands of viral DNA were detected in HindIII-, EcoRI-, and Xba I-digested DNA from both tumor and nontumorous liver (Fig. 3A, lanes 2, 3, 5, and 6 and data not shown), indicating that these bands were not unique to the tumor. Viral DNA sequences in DNA fragments longer than the viral genome (3.2 kb) were detected after HindIII, Kpn I, and Xba I digestions of tumor DNA but not in undigested tumor DNA, consistent with integration of viral DNA at the same cellular DNA site in many cells of the HCC.

No viral DNA was detected in DNA extracted from the tumors of animals GL21 or RV23, either by Southern blot analysis with a sensitivity for detection of 0.1 viral genome in a single band per haploid cell genome equivalent or by slot blot hybridization with a sensitivity that could detect 0.1 pg of viral DNA per 10 μg of cell DNA. A DNA analysis could not be carried out on the extensively autolyzed tumor of Trt02.

DISCUSSION

In a colony of captive ground squirrels followed over a 5-year period, three hepatocellular carcinomas were observed in animals with GSHV markers. Two of these were trabecular carcinomas similar to HCC frequently observed in WHV-infected woodchucks and HBV-infected humans (8, 9), developing in ground squirrels infected with GSHV for at least 2.4 and 4.5 years, respectively. Integrated viral DNA was detected in DNA from one of the ground squirrel HCC, as has been observed in many of the HCC associated with HBV and WHV infection. The third HCC reported here occurred in a ground squirrel with evidence of prior GSHV infection (antibody to the virus) but without evidence of active infection (no GSHV in the blood) for most of its life. In this animal, the HCC was not trabecular, nor was viral DNA detected in the tumor. HCC appeared earliest in an animal that had been treated nearly 2 years earlier with the antiviral compound 9-(1,3-dihydroxy-2-propoxy)methylguanine. It was one of six treated with this drug, and the results raise the question of whether the drug may have contributed to tumor formation in this animal. The thymoma in a carrier squirrel is of interest in view of the demonstration of HBV DNA in human lymphoblastoid cells (21) and the frequency of hematopoietic cells in woodchuck HCC (8). The tumors in other sites were probably related to the relatively advanced age of the squirrels.

Little information is available on the occurrence of tumors in various species of squirrels, since these rodents have been little used as laboratory animals and little hunted for food in recent years. We did not observe any tumors in newly trapped ground squirrels, but only in animals held in captivity until middle or old age [the lifespan in captivity is estimated for some species of ground squirrels to be 8–10 years (22, 23)]. HCC thus may develop in the middle to latter years of the lifespan of GSHV carriers infected in early life, like HCC in human carriers, rather than after relatively shorter periods.
of chronic infection, as in WHV-infected woodchucks (3, 8, 9). The incidence of HCC in GSHV-infected ground squirrels seems to be lower than in WHV-infected woodchucks, but how it compares to the incidence in HBV-infected humans cannot be estimated from the limited data reported here.

The incidence of HCC reported here in carrier ground squirrels is not statistically different from the incidence in noncarriers; too few tumors have been seen and too few animals examined to permit a conclusion about the relative incidence. However, the similarity of histology of GSHV-carrier HCC to those of hepadnavirus-associated woodchuck and human HCC, the presence of HCC as the predominant type of tumor in aging carrier ground squirrels, and the absence of HCC in older ground squirrels with no markers of GSHV suggest that this hepadnavirus, like the well-studied HBV and WHV, may be associated with HCC.

Note Added in Proof. Since this manuscript was accepted for publication, we have observed development of HCC in five more squirrels: four were carriers of GSHV and the fifth had anti-GSHs, indicative of past infection. All five squirrels were 5 years of age or older and had been observed for at least 4.5 years. None of the animals in a cohort of GSHV-marker-free squirrels observed for the same period of time has developed HCC.

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