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# Extensive oxidative DNA damage in hepatocytes of transgenic mice with chronic active hepatitis destined to develop hepatocellular carcinoma

(hepatocytes/8-oxo-2′-deoxyguanosine/hepatitis B large envelope protein/superoxide)

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**ABSTRACT** **A transgenic mouse strain that expresses the hepatitis B virus (HBV) large envelope protein in the liver was used to determine the extent of oxidative DNA damage that occurs during chronic HBV infection. This mouse strain develops a chronic necroinflammatory liver disease that mimics the inflammation, cellular hyperplasia, and increased risk for cancer that is evident in human chronic active hepatitis. When perfused *in situ* with nitroblue tetrazolium, an indicator for superoxide formation, the liver of transgenic mice displayed intense formazan deposition in Kupffer cells, indicating oxygen radical production, and S-phase hepatocytes were commonly seen adjacent to the stained Kupffer cells. Similar changes were not observed in nontransgenic control livers. To determine whether these events were associated with oxidative DNA damage, genomic DNA from the livers of transgenic mice and nontransgenic controls was isolated and examined for 8-oxo-2′-deoxyguanosine, an oxidatively modified adduct of deoxyguanosine. Results showed a significant, sustained accumulation in steady-state 8-oxo-2′-deoxyguanosine that started early in life exclusively in the transgenic mice and increased progressively with advancing disease. The most pronounced increase occurred in livers exhibiting microscopic nodular hyperplasia, adenomas, and hepatocellular carcinoma. Thus, HBV transgenic mice with chronic active hepatitis display greatly increased hepatic oxidative DNA damage. Moreover, the DNA damage occurs in the presence of heightened hepatocellular proliferation, increasing the probability of fixation of the attendant genetic and chromosomal abnormalities and the development of hepatocellular carcinoma.**

Chronic inflammatory diseases affect millions of people world-wide and may be a primary factor in the development of up to one-third of all cancers (1). A major association between a persistent infection and cancer is evident in chronic active hepatitis B virus (HBV) infection (2). Epidemiological studies show that incidence of hepatocellular carcinoma (HCC) correlates strongly to geographical areas of endemic HBV infection (3). Furthermore, patients with chronic hepatitis have a much higher incidence of liver cirrhosis and HCC than individuals not infected with HBV (4). Also, HBV antigens in sera of patients are associated with HCC (5). Because the virus infects >300 million people throughout the world, HBV has been described as second only to tobacco as a known human carcinogen (6).

Despite the apparent epidemiological association of chronic active hepatitis infection and cancer, the mechanisms involved are still not entirely understood. Research has been hampered due to the long latency period between the onset of infection and cancer development, and the lack of suitable animal models that mimic chronic active human hepatitis.

HBV does not contain any known acutely transforming oncogenes, and only rarely do integrated HBV sequences activate cellular protooncogenes (7–10). In contrast, persistent inflammation associated with chronic active hepatitis can be associated with mutations in tumor suppressor genes (11) and extensive chromosomal abnormalities. Persistent HBV infection often leads to cirrhosis that often precedes the appearance of HCC (12). The necroinflammatory liver disease seen during HBV infection is thought to be mediated by the host immune response to viral antigens. Activated macrophages and other recruited leukocytes release powerful reactive oxygen species such as HOONO (from NO and $O_2^-$), HOCl, and $H_2O_2$ at sites of infection, causing areas of focal necrosis and compensatory cell division (13, 14). These oxidants not only kill target cells but may also overwhelm cellular antioxidant defenses of neighboring cells, leading to damage of important biomolecules, including DNA (15).

We hypothesize that oxidative DNA damage may be an important underlying event that leads to cancer during chronic active hepatitis and other persistent inflammatory diseases. We tested this hypothesis using a transgenic mouse model in which overexpression of the HBV large envelope protein causes a necroinflammatory liver disease that inexorably progresses to HCC. This mouse strain effectively reproduces many of the characteristics of human chronic active hepatitis, including focal hepatocellular necrosis, Kupffer cell activation, and chronic hepatocellular hyperplasia (16). We determined the relative abundance of superoxide in these mice by employing an *in situ* vascular perfusion technique, whereby nitroblue tetrazolium (NBT) was perfused through the liver to depict areas where superoxide was formed. Furthermore, oxidative DNA damage was directly measured by analyzing hepatic levels of 8-oxo-2′-deoxyguanosine ($oxo^8dG$), an adduct of deoxyguanosine.

## MATERIALS AND METHODS

**Reagents.** NBT, 5-bromo-2′-deoxyuridine (BrdUrd), hematoxylin/eosin, phorbol 12-myristate 13-acetate, and collagenase were purchased from Sigma. RNase A and T were obtained from Boehringer Mannheim. Phenol was purchased from Clontech. Methanol (HPLC grade) was from Fisher.

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Abbreviations: HBV, human hepatitis B virus; NBT, nitroblue tetrazolium; HCC, hepatocellular carcinoma; $oxo^8dG$, 8-oxo-2′-deoxyguanosine; BrdUrd, 5-bromo-2-deoxyuridine.

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Zinc/formalin was purchased from Anatek (Battle Creek, MI). Metofane inhalational anesthesia was from Pitman–Moore (Mundelein, IL). All other chemicals were reagent grade or the highest purity that could be obtained.

**Statistical Analysis.** All data was analyzed for significance using the paired Student's *t* test. All numbers are expressed as the mean ± SEM.

**Transgenic Mice.** Transgenic mice of lineage 50-4 [official designation, Tg(Alb-1 HBV)Bri 44] and nontransgenic littermates between the ages of 1 and 31 mo of age were used in this study. This transgenic mouse line contains the HBVBgl II-A DNA fragment downstream of the mouse albumin promoter and has been extensively characterized (17–20). Mice were examined periodically for signs of liver cell injury as determined by the presence of glutamate/pyruvate transaminase activity in the blood sera. Tumor development was assessed by abdominal palpation and autopsy. Liver tissue was processed for histopathologic analysis and snap-frozen in liquid nitrogen and stored at −80° until used for DNA isolation.

Sections of zinc/formalin-fixed, paraffin-embedded liver tissue were stained with hematoxylin/eosin for the presence of HBV serum antigen, as described (17). To demonstrate cellular proliferation, a thymidine analogue, BrdUrd, was used to label the hepatic and nonhepatic cells in S-phase cell cycle. The incorporated BrdUrd was demonstrated immunohistochemically with monoclonal anti-BrdUrd antibody (Becton Dickinson) by an indirect immunoperoxidase method.

Liver tissue was classified histopathologically into the following categories by described criteria (17): (*i*) livers from very young transgenic mice and from nontransgenic littermates between 1 and 31 mo of age that showed no histopathological abnormalities [designated WNL (within normal limits)]; (*ii*) livers from 2- to 4-mo-old transgenic mice where high expression of the large envelope protein resulted in a hyperplastic endoplasmic reticulum that resembled "ground glass" under microscopic examination; with advancing age, these mice displayed biochemical and histological evidence of liver cell injury including high levels of serum glutamate/pyruvate transaminase and necroinflammatory foci [designated GG/INJ (ground glass/injury)]; (*iii*) 6- to 18-mo-old transgenic mice with microscopic nodular hyperplasia reflecting the presence of dysplastic hepatocytes and preneoplastic foci (designated MNH); (*iv*) livers with MNH that also contain adenomatous neoplastic tumors (designated MNH/Adenoma); and (*v*) tissue with HCC (designated HCC). These disease processes closely resemble the pathophysiological progression seen in human chronic active hepatitis.

**NBT Perfusion Method.** Five hours before NBT perfusion, BrdUrd (100–150 mg/kg) in sterile saline was given i.p. to the animals for labeling of S-phase nuclei in liver. The mice were sacrificed by cervical dislocation, and a midlateral abdominal incision was made. A 20-gauge needle connected to a Sage Instruments (Boston) tubing pump (model 375A) was inserted into the inferior vena cava, the portal vein was cut, and the liver was perfused in a retrograde direction (flow rate 1 ml/min) with a Hanks' balanced salts solution (HBSS) (pH 7.4) at 37°C. Once blood was removed, NBT (1 mg/ml) was added to the HBSS, and the liver was perfused for 20 min at 2 ml/min. All unreacted NBT was removed from the liver by postperfusion with HBSS. The NBT-perfused liver was fixed in zinc/formalin for histologic examination for formazan deposits and for immunocytochemical demonstration of BrdUrd in S-phase nuclei. In some experiments livers were subsequently perfused with 10% (wt/vol) zinc/formalin for histological determination of BrdUrd labeling and sites of formazan deposition. Approximately 3-μm sections were processed. In selected experiments superoxide dismutase was included in the perfusate (500 μg/ml) as a specific control.

**Determination of Oxidative DNA Damage.** For determination of hepatic steady-state oxo$^8$dG, mice were sacrificed either by cervical dislocation or an overdose of anesthesia. The livers were quickly excised, snap-frozen in liquid nitrogen, and stored at −80°C. Liver samples were homogenized, and the DNA was isolated essentially as described by Blobel and Potter (21). DNA was enzymatically hydrolyzed to nucleosides as described (22). The hydrolyzates were centrifuged through 30-kDa molecular cutoff filters to remove any large particulates. Samples were then separated and analyzed by HPLC with electrochemical detection as described (22).

**Cell Isolation Studies.** Transgenic mice and nontransgenic controls were anesthetized with metofane, and hepatocytes were obtained as described (23). Briefly, the inferior vena cava was cannulated, and the portal vein was severed. Collagenase (1 mg/ml) dissolved in HBSS was perfused through the liver at 37°C to disperse hepatocytes. Isolated cells were further separated into parenchymal and nonparenchymal fractions by a series of differential centrifugations. Viability of the cells was assessed by trypan blue exclusion; typically, viability was 90% or greater. Contamination of the two cell fractions was determined by light microscopy; enrichment of the cell populations was usually 85% or greater.

## RESULTS

**Increased Oxidant Production and Cell Proliferation.** NBT is a dye that is reduced to an insoluble formazan derivative upon exposure to superoxide (24). The blue-colored formazan is readily detectable in the tissue by light microscopy. It is a valid histochemical marker for the presence of reactive oxygen species. Although superoxide reduces NBT, it primarily acts on cells as an oxidizing agent through its further conversion to $H_2O_2$ and HONOO. In the present study, we used the NBT reaction as a histological marker for the presence of reactive oxygen species in livers of transgenic mice. After perfusion, liver sections were examined for the presence of formazan staining. Tissue slices obtained from 3-mo-old transgenic mice showed that the liver was extensively stained (Fig. 1*C*). In contrast, liver slices from nontransgenic mice did not become stained after NBT perfusion (Fig. 1*A*) unless they were simultaneously perfused with phorbol 12-myristate 13-acetate (Fig. 1*B*), which is known to activate hepatic macrophages (Kupffer cells) (25, 26). These results indicate that livers in transgenic mice contain higher concentrations of oxygen radicals and are, therefore, under an increased oxidative stress.

**Cell Proliferation in the Presence of Reactive Oxygen Species.** After NBT perfusion, sections of tissue were taken for histological examination. Microscopic analysis revealed traces of formazan crystal deposition at the surface of both transgenic and nontransgenic hepatocytes, suggesting that it may be a by-product of normal cellular metabolism (data not shown). However, intense deposition was seen in many of the nonparenchymal cells in transgenic liver tissue (Fig. 2*A*) but not in controls. Microscopically, the formazan crystals were seen sparingly on the hepatocyte surface and abundantly in association with sinusoidal littoral cells. The density of formazan deposits reflects the amount of superoxide


FIG. 1. Formazan staining in livers from 3-mo-old transgenic and nontransgenic mice after perfusion with NBT. Livers were perfused with NBT (1 mg/ml) followed by perfusion with HBSS to remove unreacted NBT. (*A*) Nontransgenic mouse liver. (*B*) Nontransgenic mouse liver simultaneously perfused with phorbol 12-myristate 13-acetate. (*C*) Transgenic mouse liver.

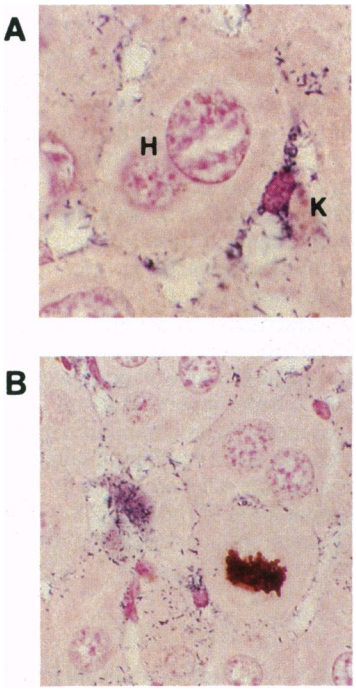


FIG. 2. (*A*) Formazan-stained section of NBT-perfused transgenic mouse liver. After NBT perfusion, 3-$\mu$m liver sections from a 3-mo-old transgenic mouse were fixed with zinc/formalin and stained with hematoxylin/eosin. Shown is a typical thin section, indicating intense formazan deposition (purple stain) surrounding activated Kupffer cells. (*B*) Histologic analysis of cell division and oxidant production in transgenic mouse liver. After NBT perfusion of a 3-mo-old transgenic mouse that was previously injected with BrdUrd, liver sections were treated with zinc/formalin, hematoxylin/eosin, and antibodies to BrdUrd as described. Note the BrdUrd-positive mitotic figure (red stain) in a hepatocyte in close proximity to nonparenchymal cells displaying oxidant formation (purple stain). H, hepatocyte; K, Kupffer cell. (×55.)

generated locally (Fig. 2*A*). Formazan deposits were much lower in transgenic mouse liver that was perfused with superoxide dismutase that was added to the NBT perfusate (data not shown). These nonparenchymal cells were histologically compatible with Kupffer cells, the resident macrophages of the liver (Fig. 2*A*). Thus, formazan staining evident in the transgenic mouse livers is derived from the presence of high levels of superoxide, presumably released from activated Kupffer cells.

To examine the relationship between heightened cell proliferation and increased oxidant production, transgenic mice and controls were injected with BrdUrd to stain cells undergoing DNA replication (27, 28). Both groups of mice at 1 mo of age had a high percentage of BrdUrd-stained cells that had declined significantly by 3 mo in nontransgenic livers and remained low thereafter (Table 1). BrdUrd labeling in transgenic livers also declined initially but by 6 mo had again markedly increased. In transgenic mice >9 mo old, proliferation was 80-fold higher than in nontransgenic littermates. Therefore, transgenic mice used in this study display greatly increased rates of hepatocellular proliferation than nontransgenic mice as a consequence of their underlying liver disease. As shown in Fig. 2*B*, the BrdUrd-labeled liver cells were seen adjacent to activated Kupffer cells. These findings may suggest a high degree of vulnerability of mitotic cells to oxidative stress in an oxygen-radical-rich milieu.

Table 1. BrdUrd-labeling index of hepatocytes in transgenic and nontransgenic mice

| Age, mo | Transgenic | Nontransgenic |
|---|---|---|
| 1 | 2.50 ± 1.10 (3) | 2.7 ± 1.9 (11) |
| 3 | 0.30 ± 0.40 (4) | 0.08 ± 0.06 (5) |
| 6 | 1.24 ± 0.51 (4) | 0.06 ± 0.03 (7) |
| >9–27 | 1.65 ± 1.55 (15) | 0.02 ± 0.01 (4) |

Numbers in parentheses denote *n* values.

**Oxidative DNA Damage.** To determine whether the associated inflammation evident in transgenic mice resulted in increased oxidative DNA damage, mice exhibiting different stages of liver injury were sacrificed, and hepatic oxo$^8$dG values were determined. Oxo$^8$dG values in old nontransgenic mice ($n$ = 21) was 16.1 ± 2.6 fmol/$\mu$g of DNA (Fig. 3*A*). Livers of transgenic mice with no lesions had similar levels of oxidative DNA damage [15.6 ± 2.4 fmol/$\mu$g ($n$ = 16)]. However, transgenic mice with liver cell injury had substantially increased hepatic oxo$^8$dG levels, which became more pronounced as the level of injury increased ($P < 0.01$; Fig. 3*A*). There was no statistical difference in oxo$^8$dG values in transgenic mice with no lesions and those having ground glass morphology with or without focal necrosis and inflammation. The greatest increase in oxo$^8$dG levels occurred between samples with "ground glass" injury and mice with evidence of microscopic nodular hyperplasia [25.4 ± 7.4 ($n$ = 12) versus 115.9 ± 23.1 ($n$ = 12)]. DNA damage remains relatively constant thereafter, although the livers with micro-

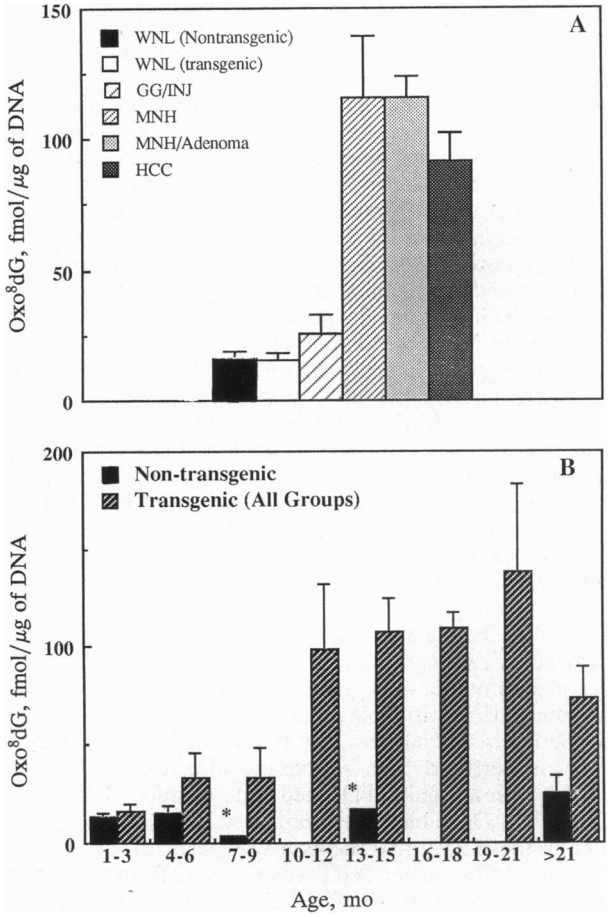


FIG. 3. Oxidative DNA damage in hepatitis B transgenic mice. DNA was isolated from livers of transgenic mice or nontransgenic controls. Animals studied ranged from 1 to 31 mo of age ($x$ axis) and represented all stages of liver disease. (*A*) Oxidative DNA damage in nontransgenic and transgenic mice with increased degrees of liver injury. Oxo$^8$dG is markedly increased in animals with evidence of microscopic nodular hyperplasia, adenoma, and HCC. WNL, within normal limits; GG/INJ, ground glass/injury; MNH, microscopic nodular hyperplasia (see text). (*B*) Oxidative DNA damage as a function of age. *, $n$ = 2.

Table 2. Steady-state oxo$^8$dG levels in parenchymal and nonparenchymal cells

| | Nonparenchymal fraction | | Parenchymal fraction | |
|---|---|---|---|---|
| Age, mo | Transgenic | Nontransgenic | Transgenic | Nontransgenic |
| 1–2 | 32.8 ± 8.4 (4) | 9.4 ± 1.2 (3) | 16.0 ± 4.9 (4) | 15.3 ± 2.3 (3) |
| 4–6 | 26.3 ± 3.3 (5) | 17.9 ± 5.8 (3) | 73.9 ± 37.9 (5) | 12.1 ± 0.2 (3) |

Numbers in parentheses denote *n* values.

scopic nodular hyperplasia and adenomas showed the highest steady-state values [115.9 ± 23.1 ($n$ = 12) and 115.9 ± 7.6 ($n$ = 24), respectively], which were almost 7-fold higher than nontransgenic mice. DNA damage in livers with HCC appeared to be lower than adenomatous tissue [91.4 ± 11.1 ($n$ = 23)] but was still significantly higher than transgenic mice where no lesions were evident (Fig. 3*A*). These results indicate oxidative DNA damage significantly increases only after substantial tissue damage occurs.

Previous studies showed that oxidative DNA damage (as measured by appearance of oxo$^8$dG) increases in liver during aging (29). Because progression of liver damage from no discernible lesions to HCC occurred over the lifespan of the mouse, it was necessary to determine whether the increased oxo$^8$dG was due to chronic inflammation or merely reflected an age-associated accumulation in this particular mouse strain. Oxo$^8$dG in 1- to 3-mo-old nontransgenic mice was 13.1 ± 1.7 fmol/$\mu$g of DNA ($n$ = 9). This concentration is similar to previously reported oxo$^8$dG values in rodents of similar age (29). Essentially no age-associated increase in oxo$^8$dG was observed in nontransgenic mice, except in animals >21 mo of age (Fig. 3*B*), where the mean oxo$^8$dG content increased to 25.0 ± 9.2 ($n$ = 5). In contrast to nontransgenic mice, oxo$^8$dG in livers of transgenic animals became significantly elevated beginning ≈4 to 6 mo of age ($P < 0.1$), and extremely high levels of oxidative DNA damage were observed throughout their lifespans (Fig. 3*B*). The age at which oxo$^8$dG becomes significantly elevated is typically about the age that micronodular hyperplasia becomes evident. These results indicate that nontransgenic mice undergo only a nominal age-associated increase in oxo$^8$dG and that the large increases in oxo$^8$dG concentrations seen in transgenic mice cannot be accounted for solely due to the aging process.

While these data presented in Fig. 3 suggest that oxidative DNA damage is extensive, they do not indicate the liver cell type that is affected. In view of the evidence implicating Kupffer cells as the source of oxidants in this model (Fig. 2), we compared the oxo$^8$dG levels in hepatic parenchymal and nonparenchymal cells from 1-mo-old transgenic mice with normal livers and 4- to 6-mo-old mice with elevated serum glutamate/pyruvate transaminase levels indicative of tissue injury. The same cellular populations from nontransgenic mice were studied as controls.

At 1 to 2 mo of age, both cell populations from nontransgenic mice displayed approximately the same oxo$^8$dG levels (Table 2). In contrast, the hepatic nonparenchymal cells from the 1-mo-old transgenic mice displayed significantly higher oxo$^8$dG concentrations as compared with the corresponding parenchymal cells or to either fraction from nontransgenic mice. Interestingly, as the mice aged, the oxo$^8$dG concentration of the transgenic parenchymal cells increased nearly 5-fold relative to the 1-mo-old transgenic mice and >6-fold relative to their age-matched nontransgenic controls. The oxo$^8$dG content of the nonparenchymal cells in both groups did not change substantially as they aged. These observations suggest that the transgenic nonparenchymal cells sustain oxidative DNA damage early in the disease process, which does not appear to worsen as the disease progresses. In contrast, the transgenic parenchymal cells display much more severe oxidative DNA damage than the nonparenchymal cells, but the onset of the changes are delayed until after the onset of chronic hepatitis.

## DISCUSSION

The characteristics of the transgenic mouse strain used in this study provided us the chance to examine oxidative DNA damage that might occur during a chronic necroinflammatory disease associated with sustained cell division. The mechanisms responsible for chronic hepatitis in these mice have been extensively studied (16–20). The mice overproduce the HBV large envelope polypeptide that accumulates in the endoplasmic reticulum, causing a storage disease characterized by hepatocellular necrosis and inflammation that progresses to the development of microscopic preneoplastic hyperplastic foci and eventually to the development of benign (adenomatous) and malignant (HCC) hepatic tumors that ultimately destroy the liver and kill the mouse. This model, therefore, closely mimics many of the events that precede the onset of HCC in human chronic active hepatitis.

This report shows that the livers of these transgenic mice display marked increases in the production of reactive oxygen species and the development of oxidative DNA damage long before the development of HCC. While we do not know why the nonparenchymal cells are activated in these mice, we suspect that this might reflect either the phagocytosis of HBV serum antigen particles secreted by the hepatocytes or a response to the death of hepatocytes that begins during the first 2 mo of life in these animals. Importantly, the high level of oxidants produced by the nonparenchymal liver cells in these animals correlates well with their increased content of oxidatively damaged DNA, as shown in our cell-isolation studies. Most importantly, this was followed by a much greater degree of oxidative DNA damage in the hepatic parenchymal cells in these animals, as much as a year before the onset of HCC. In whole-liver DNA preparations, the steady-state oxo$^8$dG values were almost 7-fold higher in transgenic mice (MNH/Adenoma group) versus controls.

We measured oxo$^8$dG in the present study because it is thought to arise via hydroxyl-radical attack on the C-8 position of deoxyguanosine and is thus a specific marker for oxidative damage (30–32). oxo$^8$dG also causes G·C → A·T transversions (33), in *in vitro* experiments, suggesting that its accumulation in these transgenic mice may increase the possibility of mutation. Because oxo$^8$dG is only 1 of 20 known adducts formed from oxidative injury to DNA, the high levels of oxo$^8$dG indicate that transgenic mice are undergoing massive oxidative injury.

Glycosylases quickly remove the mutagenic lesion, oxo$^8$Gua, from DNA (34). The fact that oxo$^8$dG accumulates to such high levels in the hepatocytes of these animals, therefore, suggests that cellular antioxidant and DNA-repair defenses may be overwhelmed by the oxidative environment of the inflamed liver or that they may actually be functionally compromised in the HBV serum antigen-positive hepatocyte. The former concept is supported by a recent report by Yamamoto *et al.*‖ which showed that patients with chronic

‖Yamamoto, Y., Iida, N., Nagata, Y. & Niki, E., Sixth International Conference on Superoxide and Superoxide Dismutase, October 11–15, 1993, Kyoto.

active hepatitis in Japan had significantly lower ubiquinol levels in their sera and a correspondingly high level of oxidized ubiquinol. This result suggests that certain antioxidants might be depleted during chronic hepatitis, thereby diminishing the ability of cells to scavenge free radicals generated by the host immune response. Interestingly, in preliminary experiments we have shown that hepatocellular catalase activity is markedly decreased in these transgenic mice (47). At present, however, we do not know the extent to which such deficiencies in antioxidant systems or defects in the DNA-repair machinery in the mice used in this study also contribute to the elevated oxo$^8$dG values seen.

Because the livers of the transgenic mice display hepatocellular necrosis and inflammation, it is possible that some of the oxo$^8$dG was present in necrotic tissue. Although this would still be an indicator of the level of oxidant-induced DNA damage in these mice, it would cause us to overestimate the amount of oxidative DNA damage in viable tissue that could be ultimately converted to mutations. While this complication cannot be dismissed, it is evident from the isolated cell studies that viable cells also contain substantially higher oxo$^8$dG concentrations. Thus, oxo$^8$dG from necrotic tissue could not account for all of the increase in DNA damage reported in this study.

The transgenic mice used in this study display a strong, sustained increase in cell proliferation. The results presented in this manuscript strongly suggest that tissue necrosis and compensatory cell division in an environment rich in oxidants may be the primary cause for neoplastic transformation in these mice and, by extension, in human chronic active hepatitis. Compensatory cell division causes unwinding of double-stranded DNA and removal of protecting histones, which allows greater exposure of DNA to reactive oxygen species. Also, rapidly dividing cells may allow insufficient time to repair DNA damage, which would then be converted into mutations in daughter cells. Thus, chronic cell injury is similar to ionizing radiation regarding its effects on DNA mutation.

As this manuscript was being prepared, a study was published showing that liver samples from humans with chronic hepatitis had significantly increased levels of oxo$^8$dG (35). Moreover, oxo$^8$dG content correlated with serum alanine transaminase activity, a sensitive indicator of liver cell injury. These results, coupled to our present study, make a compelling argument that heightened oxidative DNA damage occurs in the liver in chronic HBV infection.

Finally, oxidative DNA damage may be an important risk factor for cancer in other chronic inflammatory diseases. Chronic viral (hepatitis C and D; refs. 36 and 37) and fluke (*Opistorchis* sp., *Chlonorchis* and *Schistosoma*; refs. 38–42) infections of the liver markedly increase the likelihood of liver cancers. Furthermore, the necroinflammatory bowel disease, ulcerative colitis, carries a high risk for development of colon cancer (43). *Helicobacter pylori* infection of the stomach correlates to increased risk for stomach cancer (44), whereas inhalational irritants (silica and asbestos; refs. 45 and 46) markedly increase the chance for developing lung cancers, even in nonsmokers. Although the pathogens that cause these diseases are diverse and unrelated, all of these agents cause a sustained inflammatory response and increased cell replication, similar to the current model.

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