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Interferon-γ-mediated hepatocarcinogenesis in mice treated with diethylnitrosamine

Mitsuru Matsuda[1], Yasunari Nakamoto[1], Satoshi Suzuki[2], Tomomitsu Kurata[3] and Shuichi Kaneko[1]

[1]Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, Kanazawa, Ishikawa, Japan; [2]Research Laboratories, HAB Research Organization, Ichikawa, Chiba, Japan and [3]Department of Pharmacology, School of Medicine, Showa University, Shinagawa, Tokyo, Japan

Hepatocarcinogenesis is a complex multifactorial process in which continuous intrahepatic inflammation plays a major role. Although inflammatory cell infiltration is observed in the process of chemical-induced hepatocarcinogenesis, the pathophysiological role of the inflammatory response is not well defined. To approach this question, molecular and cellular responses were monitored during the development of liver tumors in mice exposed to a chemical hepatocarcinogen, diethylnitrosamine (DEN), in drinking water (50 μg/l). Intrahepatic type I and type II interferon (IFN-β and IFN-γ, respectively) mRNA expression was found to be induced 2 months before the appearance of hepatocellular carcinomas. The pathogenetic importance of IFNs was determined by monitoring tumor development in mice genetically deficient in the IFN-α/β receptor (IFN-α/βR KO) or the IFN-γ receptor (IFN-γR KO). IFN-γR KO mice developed fewer tumors than IFN-α/βR KO and wild-type (wt) mice, although the tumor diameters did not differ significantly among the three lineages. Interestingly, immunohistochemical studies demonstrated that the percentage of monocytes/macrophages in infiltrating mononuclear cells was reduced greatly in the livers of IFN-γR KO mice, which is consistent with the facts that intrahepatic cytokine expression was diminished and oxidative DNA damage was induced to a lesser extent. In conclusion, type II IFN, but not type I IFNs, may be involved critically in the initiation stage, but not the promotion stage, of DEN-induced hepatocarcinogenesis by enhancing monocytes/macrophages activation and eventual hepatocyte DNA damage.

Laboratory Investigation (2005) **85,** 655–663, advance online publication, 14 March 2005; doi:10.1038/labinvest.3700257

Keywords: hepatocarcinogenesis; diethylnitrosamine; interferon-γ; gene knockout mice; oxidative DNA damage

Hepatocellular carcinoma (HCC) occurs after many years of chronic hepatitis. Prolonged inflammation is thought to set up a cycle of liver cell destruction and regeneration, resulting in a mitogenic and mutagenic environment that precipitates random genetic and chromosomal damage and leads to the development of HCC.[1–5] Recently, continuous intrahepatic inflammation has been reported to be the principal oncogenic factor in hepatocarcinogenesis on the basis of experiments with a hepatitis B virus (HBV) transgenic mouse model.[6,7] Interestingly, in that study, malignant transformation was induced by immune-mediated mechanisms in the absence of viral transactivation, insertional mutagenesis, or genotoxic chemicals.

Diethylnitrosamine (DEN) is an experimental hepatocarcinogen found in a variety of products to which humans may be exposed, for example, tobacco smoke, meat, and whiskey. Currently, the mechanism of DEN-induced hepatocarcinogenesis is thought to be as follows:[8] DEN is hydroxylated by cytochrome *P*-450 isozymes in the liver, through an alkylation mechanism, to become bioactive. Subsequently, bioactivated DEN reacts with DNA, causing ethylation of the bases. The ethyl DNA adducts can interrupt base pairing, resulting in mutations and the activation of proto-oncogenes, for example, *ras*,[9,10] and inhibition of tumor–suppressor genes, for example, *p53*,[11] which often result in HCC. While the previous studies reported that continuous intrahepatic necroinflammatory changes were observed during the process of DEN-induced hepatocarcinogenesis,[12,13] the pathogenetic importance of the inflammatory response has not been well defined.

To approach this question, mice were exposed in this study to DEN in drinking water. Liver tumors

appeared in 46% of the mice after 3 months of DEN exposure, and intrahepatic type I and type II interferon (IFN-β and IFN-γ, respectively) mRNA expression was induced 2 months before the appearance of tumors. Furthermore, to define the role of type I and II IFNs in the process of chemical carcinogenesis, mice genetically deficient in the IFN-α/β receptor (IFN-α/βR KO) or IFN-γ receptor (IFN-γR KO) and wild-type (wt) syngeneic controls were treated with DEN, and tumor development was monitored. The results indicate that type II IFN, but not type I IFNs, may be involved critically in the initiation stage, but not the promotion stage, of hepatocarcinogenesis in mice exposed to DEN in drinking water.

Materials and methods

Mice and Animal Experiments

129SV wt mice, IFN-α/β receptor knockout (IFN-α/βR KO), and IFN-γ receptor knockout mice (IFN-γR KO) on a 129SV background were purchased from B&K Universal (East Yorkshire, UK). They were maintained on a 12-h light/dark cycle at 22°C. They were fed a standard laboratory diet, CRF-1, from Charles River Japan (Kanagawa, Japan) and given tap water *ad libitum*. All animal experiments were conducted in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals (NIH publication 86-23, revised 1985).

Male 4–5-week-old mice were used in this experiment. Mice were exposed to DEN (Wako, Tokyo, Japan) at 50 μg/l in drinking water throughout the period of study. On a monthly basis for 5 months, 8–17 mice were killed and tumors that had developed on the liver surface were observed. The numbers of liver tumors and diameters of liver tumors were evaluated. Inflammatory cell infiltration was quantitated in 100 high-power (×400) fields representing 4 mm² of the liver tissue stained with hematoxylin and eosin.

Western Blot Analysis of Cytochrome *P*-450 2E1 (CYP2E1) Expression in the Liver

Mouse liver washed with ice–cold buffer (250 mM sucrose, 1 mM EDTA, and 3 mM Tris-HCl, pH 7.4) was weighed and homogenized in three volumes (v/w) of the buffer. The homogenate was centrifuged at 9000 g for 20 min, and the supernatant was ultracentrifuged at 105 000 g for 60 min. The microsomal pellet was suspended in the homogenizing buffer and again centrifuged at 105 000 g for 60 min at 4°C. The microsome was suspended in suspension buffer (20% glycerol, 1 mM EDTA, and 100 mM Tris-HCl buffer, pH 7.4) and stored at −80°C until use. CYP2E1 activity was determined as described.[14] Briefly, CYP2E1 activity was determined using chlorzoxazone (100 μM) as a substrate. Specifically,

the incubation mixture contained 1 mg/ml of microsome. The mixture was incubated at 37°C for 15 min. All reactions were then terminated and subjected to HPLC Gulliver 1500 analysis (JASCO, Tokyo, Japan) with a reversed-phase analytical column. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots analysis were carried out on pig liver microsomes, and the immunoblots were probed with antibody to rat CYP 2E1 (Gentest, Discovery Labware, BD Biosciences, Massachusetts, USA).

Immunohistochemical Analysis

Expression of glutathione S-transferase placental form (GST-p) and alpha-fetoprotein (AFP) were studied using rabbit anti-mouse GST Yp antibodies (Biotrin International, Dublin, Ireland) and rabbit anti-mouse AFP antibodies (ICN Biomedicals, Inc., Aurora, USA), respectively. Monocytes/macrophages in inflammatory infiltrates were detected using a rat monoclonal antibody to mouse macrophages (Caltag Laboratories, Burlingame, USA). Liver tissues obtained from the mice were fixed in formalin and embedded in paraffin. Sections of 2 μm were assayed for GST-p, AFP, and macrophage markers by immunoperoxidase staining using the avidin–biotin complex (ABC) method (Vector Laboratories Inc., Buringame, USA). Sections were deparaffinized and dehydrated with xylene and a graded alcohol series. To quench endogenous peroxidase activity, sections were incubated for 30 min in 0.3% H₂O₂ in water. To block nonspecific staining, sections were incubated for 30 min in 10% normal blocking serum in phosphate-buffered saline (PBS). Then, sections were covered with rabbit anti-mouse GST Yp antibody (1:100 diluted) or rabbit anti-mouse AFP antibody (1:10 diluted) for 30 min at room temperature. After washing, sections were covered with biotinylated antibody for 30 min at room temperature, washed, and then incubated with conjugated streptavidin for 30 min at room temperature. After washing, sections were incubated with 0.01% diaminobenzidene/0.01% H₂O₂ and counterstained with hematoxylin.

RNA Extraction and RNase Protection Assay

Frozen nontumorous liver tissues (200 mg) from representative mice, killed at the indicated time points, were homogenized using an ultrasonicator and total RNA was extracted by the guanidium isothiocyanate/silica-gel-based membrane method using RNeasy Midi Kits (QIAGEN, Hilden, Germany). To monitor the expression of a panel of inflammatory cytokines, 15 μg of total RNA was subjected to RNase protection assay using a Ribo-Quant Multi-Probe RNase Protection Assay System (Pharmingen, San Diego, USA). Template set mCK-3b (Pharmingen), containing subclones of mTNF-β,

mLT-β, mTNF-α, mIL-6, mIFN-γ, mIFN-β, mTGF-β1, mTGF-β2, mTGF-β3, mMIF, mL32, and mGAPDH, was used as templates for T7 polymerase-directed synthesis of [^{32}P] antisense RNA probes. After overnight hybridization at 56°C and digestion with RNase A/RNase T1 mix at 45°C for 30 min to degrade unhybridized probe, the hybridized fragments were separated on a 5% polyacrylamide gel. Gels were dried and RNA bands were visualized by autoradiography and analyzed on a BAS 1000 image analyzer (Fuji Photo Film, Tokyo, Japan).

Quantitation of 8-Hydroxydeoxyguanosine in Liver

Frozen nontumorous liver tissues (350 mg) from representative mice killed at the indicated time points were homogenized using an ultrasonicator and total genomic DNA was isolated using a Blood & Cell Culture DNA Maxi Kit (QIAGEN). To evaluate oxidative DNA damage, 200 μg of genomic DNA was subjected to measurement of 8-hydroxydeoxyguanosine (8-OHdG) by enzyme-linked immunosorbent assay (ELISA) using an anti-8-OHdG monoclonal antibody (8-OHdG Check, JICA, Shizuoka, Japan).

Statistical Analysis

All numbers are expressed as the mean ± s.e. All data were analyzed for significance using the paired Student's t-test or Mann–Whitney's U-test. A P-value < 0.05 was considered to indicate a statistically significant difference.

Results

Liver Tumor Development and Intrahepatic Cytokine mRNA Expression

129SV wt mice were exposed to DEN (50 μg/l) in drinking water, and the numbers and diameters of liver tumors were monitored. Liver tumors appeared in 46% of the mice after 3 months of DEN exposure and in all mice after 4 months exposure (Figure 1a). The numbers and diameters of the tumors increased during the period of exposure to DEN (Figure 1b and c). To define the histological features of liver tumors, liver samples collected at the indicated time points were evaluated immunohistochemically. The tumors displayed the features of a solid pattern of HCC that compresses the adjacent hepatic parenchyma (Figure 2a and b), and stained immunohistochemically positive for GST-p (Figure 2c) and negative for AFP (Figure 2d). On the basis of these observations, the liver tumors were classified histologically as HCC-expressing GST-p. Furthermore, because the surrounding hepatic parenchyma displayed infiltration of inflammatory cells (Figure 2b), the numbers of infiltrating mononuclear cells were counted during DEN exposure period. Inflammatory cell infiltration was not observed after 1 month of
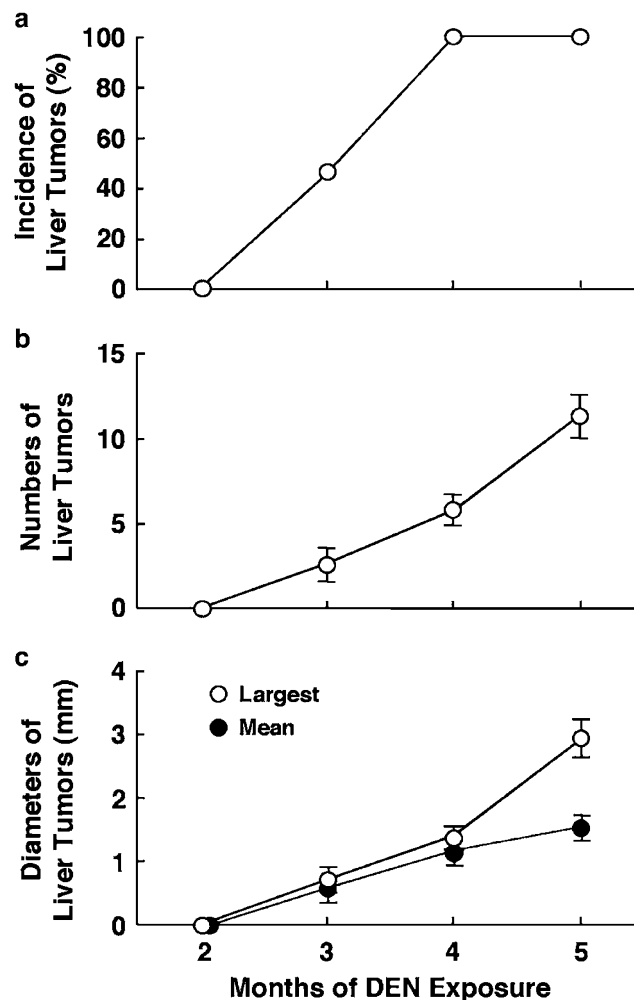

Figure 1 Tumor development in the livers of wt mice exposed to diethylnitrosamine (DEN) in drinking water. Rate of incidence (**a**) and numbers (**b**) of liver tumors and diameters of the largest liver tumors and mean diameters of the liver tumors (**c**) were monitored at autopsy monthly for the period of study. (**a**) Liver tumors appeared in 46% of the mice after 3 months of DEN exposure and in all mice after 4 months exposure. (**b** and **c**) The numbers of liver tumors, the diameters of the largest tumors, and the mean diameters of the liver tumors increased during DEN exposure period (3 months, 2.6 ± 1.0/liver, 0.7 ± 0.2 mm, 0.6 ± 0.2 mm; 4 months, 5.8 ± 0.9/liver, 1.4 ± 0.1 mm, 1.1 ± 0.1 mm; and 5 months, 11.4 ± 1.3/liver, 2.9 ± 0.3 mm, 1.5 ± 0.1 mm, respectively). $n = 13$ (2 months), 13 (3 months), 17 (4 months), and 17 (5 months).

DEN exposure, but was observed after 2 months of exposure and its extent increased thereafter (2 months, 246 ± 128/mm^2; and 3 months, 1597 ± 502/mm^2). To understand the activation status of the inflammatory infiltrates, the production of inflammatory cytokines was evaluated in an RNase protection assay. In wt mice, intrahepatic expression of LT-β, TNF-α, IFN-γ, IFN-β, TGF-β1, and TGF-β3 mRNA appeared after 1 month of DEN exposure and remained elevated until 5 months of exposure (Figure 3). The results suggest unexpectedly that intrahepatic expression of the cytokines
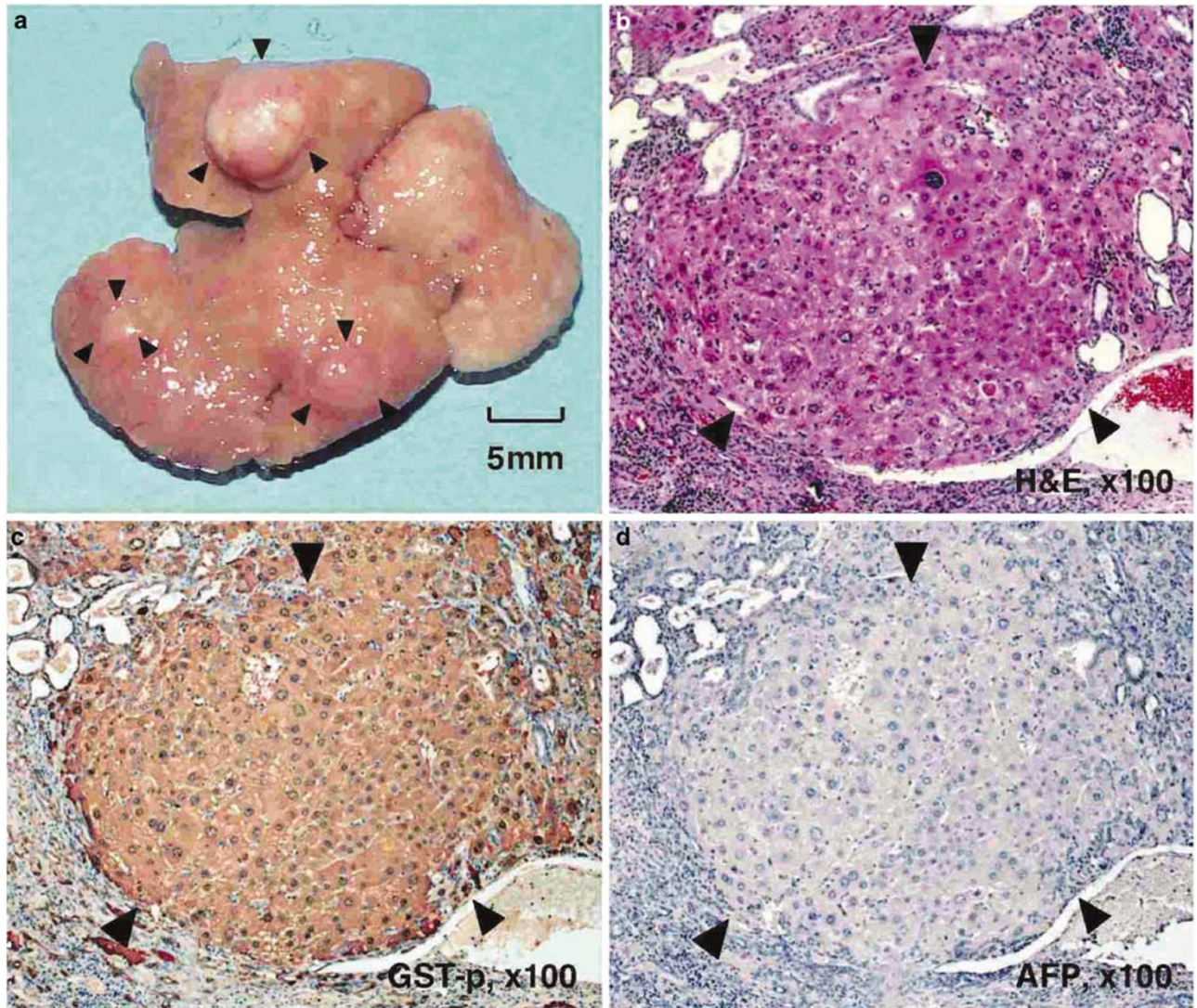
Figure 2 Gross and microscopic evidence of hepatocellular carcinoma (HCC) in wt mice after 5 months of diethylnitrosamine (DEN) exposure. (**a**) A multinodular liver from a representative mouse. Liver nodules are indicated by arrowheads. (**b**) Classical histological features of solid HCC consisting of relatively giant hepatocytes with large hyperchromatic nuclei that display increased mitotic activity, compress the adjacent hepatic parenchyma, and display areas of hemorrhage and necrosis (not shown). Inflammatory infiltrates are observed in the region surrounding the nodule. Hematoxylin and eosin; original magnification × 100. Serial sections of the specimen described in (**b**) were analyzed immunohistochemically for expression of glutathione S-transferase placental form (GST-p) (**c**) and alpha-fetoprotein (AFP) (**d**). Tumor cells in the liver nodule displayed strong cytoplasmic staining in brown for GST-p, while no staining for AFP. (Original magnification: **b–d**, × 100.)

preceded inflammatory cell infiltration and tumor development.

Tumor Development in the Livers of IFN-α/βR KO and IFN-γR KO Mice

Among the cytokines induced prior to inflammatory cell infiltration, type I IFNs are known to inhibit the growth of neoplastic cells by inducing apoptosis[15] and type II IFN is the major proinflammatory cytokine that regulates macrophage function and contributes critically to the establishment of chronic inflammation.[16] To define the role of type I and II

IFNs in the process of chemical hepatocarcinogenesis, IFN-α/βR KO mice, IFN-γR KO mice, and wt mice were treated with DEN and tumor development was monitored. IFN-γR KO mice developed fewer tumors than IFN-α/βR KO or wt mice 5 months after DEN exposure ($P < 0.05$, Figure 4a). However, the sizes in diameter of the largest liver tumors and mean diameters of liver tumors were not significantly different (Figure 4b, c and d) and the histological features were similar among the three lineages. Furthermore, CYP2E1 activity in the liver was measured to evaluate the capacity to activate DEN via members of the CYP450 family of enzymes. After 3 months of DEN exposure, CYP2E1 activities
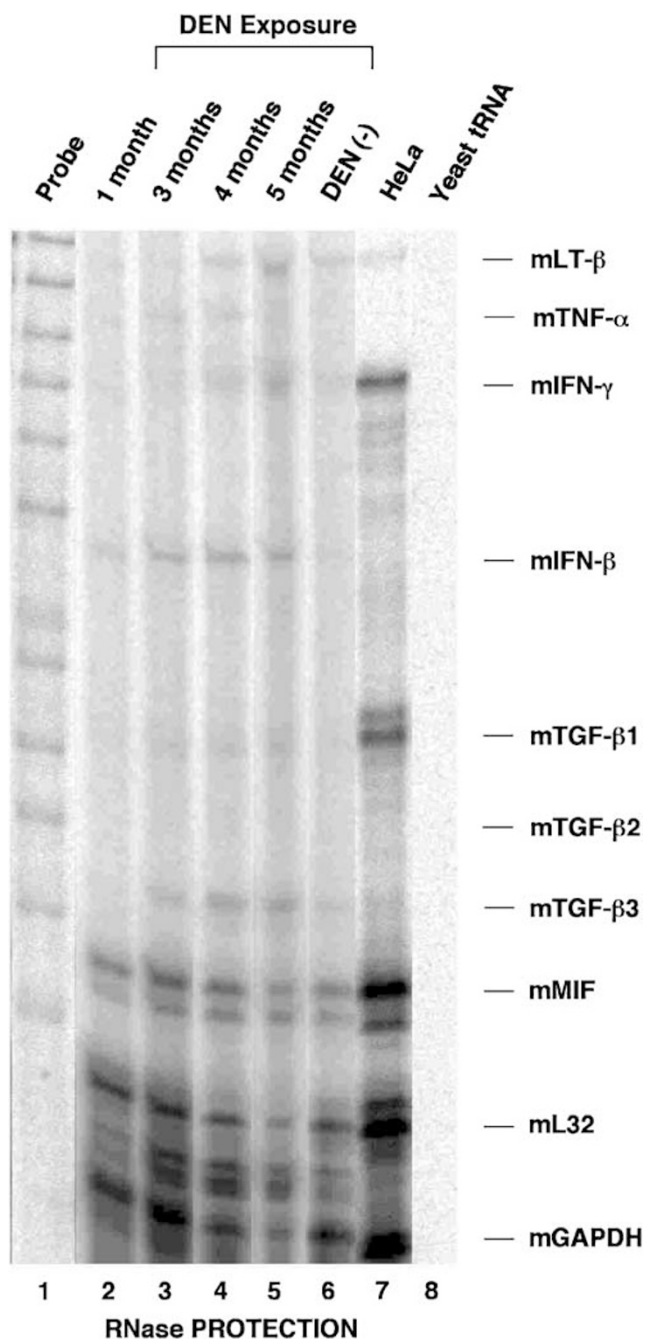
Figure 3 Analysis of intrahepatic cytokine mRNA expression in wt mice after diethylnitrosamine (DEN) exposure. Total RNA (15 μg) was extracted from nontumorous liver tissue of representative mice killed and subjected to RNase protection assay to monitor the expression of a panel of inflammatory cytokines. Intrahepatic LT-β, TNF-α, IFN-γ, IFN-β, TGF-β1, and TGF-β3 mRNA appeared after 1 month of DEN exposure and remained elevated. The housekeeping genes, ribosomal protein light 32 (L32), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to normalize the amount of RNA loaded in each lane.
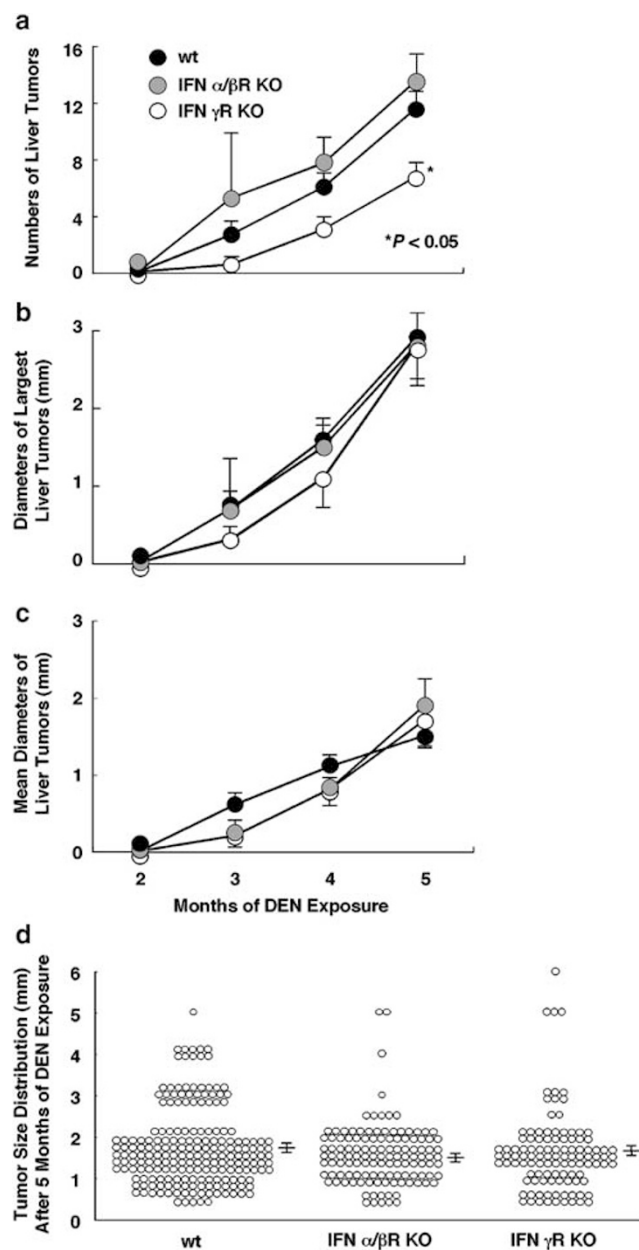

Figure 4 Tumor development in the livers of IFN-γR KO, IFN-α/βR KO, and wt mice exposed to diethylnitrosamine (DEN) in drinking water. Numbers of liver tumors, diameters of the largest liver tumors, and the mean diameters of the liver tumors were determined at autopsy. (**a**) At 5 months after DEN exposure, IFN-γR KO mice developed fewer tumors than IFN-α/βR KO and wt mice ($P < 0.05$). (**b**) The diameters of the largest liver tumors were not significantly different among the three lineages. (**c**) The mean diameters of the liver tumors were not significantly different among the three lineages. (**d**) Tumor size distribution after 5 months of DEN exposure was not significantly different among the three lineages. At 2 months, $n = 13$, 5, and 12 in wt, IFN-α/βR KO, and IFN-γR KO mice, respectively; 3 months, $n = 13$, 3, and 10, respectively; 4 months, $n = 17$, 12 and 15, respectively; 5 months, $n = 17$, 9 and 16, respectively.

were comparable between IFN-γR KO and wt mice (CYP2E1 activities ($\pm$s.d.) (pmol/mg/min): 746.4$\pm$291.7 and 424.1$\pm$76.4, respectively; not significant). In addition, CYP2E1 activities of IFN-γR KO and wt mice without exposure to DEN were

also comparable (CYP2E1 activities ($\pm$s.d.) (pmol/mg/min): 3005.6$\pm$286.6 and 2530.6$\pm$305.0, respectively; not significant). These data suggest that these

strains may have similar capacity to activate DEN in their liver tissues.

Inflammatory Reactions in the Livers of IFN-α/βR KO and IFN-γR KO Mice

To understand the effect of IFNs on inflammatory cell infiltration during the process of chemical hepatocarcinogenesis induced by DEN, liver samples collected from IFN-α/βR KO mice, IFN-γR KO mice, and wt mice were stained and the numbers of infiltrating mononuclear cells were counted. Numbers of infiltrating mononuclear cells were similar among three lineages (3 months; wt mice, $1597 \pm 502/mm^2$; IFN-α/βR KO mice, $640 \pm 280/mm^2$; IFN-γR KO mice, $890 \pm 122/mm^2$; not significant). Furthermore, liver section samples were stained immunohistochemically with a rat monoclonal antibody to mouse macrophages. In wt mice, the proportion of macrophages increased over the period studied (2 months, $13.3 \pm 3.3\%$; 3 months, $16.7 \pm 3.3\%$; 4 months, $20.0 \pm 0.1\%$; and 5 months, $32.5 \pm 10.3\%$). In contrast to IFN-α/βR KO and wt mice, IFN-γR KO mice had reduced proportions of macrophages in the liver (Figure 5). In addition, to evaluate the activation status of inflammatory infiltrates, intrahepatic expression of inflammatory cytokines was analyzed using an RNase protection assay. Intrahepatic expression of TNF-α, IFN-γ, and IFN-β was lower in IFN-γR KO mice than wt mice

(Figure 6). Collectively, the data suggest that IFN-γ-induced activation of macrophages may be involved in the process of DEN-induced chemical hepatocar-
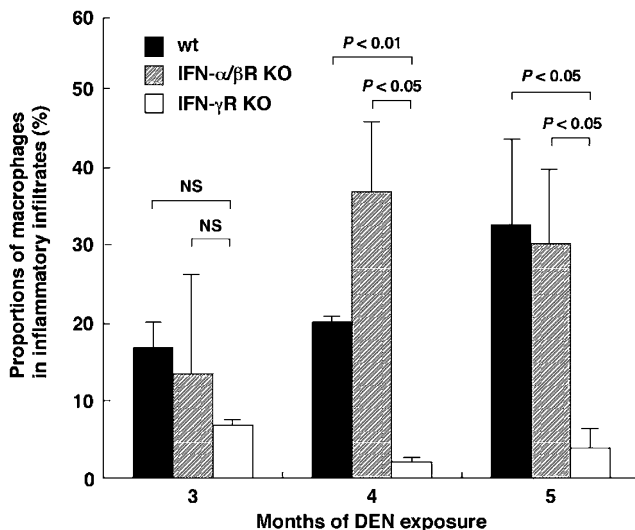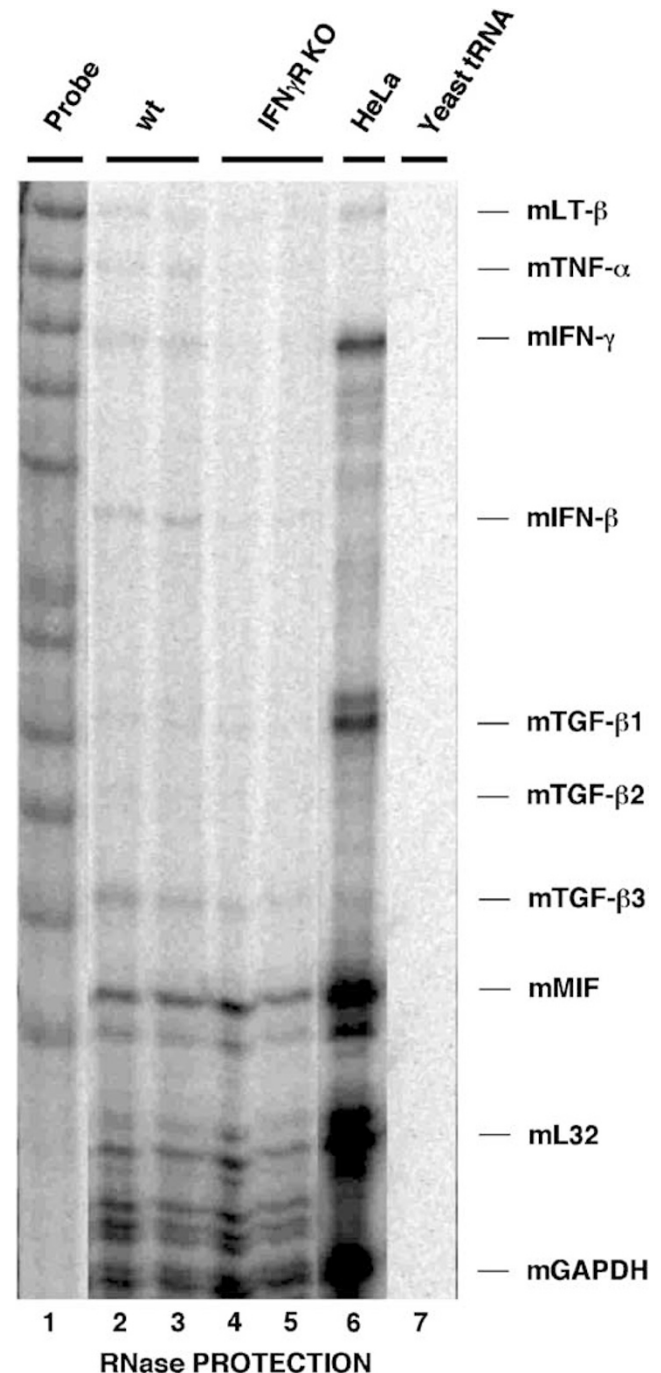

Figure 5 Quantitative immunohistochemical analysis of inflammatory infiltrates in the livers of IFN-γR KO, IFN-α/βR KO, and wt mice after diethylnitrosamine (DEN) exposure. Mice were killed at the time points indicated. Liver samples were stained immunohistochemically with rat monoclonal antibody to mouse macrophage. A total of 100 high-power ($\times 400$) fields representing $4 mm^2$ of liver tissue were examined. The proportions (%) of macrophages are expressed as means $\pm$ s.e. In contrast to IFN-α/βR KO and wt mice, the proportions of macrophages were greatly reduced in IFN-γR KO mice.

Figure 6 Analysis of intrahepatic cytokine mRNA expression in wt and IFN-γR KO mice after 3 months of diethylnitrosamine (DEN) exposure. Total RNA ($15 \mu g$) was extracted from nontumorous liver tissue of representative mice and subjected to RNase protection assay to monitor the expression of a panel of inflammatory cytokines. Intrahepatic expression of LT-β, TNF-α, IFN-γ, IFN-β, and TGF-β3 was weaker in IFN-γR KO mice than in wt mice. The housekeeping genes, ribosomal protein light 32 (L32), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to normalize the amount of RNA loaded in each lane.

cinogenesis because the proportion of infiltrating macrophages was diminished and the activation status was reduced in IFN-γR KO mice, in which the total numbers of liver tumors were lower than wt mice.

Amounts of 8-OHdG Detected in the Liver

To estimate the oxidative DNA damage in chemical hepatocarcinogenesis, genomic DNA was extracted from nontumorous liver tissue of representative mice treated with DEN for 3 months and subjected to measurement of 8-OHdG by ELISA. Lower amounts of 8-OHdG were detected in the livers of IFN-γR KO mice than of IFN-α/βR KO and wt mice (Figure 7). The results indicate that the oxidative DNA damage caused during DEN exposure period was reduced in IFN-γR KO mice, suggesting that IFN-γ may contribute to the enhancement of the oxidative damage and allow cells that carry

damaged DNA to survive in the process of DEN-induced hepatocarcinogenesis.

Discussion

The current study shows that type II IFN, but not type I IFNs, may be involved critically in the initiation stage, but not the promotion stage, of hepatocarcinogenesis in mice treated with DEN. IFN-γR KO mice developed fewer HCCs than IFN-α/βR KO and wt mice, although the diameters of liver tumors were not significantly different among the three lineages. In IFN-γR KO mouse livers, the proportion of monocytes/macrophages was greatly reduced and their activation status was reflected by the lower levels of intrahepatic cytokine expression, suggesting that IFN-γ may contribute to the activation of monocytes/macrophages seen during the process of DEN-induced hepatocarcinogenesis. The activated state of the monocytes/macrophages was
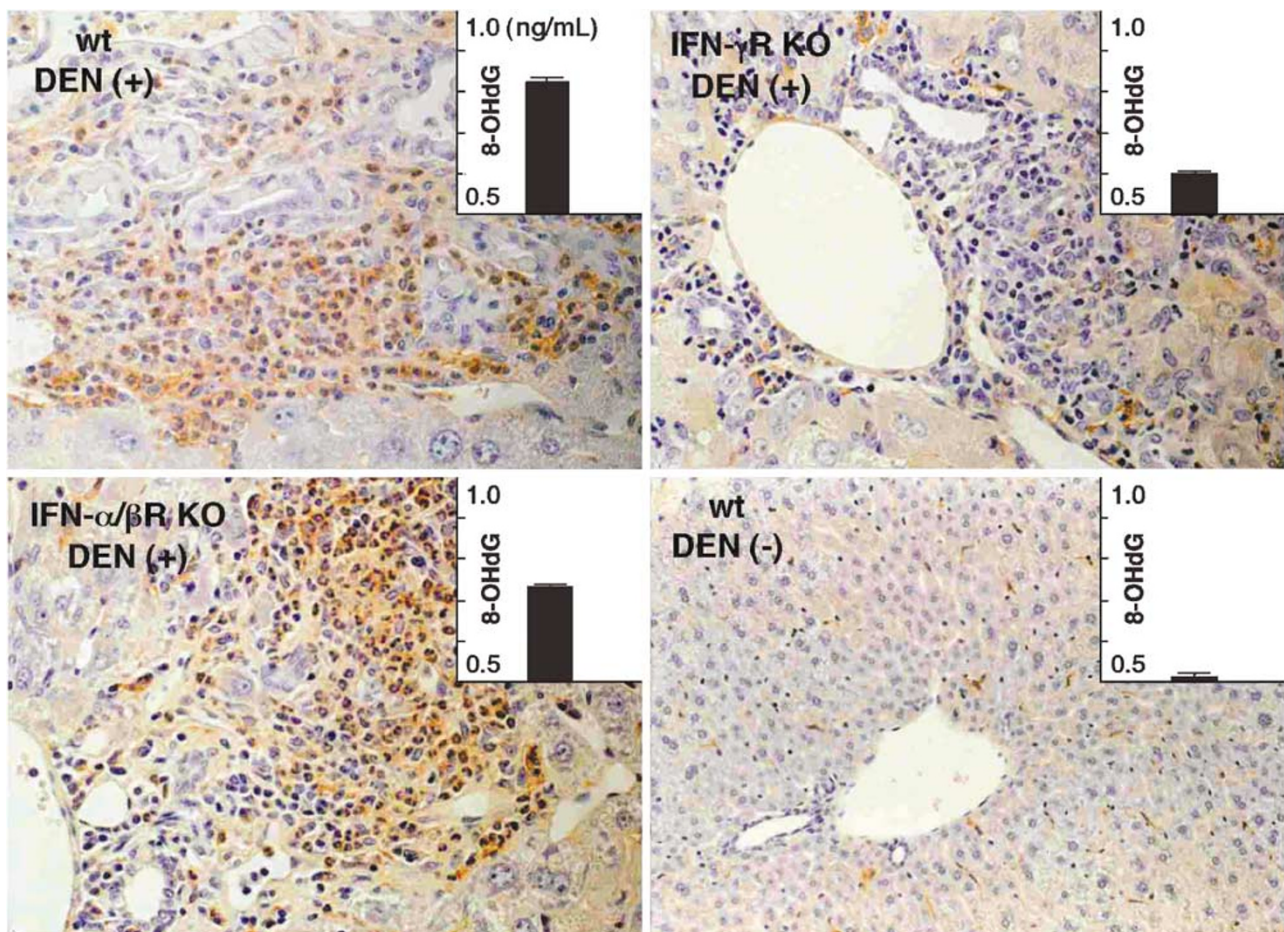

Figure 7 Immunohistochemical analysis of inflammatory infiltrates and 8-hydroxydeoxyguanosine (8-OHdG) amounts detected in the livers of IFN-γR KO, IFN-α/βR KO, and wt mice exposed to diethylnitrosamine (DEN) for 3 months. Liver samples were stained immunohistochemically with rat monoclonal antibody to mouse macrophage (in brown). In contrast to the subsets of infiltrating inflammatory cells in IFN-α/βR KO and wt mice liver, the proportions of macrophages were greatly reduced in IFN-γR KO mice. For measuring 8-OHdG levels, four samples from each strain were used. The amounts of 8-OHdG are expressed as means ± s.e. The amounts of 8-OHdG, indicated in each inset, were in lesser extent in the livers of IFN-γR KO mice than in those of IFN-α/βR KO and wt mice ($P < 0.05$).

reflected by the extent of intrahepatic cytokine expression in the mice. Furthermore, on the basis of amounts of 8-OHdG detected, oxidative DNA damage was induced to a lesser extent in livers of IFN-γR KO mice than in those of IFN-α/βR KO and wt mice, leading to a difference in the mutagenic potential and the development of HCC.

The pathogenetic importance of proinflammatory cytokines in DEN-induced hepatocarcinogenesis was evaluated in this study. Intrahepatic IFN-β and IFN-γ mRNA expression, as well as TNF-α, LT-β, TGF-β1, and TGF-β3, were induced after 1 month of DEN exposure and remained high for the period of study. Importantly, the induction of cytokines began 2 months earlier than the appearance of the liver tumors, suggesting that the immune responses may precede the development of HCC during the process of chemical carcinogenesis. Previous studies demonstrated that type I IFNs inhibit the growth of neoplastic cells by inducing apoptosis[15] and that type II IFN is the major proinflammatory cytokine that regulates macrophage function and contributes critically to the establishment of chronic inflammation.[16] However, the pathophysiological role of the cytokines in DEN-induced hepatocarcinogenesis was not well determined.

To define the pathophysiological role of the IFNs in the process of DEN-induced hepatocarcinogenesis, mice genetically deficient for the IFN-α/β or IFN-γ receptor were exposed to DEN, and tumor development was monitored. The results indicate that IFN-γ, but not IFN-α/β, may be involved critically in the initiation stage, but not the promotion stage, of hepatocarcinogenesis in mice treated with DEN. The results are consistent with the reports in which IFN-γ was suggested to promote tumor initiation of cholangiocarcinoma and lung cancer by causing activation of inducible nitric oxide synthase (iNOS) and excess production of nitric oxide (NO), increased manganese superoxide dismutase (Mn-SOD) and decreased catalase, and subsequent DNA damage.[17,18]

Further, in contrast to the subsets of infiltrating inflammatory cells in IFN-α/βR KO and wt mouse livers, the proportion of monocytes/macrophages was greatly reduced in IFN-γR KO mouse livers. The reduction in the percentage of monocytes/macrophages in inflammatory infiltrates in IFN-γR KO mice reflected a deficiency of IFN-γ stimulation because IFN-γ is known to promote macrophage chemotaxis indirectly and to induce its activation directly. Its indirect chemotactic activity is reported to be mediated by macrophage-attracting chemokines, for example, macrophage inflammatory protein (MIP)-1α and monocyte chemoattractant protein (MCP)-1.[19] Recruited and activated macrophages may induce oxidative DNA damage in hepatocytes, probably by NO production,[20,21] which is consistent with our observation that oxidative DNA damage was induced, on the basis of the amounts of 8-OHdG detected, to a lesser extent in the livers of

IFN-γR KO mice than in those of IFN-α/βR KO and wt mice.

The current observations emphasize the pathogenetic potential of IFN-γ during the course of chemical hepatocarcinogenesis. Interestingly, IFN-γ influences the initiation stage, which is associated with the recruitment/activation of monocytes/macrophages and the induction of oxidative DNA damage in hepatocytes, and not the promotion stage of hepatocarcinogenesis.

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