Increased intrahepatic cyclooxygenase 2, matrix metalloproteinase 2, and matrix metalloproteinase 9 expression is associated with progressive liver disease in chronic hepatitis C virus infection: role of viral core and NS5A proteins

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Cyclooxygenases catalyse the first step in the biosynthesis of various prostaglandins (PG) and thromboxanes.1 2 There are two cyclooxygenase (COX) isoforms: COX-1 is constitutively expressed in a number of cell types and is involved in the homeostatic functions of PG whereas COX-2 is induced by a variety of proinflammatory stimuli, such as cytokines and lipopolysaccharide (LPS).3 10 COX-2 has been implicated in inflammation, fibrogenesis, and carcinogenesis.7 11 In this regard, different authors have reported that increased COX-2 expression and PG production may contribute to liver damage and tumorigenesis in distinct animal models11 as well as to fibrosis and hepatocellular carcinoma (HCC) in humans.11 Moreover, in vitro pharmacological inhibition of COX-2 has been shown to be effective in modulating hepatic stellate cell activation14 and inhibiting proliferation of human hepatoma cells,15 suggesting a potential therapeutic role for this strategy in chronic liver diseases.

Hepatitis C virus (HCV) infection is the most frequent cause of chronic liver disease in Western countries16 and 20–30% of these patients will develop cirrhosis with the risk of HCC.17 As HCC occurs mainly in patients with cirrhosis,18 a common pathogenic basis at the molecular level between hepatic fibrogenesis and carcinogenesis must exist. A potential link may be activation of the proteolytic enzymes termed matrix metalloproteinases (MMPs) which play a central role in extracellular matrix remodelling and are involved in a variety of pathophysiological processes, such as fibrogenesis and carcinogenesis.19 The clinical relevance of these MMPs, in particular MMP-2 and MMP-9, has been recently highlighted by the fact that increased hepatic gelatinase levels were associated with the fibrotic index in patients with HCV induced liver disease and with tumour progression and recurrence in HCC patients.20 21 In this context, we have recently shown that COX-2 expression promotes the release of MMP-2 and MMP-9 in rat fetal liver cell lines.22

Abbreviations: COX, cyclooxygenase; CT, controls; DFU, 5,5-dimethyl-3(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(1H)-furanone; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LPS, lipopolysaccharide; MCH, mild chronic hepatitis; MMPs, matrix metalloproteinases; NOS, nitric oxide synthase; PG, prostaglandin; PMA, phorbol myristate acetate; RT-PCR, reverse transcription-polymerase chain reaction; SCH, severe chronic hepatitis; SLC, sinusoidal lining cells; MNC, mononuclear cells; HBsAg, hepatitis B surface antigen; HIV, human immunodeficiency virus; TNF-a, tumour necrosis factor a; IL, interleukin; CK, cytokine mixture; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; CIR, cirrhosis
suggesting that COX-2 may play a pivotal role in the secretion of active MMPs by liver cells.

The aims of this work were to determine the expression pattern of COX-2 and MMPs in liver tissue from patients with HCV induced chronic liver disease, and to assess whether HCV proteins induce COX-2 and MMP-9 expression in cultured human hepatocyte derived cells.

METHODS

Patient characteristics

The study included 32 patients with biochemical, virological, and histological findings compatible, according to internationally accepted criteria, with HCV related chronic liver disease (27 with chronic hepatitis and five with end stage cirrhosis). Baseline characteristics of HCV patients are shown in Table 1. In addition, six non-alcoholic patients with isolated steatosis and four patients with histologically normal liver were also studied and used as controls (CT). These CT subjects were seronegative for antibodies to HCV, and all HCV+ and HCV− patients included in this study were seronegative for hepatitis B surface antigen (HBsAg) and for antibodies to human immunodeficiency virus (HIV). Written informed consent was obtained from each patient, and approval for the study was granted by the institutional ethics committee.

Viral markers

HBsAg and antibodies to HIV were determined by commercially available enzyme immunoassay kits (Abbott Laboratories, North Chicago, Illinois, USA). Antibodies to HCV were detected using a commercially available second generation enzyme linked immunoassay kit (Ortho Diagnostic Systems, Raritan, New Jersey, USA). HCV RNA was detected by a nested polymerase chain reaction technique using primers from the highly conserved 5′ non-coding region of the HCV genome, as described elsewhere.24

Chemicals

LPS from Salmonella typhimurium and cytokines (tumour necrosis factor α (TNF-α) and interleukin (IL)-1β) were from Sigma Chemical Co. (St Louis, Missouri, USA) and Roche Diagnostics (Mannheim, Germany). Antibodies were obtained from Santa Cruz Laboratories (Santa Cruz, California, USA) and Cayman Chemical (Ann Arbor, Michigan, USA). DFU [(5, 5-dimethyl-3-(3-fluorophenyl)-4-(4-methyl sulphonyl)phosphoryl]-2(5H)-furanone] is a highly selective COX-2 inhibitor25 from Merck (Rahway, New York, USA). Reagents for electrophoresis were obtained from Bio-Rad (Hercules, California, USA). Tissue culture dishes were from Falcon (Lincoln Park, New Jersey, USA). Tissue culture media were from BioWhittaker (Walkersville, Maryland, USA).

DNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA of liver biopsy samples from all HCV+ and HCV− patients included in the study was extracted by the guanidinium isothiocyanate method using TRIZol reagent.26 RNA (1 μg) was reverse transcribed using oligo (dT)16 sequence as primer and 50 U of expand reverse transcriptase (Roche Diagnostics, Mannheim, Germany). PCR amplification of the reverse transcribed RNA was carried out with 1 unit of DNA polymerase in a 50 μl reaction mixture containing 200 μM of each dNTPs, 0.3 μM of specific primers, and 1X reaction buffer using a DNA Thermal Cycler. Cycling conditions were three minutes at 94°C, 20 seconds at 94°C, 20 seconds at 58°C, 30 seconds at 72°C, 35 cycles, and a final elongation for five minutes at 72°C. Amplified DNA fragments were analysed by electrophoresis in a 1.5% agarose gel. Quantification of the bands was performed by laser densitometry after normalisation with 18S ribosomal RNA.

The primers specific for COX-2 were based on published sequences.27 The primers specific for MMP-9 were 5′-CCA GCA TCT GTA TGG TCG TG-′3′ (sense) and 5′-CAG AAC GAC CAC CAG TAG GG-′3′ (antisense) and for 18S rRNA were 5′-GCA ATT ATT CCC CAT GAA CGA (sense) and 5′-CAGG AGC GGA GGG ACT TAA TCA A-3′ (antisense). The
specificity of the amplified bands was validated by their predicted size.

**Determination of PGE₂ and MMP-9 levels**

Liver biopsy samples from all HCV+ and HCV− patients were homogenised in assay buffer: 0.1 mol/l phosphate buffer, pH 7.5, containing 0.9% (v/v) bovine serum albumin. After treating the homogenates with a 1:4 water:ethanol solution and 10 µl of glacial acetic acid, samples were purified through Amprec C18 minicolumn, eluted with ethyl acetate, and evaporated to dryness under nitrogen. Then, PGE₂ levels were determined using a specific enzyme immunoassay system (Amersham Biosciences UK Ltd, Buckinghamshire, UK). For MMP-9 activity, another piece of the same liver biopsy samples used for PGE₂ determination was homogenised in 50 mmol/l Tris-CIH, pH 7.4, with 1 mmol/l glycerol. After centrifugation for 10 minutes at 5000 g, the supernatant was used in an assay system with a specific chromogenic peptide substrate.

**Immunohistochemical analysis**

Liver biopsy samples from all HCV+ and HCV− patients were assessed using an indirect immunoperoxidase staining technique, as detailed elsewhere. Briefly, cryostat liver sections (5 µm thick) were incubated with a rabbit serum that recognises a peptide fragment corresponding to amino acids 584–598 from the C terminus region of human/murine COX-2 (Cayman Chemical) at a working dilution of 1:300. In parallel, each tissue section was also incubated with a rabbit antimouse antiserum (Dakopatts, Copenhagen, Denmark), at a working dilution of 1:300, and was used as a negative control.

**Cell culture studies**

**Plasmid constructs**

The expression vector pEF1α has been described previously as pCDEF. The expression vector pEF-croc was obtained by subcloning into pEF1α a PCR fragment encoding full length core protein from HCV genotype 1b. The plasmid pcDNA3.1-NS5A was obtained by subcloning into pcDNA3.1 (Invitrogen Corp., Carlsbad, California, USA) a PCR fragment encoding full length NS5A protein and was kindly provided by Dr Lai (University of Southern California, Los Angeles, California, USA). The construct pUHD10-3-croc was generated by subcloning a DNA fragment coding for core protein into the blunt ended XbaI site of the tetracycline controlled expression plasmid pUHD10-3. The expression vector pUHD10-3-Flag-NS5A was obtained by subcloning a DNA fragment coding for Flag-NS5A protein into a blunt ended EcoRI site of pUHD10-3. The reporter plasmid harbouring the human COX-2 promoter (−1796 to +104) linked to the luciferase gene has been described previously.

**Stable transfectants**

Polyclonal transfectants Chang Liver (CHL) cells core and CHL-NS5A were generated by stable transfection of CHL cells (CCL13; American Type Culture Collection, Manassas, Virginia, USA) with pCDEF and pcDNA3.1-NS5A expression vectors, respectively. Expression of core protein was confirmed by western blot assay (anticore MA1080 from Affinity BioReagents) and that of NS5A by RT-PCR. We also generated transfectants CHL-croc by stable transfection of CHL cells with a vector which drives expression of the bacterial chloramphenicol acetyltransferase (cat) gene, which were used as controls. Where indicated, cells were stimulated for 24–48 hours with 0.5 µg/ml of LPS, a cytokine mixture (CK) (0.5 µg/ml of LPS, 20 ng/ml of TNF-α, 20 ng/ml of IL-1β), or 10 nM of phorbol myristate acetate (PMA). In some experiments cells were treated with 50 µM DUF (Merck, Rayway, New York, USA) in order to inhibit COX-2 activity.

Polyclonal transfectants AML12-core and AML12-NS5A, which express the viral proteins in a tetracycline (tet)-off inducible manner, were generated by stable transfection of the previously reported clone 3 of the murine hepatocyte cell line AML12 with pUHD10-3-core and pUHD10-3-Flag-NS5A, respectively.

**Transient transfection and luciferase assays**

CHL cells were transfected with 0.1 µg of COX-2 promoter containing plasmid and increasing amounts (µg) of pEF-croc or pcDNA3.1-NS5A employing the DOSPER liposomal reagent (Roche Diagnostics). A snail gene promoter was used as a control for specificity in all luciferase assays. Transfected cells were grown in Dulbeco’s modified Eagle’s medium (DMEM) with 2% fetal calf serum (FCS) for...
24 hours. Cells were lysed and cell extracts were assayed for luciferase activity using a Lumat LB9501 luminometer.

Protein extraction and Western blot analysis
The experimental procedure used was identical to that described for liver biopsy samples.

RT-PCR assay
AML12-core and AML12-NS5A cells were grown, in the presence or absence of tet, for 24 hours in 1% FCS. RNA isolation and cDNA synthesis were then performed as described for liver biopsy samples. PCR conditions for COX-2 were five minutes at 95°C, 30 seconds at 95°C, 30 seconds at 60°C, 20 seconds at 72°C, 31 cycles, and a final elongation for five minutes at 72°C. PCR conditions for β-actin were five minutes at 95°C, 30 seconds at 95°C, 45 seconds at 60°C, one minute at 72°C, 25 cycles, and a final elongation for five minutes at 72°C. Amplified DNA fragments were analysed by electrophoresis in a 1.5% agarose gel. The primers specific for murine COX-2 were 5'-TGA GTA CCG CAA ACG CTT CTC-3' (sense) and 5'-TGG ACG AGG TTT TTC CAC CAG-3' (antisense) and yielded a 151 bp PCR product. The primers for β-actin were 5'-ATC TGG CAC ACC TTC TAC AAT GAG CTG CG-3' (sense) and 5'-CGT CAT ACT CCT GCT GTC TGG TCA TCC ACA TCT GC-3' (antisense).

Determination of culture supernatant PGE2 levels
Experiments were carried out following the same protocol used for liver biopsy samples but without the purification step.

Statistical analysis
The significance of differences was determined using the Kruskal-Wallis ANOVA test for data from COX-2 and MMPs experiments of protein, mRNA, and enzyme activity. Statistical significance was established at a p value <0.05.

RESULTS
Increased intrahepatic COX-2 expression in HCV-induced chronic liver disease
Western blot analysis from liver biopsy extracts of all HCV+ patients included in this study demonstrated that COX-2 expression levels were significantly higher in MCH (n = 22, mean 2.4-fold), SCH (n = 5, mean 3.3-fold), and cirrhosis (CIR) (n = 5, mean 3.9-fold) than in HCV CT samples (n = 10) (fig 1A, 1B top). When PGE2 levels were measured (fig 1B bottom), a statistically significant increase was found in liver samples from HCV+ SCH (n = 5, mean 1.9-fold) and CIR (n = 5, mean 3.3-fold) compared with CT samples (n = 10). Likewise, COX-2 mRNA levels were significantly higher in MCH (n = 22, mean 2.6-fold), SCH (n = 5, mean 3.5-fold), and CIR (n = 5, mean 5.3-fold) than in CT liver biopsy samples (n = 10) (fig 1C). We also determined NOS-2 protein by western blot as a surrogate marker of inflammation (fig 1D), showing that NOS-2 expression was significantly higher in MCH (n = 22, mean 1.6-fold), SCH (n = 5, mean 2.0-fold), and cirrhosis (n = 5, mean 3.2-fold) than in CT liver biopsy samples (n = 10).

Immunohistochemical experiments performed on liver biopsy sections from patients with HCV+ end stage cirrhosis detected COX-2 expression in a number of cell types, including hepatocytes, which showed a predominant cytoplasmic staining, and although some hepatocytes were observed at the edge of hepatic lobules, the majority were mainly restricted to the regenerative nodules (fig 2A, 2B). In contrast, in patients with SCH, hepatocellular COX-2 immunoreactivity was observed in some hepatocytes located in periporal areas (fig 2C). Notably, hepatocytes from patients with MCH were negative for COX-2, with the positive cells being restricted to sinusoidal lining cells and scattered mononuclear cells (fig 2D). In all liver sections from patients with non-alcoholic steatosis (fig 2E) and with a normal liver (fig 2F), no hepatocellular COX-2 staining was observed, being detected only in scattered sinusoidal lining cells.
Table 2  Comparative analysis of intrahepatic expression levels of COX-2, MMP-2, and MMP-9 between high and low viral load patients

<table>
<thead>
<tr>
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<th>High viral load</th>
<th>Low viral load</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-2</td>
<td>0.48 (0.16)</td>
<td>0.58 (0.16)</td>
<td>0.13</td>
</tr>
<tr>
<td>MMP-2</td>
<td>1.63 (0.8)</td>
<td>1.56 (0.76)</td>
<td>0.83</td>
</tr>
<tr>
<td>MMP-9</td>
<td>0.43 (0.3)</td>
<td>0.26 (0.2)</td>
<td>0.15</td>
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Enhanced MMP-2 and MMP-9 expression and activity in HCV-related chronic liver disease

A statistically significant increase in MMP-9 and MMP-2 proteins was observed in MCH (n = 22, mean 2- and 3.2-fold, respectively), SCH (n = 5, mean 2.6- and 4.7-fold), and CIR (n = 5, mean 6.6- and 6.9-fold) compared with CT samples (n = 10) (fig 3A, 3B). Moreover, a significant increase in MMP-9 activity was found in SCH (n = 5, mean 2-fold) and CIR (n = 5, mean 2.8-fold) compared with CT samples (n = 10) (fig 3C). RT-PCR analysis demonstrated that intrahepatic MMP-9 mRNA levels were significantly higher in MCH (n = 22, mean 2.6-fold), SCH (n = 5, mean 3.6-fold), and CIR (n = 5, mean 4.5-fold) than in CT samples (n = 10) (fig 3D). A comparative analysis of COX-2, MMP-2, and MMP-9 expression levels in liver biopsies from all HCV patients was carried out. Interestingly, the intrahepatic content of these proteins increased in parallel with the histofibrotic stage of HCV induced liver disease, being maximal in cirrhosis (fig 3E). However, no relationship between intrahepatic expression levels of COX-2, MMP-2, and MMP-9 and viral load of HCV patients studied was found (see table 2).

HCV induced COX-2 upregulation in hepatocyte derived cultured cells

Experiments performed in control CHL-cat cells showed that COX-2 expression was only detected after 48 hours of treatment with PMA. In contrast, weak COX-2 expression in untreated CHL-core polyclonal transfectants was already observed, its expression being markedly enhanced after treatment with LPS, CK, and PMA (fig 4A, 4B). Similar results, although to a lesser extent, were obtained in CHL-NS5A polyclonal cells. COX-2 protein was functionally active, as demonstrated by the increase in PGE2 concentrations in culture supernatants from control CHL-cat cells stimulated with PMA. Noteworthy, there was a further increase in PGE2 levels in culture supernatants from CHL-core and CHL-NS5A cells treated under the same conditions. However, PGE2 concentrations were lower than expected for COX-2 expression levels observed in core and NS5A transfected CHL cells, particularly in those treated with LPS and CK compared with PMA treated cells (fig 4C).

As shown in fig 5A, both viral proteins were able to activate the Cox-2 gene promoter in a dose dependent manner. The set-off mediated expression of core and NS5A resulted in upregulation of COX-2 mRNA (fig 5B).

Resting control CHL-cat cells expressed basal levels of MMP-9 protein but its expression was enhanced by the effect of LPS, CK, and PMA. Interestingly, a maximal increase in MMP-9 expression was observed in CHL-core cells, independent of the stimuli used (fig 6A, 6B) whereas NS5A did not exert any effect on MMP-9 expression (data not shown), suggesting different roles for both viral proteins on MMP-9 expression. Noteworthy, DFU apart from inhibiting PGE2 levels (not shown) decreased MMP-9 expression induced by LPS, CK, and PMA in control CHL-cat cells, demonstrating a clear relationship between COX-2 expression and MMP-9.
production. In contrast, inhibition of MMP-9 protein levels detected after DFU addition to CHL-core cells was markedly lower than that observed in control CHL-cat cells (fig 6A, 6B), suggesting a direct effect of core protein on regulation of MMP-9 protein synthesis.

DISCUSSION

The results of the present study provide evidence that COX-2 is overexpressed in the liver tissue of patients with chronic HCV infection, showing a positive correlation with the fibrotic stage of HCV induced liver disease. Interestingly, intrahepatic COX-2 expressed in HCV infected patients was functional, as demonstrated by the parallel increase in PGE$_2$ levels detected in liver extracts from the same patients.

The molecular basis for hepatocellular COX-2 expression in patients with chronic HCV infection remains to be defined but there is increasing experimental evidence that certain HCV proteins, such as core and NS5A, can function as transcriptional transactivators for a wide number of cellular genes. To assess the role of HCV proteins on COX-2 expression during chronic HCV infection, we performed in vitro experiments using cultured hepatocyte derived cells stably and transiently transfected with core or NS5A proteins. We found that maximal hepatocellular COX-2 expression occurred by the synergistic inducing effect exerted by HCV proteins plus several inflammatory mediators, including TNF-$\alpha$ and IL-1$\beta$, which are well known factors involved in the fibrotic process, supporting our in vivo findings that, independent of viral load, intrahepatic COX-2 expression increased in parallel with the stage of HCV induced chronic liver disease.

It has been reported that core and NS5A proteins are able to activate different transcription factors, such as nuclear factor $\kappa$B, AP-1, SRE, and STAT-3, suggesting their potential role in upregulation of COX-2 gene expression. Moreover, both viral proteins alter intracellular calcium levels and it is well known that the NF-AT family of transcription factors, which are calcium regulated, is essential for activation of COX-2 gene expression. Alternative candidates transactivated by HCV proteins are PPAR-$\alpha$ ligands, which are able to induce COX-2 expression in hepatocytes. Transcriptional regulation by PPARs is achieved through PPAR-retinoid X receptor heterodimers and interaction of core protein with retinoid X receptor $\alpha$ modulates transcriptional activity. The role of C/EBP-$\alpha$ transcription factor in the regulation of COX-2 expression in hepatocytes has also been described, providing another potential target to interact with HCV proteins.

Proteolytic enzymes, known as matrix metalloproteinases (MMPs), play a central role in cell migration by degrading cellular and extracellular components during cancer invasion, morphogenesis, organ development, and tissue damage. The relevance of MMPs for liver extracellular matrix remodelling can be deduced from the observation that pro-MMP-2 and pro-MMP-9 are activated during rat liver regeneration after partial hepatectomy, and both contribute to priming hepatocyte proliferation. In the present study, we showed that the intrahepatic content of MMP-2 and MMP-9 is increased in HCV infected patients, showing a close relationship with fibrotic stage of chronic liver disease, in a similar manner to that observed for COX-2 protein. In addition, an inducing effect on MMP-9 synthesis in human liver cells is exerted by core protein, but not by NS5A, this induction being partially abrogated by a specific COX-2 inhibitor. Our results from HCV infected patients as well as from HCV transfected human hepatic cells are in accordance with our previous work demonstrating that COX-2 expression promotes the release of active MMP-2 and MMP-9 in fetal rat hepatocytes, favouring the notion that COX-2 may also be important in secretion of active MMPs by human liver cells.
We showed here that progressive hepatic fibrosis but not viral load is associated with increased intrahepatic COX-2 and MMPs expression during chronic HCV infection and that in vitro pharmacological inhibition of COX-2 partially reversed these processes. On the basis of these data, it is tempting to hypothesise that selective COX-2 inhibitors, in association with antiviral therapy, might be useful as antifibrotic drugs in chronic HCV infection.

In conclusion, we have shown that intrahepatic COX-2, MMP-2, and MMP-9 overexpression is associated with progressive liver disease in chronic HCV infection, suggesting their pathogenic role in the fibrotic process. We also demonstrated that HCV core and NS5A proteins, alone and with the synergistic effect of endotoxin and proinflammatory cytokines, were able to upregulate COX-2 and MMP-9 gene expression in cultured human hepatic cells, thus providing a potential mechanism by which chronic HCV infection could lead to liver fibrosis.

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REFERENCES


20. Lichtinghagen R, Michels D, Haberkorn C, et al. Matrix metalloproteinase (MMP-2, MMP-9), and tissue inhibitor of metalloproteinase-1 are closely related to the fibroproliferative process in the liver during chronic hepatitis C. J Hepatol 2001;34:239–47.


