Cyclooxygenase-2 inhibitors suppress the growth of human hepatocellular carcinoma implants in nude mice

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Cyclooxygenase (COX)-2 is expressed in hepatocellular carcinomas (HCCs) and HCC cell lines. COX-2 inhibition strongly suppresses growth of HCC cells in vitro by inducing apoptosis and reducing proliferation. Here, we evaluate the in vivo effects and mechanism of COX-2 inhibition of human HCC cell line derived xenotransplanted tumors in nude mice. Firstly, nude mice were treated with a COX-2 specific inhibitor (meloxicam) or a non-specific inhibitor (sulindac) starting 5 days prior to tumor cell injection. After 35 days, mice were killed and tumors were analyzed morphologically and assayed for proliferation (Ki67), apoptosis (M30) and COX-2 expression. Secondly, mice were treated with meloxicam or sulindac after tumors had reached a diameter of at least 0.2 cm. COX-2 expression was maintained in implant tumors at levels comparable with parental cells. Selective COX-2 inhibition led to a significant reduction of tumor growth and weight. COX-2 inhibition had a significant anti-proliferative and pro-apoptotic effect on tumor cells. These results demonstrate that under experimental conditions selective COX-2 inhibition suppresses solid HCC growth in vivo and, therefore may have preventive and therapeutic potential for human HCCs.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, accounting for ~6% of all human cancers and up to 1 million deaths per year (1). A recent study demonstrates that the HCC-incidence has increased by 71% over the last 25 years in the US (2). Clearly, HCC is a growing health problem and no efficient secondary prevention or systemic treatments are available currently.

A growing body of evidence indicates that non-steroidal anti-inflammatory drugs (NSAIDs) possess antitumorigenic properties (3). Initially demonstrated for colorectal carcinogenesis, these findings have been extended recently to other carcinomas, including breast, prostate, pancreatic cancers and HCC (3–13).

Cyclooxygenases catalyze the enzymatic conversion of arachidonic acid to prostaglandins [e.g. prostaglandin E2 (PGE2)] and represent specific targets of NSAIDs. At least two cyclooxygenases are present in humans: cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). COX-1 is constitutively expressed in many tissues and is important for the synthesis of cytoprotective gastrointestinal prostaglandins and platelet aggregation (14–16). In contrast, COX-2 is inducible, and increased COX-2 concentrations have been observed in inflamed and tumorous tissues (17,18). It promotes cell growth (19) and angiogenesis (20), and reduces apoptosis (21). Non-selective NSAIDs, such as acetylsalicylic acid and sulindac, inhibit both COX-1 and COX-2, and therefore reduce the synthesis of cytoprotective as well as pro-inflammatory prostaglandins. Selective COX-2-inhibitors, such as meloxicam, specifically down-regulate the pro-inflammatory prostaglandins (e.g. PGE2), and are thought to cause less unwanted side effects in long term treatments.

Recently, we and others have demonstrated that selective inhibition of COX-2 has marked anti-neoplastic effects in different HCC cell lines, which were significantly attributable to the induction of apoptosis and reduction of proliferation (13,22). Furthermore, increased expression of COX-2 in human HCCs has been demonstrated by independent studies (10,12).

Nevertheless, the relevance of COX-2 and the effects of specific COX-2 inhibitors in HCCs under in vivo conditions are unknown. The present study investigates the preventive and therapeutic potential of COX-2 inhibition in solid HCCs generated in nude mice. Our data demonstrate that treatment with COX-2 inhibitors significantly reduces the growth of HCCs in vivo by reducing proliferation and enhancing apoptosis of the tumor cells.

Materials and methods

Animals

Six to eight weeks old female athymic nude (nu/nu) mice (20–25 g), from Harlan Winkelmann (Borchen, Germany), were used in all experiments. The animals were kept under pathogen-free conditions in a 12 h light-dark cycle at 21.5°C and 60% relative humidity. The mice were given an autoclaved standard diet ‘Altromin’ (Altromin, Lage/Lippe, Germany) and water ad libitum. The indicated concentrations of COX inhibitors sulindac (Sigma, St Louis, MO) and meloxicam (Mobeck, Boehringer Ingelheim, Ingelheim, Germany) were mixed with the diet by Altromin. All animal experiments were performed according to the guidelines and with approval of the local institutional animal care committee.

Cell culturing and preparation for transplantation

The human liver tumor cell line HuH-7 was kindly provided by R.Bartenschlager (Institute of Virology, University of Mainz, Germany) and cultured in RPMI medium (Biochrom, Cambridge, UK) supplemented with fetal calf serum (FCS, 10% [v/v]), non-essential amino acids (0.1 mM), 1-glutamine (5 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Sigma) in an atmosphere containing 5% CO2. Prior to injection, HuH7-cells were harvested in the exponential growth phase and washed three times in serum-free medium. In a pathogen-free environment, 4 × 107 cells per 100 μl of phosphate buffered saline (PBS) were injected subcutaneously into the back (right and left sides of the spine) using 28-gauge needles.

Abbreviations: COX-1, COX-2, cyclooxygenase-1, -2; NSAIDs, non-steroidal anti-inflammatory drugs; HCC, hepatocellular carcinoma; PGE2, prostaglandin E2.
Experimental design and monitoring of tumor growth

Preventive approach. Animals (n = 40) were divided into four groups consisting of 10 mice each. Mice received the standard diet supplemented either with sulindac (non-selective COX-inhibitor) (group 1, 400 p.p.m.; n = 10) or with meloxicam (selective COX-2 inhibitor) (group 2, 54 p.p.m.; n = 10) starting 5 days prior to tumor cell injection and throughout the whole experiment. Group 3 was pre-treated a standard diet supplemented with 162 p.p.m. meloxicam and after tumor cell injection the dose was changed to 54 p.p.m. Untreated mice served as controls (n = 10 for groups 1 and 2; n = 10 for group 3). Tumor growth was monitored constantly by two-dimensional measurements using a vernier calliper. Tumor nodules were identified in 90% of the untreated mice, ~20 days after injection.

Therapeutic approach. HuH7 cells were inoculated s.c. on day 0. The animals were monitored as described above. When tumors were palpable, which corresponded to a diameter of at least 0.2 cm, the animals were randomly distributed either into one control group (n = 4) or treatment groups, receiving meloxicam (54 p.p.m.; n = 10) or sulindac (400 p.p.m.; n = 5). The diets of the treatment groups were changed to the diets containing the respective drugs immediately. Body weights and the maximal two-dimensional tumor surface were recorded in 2-day intervals. To control for minor differences in starting volumes between the different treatment groups the percentage of tumor growth is given for each treatment group in comparison with the tumor size at the time point of treatment initiation.

The formula \( \frac{L}{2} \times \pi \left( \frac{W}{2} \right)^2 \) was used to calculate the maximal tumor surface areas. Mice were killed by cervical dislocation before the tumor burden caused any obvious morbidity. The final tumor size and weights were registered and all tumors were divided in half for formalin fixation and paraffin embedding or frozen storage for protein extraction.

Histology

Tumors were fixed in 10% neutral buffered formalin and embedded in paraffin. Four micrometre sections were stained with H&E. All tumors were examined for cyto- and histomorphology, intratumoral bleeding and necroses.

Immunohistology

Four micrometre paraffin sections were prepared, deparaffinized in xylene and rehydrated with graded ethanol. Antigen retrieval was performed by microwave pre-treatment. Citric acid buffer (10 mM) (pH 6) was pre-heated by incubation in a microwave oven at 650 W for 3 × 5 min. After cooling, the slides were first treated with 30% hydrogen peroxide in PBS to block the endogenous peroxidase. Non-specific antibody-binding was blocked by normal rabbit serum. Primary M30 antibody (CytoDeath, Boehringer Mannheim, Mannheim, Germany; dilution 1:50) was applied and incubated in a moist chamber at 4°C overnight. Monoclonal biotinylated rabbit anti-mouse IgG antibodies (Dako, Glostrup, Denmark) were applied at a dilution of 1:200, followed by a streptavidin–biotin complex and incubated with a solution containing 3-amino-9-ethylcarbazole and hydrogen peroxide for signal detection. M30-staining was counted in at least six different fields of vision (magnification 400×). The percentage of positive tumor cells compared with the total tumor cell number was calculated.

Histology

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Statistical analysis

Statistical significance was assessed by the non-parametric Mann–Whitney U test and a P-value of <0.05 was considered significant.

Results

Selective COX-2 inhibition reduces growth of xenotransplanted HuH7 tumors in nude mice

Expression of COX-2 in HuH7-derived tumors was well-detectable by western immunoblot and immunohistology of tumor tissues and appeared to be comparable with the expression determined in the parental HuH7 cell line (Figure 1A and B).
Furthermore, the COX-2 expression levels were comparable within and between the different treatment groups (data not shown).

We next determined, whether COX-inhibition modulated tumor cell growth in vivo. Injection of human HuH7 cells led to visible tumor development at day 22 after tumor cell injection. Almost 97% of the mice developed tumors on both sides. Continuous treatment of mice with sulindac (400 p.p.m., group 1) or meloxicam (54 p.p.m., group 2) started 5 days prior to s.c. injection. The tumor growth rates and the tumor weights were reduced in the sulindac- and meloxicam-treated groups at the time of death (Figure 2A and B). The effect was slightly higher in the meloxicam-treated group but did not reach statistical significance in any of the two groups under the selected experimental conditions (mean tumor weight in the sulindac-treated group 0.71 ± 0.11 g and meloxicam-treated group 0.68 ± 0.09 g versus controls 0.87 ± 0.15 g). Since growth reduction was higher in the meloxicam-treated group, we modified the meloxicam treatment by administering a higher dose of meloxicam (162 p.p.m., group 3) during the 5 days pre-treatment period.
At the time of HuH7-cell inoculation the drug treatment was reduced to 54 p.p.m. meloxicam, since preliminary tests had shown that long-term treatment with 162 p.p.m. meloxicam resulted in a weight reduction of the animals. In each animal the tumor with the greater diameter was evaluated. A significant reduction of tumor growth was observed in the animal group fed with meloxicam (group 3) in comparison with the control group from day 32 on (Figure 2A, C and D). In addition the mean tumor weight at the end of the experiment was significantly lower in the meloxicam treated group when compared with the controls (0.13 versus 0.35 g, respectively, $P < 0.05$) (Figure 2A, right column). The average tumor surface in meloxicam-treated mice (0.14 cm$^2$) was significantly reduced by 70% as compared with control mice (0.435 cm$^2$; $P < 0.05$) (Figure 2C).

In a further experiment meloxicam and sulindac treatments were initiated in mice with already established HCCs, when tumor nodules had reached at least 0.2 cm in diameter. Again a reduction of tumor growth was observed in animals treated with sulindac (group 5) as well as meloxicam (group 4) when compared with non-treated control animals (Figure 2E); however, the differences were just beyond the level of significance ($P = 0.07$).

**Tumor analyses**

All tumors remained well circumscribed and no metastases were found. Tumor histo- and cytomorphology was comparable between the different treatment groups. The extent of tumor necrosis and intratumoral bleeding was highly variable (from 2.5 to 17%), however, without obvious differences between the different groups (data not shown). Next we compared the meloxicam-treated and the non-treated control mice regarding tumor cell proliferation (Ki-67) and apoptosis (M30). Tumor cell proliferation was lower in the meloxicam-treated
animals when compared with untreated controls [20.2 versus 37.4\% (group 3)] \((P < 0.05)\) and [25.3 versus 38.3\% (group 4)] and the difference was significant in the preventive experiment (Figure 3A and B). Moreover, in tumors from meloxicam-treated mice a significant increase of M30-positive cells [3.24-fold (group 3) and 2.02-fold (group 4)] was observed when compared with the respective controls \((P < 0.05)\) (Figure 4A and B).

**Discussion**

The increasing incidence, limited treatment options and poor prognosis of HCC emphasize the need to explore new strategies of chemoprevention and treatment. It is well documented that expression of COX-2 is found in colon carcinogenesis, but also other carcinomas. Recently, two reports have demonstrated increased COX-2 expression in limited series of HCCs (10,11).

Human HCC cell lines, including HuH7 cells, express significant concentrations of COX-2 (13,23), and these expression levels are maintained in implant tumors. The mechanism leading to increased COX-2 expression in HCCs is still not clear, but several different possibilities have to be discussed. As demonstrated by numerous CGH studies, 1q25.2-q25.3, the chromosomal location of the COX-2 gene and cytosolic phospholipase A2 (cPLA2), is over-represented in ~60\% of all HCCs (24,25), suggesting a gene dosage effect. Since cPLA2 is co-regulated with COX-2 (26,27), it may increase the amount of arachidonic acid, the substrate for COX-2. Furthermore, COX-2 is inducible by a variety of diffusible factors that are frequently activated during carcinogenesis, including cytokines, growth factors and tumor promoters (28). For example, the growth factor IGF-II, which is over-expressed in ~30–40\% of HCCs, may induce up-regulation of COX-2 expression through activation of the IGF-I receptor as demonstrated in the human colon carcinoma cell line Caco-2 (29).

We have demonstrated previously that selective COX-2 inhibitors (meloxicam and SC-58635) exhibit potent anti-neoplastic properties in human HCC cell lines in vitro, which correlated with enhanced tumor cell apoptosis and reduced mitogenesis. The current study demonstrates, that COX-2 inhibitors also significantly reduce the growth of solid
xenotransplanted HCCs in vivo. Growth reduction was associated with significantly enhanced tumor cell apoptosis and reduced mitogenesis.

In vitro reversal of COX-2 inhibitor effects by the administration of PGE₂ and a significant reduction of synthesized PGE₂ after COX-2 inhibitor treatment in HCC cell lines suggest that the effects are indeed due to the inhibition of COX-2 activity (13). Although this association has not been proven in the current in vivo study, it is suggested by the fact that HuH7-derived tumors express COX-2 at levels comparable with the parental cell line, that the in vivo effects are comparable with the in vitro data and appear to be more pronounced when selective COX-2 inhibitors are administered.

The mechanisms by which NSAID-induced apoptosis is regulated and executed are still unclear. However, under in vitro conditions, initiation (caspase-9) as well as downstream execution-caspases (caspase-3 and caspase-6) are rapidly activated by intramolecular cleavage after inhibition of COX-2 in liver tumor cells (13). Although caspase-activation is sufficient to explain apoptosis execution, the nature of the responsible upstream initiating events has not been defined so far. Modulation of AKT/PKB- and BAD-phosphorylation, the RAS-signaling pathway (30), p53 expression (31), other members of the BCL-2 family such as MCL-1 (32), activation of the sphingomyelin-ceramide pathway (33) and modulation of the NFκB pathway (34) have been described in different tumor entities previously, and the mechanisms may even vary between different tumor types. Regarding the anti-proliferative effect of NSAIDs, it has been shown in colon carcinoma cells that COX-inhibition resulted in G₁ arrest in proliferating colon carcinoma cell lines as well as in HCC cell lines (22,31).

The current nude mouse tumor implant approach is still limited; it is not an orthotopic model and does not allow us to study the role of the immune reactions, homotypic tumor vascularization and the effects on the sequential hepatocarcinogenic cascade. Thus, effects of COX-2 inhibition on HCCs may be even more pronounced in a setting that resembles the human situation more closely. It is not clear, why COX-2 inhibitors appeared to be somewhat less efficient when treatment was initiated after tumors were already established. The model employed in this study uses fully developed rapidly growing subcutaneously xenografted HCC cells with a tumor doubling time of ~4 days. One possibility may well be that inhibitory intratumorous COX-2 inhibitor concentrations were not built up sufficiently to inhibit rapid tumor progression comparably. Inhomogeneous and inefficient substance penetration of the tumor tissue or secondary cellular resistance may render larger tumors partially refractory (35). In addition, in previous studies the highest expression of COX-2 was demonstrated in well-differentiated HCC (36). Therefore, selective COX-2 inhibitors may be even more efficient during the early stages of hepatocarcinogenesis.

In conclusion, selective COX-2 inhibition suppressed the growth of solid HCCs derived from human liver tumor cells in conjunction with reduced proliferation and significant induction of apoptosis in vivo. These results suggest COX-2 inhibition as a potential preventive as well as therapeutic strategy against human hepatocellular carcinoma.

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