COX-2 in human hepatocellular carcinoma in cirrhotic and non-cirrhotic livers

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Abstract

**Background:** Hepatocellular carcinoma (HCC) is one of the classical examples of inflammation-linked cancer and is described as a highly angiogenic tumor. Cyclooxygenases-2 (COX-2) is a potent mediator of inflammation and is considered to upregulate angiogenesis.

**Aim of the study:** To correlate COX-2 expression with angiogenic factors relevant for sprouting angiogenesis.

**Patients and methods:** Tissue samples of HCC and adjacent liver parenchyma of 21 patients with HCC in noncirrhotic liver and 34 patients with HCC in cirrhotic liver were analyzed for COX-2 expression at the mRNA level (real time RT-PCR) and at the protein level by Western blot and immunohistochemistry. The angiogenic factors VEGF-A, VEGFR-1, VEGFR-2, Ang-1, Ang-2 and Tie-2 were correlated with COX-2 levels separately in HCC and liver parenchyma.

**Results:** At the protein level no correlation was found between COX-2 expression and the levels of the angiogenic factors. At the mRNA level COX-2 was correlated with mRNA levels of the angiogenic factors VEGFR-1, Ang-1 and Tie-2 (Spearman’s rho ≥.58, p<0.006). COX-2 mRNA and protein expression was higher in adjacent liver parenchyma than in HCC both in cirrhotic and noncirrhotic liver. COX-2 protein localized mainly in vascular and sinusoidal endothelial cells and in Kupffer cells. No correlation between COX-2 mRNA and COX-2 protein was found.

**Conclusion:** COX-2 expression is not associated with angiogenic factors which play a role in sprouting angiogenesis in clinically detectable HCC.
Introduction:

More than 80% of hepatocellular carcinomas (HCC) are associated with chronic infection caused by either hepatitis B or C virus.\(^1\) In well-developed countries with a high incidence of obesity and diabetes mellitus the risk of developing HCC rises proportionally with increasing body mass index (BMI) and the duration of diabetes.\(^2,3\) These conditions are frequently associated with non-alcoholic steatohepatitis (NASH).\(^4,5\) The common denominator in viral hepatitis associated liver diseases and NASH is presumed to be the chronic inflammation leading to fibrosis and cirrhosis and ultimately to HCC. HCC is thus one of the classical examples of inflammation-linked cancer.\(^6\) The cyclooxygenases-2 (COX-2)-prostanoid pathway plays a pivotal role in inflammation as well as in the pathophysiology of many liver diseases and associated conditions, e.g. fibrosis, portal hypertension, cirrhosis and HCC.\(^7\) COX-2 is an inducible immediate-early gene originally found to be induced by various stimuli including mitogens, cytokines and growth factors.\(^8\) COX-2 might favor tumor growth by various mechanisms including stimulation of angiogenesis, evasion of apoptosis and propensity to metastatic behavior and invasion. COX-2 inhibitors can block these mechanisms by various pathways and selective COX-2 inhibitors have been evaluated for their effect on HCC cell growth and invasion using animal models of hepatocarcinogenesis.\(^9,10\)

Apart from the development of HCC in cirrhotic liver, HCC can also arise in noncirrhotic livers with normal histology and no signs of inflammation or previous viral hepatitis. A common characteristic of both types of HCC is the highly vascularized nature, which is one of the hallmarks for diagnosing HCC. The occurrence of both types of HCC in humans offers a unique opportunity to study the possible relation between inflammation and angiogenesis, in which angiogenic characteristics can be analyzed in the presence (cirrhotic HCC) or absence (noncirrhotic HCC) of chronic inflammation. Unraveling of the underlying mechanisms might offer in the future more efficacious treatment modalities both for (chemo)prevention and possibly also for treatment of already established HCC.

The aim of the present study is to correlate COX-2 expression with the expression of angiogenic factors in human HCC in cirrhotic and noncirrhotic livers. We investigated gene and protein expression levels of COX-2, VEGF-A, VEGFR-1 (Flt-1) and VEGFR-2 (KDR), and Angiopoietin (Ang)-1, Ang-2, and their receptor Tie-2 using quantitative RT-PCR and Western blot analysis in HCC, adjacent liver parenchyma and normal liver parenchyma. The cellular localization of COX-2 was studied by immunohistology.
Patients and methods

Patients

All (anonymised) tissue samples were collected in the University Medical Center Groningen, the Netherlands and were processed according to national guidelines of the Dutch government. Tissue samples of 55 HCC patients were included; 21 noncirrhotic HCC were obtained from partial liver resection specimens and 34 cirrhotic HCC were obtained from liver explants during liver transplantation. A sample of adjacent, non-tumorous liver tissue was also included in the study. None of the HCC patients had been previously treated by local ablation, chemoembolisation or chemotherapeutic drugs before surgery. We also included 9 samples of histologically normal liver collected from surplus material of donor livers, or from partially resected livers from patients with a hemangioma in the liver or a traumatic liver rupture.

RNA extraction and real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated with the RNAeasy Mini Kit (Qiagen, Leusden, Netherlands) with subsequent DNA removal using the RNase-free DNase set (Qiagen), both according to the protocol of the manufacturer. RNA was analyzed qualitatively by gel electrophoresis and quantitatively with Nanodrop ND-100 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). Reverse transcription and real-time PCR were performed as described previously. Briefly, one microgram of total RNA was used for the synthesis of first-strand cDNA and 10ng cDNA was used for each PCR reaction. Exons overlapping primers and minor groove binder (MGB) probes were purchased as Assay-on-Demand from Applied Biosystems (Nieuwekerk a/d IJssel, The Netherlands): housekeeping gene GAPDH (assay ID Hs99999905_m1), COX-2 (assay ID Hs00153133_m1), VEGF (Hs00173626_m1), EGFR-1 (Hs00176573_m1), VEGFR-2 (Hs00176676_m1), Tie2 (assay ID Hs00176096_ml), Ang-1 (assay ID Hs00181613_ml), Ang-2 (assay ID Hs00169867_ml). Control samples of distilled water and at random selected samples of RNA not subjected to reverse transcriptase were consistently found to be negative. TaqMan RT-PCR was performed in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems) with the following cycling conditions: 2 min at 50°C, 10 min at 95°C, and 40 two-step cycles of 15 s at 95°C and 60 s at 60°C. Triplicate real-time PCR analyses were executed for each sample, and the obtained threshold cycle values (Ct) were averaged. Gene expression was normalized to the expression of the housekeeping gene GAPDH, yielding the relative gene expression level.

Western blot

Of each frozen tissue block, twenty 5μm thick tissue slices were lysed in
radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% nonidet P-40, 0.25% Na-deoxycholate, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM sodium fluoride, 1 mM sodium orthovanadate, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin (Sigma), and 1 μg/ml leupeptin (Roche), and 1 μg/ml pepstatin (Roche)]. Cell debris was removed by centrifugation at 10,000g for 15 minutes, and protein concentration was measured using pyrogallol red-molybdate solution. Indicated amounts of lysates were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes (0.45 μm, Bio-Rad laboratories; Hercules, CA). The membranes were next probed with mouse anti-COX-2 1:500 (BD Biosciences Pharmingen, #610204) diluted in 5% non-fat milk/TBST. Then followed by incubation with peroxidase-labeled rabbit anti-mouse Ig (DAKO, 1:1000 diluted in 5% non-fat milk/TBST ) and treated with an enhanced chemiluminescent substrate for detection of HRP (Amersham Life Science, London, UK). After stripping with 25mM glycine/1%SDS (PH=2.0), β-actin was detected on the membrane, using the same method (mouse anti- β-actin, Abcam, ab8226). Protein expression observed as electrophoretic bands was quantified using image analysis software (Quantity One, Bio-Rad), which calculated the volume of bands (intensity x mm2). The COX-2 volume of each sample was divided by the control (β-actin), yielding the protein expression value presented. The bands of COX-2 protein were located at about 70KD as expected according to the datasheet of the antibody.

**Immunohistological examination of COX-2 expression**

4μm tissue sections were deparaffinized and treated with 0.08% H2O2 for 30 minutes to block endogenous peroxidase. Slides were incubated with mouse anti-COX-2 (BD Biosciences Pharmingen, #610204) 1:50, diluted in 1%BSA/PBS, at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse Ig and goat anti-rabbit Ig (DAKO), both 1:100 diluted in 1%BSA/1% albumin/PBS. Diaminobenzidin was used to develop the staining reaction and nuclear counterstaining was performed with hematoxylin. As a positive control a sample of a colon adenocarcinoma was included.

**Analysis of correlation between COX-2 protein and mRNA level**

In order to correlate the COX-2 mRNA expression with the corresponding COX-2 protein levels the Pearson correlation coefficient was calculated separately for HCC and liver parenchyma. To get a more detailed picture of this correlation we calculated the COX-2 protein:COX-2 mRNA ratio for each individual case; this method has been used for identification of the strength of correlation of mRNA expression and protein level of individual genes by Pradet et al. (12) This ratio is depicted in a scatter plot as arbitrary units (protein:mRNA
ratio) on the y-axis and the individual cases on the x-axis.

**Correlation of COX-2 with Ang-2:Ang-1 ratio**

Although in many tumors both Ang-1 and Ang-2 are elevated, the pro-angiogenic state of hypervascular tumors is characterized by an overexpression of Ang-2 in relation to Ang-1 thus creating a shift in the Ang-2:Ang-1 ratio in favor of Ang-2.\(^{13}\) Therefore we analyzed the correlation between COX-2 mRNA/protein expression and the Ang-2:Ang-1 ratio separately in HCC and liver parenchyma.

**Statistical analysis**

Quantitative data were expressed as median and interquartile range (IQR). A two-tailed nonparametric test was used for comparison of groups; either the Mann-Whitney-U test for non-related samples (comparison between cirrhotic and noncirrhotic samples) or the Wilcoxon test for related samples (comparison between tumor and adjacent liver tissue). Correlation statistics between COX-2 and angiogenic variables were performed using the non-parametric Spearman’s rho test. For all statistical analyses, the level of significance was set at 0.05.

**Results**

**Correlation of COX-2 gene expression and protein level with angiogenic factors**

Table 1 shows that COX-2 mRNA levels in HCC in both cirrhotic and noncirrhotic liver were significantly correlated with mRNA levels of VEGFR1, Ang-1 and Tie-2. The strongest correlation was obtained between COX-2 mRNA and Tie-2 mRNA; Spearman’s rho was around .80. Only in HCC in noncirrhotic liver a significant correlation between COX-2 mRNA and VEGFR2 mRNA was obtained (rho .72, p < 0.001). COX-2 protein was correlated with VEGF mRNA in noncirrhotic HCC, whereas in cirrhotic HCC the mRNA levels of COX-2 were correlated with VEGFA in the tumor. No correlations were found between COX-2 expression and protein levels of the angiogenic factors (data not shown). We found that neither COX-2 mRNA nor COX-2 protein correlated with protein levels of the angiogenic factors. The Ang-2:Ang-1 ratio was not correlated with COX-2 protein or mRNA levels in normal liver (data not shown) or in liver parenchyma adjacent to HCC in both cirrhotic and noncirrhotic liver (table 1). In HCC tumor tissue no statistically significant correlation between the Ang-2:Ang-1 ratio and COX-2 mRNA/protein was found except for a negative correlation between the Ang-2:Ang-1 ratio and COX-2 mRNA in noncirrhotic HCC (rho -.58, p = 0.006, Table 1)
Table 1. Correlation between COX-2 mRNA and protein level with mRNA level of various angiogenic factors. Spearman’s rho and p-value are presented in tumor and adjacent cirrhotic and noncirrhotic liver separately. The significant correlations are represented in bold.

<table>
<thead>
<tr>
<th>mRNA level</th>
<th>COX-2 mRNA</th>
<th>COX-2 protein</th>
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<td>adjacent</td>
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**COX-2 expression in normal liver parenchyma, tumor-adjacent liver parenchyma and HCC in cirrhotic versus noncirrhotic liver**

No difference in COX-2 mRNA and COX-2 protein expression was found in normal liver parenchyma and parenchyma adjacent to tumor (Fig 1,2). COX-2 expression was observed in both vascular and sinusoidal endothelial cells as well as in Kupffer cells in normal liver and in parenchyma adjacent to HCC; no expression was found in hepatocytes or in cholangiocytes (Figure 3).

A comparison between COX-2 mRNA and protein expression in cirrhotic versus noncirrhotic HCC revealed no differences, although a trend towards higher protein levels in HCC in cirrhotic liver was seen (p = 0.07, Fig 1/2). Using immunohistochemistry, COX-2 expression in HCC was mainly found in endothelial cells lining the sinusoid-like spaces in the tumor but less conspicuous than in vascular endothelial cells at the interface between tumor and adjacent liver parenchyma. (Figure 3).

As a positive control of COX-2 immunohistological staining we included a colon carcinoma sample which was strongly positive (Figure 4).
COX-2 expression in HCC compared with tumor-adjacent liver parenchyma

Liver parenchyma adjacent to the tumor demonstrated a higher COX-2 expression than the tumor, both at the mRNA and protein level and both in cirrhotic and noncirrhotic liver (Figure 1/2). Median COX-2 mRNA level in tumor-adjacent liver parenchyma was about tenfold higher than in the tumor in noncirrhotic liver (p = 0.01). In cirrhotic liver, tumor-adjacent parenchyma had a six-fold higher COX-2 mRNA content (p < 0.001). Protein levels of COX-2 were approximately 370 fold higher in adjacent liver parenchyma as compared to HCC in noncirrhotic liver. In cirrhotic liver, adjacent parenchyma had a 30% higher COX-2 protein level (p < 0.02) than the tumor. Although median COX-2 expression in HCC was less than in the surrounding parenchyma, individual cases demonstrated variable results as can also be seen in the Western blots of COX-2 (Figure 5).
Correlation of COX-2 mRNA expression and protein level

A non-significant (p=0.65) correlation between COX-2 mRNA expression and COX-2 protein level in HCCs was found (Pearson rho -.81). Also no correlation was found when analyzing COX-2 protein and mRNA in liver parenchyma from both normal livers and HCC tumor livers (p=.14, Pearson rho 0.23). In scatter plots (Figure 6; upper panel adjacent parenchyma; lower panel HCC) the correlation between the expression levels of COX-2 mRNA and the corresponding protein is depicted as protein:mRNA ratio (arbitrary units) for the individual cases. The data demonstrate a poor correlation (r = 0.18 in HCC and r = 0.23 in liver parenchyma, both p > 0.05) between COX-2 mRNA and protein level. As can be seen in figure 6 (lower panel) protein expression can vary between around 0 (first case lower left corner) and more than 37,000 (last case upper right corner) in relation to a given mRNA expression level in HCC. In liver parenchyma (both normal and tumor-adjacent) the COX-2 protein:COX-2 mRNA ratio has a lower range, varying from 100 to more than 7,000 (Figure 6 upper panel)
Chapter 5

Figure 5. Representative samples of Western blot of COX-2 in matched (pairs of 11 noncirrhotic (upper panel) and 8 cirrhotic (lower panel) samples of HCC and tumor-adjacent liver parenchyma. (Adj: tumor-adjacent liver parenchyma; T: tumor (HCC)).

Figure 6. Correlation between COX-2 mRNA (real time RT-PCR) and corresponding COX-2 protein level (Western blot) in liver parenchyma (right panel) and hepatocellular carcinoma (left panel). Samples of parenchyma were obtained from normal liver (n = 9), or parenchyma adjacent to HCC in noncirrhotic liver (n = 11) or cirrhotic liver (n = 22). Tumor samples (left panel) were obtained from cirrhotic or noncirrhotic HCC. The horizontal lines represent the median values. (○: normal liver, □: noncirrhotic liver, ▽: cirrhotic liver)

Discussion

In this study we tried to evaluate in human HCC whether COX-2 -an important mediator of inflammation- is related to the expression of factors which have a role in tumor angiogenesis. Four main conclusions can be drawn from this study.

First, we were not able to identify a correlation between COX-2 protein and the protein levels of the angiogenic factors neither in the tumor nor in adjacent liver parenchyma. This finding holds true both for cirrhotic and noncirrhotic liver. Translation of the results of this correlative study into a conclusion of a causal relation between COX-2 and angiogenic factors should be done very cautiously, if at all. However, this lack of correlation suggests that
COX-2 protein is not pivotal as a mediator of angiogenesis.

The second conclusion is that although COX-2 mRNA expression is positively correlated with the expression of the VEGF-Receptor1 (VEGFR-1) and Ang-1 and Tie-2 in HCC this also does not support an important role for COX-2 as an initiator of angiogenesis. This is because only a positive correlation with the vessel stabilizing growth factor Ang-1 was found and not with the vessel destabilizing growth factor Ang-2. Thus, this finding suggests that COX-2 expression in HCC occurs at a stage in which vessel stabilization, more than destabilization, is the prominent feature. Current knowledge with respect to angiogenesis states that Ang-1, which is widely expressed in human tissues, constitutively activates Tie-2, which is almost exclusively expressed in endothelial cells, and thereby maintains blood vessel quiescence. Ang-2 is mainly produced by endothelial cells and is active at sites of vascular remodeling like the female reproductive organs – especially placenta- and certain tumors. Upregulation of Ang-2 results in binding to Tie-2 and destabilization of vessels which subsequently can develop in two ways depending on the presence of other cytokines; in the presence of proangiogenic cytokines, like VEGF, angiogenesis will occur, whereas in the absence of proangiogenic activity vessel regression will follow. When we apply the concepts of the angiogenic balance to the findings in the present study, an active role for COX-2 as a mediator of angiogenesis in human HCC is questionable. The reason is that although a positive correlation between COX-2 and VEGF-A and VEGFR-1 was found (suggesting preparation for angiogenesis), it is unlikely that VEGFA can exert its angiogenic action because concomitantly COX-2 is positively correlated with Ang-1/Tie-2 (favoring vessel quiescence) and COX-2 is not correlated with Ang-2 expression, the upregulation of which is a prerequisite for vessel destabilization and angiogenesis. The pro-angiogenic switch, characterized by a Ang-2:Ang-1 ratio in favor of Ang-2, has been found in many tumor types including HCCs. But even by analyzing this pro-angiogenic Ang-2:Ang-1 ratio in relation to COX-2 expression in HCC and adjacent liver parenchyma we could not obtain a positive correlation, again suggesting that COX-2 is not acting as a pro-angiogenic mediator via the Ang-1/2 and Tie-2 system. Although causative relations cannot be drawn from this correlative study the suggestion is raised that COX-2 is not a mediator in angiogenesis because it is not only positively correlated with the expression of the angiogenic cytokine VEGFA and its receptor (VEGFR-1) but also with that of the vessel stabilizing Ang-1/Tie-2 system.

The third conclusion that can be drawn from this study is that the chronic inflammation in cirrhotic liver parenchyma is not associated with an increased COX-2 expression. The current dogma describes that COX-1 is constitutively expressed and COX-2 is only expressed as a result of induction by a wide range of stimuli. In a recent autopsy study on trauma victims, this concept
was seriously questioned, because several healthy tissues, including the normal liver were shown to express COX-2.\(^{(17)}\) We also found COX-2 expression in normal liver parenchyma; this was comparable to COX-2 expression in liver parenchyma adjacent to HCCs. In parenchyma adjacent to HCC we found that COX-2 expression was even higher than in HCC itself. Our data are in agreement with findings of Morinaga et al. who found a 2.5-fold higher COX-2 mRNA expression in adjacent liver parenchyma as compared to HCC.\(^{(18)}\) In their study COX-2 protein expression -determined by immunohistochemistry- seemed to parallel mRNA expression although it was evaluated in only 8 of 22 samples.

The fourth finding is the strongly inconsistent correlation between COX-2 and angiogenic factors with respect to mRNA and protein levels. At the mRNA level a positive and significant correlation between COX-2 and the angiogenic factors VEGFR-1, Ang-1 and Tie-2 was found. Thus one would also expect a comparable correlation between COX-2 and the same angiogenic factors at the protein level. However, at the protein level none of the angiogenic factors correlated with COX-2 protein expression. It turned out that COX-2 mRNA levels were not correlated with COX-2 protein levels both in liver parenchyma and in HCCs. We more specifically analyzed this discrepancy by calculating the ratio COX-2 mRNA:COX-2 protein level for the individual samples of both liver parenchyma and HCC as suggested previously.\(^{(12)}\) These ratios revealed a range of 0–7,000 and 0–37,000 in liver parenchyma and HCC respectively. This discordance between COX-2 mRNA and protein level is likely to be explained by post-transcriptional mechanisms, like transcript decay and translation rate, but also by post-translational modifications which determine protein turnover and thereby protein expression levels.\(^{(19)}\) In a pivotal study, analyzing 106 genes of the yeast \textit{S. cerevisiae}, Gygi et al. found an overall good correlation between mRNA and protein level, but in a subset of 73 low abundance proteins the correlation was weak.\(^{(20)}\) In that study a non-significant Pearson correlation coefficient as low as 0.10 was found for the lowest abundance proteins.\(^{(20)}\) For some genes these authors found a more than 20-fold difference in protein expression for mRNA levels of the same value. They concluded that mRNA transcript levels provide little predictive values for the level of protein expression, especially for the low abundance proteins to which COX-2 belongs. Also in humans highly discrepant results between mRNA and protein expression were encountered; in a study of human lung adenocarcinomas only 17% (28/165) of the analyzed proteins demonstrated a significant correlation between mRNA and protein levels within the same tumor.\(^{(21)}\) Another study on 47 human colon carcinoma samples revealed no correlation between mRNA levels and protein levels of dihydropyrimidine dehydrogenase, the enzyme metabolizing the cancer drug 5-fluouracil.\(^{(22)}\) Taken together, mRNA abundance does not necessarily translate into the detection of the corresponding protein with the currently available techniques.
and conclusions based solely on protein expression even if in combination with mRNA expression should be drawn cautiously.\(^{(12)}\)

The question rises whether these precautions also apply for COX-2 expression in HCC. Several pathways regulate post-transcriptional COX-2 expression, many of which are of importance in HCC as well.\(^{(23,24)}\) Alterations in these pathways might have an influence on COX-2 expression. It is also important to realize that COX-2 protein has a short half-life. In various cell lines COX-2 protein degradation varies from 2 to 7 hours.\(^{(16)}\) Also relevant in this respect is the recent discovery of a second pathway for COX-2 protein degradation which can be stimulated by the addition of the substrate arachidonic acid.\(^{(25)}\)

From this study in human HCCs we conclude that COX-2 is expressed to a similar extent in cirrhotic and noncirrhotic background. Additionally, we conclude that COX-2 mRNA expression in HCC is correlated with angiogenic factors playing a predominant role in vessel stabilization and as such it is unlikely that COX-2 is associated with sprouting angiogenesis. With the current working concept of the angiogenic balance, it is unlikely that COX-2 acts as an angiogenic mediator in advanced human HCC.

A further unraveling of transcriptional and post-transcriptional processing of COX-2 will hopefully shed light on the discrepancies between COX-2 mRNA and COX-2 protein levels. The clinical usefulness of COX-2 inhibitors especially in the setting of chemoprevention for the development of human HCC can only be based on randomized clinical trials since the precise regulatory mechanisms of COX-2 are far from elucidated.\(^{(26-28)}\)

Although the concept of the angiogenic balance is considered to be essential for tumor vessel development, tumor heterogeneity and angiogenic escape pathways exist which make tumor angiogenesis far more complex and less predictable and could probably also explain unresponsiveness of some tumor types to anti-angiogenic treatment.\(^{(29,30)}\)
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