LIVER DISEASE

Expression of a cyclo-oxygenase-2 transgene in murine liver causes hepatitis

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Background: It has been proved that cyclo-oxygenase-2 (COX-2) is rapidly induced by inflammatory mediators. However, it is not known whether overexpression of COX-2 in the liver is sufficient to promote activation or secretion of inflammatory factors leading to hepatitis.

Aim: To investigate the role forced expression of COX-2 in liver by using inducible COX-2 transgenic (TG) mice.

Methods: TG mice that overexpress the human COX-2 gene in the liver using the liver-specific transthyretin promoter and non-TG littermates were derived and fed the normal diet for up to 12 months. Hepatic prostaglandin E₂ (PGE₂) content was determined using enzyme immunoassay, nuclear factor kappaB (NF-κB) activation by electrophoretic mobility shift assays, apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labelling and proliferation by Ki-67 immunohistochemistry.

Results: COX-2 TG mice exhibited strongly increased COX-2 and PGE₂, elevated serum alanine aminotransferase level and histological hepatitis. Hepatic COX-2 expression in the TG mice resulted in activation of NF-κB and inflammatory cytokine cascade, with a marked expression of the proinflammatory cytokines tumour necrosis factor (TNF)-α (9.4-fold), interleukin (IL)-6 (4.4-fold), IL-1β (3.6-fold), and of the anti-inflammatory cytokine IL-10 (4.4-fold) and chemokine macrophage inflammatory protein-2 (3.2-fold). The inflammatory response of the COX-2 TG mice was associated with infiltration macrophages and lymphocytes, increased cell proliferation and high rates of cell apoptosis. Administration of the COX-2 inhibitor celecoxib in TG mice restored liver histology to normal.

Conclusion: Enhanced COX-2 expression in hepatocytes is sufficient to induce hepatitis by activating NF-κB, stimulating the secretion of proinflammatory cytokines, recruiting macrophage and altering cell kinetics. Inhibition of COX-2 represents a mechanism-based chemopreventive approach to hepatitis.

isoforms of cyclo-oxygenase (COX), constitutive COX-1 and inducible COX-2, catalyse the production of prostaglandins from arachidonic acid.¹ In many tissues, including the liver, COX-1 is expressed constitutively, whereas COX-2 is induced as an immediate-early gene by a variety of stimuli such as growth factors, cytokines, tumour promoters, peroxisomal proliferators and carcinogens. COX-2-induced production of prostanoids is often implicated in inflammatory diseases and carcinogenesis, characterised by recruitment of inflammatory cells and injury of tissue.^{2–5} Thus, COX-2 is thought to act as a proinflammatory emergency enzyme.^{4,5} Previous studies have implicated up-regulation of COX-2 in the livers of patients with chronic virus hepatitis,⁶ cirrhosis⁷ and hepatocellular carcinoma,^{7,8} and shown that COX-2 plays a key proinflammatory role in the evolution of nutritional steatohepatitis in mice.⁹ However, it remains unclear whether COX-2 alone is sufficient to initiate liver inflammation. One way to address this question is to use transgenic (TG) mice that offer a unique opportunity to study tissue-specific effects by expressing putative gene in an in vivo system. In this study, the functional consequences of TG COX-2 expression were achieved specifically in the liver driven by a transthyretin (TTR) promoter. The direct role and potential mechanisms by which COX-2 contributes to the development of hepatitis were elucidated.

MATERIALS AND METHODS

Generation of TG mice

The TTR was kindly provided by Dr Terry van Dyke (University of Pittsburgh, Pittsburgh, Pennsylvania, USA). Briefly, the TTR

exV3 vector was derived from pTTR1¹⁰ by making several point mutations in the first and second exons, which destroy ATGs and introduce unique cloning sites. pTTR1 has been tested extensively in TG mice, and is found to express high transcript levels in the liver (hepatocyte only) in a copy that is number-independent.¹⁰ A 3.2 kb human COX-2 complementary DNA (cDNA) was subcloned downstream of the TTR promoter (fig 1A). Hybrid vigour F1CC mice were generated by crossing CBA/N male with C57BL/6 female mice. Fertilised eggs were obtained from the mating of superovulated F1CC female mice with F1CC male mice. Vector-free human TTR-COX2 was obtained by *StuI* digestion of the plasmid. The DNA fragment was then microinjected into the pronuclei of the fertilised eggs, using a standard microinjection procedure. Surviving zygotes were transferred into the oviducts of foster ICR mothers. TG mice were identified by PCR of the tail DNA. Male founders were then mated with female C57BL/6 mice to derive F1 and F2 hemizygotic TG mice. F2 offspring were used for experiments. For sequential morphological observation in the liver of the TG mice, liver tissue samples were taken at 3, 6, 9 and 12 months of age from at least 4 animals at 3, 6 and 9 months, respectively, and 14 animals at 12 months. Wild-type (WT)

Abbreviations: ALT, alanine aminotransferase; cDNA, complementary DNA; COX, cyclo-oxygenase; IL, interleukin; IP-10, interferon-γ-inducible protein-10; MIP-2, macrophage inflammatory protein-2; NF-κB, nuclear factor-kappaB; PI, proliferation index; PGE₂, prostaglandin E₂; TG, transgenic; TGF-β1, transforming growth factor-β1; TNF-α, tumour necrosis factor-α; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labelling; WT, wild type

littermates were analysed in parallel as controls. Animals were killed under anaesthesia, livers were harvested and weighed, fixed and embedded for histopathological and immunohistochemical examinations. A portion of the liver samples was snap-frozen in liquid nitrogen and stored at -80°C until required. All protocols and procedures were approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong.

Histopathology and immunohistochemistry

H&E-stained sections of paraffin-embedded liver tissue were examined by two independent investigators blinded to the study, and the severity of liver pathology (necroinflammation) was scored as 0, absent; 1, mild; 2, moderate; and 3, severe.

COX-1, COX-2, CD4 for T-helper cells, PAX-5 for B lymphocyte, F4/80 for macrophage staining and Ki-67 for proliferation staining were detected in paraffin-embedded liver sections using the specific antibodies and an avidin-biotin complex immunoperoxidase method. Briefly, endogenous peroxidase activity was blocked by treating sections with 3% hydrogen peroxide. After blocking with 10% non-immunised goat serum, the primary specific antibody for COX-1 and COX-2 (Santa Cruz Biotechnology, Santa Cruz, California, USA), for CD4 (eBioscience, San Diego, California, USA), for PAX-5 (Biocare Medical, Concord, California, USA), for F4/80 (Serotec, Kidlington, Oxford, UK) and for Ki-67 (Abcam, California, USA) were applied. Primary antibodies were omitted and non-immunised goat serum was used for negative controls. After extensive rinsing, the biotinylated secondary antibody and ABCComplex/HRP (Dako A/S, Glostrup, Denmark) were applied. Peroxidase activity was visualised by applying diaminobenzidine to the sections, which were then counterstained with haematoxylin.

Neutrophil staining was performed using the Naphthol AS-D Chloroacetate Esterase Kit (Sigma-Aldrich, St Louis, Missouri, USA), according to the manufacturer's instructions.

Biochemical assays

Serum alanine aminotransferase (ALT) levels were measured using automated techniques in Hong Kong X-ray and Laboratory (Hong Kong).

PGE₂ assay

PGE₂ contents were determined using an enzyme immunoassay kit (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). Briefly, approximately 20 mg of snap-frozen liver tissues were homogenised in 20 volumes of 15% ethanol and centrifuged. The supernatant was then applied to a preprimed Amprep C18 mini column (Amersham Pharmacia Biotech). The quantity of eluted PGE₂ normalised to protein weight in supernatants, was determined using ELISA.

Determination of NF- κ B binding activity

Nuclear factor κ B (NF- κ B) binding activity was determined by electrophoretic mobility shift assay. Briefly, nuclear extracts were prepared by ultrasound disruption of cell membranes, followed by high salt extraction. In total, 10 μ g of nuclear protein was mixed with a double-stranded oligonucleotide corresponding to an NF- κ B binding motif (Cat 14937901; Promega, Madison, Wisconsin, USA) and labelled with [³²P]dATP using oligonucleotide kinase. After binding for 20 min, samples were separated by electrophoresis on non-denaturing 4% polyacrylamide gel and exposed to x ray films.

To confirm that the NF- κ B pathway is active, the quantification of DNA-binding activity of NF- κ B was measured by an ELISA assay, using the NF- κ B p50/p65 Transcription Factor

Figure 1 (A) Scheme of the TTR1-COX2 transgene. The TTR1-COX2 DNA transgene consisting of a transthyretin (TTR) promoter, a human COX-2 cDNA and a TTR1 exV3 genomic sequence. The TTR1 promoter was cloned upstream of the human COX-2 gene, and the resulting construct was used to derive transgenic (TG) mice, as described in the Materials and methods section. (B) Immunohistochemistry for COX-2 in livers of wild-type (WT) and TG mice. (B1) Cyclo-oxygenase-2 (COX-2) was not found in WT mice and liver appearances were normal. (B2). There was strong and diffuse staining for COX-2 in the hepatocytes of TG mice. Liver showed numerous foci of necroinflammation (arrow) (representative sections: $\times 200$ magnification). (C) Effect of COX-2 overexpression on generation of prostaglandin E₂ (PGE₂) in the livers of TG mice. Hepatic PGE₂ was determined (Materials and methods) in triplicate. Values are mean (SD) (WT: n=10; TG: n=14). * $p < 0.001$, TG compared with WT mice; # $p < 0.001$, TG+celecoxib compared with TG mice.

Assay Colorimetric kit (Chemicon, Temecula, California, USA), according to the instructions of the manufacturer.

cDNA synthesis and RT-PCR

Total RNA was extracted from the frozen liver tissues by using RNA Trizol reagent (Invitrogen, Carlsbad, California, USA), according to the manufacturer's protocol. In total, 5 µg of total RNA was reverse transcribed into cDNA. β-Actin served as an internal control for total cDNA content. RNA levels of COX-1, COX-2, inflammatory factors, chemokines and apoptosis-related genes (table 1) were quantified by real-time RT-PCR using SYBRGreen Master Mix (Applied Biosystems, Foster City, California, USA).

Detection of cytokine protein expression

Frozen liver tissues (~60 mg) were homogenised in 1 ml ice-cold protein extraction reagent (Cytobuster, Novagen, San Diego, California, USA) consisting of protease inhibitor cocktail. The homogenates were centrifuged and the supernatants were used for assay. The concentration of cytokines tumour necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL)-12 were calculated with reference to a standard curve using cytometric bead array kits (BD Biosciences, San Diego, California, USA), as described previously.¹¹ Results were

expressed as picograms of cytokine per milligram of tissue, calculated from the known weight of the tissue sample and the volume of lysis buffer in which the sample was homogenised.

Determination of apoptotic index

Terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labelling (TUNEL) assay was performed on paraffin sections (4 mm) with a DeadEnd™ Colorimetric TUNEL System (Promega) according to the manufacturer's instructions. Sections were counterstained with haematoxylin. The percentage of apoptotic cells was calculated from randomly selected fields. At least 1000 cells were counted in five random fields, and the percentage of TUNEL-positive cells was then calculated (apoptotic index).

Determination of proliferation index (PI)

Proliferation was assayed by immunoperoxidase staining for Ki-67 as described above. At least 1000 cells were counted in five random fields, and the PI was expressed as a percentage of the ratio of Ki-67-positive nuclei to the total nuclei counted.

Treatments of TG mice with celecoxib

To study the effect of COX-2 inhibition on the transgene-induced phenotype, we administered the COX-2-selective inhibitor celecoxib (1500 ppm) in the diet for 4 weeks at a dose previously shown to inhibit PGE₂ synthesis in murine epidermis.⁹

Statistics

Data were presented as mean (SD). Results from experiments were analysed by the Student's t-test. A two-sided p value of <0.05 was considered statistically significant.

RESULTS

COX-2 is specifically expressed in the liver of TG mice

To achieve forced expression of COX-2 specifically in the liver, we generated TG C57BL6/J mice carrying the human isoform of COX-2 under the control of the TTR promoter (fig 1A). Tissue specificity of transgene expression of COX-2 was examined by isolating total RNAs from various organs (liver, oesophagus, stomach, duodenum, intestine, colon, pancreas, gall bladder, lung, brain, kidney, testis, lymph node, cardiac muscles, smooth muscle, trachea, skin, thymus and blood) of TG offsprings. As expected, human COX-2 transgene mRNA was only expressed in the liver. COX-2 mRNA was barely detectable in livers of WT mice, but forced COX-2 mRNA was approximately 4000-fold (3919 (2034) vs 1 (1.2), p<0.001) increased in the livers of TG mice.

COX-2 protein was noted only occasionally in hepatocytes in livers from WT mice (fig 1B1), whereas strong and intense COX-2 immunoreactivity was detected in hepatocytes from TG mice (fig 1B2). Hepatocyte COX-2 immunostaining was found primarily in the cytoplasm. However, COX-1 mRNA and protein expression were similar in livers from TG and WT (fig 2).

PGE₂ levels were elevated in the liver of TG mice

Because COX-2 is an enzyme with a measurable product, we sought to confirm its functional activity by measuring its downstream metabolite, PGE₂. The elevated expression of COX-2 resulted in a 27-fold increase in PGE₂ contents in the livers of COX-2 TG mice compared with WT controls (fig 1C), indicating that COX-2 protein was enzymatically active.

COX-2 TG mice develop hepatitis

Up to 3 months of age, no recognisable histological change was observed in the liver of TG mice in comparison with WT littermates. During 6–9 months, necroinflammation began to

Table 1 Primers for quantitative real-time PCR analysis

Gene	Genbank accession no	Primer sequence
Bcl-2	NM_009741	F 5'-CCTGTGGATGACTGAGTACC-3' R 5'-GAGACAGCCAGGAGAAAT-3'
Caspase 3	NM_009810	F 5'-AGCAGCTTTGTGTGTGATTCT-3' R 5'-AGTTTCGGCTTCCAGTCAGAC-3'
COX-1	BC023322	F 5'-GTCATCAAGGAGTCCCAG-3' R 5'-CCAGTTCTTCAGTGAGGC-3'
COX-2	NM_011198	F 5'-CACACTATCACTGCGACC-3' R 5'-CTCTCTGCTCTGGTCAATGG-3'
Fas	NM_007987	F 5'-GCAAACAGACTTCTACTGCG-3' R 5'-TTTGATTGCTGGTTGCTGTG-3'
Fas ligand	NM_010177	F 5'-CAGCAGTGCCACTTCATCTTG-3' R 5'-TCCTTGGTCCGGCCCTCT-3'
GADPH	XM132897	F 5'-AACGACCCCTTCATTGAC-3' R 5'-TCCACGACATACTCAGCA-3'
ICAM-1	NM010493	F 5'-CCTGCCTCTGAAGCTCGGAT-3' R 5'-ACAGGAACCTTCCCGCCACC-3'
IFN-γ	AK089852	F 5'-TGGCTCTGCAGGATTTTCATG-3' R 5'-TCAAGTGGCATAGATGTGGAAG-3'
IL-1β	NM_008361	F 5'-TCAGGCAGGCAGTATCACTATT-3' R 5'-GGAAGGTCCACGGGAAAGA-3'
IL-6	NM031168	F 5'-ACAACCACGGCCTTCCCTACT-3' R 5'-GTGTAATTAAGCCTCCGACT-3'
IL-10	NM_010548	F 5'-CTTGCACTACCAAGCCACA-3' R 5'-TAAGAGCAGGCAGCATAGCA-3'
IL-12	NM_008352	F 5'-AGCAGTAGCAGTTCCTCGA-3' R 5'-CGGCAGTTGGCAGGTGACA-3'
IP-10	NM_021274	F 5'-AAGTGCTGCGCTCATTTCT-3' R 5'-GTGGCAATGATCTCAACACG-3'
MIG	NM_008599	F 5'-TGAATTCCTTGCCACCTTC-3' R 5'-GCCCTGATCTTCCATTCA-3'
MIP-2	NM_009140	F 5'-ATCCAGAGCTTGAGTGTGACGC-3' R 5'-AAGGCAAACTTTTGACCGCC-3'
LIX	U27267	F 5'-CTCGCCATTATGCGGAT-3' R 5'-CTTCAGCTAGATGCTGCGGC-3'
TGF-β1	NM_011577	F 5'-GGTAACCGGCTGCTGACC-3' R 5'-GCCCTGTATTCCGTCTCTCTT-3'
TNF-α	M13049	F 5'-CGTGCTCTCACCCACAC-3' R 5'-GGGTCATACCAGGGTTGA-3'

COX, cyclo-oxygenase; GADPH, glyceraldehyde-3-phosphate dehydrogenase; ICAM, intercellular adhesion molecule; IFN, interferon; IL, interleukin; IP-10, interferon-γ-inducible protein-10; LIX, lipopolysaccharide-induced CXC chemokine; MIP-2, macrophage inflammatory protein-2; MIG, monokine-induced by γ-interferon; TGF, transforming growth factor; TNF, tumour necrosis factor.

Figure 2 Hepatic cyclo-oxygenase-1 (COX-1) mRNA and protein expression in COX-2 transgenic (TG) mice and wild-type (WT) mice. (A) COX-1 mRNA levels were determined by real-time PCR, normalised to glyceraldehyde-3-phosphate dehydrogenase, and are expressed as fold induction (arbitrary unit). Data are mean (SD; WT: n = 10; TG: n = 14). (B) COX-1 protein expression was highly and almost equally expressed in WT (B2) and TG (B3) mice. B1 is negative control, and phosphate-buffered saline was used in place of the primary antibodies.

appear randomly in the lobules. This change became prominent in 12 months, reflected in the serum transaminase level. A marked elevation of serum ALT was observed only in 12-month-old TG mice as compared with the WT mice (mean (SD), 137 (129) vs 57 (8) IU/L, $p < 0.05$). Histological examination of COX-2-expressing livers had revealed mostly moderate spontaneous hepatic inflammation, but without signs of fibrosis. Inflammatory cells were recruited and arranged in inflammatory foci within COX-2-expressing areas (fig 1B2). In contrast, inflammatory infiltrates were absent from WT mouse livers (fig 1B1). Therefore, overexpression of COX-2 in the liver is associated with the development of inflammation. TG mice did not show any gross or histopathological changes in other organs, including lymphoid tissues, kidneys, stomach, pancreas, bladder, intestine, brain, heart or lungs.

NF- κ B was activated in the liver of COX-2 TG mice

To determine the effect of forced expression of COX-2 on the inflammatory mediator that caused hepatic inflammation, we examined the activation of transcription factor NF- κ B by electrophoretic mobility shift assay (fig 3A) and confirmed it by an ELISA-based assay (fig 3B). The nuclear translocation of NF- κ B subunits p50 and p65 was increased in the livers of COX-2 TG mice, but not in the livers of WT mice (fig 3). Thus, COX-2 produced a striking translocation of NF- κ B p50 and p65 from the cytoplasm to the nucleus in the livers of COX-2 TG mice.

Inflammatory cytokines, chemokines and growth factors are induced in the liver of COX-2 TG mice

We next determined mRNA levels for various proinflammatory cytokines, chemokines and growth factor (transforming growth factor- β 1 (TGF- β 1)) in the liver tissues. Among them, the levels

of cytokines TNF- α , IFN- γ , IL-1 β , IL-6 and IL-10 and chemokine macrophage inflammatory protein-2 (MIP-2) were increased significantly in TG mice compared with that in the WT mice (fig 4). In contrast, there was no significant increase in hepatic mRNA levels of IL-12, TGF- β 1 and IFN- γ -inducible chemokines IP-10 and MIG (monokine induced by γ -interferon) in TG mice (fig 4). Similarly, the results of protein analysis confirmed that TNF- α (3.5 (2.2) vs 1.6 (1.1) pg/mg tissue, $p < 0.05$), IFN- γ (3.7 (3.6) vs 0.4 (0.8) pg/mg tissue, $p < 0.05$), but not IL-12 (0.8 (0.7) vs 0.6 (0.5) pg/mg tissue) protein, were expressed at higher levels in the livers of TG mice than in liver tissues of WT mice.

Increased recruitment of macrophages and lymphocytes in the liver of COX-2 TG mice

The presence of inflammatory cytokines in COX-2-expressing liver tissue may result in attraction of macrophages and neutrophils, which are well known to respond to these mediators. In the WT mice, no or few macrophages were observed in the liver (fig 5A1). In contrast, a heavy macrophage infiltration was detected in the livers of 12-month-old TG mice (fig 5A2), but infiltration of macrophages at early ages (3, 6 and 9 months) in TG mouse livers was not apparent. Furthermore, we found that macrophages were mainly present in the inflammatory foci adjacent to the COX-2-producing hepatocytes. This suggested that COX-2 may be involved in the recruitment of macrophages in the TG liver. The macrophages in the inflammatory sites were the infiltrating blood-borne monocytes/macrophages or Kupffer cells, or a mix of both. On the contrary, there were no neutrophils in the livers of WT mice (fig 5B1) but only a slight increase in the numbers of neutrophils in the inflammatory foci of TG mice (fig 5B2). The recruitment of lymphocytes to the liver was assessed using

Figure 3 Activation of nuclear factor (NF)- κ B in cyclo-oxygenase-2 (COX-2) transgenic (TG) mice. (A) Elevated levels of NF- κ B p50 and p65 DNA-binding activity were detected in TG mice, but not in wild-type (WT) mice, using electrophoretic mobility shift assay (EMSA). (B) NF- κ B p50 and p65 activities determined by using ELISA-based assay. The specific nuclear p50 and p65 binding activity was quantified using different antibodies. Similar to EMSA, activation of NF- κ B p50 and NF- κ B p65 was significantly higher in COX-2 TG mice than in WT mice. Data are mean (SD); * $p < 0.05$ for TG mice compared with WT. OD, optical density.

immunohistochemical analysis; TG livers contained massive infiltration of Hax-5+ B lymphocytes (fig 5D), with lesser numbers of CD4 helper T cells (fig 5C).

Increased liver cell apoptosis is evident in the TG livers

We further investigated whether COX-2-induced hepatitis is caused by necrosis or apoptosis. As shown in fig 6, many TUNEL-positive cells were found in the livers of 12-month-old TG mice (fig 6A2), whereas only a few TUNEL-positive nuclei were noted in the liver sections from WT mice (fig 6A1). Consistent with this, the expression of Fas ligand and caspase 3 transcripts was significantly increased in the liver tissue from TG mice but not in the livers of WT mice (fig 6B). Quantitation of TUNEL-positive cells from 3- to 12-month-old TG mice revealed a significant increase in mean AI in the liver sections of TG compared with those of WT mice at the age of 6 months, and a further increase was observed thereafter (fig 6C).

Cell proliferation is induced in the TG mice

The cell proliferation rate was determined by Ki-67 staining. In the liver of WT mice, proliferating cells were detected only occasionally (fig 7A1). In the TG mice, however, the Ki-67 labelled cells were found frequently in the liver (fig 7A2). The PI in the liver of TG mice was three times higher than that in the WT mice (fig 7B).

Administration of a COX-2 inhibitor restores liver histology

When TG mice were fed the COX-2-selective inhibitor celecoxib for 4 weeks, expression of COX-2 was blunted (fig 1B3) and PGE₂ accumulation in liver was reduced to WT levels (fig 1C). Moreover, the transgene-dependent phenotype for the hepatic inflammation—that is, ALT level (66 (8) U/l) and hepatic necroinflammation—was restored to normal under celecoxib treatment (fig 1B3).

Figure 4 mRNA levels for cytokines, chemokines and growth factor in liver tissues. Quantitative analysis of real-time PCR products indicated that the mRNA levels for mediators of tumour necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, interferon- γ (IFN- γ), IL-10 and macrophage inflammatory protein-2 (MIP-2) were dramatically increased in cyclo-oxygenase-2 transgenic (TG) mice than in wild-type (WT) mice. β -Actin was used as the internal standard. Each column represents mean (SD) (arbitrary unit), * $p < 0.05$. TGF, transforming growth factor- β ; IP-10, interferon- γ -inducible protein-10; MIG, monokine-induced by γ -interferon; LIX, lipopolysaccharide-induced CXC chemokine.

Figure 5 (A) Hepatic macrophages and lymphocytes were recruited in transgenic (TG) mice. (A1) There is no macrophage in livers from wild-type (WT) mice. (A2) Liver sections from TG mice showed abundant F4/80-positive macrophages (arrows), many of which were present in inflammatory foci (representative sections: $\times 200$ magnification). (B) Hepatic neutrophils were increased in TG mice. (B1) None of the neutrophils was found in the livers from WT mice. (B2) There were few neutrophils (arrows) in the livers of TG mice (representative sections: $\times 200$ magnification). (C) The recruitment of CD4 T cells and (D) massive infiltration of Hax-5 B lymphocytes in TG mice (representative sections: $\times 200$ magnification).

DISCUSSION

We demonstrated that forced local production of COX-2 is sufficient to initiate a liver-specific inflammatory disease. TG mice showed elevation of serum transaminases, indicating the presence of liver cell damage. Histological analysis revealed the evidence of chronic active hepatitis with necroinflammatory change, together with inflammatory cell infiltration, at the COX-2-rich area within the liver. Feeding TG mice with the selective COX-2 inhibitor (celecoxib) completely restored liver histology and ALT levels. The results confirm that the development of the TG phenotype was causally related to COX-2 and COX-2-dependent PGE₂ synthesis. However, the pathological changes in the liver were modest. Specifically, fibrosis is lacking in TG mice compared with humans. It is possible that inflammation is mainly induced by COX-2, but fibrosis will require another mediator, as COX-2 suppression does not prevent fibrosis.¹² Alternatively, this could be due to the remarkable regenerative capacity of mouse liver cells.

In our study, a significant increase in PGE₂ levels was observed in the livers of TG mice compared with WT mice. Production of PGE₂, a representative COX-2 metabolite, is stimulated by forced COX-2 expression, because COX-1 level was not changed in both WT and TG strains. COX-2-derived prostanoids are likely to play a role in the cascade of events that

lead to the inflammation of liver in the TG mice, as PGE₂ has been reported to act as a proinflammatory factor in many aspects: to recruit macrophage infiltration in liver¹³ and in gastric mucosa,¹⁴ to modulate cytokine expression in liver¹⁵ and in other tissues,¹⁵⁻¹⁷ and to stimulate proliferation of hepatocytes.¹⁸ Thus, COX-2-induced PG production could be a causative factor in the development of hepatitis. Further analysis of the functions of prostaglandins present in COX-2 liver tissue will be necessary to clarify the mechanisms behind the observed inflammatory response. As macrophages are found adjacent to COX-2-expressing cells, it is conceivable that COX-2 and/or COX-2-derived PGE₂ may be the key factors for chemotaxis of macrophage in TG mice. It was reported that increased PGE₂ levels in the liver enhances recruitment of macrophages derived from monocytes.¹³ Activated macrophages in the inflammatory sites may supply cytokines and growth factors to the liver cells,^{14, 19} to set up the cascade of inflammation in the liver. It appears that COX-2/PGE₂-dependent macrophage infiltration, together with cytokine signals, is important for inflammation and liver injury in this model.

It remains to be determined as to which inflammatory factors are most important for the development of COX-2-induced hepatitis. NF- κ B activation plays a critical role in the

Figure 6 Assessment of apoptosis in liver tissues. (A1) Fewer apoptotic cells were observed in the wild-type (WT) mice. (A2) Livers from 12-month-old transgenic (TG) mice had a prominent population of cells with condensation or fragmentation of nuclei and cytoplasm shrinkage (arrows), indicating apoptosis ($\times 200$ magnification). (B) An increased expression of apoptotic genes Fas ligand and caspase 3 was evident in TG mice, compared with 12-month-old WT mice. (C) An increase in the percentage of terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labelling-positive cells (arrows) is observed in TG mice at 6, 9 and 12 months of age. β -Actin was used as the internal standard. Data are mean (SD); * $p < 0.05$, ** $p < 0.001$ for TG compared with WT mice.

pathogenesis of liver inflammation by controlling the coordinated expression of genes involved in inflammation, apoptosis and tissue remodelling.^{9 20 21} NF- κ B might affect production of COX-2-derived proinflammatory mediators by binding to the promoter region of COX-2, which contains two NF- κ B motifs.^{22 23} However, we demonstrated here that forced expression of the COX-2 in liver resulted in nuclear translocation of NF- κ B. Whether this is a direct or an indirect action remains to be determined. However, this activation was critically dependent on the signalling function of COX-2. Our results suggest that, in vivo, forced COX-2 expression can have a downstream

effect on signal generation to induce the activation of NF- κ B. Activated NF- κ B may enhance inflammation and cell proliferation by inducing cytokines, chemokines and growth factors, leading to liver injury.^{9 24–26}

To further elucidate how the inflammatory response during hepatitis may be initiated in the forced expression of COX-2, we assessed the macrophage-derived production of proinflammatory and anti-inflammatory cytokines. We found significant increase in the levels of proinflammatory cytokines (TNF- α , IL-1 β , IL-6 and IFN- γ) and anti-inflammatory cytokine IL-10 in livers from COX-2 TG mice. The level of TNF- α was grossly

Figure 7 Assessment of proliferation in liver tissues. (A1) Few Ki-67-positive cells were seen in the wild-type (WT) mice. (A2) An increase in the percentage of Ki-67-positive cells (arrows) was seen in transgenic (TG) mice ($\times 200$ magnification). (B) An increased cell proliferation was seen in TG mice than in WT mice. Data are mean (SD); * $p < 0.001$ for TG compared with WT mice.

elevated among the cytokines investigated. TNF- α has been shown to be involved in many different processes, including inflammation, cell proliferation and apoptosis. TNF- α expression has been documented in a variety of liver inflammatory injuries.^{27–29} Thus, it is conceivable that TNF- α contributes to the inflammatory process in COX-2 TG mice. Moreover, TNF- α may also induce liver injury by mediating neutrophils.³⁰ IL-1 β is a proinflammatory cytokine that facilitates the activation of T cells.³¹ IL-6 is a major cytokine that influences the growth of B cells.³² IFN- γ is a cytokine involved in the activation of macrophages to produce TNF- α .^{33–34} The anti-inflammatory cytokine IL-10 is well known to be produced by macrophages.³⁵ IL-10 has been shown to inhibit inflammatory cytokine production via inhibition of NF- κ B function.^{22–36} TGF- β 1 is a potent stimulator of fibroblast proliferation.³⁷ However, there was no increase in TGF- β 1 expression in the COX-2 TG mouse. Thus, although the liver of this TG mouse is chronically injured, liver fibrosis was not prominent in this model.

The chemokine MIP-2 (CXCL2) is an NF- κ B-responsive gene,³⁸ and is considered the mouse functional equivalent of human IL-8. Several studies have reported that expression of MIP-2 was elevated in inflammatory liver injury.^{39–40} We found that the mRNA levels of MIP-2 were significantly increased in COX-2 TG mice compared with WT mice (fig 4), indicating that MIP-2 was implicated as a mediator of hepatic inflammation in this COX-2 TG model. However, other CXC chemokines, including IFN- γ -mediated chemokines (MIG, IP-10) and lipopolysaccharide-induced CXC chemokine, were not induced in this TG model, suggesting that COX-2-mediated hepatitis is not through enhancing CXC chemokines (MIG, IP-10 and lipopolysaccharide-induced CXC chemokine), which may explain the modest damage in this model. To sum up, transcription of COX-2 induces NF- κ B activation, upregulation of TNF- α and other inflammatory cytokines (IL-1 β , IL-6 and TNF- α) and chemokine MIP-2, but does not induce TGF- β 1-induced hepatic stellate cell activation and fibrogenesis. In addition, COX-2 induced inflammation may not alter Th1/Th2 cytokine balance in the liver, as both Th1 cytokine (TNF- α and IFN- γ) and Th2 cytokine (IL-10) are upregulated in the livers of TG mice.

Apart from the striking alterations of cytokines, COX-2 TG mice were associated with a dramatic increase in cell apoptosis, which was consistent with the apparent inflammation. In addition, the expression of the apoptotic genes Fas ligand and caspase 3 were elevated, which coincided with increased COX-2 and PGE₂. Thus, COX-2/PGE₂ may regulate the expression of these death-regulatory genes, and thus induce apoptosis and contribute to inflammation.^{41–43} On the other hand, PGE₂, IFN- γ and TNF- α were shown to stimulate proliferation via growth factors in a number of tissues and cell types.^{44–47} Indeed, the liver of COX-2 TG mice exhibited a significant increase in cell proliferation. Our observations suggest that local COX-2-mediated PGE₂ production triggers cell proliferation.

COX-2, PGE₂, macrophage infiltration and inflammatory factor upregulation and cell kinetics are often closely linked to each other, and it is difficult to establish a causal relationship between the inflammatory events and the apoptosis. However, due to the presence of higher levels of apoptotic cells in the liver of COX-2 TG mice at an early age of 6 months, and enhanced macrophage infiltration being apparently late, at 12 months, it is conceivable that macrophage infiltrations are positively secondary to changes in the liver apoptosis. In our COX-2 TG mouse model, it is likely that COX-2-generated PGE₂ promotes cellular injury, death or apoptosis.^{13–18–42} Cell apoptosis and PGE₂ may in turn stimulate the production of cellular mediators causing inflammation and macrophage recruitment,⁴⁸ which further contribute to the activation of proinflammatory cytokine cascade and cell

proliferation. The cytokine upregulation might facilitate damage of further tissues and promote inflammation. The recruitment of helper T cells may play a role in cell-to-cell interactions and in releasing IFN- γ and TNF- α ,⁴⁹ whereas the massive infiltration of B lymphocytes may be involved in producing inflammatory cytokines and contribute to hepatocyte injury in COX-2-mediated hepatitis.⁵⁰

In conclusion, results from our study suggest that TG overexpression of COX-2 in itself is sufficient to cause hepatitis in the absence of noxious stimuli. This is the first piece of evidence for a new insight into the functional role of COX-2 in triggering liver inflammation. These findings support the notion that COX-2 plays a critical role in inflammatory liver diseases and may be an important therapeutic target in preventing liver injury.

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EDITOR'S QUIZ: GI SNAPSHOT

Answer

From question on page 957

The diagnosis is liver, spleen and renal infarction caused by bacterial endocarditis. The patient had infarctions involving multiple organs including the brain, liver, spleen and kidney, suggesting the presence of cardiogenic embolism. Echocardiography confirmed the presence of mitral valve vegetation and four sets of blood cultures yielded *Staphylococcus aureus*. Despite antibiotic therapy, the patient died of multiple organ failure 1 week after admission.

Unlike splenic and renal infarction, infarction of the liver is rare and is usually diagnosed at autopsy before the wide use of cross-sectional image modalities such as CT or MRI. A common cause of liver infarction is hepatic artery thrombosis following transplantation, hepatic artery injury during laparoscopic cholecystectomy, hepatic artery disruption during motor vehicle injury or complications following transcatheter embolisation of hepatic tumours. Rarely, pre-eclampsia, shock or polyarteritis nodosa have been reported as a rare cause of liver infarction. Infarctions involving multiple organs in this case lead to the diagnosis of endocarditis despite its atypical presentation. Patients with liver infarction may have abdominal pain, fever, chills or abnormal liver function. Ultrasound examination of the liver may show poorly defined inhomogeneous or hypoechoic zones. CT scan typically discloses a well-circumscribed, peripheral and wedge-shaped lesion. Management of liver infarction requires correction of the underlying disease. Serial CT or ultrasound examinations are usually required to monitor its regression or treat infectious complications.

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