Cyclooxygenase-2 Inhibition Induces Apoptosis Signaling via Death Receptors and Mitochondria in Hepatocellular Carcinoma

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Abstract

Inhibition of cyclooxygenase (COX)-2 elicits chemopreventive and therapeutic effects in solid tumors that are coupled with the induction of apoptosis in tumor cells. We investigated the mechanisms by which COX-2 inhibition induces apoptosis in hepatocellular carcinoma (HCC) cells. COX-2 inhibition triggered expression of the CD95, tumor necrosis factor (TNF)-R, and TNF-related apoptosis-inducing ligand (TRAIL)-R1 and TRAIL-R2 death receptors. Addition of the respective specific ligands further increased apoptosis, indicating that COX-2 inhibition induced the expression of functional death receptors. Overexpression of a dominant-negative Fas-associated death domain mutant reduced COX-2 inhibitor-mediated apoptosis. Furthermore, our findings showed a link between COX-2 inhibition and the mitochondrial apoptosis pathway. COX-2 inhibition led to a rapid down-regulation of myeloid cell leukemia-1 (Mcl-1), an antiapoptotic member of the Bcl-2 family, followed by translocation of Bax to mitochondria and cytochrome c release from mitochondria. Consequently, overexpression of Mcl-1 led to inhibition of COX-2 inhibitor-mediated apoptosis. Furthermore, blocking endogenous Mcl-1 function using a small-interfering RNA approach enhanced COX-2 inhibitor-mediated apoptosis. It is of clinical importance that celecoxib acted synergistically with chemotherapeutic drugs in the induction of apoptosis in HCC cells. The clinical relevance of these results is further substantiated by the finding that COX-2 inhibitors did not sensitize primary human hepatocytes toward chemotherapy-induced apoptosis. In conclusion, COX-2 inhibition engages different apoptosis pathways in HCC cells stimulating death receptor signaling, activation of caspases, and apoptosis originating from mitochondria. (Cancer Res 2006; 66(14): 7059-66)

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent malignancies worldwide, accounting for ~6% of all human cancers and 1 million deaths annually (1–3). The incidence of HCC in the United States has increased significantly over the past two decades (4, 5). Clearly, HCC is a growing health problem; currently, no effective systemic (chemotherapeutic or chemopreventive) treatments are available.

A growing body of evidence indicates that the new generation of cyclooxygenase (COX)-2–selective nonsteroidal anti-inflammatory drugs possesses antitumorigenic properties. Initially shown for colorectal carcinogenesis, these findings have been extended recently to other carcinomas, including breast, prostate, and pancreatic cancers, as well as to HCCs (6–10). COXs catalyze the enzymatic conversion of arachidonic acid into prostaglandins [e.g., prostaglandin E2 (PGE2)]. At least two COX isoforms have been identified thus far: the constitutively expressed COX-1 and COX-2, which is inducible under pathological conditions and of which increased concentrations have been observed in inflamed and tumorous tissues (11).

The tumorigenic potential of COX-2 overexpression has frequently been associated with resistance to apoptosis in certain cell types (11). As we and others have recently shown, selective COX-2 inhibition elicits a marked antineoplastic effect on HCC cells in vitro as well as in vivo, which has been associated with significant induction of apoptosis and rapid activation of effector caspases (caspase-9, caspase-3, and caspase-6; refs. 9, 10, 12). Because treatment of HCC cells with selective COX-2 inhibitors leads to the reduction of synthesized PGE2 and the antineoplastic effect of selective COX-2 inhibition is reversible by PGE2 substitution, a COX-2-dependent mechanism in HCCs is suggested (10).

However, the detailed underlying signaling mechanism by which COX-2 inhibition modifies the intracellular apoptosis network in HCC cell lines has not been explained in detail thus far. Here, we report that COX-2 inhibitors activate major apoptosis pathways by triggering signaling via death receptors and mitochondria and thus sensitize liver cancer cells toward chemotherapy. Furthermore, we show that down-regulation of myeloid cell leukemia-1 (Mcl-1) is a key event in the initiation of COX-2 inhibitor-mediated apoptosis. COX-2 inhibitors act synergistically with different chemotherapeutic drugs in the induction of apoptosis of HCC cells, which is of particular clinical relevance for a multimodal chemotherapeutic strategy against HCC.

Materials and Methods

Cell lines. The following cell lines were used: (a) HepG2 cells (American Type Culture Collection, Manassas, VA) derived from a human...
hepatoblastoma (13) and (b) Huh7 cells (Japan Health Science Foundation, Osaka, Japan) derived from a HCC (14).

HepG2 and Huh7 cells were maintained in DMEM (Invitrogen, Karlsruhe, Germany) containing 10% FCS, 5 mmol/L L-glutamine, and 100 μg/mL gentamicin (all from Invitrogen). In all experiments, cells were treated without antibiotics.

**Isolation of primary human hepatocytes.** Primary human hepatocytes (PHH) were isolated from fresh surgical specimens of patients undergoing partial hepatectomy. Informed consent was obtained from each patient, and the procedure was approved by the Ethics Committee, University of Heidelberg (Heidelberg, Germany). To isolate PHHs, a modified two-step collagenase perfusion was done as described (15). Isolated PHHs were seeded and cultured as described (16).

**Immunocytochemistry.** Chamber slides with subconfluent Huh7 and HepG2 cells were washed with PBS and fixed with ice-cold methanol for 10 minutes, washed thrice with PBS, and incubated with 0.3% hydrogen peroxide in methanol for 20 minutes. Cells were probed with anti-human COX-2 antibody (Santa Cruz Biotechnology, Inc.), and cytochrome c (Becton Dickinson), Mcl-1, and Bax (all at 1:1,000; Cell Signaling Technology, Beverly, MA). To ensure equivalent loading and transfer, membranes were stripped and reprobed with anti-human α-tubulin (Santa Cruz Biotechnology, Inc.), and cytochrome c oxidase subunit II (1:2,000; Molecular Probes, Eugene, OR).

**Western immunoblot.** Preparation of total protein lysates (10) and isolation of mitochondrial and cytosolic protein fractions (17) have been described previously. In brief, respective protein lysates (20 μg) were separated in SDS-polyacrylamide gels (10-15%) by electrophoresis, transferred to a polyvinylidene difluoride membrane, and probed with antibodies against anti-human COX-2 (1:2,500; Becton Dickinson, Heidelberg, Germany), cytochrome c (Becton Dickinson), Mcl-1, and Bax (all at 1:1,000; Cell Signaling Technology, Beverly, MA). To ensure equivalent loading and transfer, membranes were stripped and reprobed with anti-human actin (1:10,000; Oncogene Research Products, Boston, MA), α-tubulin (Santa Cruz Biotechnology, Inc.), and cytochrome c oxidase subunit II (1:2,000; Molecular Probes, Eugene, OR).

**Treatment with celecoxib.** Cells were treated with celecoxib (SC 58635), which was kindly supplied by Searle Research and Development (St. Louis, MO) at 10, 50, or 100 μmol/L for the indicated times.

**Treatment with cytostatic drugs.** HepG2 and Huh7 cells and PHHs were treated with bleomycin (3 and 30 μg/mL), doxorubicin (0.05 and 0.5 μg/mL), or mitoxantrone (0.1 and 1 μg/mL) alone and in combination with 100 μmol/L celecoxib for 48 hours. The serum concentrations relevant for therapy are 1.5 to 3 μg/mL for bleomycin, 0.001 to 0.02 μg/mL for doxorubicin, and 0.03 to 0.5 μg/mL for mitoxantrone (18).

**Transfections and plasmids.** HepG2 and Huh7 cells were transfected by the use of a calcium phosphate or a Fugene Transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the respective manufacturer’s instructions. The Mcl-1 expression plasmid pEF4/Mcl-1, the corresponding control plasmid (pEF4), and the ΔFas-associated death domain (FADD) plasmid have been described previously (16, 19, 20).

**Detection of apoptosis.** The quantification of DNA fragmentation was done by fluorescence-activated cell sorting (FACS) analysis of propidium iodide–stained nuclei (21, 22) carried out in a FACScan flow cytometer (Becton Dickinson) using the CellQuest software system.

Specific apoptosis of PHHs was calculated as follows: experimental group/apoptosis control – spontaneous apoptosis (the following caspase inhibitors were applied: (a) ZVAD-FMK (z-Val-Ala-Asp-fluoromethylketone; broad spectrum caspase inhibitor; Bachem, Bubendorf, Germany), (b) DEVD-FMK [z-Asp(OCH3)2-Glu(OCH3)2-Val-Asp(OCH3)2-FMK; inhibitor of caspase-3 as well as caspase-6, -7, -8, -9, and -10], (c) Z-IELD-FMK [z-Ile-Glu(Ome)-Thr-Asp(Ome)-CH2F, inhibitor of caspase-8], and (d) Z-LEHD-FMK [z-Leu-Glu(Ome)-His-Asp(Ome)-CH2F; inhibitor of caspase-4, -8, and -9] all from CalBiochem, Schwalbach, Germany).

For caspase activation assays, cells were harvested 36 and 48 hours following celecoxib treatment (caspase-3, -8, and -9), respectively; and caspase-9 fluorometric assay; R&D Systems, Minneapolis, MN).

To induce CD95 receptor-mediated apoptosis, we used the monoclonal antibody anti-APO-1 IgG3 at a concentration of 1 μg/mL (18, 23, 24) for 24 hours before harvesting. Tumor necrosis factor (TNF)-α (Sigma, Deisenhofen, Germany) was added at a concentration of 100 ng/mL together with 10 μg/mL cycloheximide (Sigma) 24 hours before harvesting. TNF-related apoptosis-inducing ligand (TRAIL; human leucine zipper (LZ)-TRAIL) was applied at a concentration of 1 μg/mL 24 hours before harvesting.

Changes in nuclear morphology were assessed after 4’,6-diamidino-2-phenylindol (DAPI; Sigma) staining of cellular DNA as described previously (18).

**Detection of the death receptors.** Cell surface expression of the CD95, the TNF-R1, the TRAIL-R1, and the TRAIL-R2 receptor was assessed by FACS as described (22, 25–27).

**Determination of mitochondrial membrane potential.** HepG2 and Huh7 cells were incubated with 5,5’,6,6’-tetrachloro-1’,3’,3’-tetraethylbenzimidazolylcarbocyanine-iode (JC-1; 5 μg/mL; Sigma) or with 3,3 dihexylcarbocyanine-iode (DiOC; Molecular Probes) at room temperature for 20 minutes (JC-1) or at 37°C in the dark for 15 minutes (DiOC), then washed, and analyzed by FACS (28, 29).

**Mcl-1 small-interfering RNA experiments.** For small-interfering RNA (siRNA)–mediated down-regulation of Mcl-1, the following siRNA sequences were applied (MWG Biotech, Ebersberg, Germany): 5’-auggauacagacgucucTT-3’ (sense) and 5’-TTCaugacagcaagcagaa-3' (antisense). As a control, green fluorescent protein (GFP) siRNA was used: 5’-ggccacagacgcagcTT-3’ (sense) and 5’-TTcaagcagagacggcagaa-3’ (antisense). HepG2 and Huh7 cells were transiently transfected with Transfectin (Bio-Rad, Hercules, CA) according to the manufacturer’s protocol and analyzed 24 and 48 hours after transfection.

**Statistical analysis and analysis of combined drug effects.** To examine whether synergy (30) between celecoxib treatment and concurrent chemotherapeutic treatment is observed, a balanced two-way ANOVA (model with fixed effects) was done. Furthermore, we applied multivariate
ANOVA and Wilcoxon's analysis to test for statistical significance. Statistical analysis was carried out using the SAS software system (SAS Institute, Inc., Cary, NC).

**Results**

**COX-2 is expressed in HCC.** COX-2 is highly expressed in Huh7 and HepG2 cells as shown by immunocytology and Western blot analysis (Fig. 1A and B). An intense COX-2 signal was detected preferentially in the perinuclear cytoplasm.

**COX-2 inhibitor-mediated apoptosis in liver tumor cells involves activation of caspases.** Treatment of Huh7 and HepG2 cells with the COX-2-selective inhibitor celecoxib elicited apoptosis in a dose- and time-dependent manner (Fig. 2A and B). In both cell lines, celecoxib-mediated apoptosis was strongly inhibited by the caspase inhibitors ZVAD-FMK, DEVD-FMK, Z-IETD-FMK, and Z-LEHD-FMK (Fig. 2C). This observation suggests the involvement of caspases in apoptosis induced by celecoxib. Involvement of caspases was further confirmed by fluorometric determination of the increased enzymatic activity of the caspase-3, caspase-8, and caspase-9 class of proteases in HepG2 and Huh7 cells following celecoxib treatment (Fig. 2D).

**COX-2 inhibition triggers the extrinsic apoptosis pathway via activation of death receptors.** To further dissect the signaling pathways involved in the mediation of celecoxib-induced apoptosis, we tested expression and did stimulation experiments of CD95, TNF-R, TRAIL-R1, and TRAIL-R2. Importantly, FACS analysis revealed significant up-regulation of CD95, TNF-R1, and TRAIL-R2 in HepG2 and of TRAIL-R1 and TRAIL-R2 in Huh7 cells following celecoxib treatment (Fig. 3A).

Addition of the respective specific antibodies and ligands (agonistic anti-APO-1 antibody, TNF-α, or LZ-TRAIL) led to a further increase of celecoxib-mediated apoptosis (Fig. 3B) in HepG2 and Huh7 cells.

Death receptor–induced apoptosis can be disrupted by the use of a dominant-negative (dn) FADD mutant (FADDm) that blocks the recruitment of caspase-8 (31). To determine whether increased death receptor expression contributed to celecoxib-induced apoptosis, we transfected HepG2 and Huh7 cells with a dn FADDm expression vector or an empty control vector (Fig. 3C). Then, we...
treated the transfectants with celecoxib and assessed the rate of apoptosis. Clearly, overexpression of a dn FADDm protected cells from celecoxib-mediated apoptosis.

This suggests that celecoxib-induced apoptosis of liver tumor cells involves activation of the extrinsic apoptosis pathway, including the CD95, the TNF, and the TRAIL receptor system.

**COX-2 inhibition induces the mitochondrial apoptosis pathway.** Because celecoxib induced caspase-9 expression and previous analyses in Jurkat T cells have shown abolition of COX-2 inhibitor-dependent apoptosis by a dn caspase-9 mutant (32), we investigated the influence of celecoxib on mitochondrial (intrinsic) apoptosis signaling pathways. FACScan analyses following JC-1 and DiOC staining revealed a rapid alteration of the mitochondrial membrane potential of HepG2 and Huh7 cells following celecoxib treatment (Fig. 4A). This phenomenon was accompanied by an accumulation of cytochrome c in the cytoplasmatic protein fraction (Fig. 4B), showing cytochrome c release from mitochondria.

Mitochondrial integrity is regulated by proapoptotic and antiapoptotic members of the Bcl-2 family, such as Mcl-1 (antiapoptotic) or Bax (proapoptotic). Mcl-1 protects cells from apoptosis induction through blockage of cytochrome c release from mitochondria by interacting with proapoptotic members of the Bcl-2 protein family [e.g., Bim (33), Bak (34, 35), and NOXA (36)]. We have shown previously (19) that Huh7, HepG2, and Hep3B cells show a significant expression of Mcl-1.

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**Figure 3.** COX-2 inhibition sensitizes hepatoma cells toward death receptor-mediated apoptosis. A, treatment with celecoxib (24, 48, and 72 hours) increased cell surface expression of CD95, TNF-R1, and TRAIL-R2 in HepG2 cells. In Huh7 cells, celecoxib led to a significant induction of cell surface expression of TRAIL-R1 and TRAIL-R2. Assays were done in triplicate. Points, mean (n = 3) of three independent experiments; bars, SD. *, P < 0.05; **, P < 0.005, ANOVA, considering all points of time. B, addition of the specific ligands following celecoxib treatment sensitized HepG2 (left) and Huh7 (right) cells toward CD95-, TNF-R-, and TRAIL-R-mediated apoptosis. Following celecoxib treatment for 24 hours, medium was removed, and the specific antibodies and ligands (agonistic anti-APO-1 antibody, TNF-α, or L2-TRAIL) were added for further 24 hours. This procedure led to a significant increase of apoptosis in both cell lines. Columns, mean (n = 3) of one of three experiments done in each cell line; bars, SD. *, P < 0.05, Wilcoxon’s test, compared with untreated control cells (next left column). C, overexpression of a dn FADDm (ΔFADD), which lacks the death effector domain and is thus unable to bind caspase-8 reduced the level of celecoxib-induced apoptosis in HepG2 and Huh7 cells. Assays were done in triplicate. Columns, mean (n = 3) of one of three experiments done; bars, SD. *, P < 0.05, Wilcoxon’s test, compared with cells treated with 100 μmol/L celecoxib only.
Following treatment of HepG2 and Huh7 cells with celecoxib, we observed a rapid down-regulation of Mcl-1 in total protein lysates as well as in the mitochondrial protein fraction that was accompanied by an increase of Bax protein levels in the mitochondrial fraction, whereas total protein levels of Bax remained unchanged, showing a translocation of Bax to mitochondria (Fig. 5A).

To find out whether down-regulation of Mcl-1 is a downstream event that contributes to COX-2 inhibitor-mediated induction of apoptosis in hepatoma cells, we tested whether enhanced Mcl-1 expression compromises the ability of celecoxib to induce apoptosis. Therefore, we transfected either a control plasmid (pEF4) or a Mcl-1 expression plasmid (pEF4/Mcl-1) in Huh7 and HepG2 cells. Enhanced Mcl-1 expression resulted in increased apoptosis resistance toward treatment with celecoxib in HepG2 as well as in Huh7 cells compared with control transfected cells. Thus, Mcl-1 down-regulation contributes to tumor cell apoptosis after celecoxib treatment (Fig. 5B).

Using a siRNA approach to inhibit Mcl-1 (Fig. 5C), we next investigated if down-regulation of physiologic levels of Mcl-1 enhances celecoxib-induced apoptosis. We had seen previously that Mcl-1 was rapidly down-regulated following COX-2 inhibition and then appreciably restored after 24 hours (Fig. 5A). Therefore, for RNA interference (RNAi), we choose times at which Mcl-1 levels had been shown to be restored following celecoxib treatment. Down-regulation of endogenous Mcl-1 function led to a significant augmentation of celecoxib-induced apoptosis in HepG2 and Huh7 cells after 24 hours (Fig. 5D). These findings indicate that down-regulation of Mcl-1 is a relevant event in the initiation of apoptosis induced by celecoxib and that Mcl-1 contributes to apoptosis sensitivity of HCC cells toward COX-2 inhibitors.

**COX-2 inhibition sensitizes liver tumor cells toward chemotherapy.** We and others have shown recently that Mcl-1 is expressed in human HCC tissue (19, 37) and that enhanced expression of Mcl-1 inhibits chemotherapy-induced apoptosis in HCC cells. Thus, we investigated whether COX-2 inhibition enhances chemosensitivity of HCC cell lines. Combined treatment of HepG2 and Huh7 cells with celecoxib and a range of chemotherapeutic drugs in clinically relevant concentrations did in fact lead to a significant increase in tumor cell apoptosis. Bleomycin, mitoxantrone, and doxorubicin showed cooperativity with celecoxib in the induction of apoptosis. A synergistic effect (30) on apoptotic cell death of these HCC cell lines was shown for the combination of celecoxib with bleomycin and for the combination of celecoxib with doxorubicin (Fig. 6A).

To determine whether COX-2 inhibitor-mediated sensitization for anticancer drug-induced apoptosis involved activation of the extrinsic apoptosis pathway, we transfected HepG2 and Huh7 cells with a dn FADDm expression vector or an empty control vector (Fig. 6A). Then, we treated the transfectants with celecoxib and bleomycin or doxorubicin or mitoxantrone and assessed the rate of specific apoptosis. Overexpression of a dn FADDm led to a significant reduction of celecoxib-mediated sensitization toward chemotherapy-induced apoptosis for all cytostatic drugs tested (Fig. 6A).

To further test the clinical relevance of these findings, we extended our studies to PHHs to investigate whether COX-2 inhibitors also sensitized normal cells toward apoptosis. Combined treatment of PHHs with celecoxib and chemotherapeutic drugs did not show any synergistic effect on apoptotic cell death (Fig. 6B).

**Discussion**

Data obtained in the present study allow us to propose a model for the regulation of apoptosis and chemosensitivity of cancer cells by COX-2-selective inhibitors.

We show that COX-2 inhibitors engage in multiple and distinct apoptosis pathways in the cell stimulating death receptor signaling, activation of caspases, and apoptosis originating from mitochondria. Furthermore, our results show a relevant role for COX-2 inhibitors in chemosensitivity of liver tumor cells. Combination of COX-2 inhibitors and chemotherapeutic drugs with different mechanisms of action in concentrations relevant for clinical use leads to a synergistic effect on the induction of apoptosis of liver cancer cells. COX-2-mediated chemosensitization involves activation of both death receptor- and mitochondria-mediated apoptosis pathways. Both mechanisms are clearly reinforced by concomitant treatment with chemotherapeutic drugs.
Our study provides several lines of evidence to show that the extrinsic death receptor pathway, particularly that involving CD95, TNF-R, and the TRAIL-R1 and TRAIL-R2 death receptors, plays a critical role in mediating COX-2 inhibitor-induced apoptosis in human liver tumor cell lines. Firstly, overexpression of a dn FADDm suppressed the ability of COX-2 inhibitors to induce apoptosis. Secondly, cell surface expression of CD95, TNF-R, and TRAIL-R2 was induced in liver tumor cell lines treated with COX-2 inhibitors. Thirdly, addition of the specific ligands led to a further increase of COX-2 inhibitor-mediated apoptosis, indicating that COX-2 inhibition induced the expression of functional death receptors on the cell surface.

The COX-2-selective inhibitor celecoxib has been shown to induce apoptosis in Jurkat T cells, which lacked caspase-8 or FADD, but did not induce apoptosis in this cellular system in the presence of a caspase-9 inhibitor and a dn caspase-9 mutant (32). Thus, in Jurkat T cells, celecoxib seems to act through a caspase-9-mediated mitochondrial signaling pathway that leads to the induction of apoptosis independent of the death receptor–mediated apoptotic pathways. In contrast, in human non–small-cell lung carcinoma cell lines, celecoxib seemed to induce apoptosis through the extrinsic death receptor pathway, inducing the expression of TRAIL-R1 and TRAIL-R2 (38). In addition to up-regulation of membrane receptors, a novel mechanism has been described recently by which COX-2 inhibitors sensitized human colon carcinoma cells to TRAIL-R2-mediated apoptosis. This involved ceramide-induced clustering of TRAIL-R2 death receptors in caveolae (39).

Here, we show that COX-2 inhibitors are involved in the activation of both the extrinsic/death receptor–mediated apoptosis pathway and the intrinsic/mitochondria-mediated apoptosis pathway in HCC cells.

Figure 5. COX-2 inhibitor-mediated apoptosis involves down-regulation of Mcl-1. A, Western blot analysis of proapoptotic and antiapoptotic proteins in celecoxib-treated HuH7 cells. A rapid decrease of Mcl-1 in total protein lysates as well as in the mitochondrial protein fraction, which is accompanied by an increase of Bax in the mitochondrial fraction. Total protein levels of Bax remained unchanged, indicating a translocation of Bax from the cytosol to the mitochondria. B, specific up-regulation of Mcl-1 induced resistance of HepG2 (top) and HuH7 (bottom) cells toward celecoxib-mediated apoptosis. HepG2 and HuH7 cells were transfected with pEF4/Mcl-1 or pEF4 as a control and concomitantly treated with celecoxib for 48 hours. Cells were analyzed by flow cytometry for apoptosis induction according to the method of Nicoletti et al. (21). Columns, mean (n = 3) of two independent experiments; bars, SD. *, P < 0.05, multivariate ANOVA, between-subject effect (Mcl-1 effect). C, top, anti-Mcl-1 and anti-actin immunoblots of HepG2 cells transfected with siRNA specific for Mcl-1 or siRNA GFP (control). siRNA directed against Mcl-1 mRNA down-regulated steady-state Mcl-1 protein levels. Bottom, HuH7 cells were transfected with pEF4/Mcl-1. siRNA directed against Mcl-1, but not siRNA directed against GFP, led to decreased Mcl-1 protein expression. D, blocking endogenous Mcl-1 function leads to a significant augmentation of celecoxib-induced apoptosis in HepG2 and HuH7 cells. Transfection of siRNA directed against Mcl-1 led to increased apoptosis after addition of celecoxib in HepG2 (top) and HuH7 (bottom) cells after 24 hours compared with transfection of siRNA directed against GFP. FACScan analysis of propidium iodide–stained nuclei was done (21). Columns, mean (n = 3) of one representative of three experiments; bars, SD. *, P < 0.05, multivariate ANOVA, between-subject effect (siRNA Mcl-1 effect).
Further more, we and others have shown recently that McI-1 is highly expressed in human HCC tissue and that enhanced expression of McI-1 inhibits drug-induced apoptosis in HCC cells (19, 37). These data suggest that McI-1 is an important survival factor for HCC and a promising target for therapeutic approaches in patients with HCC (16, 19). In the present study, we identified a decrease of McI-1 as a relevant link in the initiation of mitochondrial apoptosis induced by COX-2 inhibition. Decrease of McI-1 protein levels was followed by mitochondrial cytochrome c release and the activation of caspases as well as enhanced sensitivity to chemotherapeutic drugs. Consequently, down-regulation of endogenous McI-1 by RNAi enhanced COX-2 inhibitor-induced apoptosis. As a side note, McI-1 down-regulation occurs very rapidly within 1 hour, but apoptosis becomes evident after 1 to 3 days, indicating that the decrease in McI-1 is an early and may be a transcription-independent effect, which may jump-start the COX-2 inhibitor-mediated activation of the mitochondrial apoptosis pathway and potentially reinforce transcription-dependent apoptotic events later on. A similar delay between McI-1 down-regulation and subsequent cell death has been shown in murine lymphohematopoietic tissues following conditional deletion of the McI-1 gene in vivo (40, 41). Combining these observations suggests that McI-1 down-regulation contributes to apoptosis and initiates apoptosis by directly activating mitochondria possibly followed by transcription of genes involved in death receptor– or mitochondria-mediated apoptosis signaling pathways to sustain the apoptotic response.

Modification of McI-1 expression has been shown to trigger apoptosis sensitivity/resistance toward chemotherapeutic drugs. Overexpression of COX-2 or exposure to PGE2 can increase the apoptosis threshold in human lung adenocarcinoma cells by up-regulating the McI-1 gene. In these cells, COX-2 has been shown to promote survival by up-regulating the level of McI-1 through the phosphatidylinositol 3-kinase/Akt-dependent pathway (42). Our data clearly show that COX-2 inhibitors can directly interfere with this protumorigenic effect of COX-2 via down-regulating the expression of McI-1 protein. Thus, we describe a new mechanism of action by COX-2 inhibitors (i.e., the interference with the McI-1 resistance pathway). These data further support the use of COX-2 inhibitors in chemoprevention and chemotherapy. Further studies are warranted to determine whether COX-2 inhibitors are efficacious as “apoptosis sensitizers” in tumors, in which McI-1 accumulation causes resistance to therapy, such as chronic lymphocytic leukemia (43), relapsed acute leukemia, cholangiocarcinoma (44), and HCC (16, 19, 40, 41, 45).

Of particular clinical relevance with respect to the potential of COX-2 inhibitors for chemosensitization are our findings obtained in PHHs. COX-2 inhibitors did not sensitize normal liver cells toward chemotherapy-induced apoptosis. This implicates a therapeutic window for the combination of COX-2 inhibitors and cytostatic drugs.

In summary, COX-2 inhibitors target multiple cell survival and apoptosis signaling pathways by directly affecting death receptor–mediated apoptosis and by inducing mitochondrial apoptosis signaling. Reinforcement of the apoptosis-inducing action of multiple chemotherapeutic drugs suggests that combined treatments with COX-2 inhibitors and cytostatic agents are a therapeutically useful principle in many types of human cancer.

Acknowledgments

Received 1/26/2006; revised 4/18/2006; accepted 5/5/2006.

Grant support: Deutsche Krebshilfe (M.A. Kern and P. Schirmacher), Tumorzentrum Heidelberg/Mannheim (M.A. Kern, P.H. Krammer, P. Schirmacher, and M. Müller), Center for Molecular Medicine (ZMKM), University of Cologne (P. Schirmacher and M.A. Kern), and Köln Fortune Program of the University of Cologne (P. Schirmacher and M.A. Kern).

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We thank Katja Lorenz, Petra Hill, and Eva Eiteneuer for expert technical assistance.
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