In-vivo effects and mechanisms of celecoxib-reduced growth of cyclooxygenase-2 (COX-2)-expressing versus COX-2-deleted human HCC xenografts in nude mice

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We previously reported that celecoxib, a cyclooxygenase-2 (COX-2) inhibitor, suppresses growth of human hepatocellular carcinoma (HCC) cells through both COX-2 dependence and independence. Recently, we established COX-2-deleted human HCC cells, C2D-HuH7, and C2D-HuH7-bearing nude mice. Using this novel model, we examined the pharmacological effects and mechanisms of celecoxib on in-vivo growth of HCC xenografts in relation to COX-2 expression. After treatment with celecoxib, the mice bearing HuH7 or C2D-HuH7 xenografts were assessed for the pharmacological effects and mechanisms of celecoxib on HCC xenograft growth in relation to COX-2 expression. Celecoxib resulted in an effective and comparable growth reduction of both COX-2-expressing and COX-2-deleted HuH7 xenografts in association with decreased Ki-67 expression. These results demonstrated celecoxib's COX-2-independent in-vivo anti-HCC effects. Celecoxib increased peroxisome proliferator-activated receptor γ predominantly in HuH7 xenografts, indicating its COX-2 dependency. Celecoxib reduced p-Rb and DP1/E2F1 complex predominantly via upregulated p21/CDK4 complex in HuH7 xenograft, but p27/CDK4 complex in C2D-HuH7 xenografts. The effects of celecoxib on phosphatase and tensin homolog deleted on chromosome ten/PI3K/Akt signaling were COX-2 independent, but its effects on extracellular-regulated kinase signaling seemed COX-2 dependent. In addition, the effects of celecoxib on AC-H3, AC-H4, and histone deacetylase 2 could be both COX-2 dependent and independent. In conclusion, celecoxib suppresses growth of HuH7 xenografts regardless of COX-2 expression, which may be mediated through different signaling. *Anti-Cancer Drugs* 19:891–897 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2008, **19**:891–897

Keywords: cell cycle, cyclooxygenase-2, cyclooxygenase-2 deletion, cyclooxygenase-2 inhibitor, hepatocellular carcinoma

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Received 26 May 2008 **Revised form accepted** 2 July 2008

Introduction

Hepatocellular carcinoma (HCC) is one of the most common human cancers worldwide [1–4]. Developing an effective chemoprevention strategy is critically important in the effort of reducing this disease. Overexpression of cyclooxygenase-2 (COX-2) has been associated with carcinogenesis, including HCC [5–7]. Other authors as well as us have demonstrated that several COX-2 inhibitors can effectively inhibit HCC cell growth both *in vitro* and *in vivo* [8–14]. Although we reported that regardless of COX-2 expression, celecoxib treatment results in potent growth inhibition of human HCC cells [14], the study was carried out in different HCC cell lines. Thus, further studies are needed using a single cell origin. We recently established COX-2-deleted human HCC cells, C2D-HuH7, and developed nude mice bearing C2D-HuH7 xenograft [15]. This allows us to reliably assess the pharmacological effects and mechanisms of celecoxib on in-vivo HCC cell growth in the absence of COX-2 expression.

Even though recent studies suggested that the long-term application of a high dose of celecoxib may be associated with increased cardiovascular adverse events [16], it remains to be determined whether a low dose of this drug is safe and valuable for cancer chemoprevention. Our previous study demonstrated that celecoxib at a low dose (i.e. equivalent to 200 mg/day in humans) results in a potent in-vivo anti-HCC effect in nude mice bearing HCC xenografts [14]. This provides a rationale to further test a low dose of celecoxib alone or combined with other agents for cancer chemoprevention.

Our previous in-vitro studies have indicated that celecoxib possesses extensive pharmacological effects [14]. These include celecoxib's effects on cell proliferation and its related cell cycle modulators, apoptosis, and other cellular signaling pathways. α-Fetoprotein (AFP) has been widely used for diagnosing HCC, assessing HCC differentiation, and treatment effects of HCC on humans [17–19]. Phosphatase and tensin homolog deleted on

0959-4973 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e32830ef8ca

chromosome 10 (PTEN), phosphatidylinositol 3′-kinase (PI3K), and Akt (PTEN/PI3K/Akt) pathways have been associated with carcinogenesis [20]. Activated PI3K-Akt signaling promotes carcinogenesis [21,22]. PTEN is a negative regulator of PI3K-Akt signaling [20] and one of the most frequently inactivated genes in malignancies [23,24]. We reported that C2D reduces Akt phosphorylation in HuH7 cells [15], but it remains unknown whether PTEN/PI3K/Akt pathway is also involved in celecoxib's in-vivo effects on HCC growth.

The mitogen-activated protein kinase pathway is a mitogenic signaling cascade [25–27]. The final intermediate in the mitogen-activated protein kinase signaling cascade is extracellular-regulated kinase (ERK), which has two isoforms, ERK1 and ERK2 (ERK1, 2). The active status is phosphorylated ERK (p-ERK) which promotes cell proliferation, cell cycle progression, and oncogenesis [25–27]. It has been reported that p-ERK is increased in HCC [28], but it remains to be defined whether celecoxib-reduced HCC growth is also mediated through ERK pathway.

Histone acetylation is particularly important in controlling tumor growth. Histone acetylation modifies nucleosome structure, leads to DNA relaxation, reduces the affinity of histone complexes for DNA, enhancing the access of transcriptional factor to DNA, and promotes gene transcription. Histone deacetylases (HDACs) make histone deacetylation result in a more condensed chromatin and prevent access of transcriptional factor to DNA [29–31]. Accumulating evidence has indicated that aberrant regulation of HDACs is one of the major causes for development of human malignancies [32]. Study results indicated that HDAC2, a subtype of HDACs, is overexpressed in human gastric cancer [32], but it also remains unknown whether the change in HDAC2 and related acetylation of histone are involved in celecoxib-reduced HCC growth.

In this study, we used both COX-2-expressing HuH7 and C2D-HuH7 xenograft mice models to assess the in-vivo effects and mechanisms of celecoxib on growth of HuH7 xenografts in relation to COX-2 expression.

Materials and methods

Reagents

The reagents for cell culture were same, as previously reported [14]. Celecoxib was kindly provided by Pharmacia & Upjohn Co., (Kalamazoo, Michigan, USA). Antibody against human COX-2 was from Cayman Chemical Company (Ann Arbor, Michigan, USA). Antibody against activated caspase-3 was from Sigma Chemical Co. (St Louis, Missouri, USA). Antibodies against human cyclin D1 (CD1), DP1, phosphorylated-retinoblastoma (p-Rb), E2F1, cyclin-dependent kinase-4 (CDK-4), $p21^{waf1/cip1}$ and $p27^{kip1}$, peroxisome proliferator-activated receptor γ (PPARγ), activated caspase-9, p-Akt, p-ERK (i.e. ERK1, 2), PTEN, Ki-67, AC-H3, AC-H4, HDAC-2, and β-actin were from Santa Cruz Biotechnology (Santa Cruz, California, USA). AFP enzymatic immunoassay kit was from Monobind, Inc (Costa Mesa, California, USA). The PTEN activity assay kit was from Biomol Research Laboratories, Inc (Plymouth Meeting, Pennsylvania, USA).

Human hepatocellular carcinoma cells and culture

HuH7 cells, a human HCC cell line expressing a high level of COX-2 [14], were used as control. A pool of C2D-HuH7 cells was derived from HuH7 cells with complete deletion of COX-2 mRNA and protein, as previously reported [15]. The cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, as previously reported [8,14] and used to establish HCC xenografts in nude mice [15].

Animal experiment

The animal experiment was approved by the Institutional Animal Care and Use Committee. Four-week-old male NCRNU-M nude mice were obtained from Taconic Farms, Inc. (Usdon, New York, USA). After subcutaneous inoculation of 5×10^6/0.25 ml of HuH7 cells ($n = 10$) or C2D-HuH7 cells ($n = 10$), the two test groups received gavage of celecoxib at 50 mg/kg/day, started after 24 h of the inoculation [14,15]. Celecoxib dose was adjusted weekly based on changes in body weight. Tumor volumes were recorded weekly and the experiment lasted for 5 weeks [14,15]. At the end of the experiment, xenograft tumors were isolated and weighted after euthanasia. The blood specimens were collected from the tail vein and plasma was used to quantify AFP. Three mice bearing HCC xenografts that were closest to the mean weight of the group were selected. A 300 mg of tumor tissue from each xenograft was homogenized with lysis buffer and was centrifuged, and the clarified supernatants were stored in −80°C and used for experiments described below.

Quantification of plasma α-fetoprotein levels

The plasma levels of AFP were quantified using enzymatic immunoassay kits, as previously reported [14,15]. A standard curve was used to calculate plasma levels of AFP [14,15].

Immunoprecipitation and western blot analysis

The supernatants of xenograft lysates and western blots [14,15] were used to detect Ki-67, PPARγ, $p21^{waf1/cip1}$, $p27^{kip1}$, E2F1, cyclin D-1, CDK4, p-Rb, activated caspase-3 and caspase-9, HDAC2, AC-H3, AC-H4, p-Akt, and p-ERK. To further understand whether celecoxib can affect combining of $p21^{waf1/cip1}$, $p27^{kip1}$ with cyclin D1 with CDK4, and combining of DP1with E2F1, immonoprecipitation technique was used, as previously reported [14,15].

Phosphatase and tensin homolog deleted on chromosome 10 activity assay

PTEN protein was immunoprecipitated according to the instructions provided by the manufacturer. The phosphatase reaction was performed in 50 µl of assay buffer containing 100 mmol/l Tris–HCl pH 8.0, 10 mmol/l DTT, 200 µmol/l water-soluble diC8-PIP3, and the immunoprecipitated PTEN protein [33]. The release of phosphate from the substrate was measured in a colorimetric assay by using the PTEN activity assay kit in accordance with the instructions of the manufacturer.

Statistical analysis

The descriptive statistics was provided with mean ± SD. *t*-test was used to assess the effect (i.e. mean differences) of celecoxib treatment on AFP production, as well as the scanning data of western blot; a $P < 0.05$ was considered statistically significant.

Results

Celecoxib reduces growth of human hepatocellular carcinoma xenografts regardless of cyclooxygenase-2 expression

As shown in Fig. 1a, comparing with the corresponding untreated groups, celecoxib at the dose of 50 mg/kg/day significantly reduced the mean xenograft weight in both COX-2-expressing HuH7 group (36% decrease, $P < 0.05$) and C2D-HuH7 group (47% decrease, $P < 0.05$). Celecoxib-reduced xenograft growth was consistent with decreased Ki-67 expression by 40% in HuH7 group ($P < 0.05$) and 54% in C2D-HuH7 group (Fig. 2a, $P < 0.01$). These indicated that celecoxib suppresses growth of both HuH7 and C2D-HuH7 xenografts regardless of COX-2 expression. As shown in Fig. 2b, celecoxib increased PPARγ expression in both HuH7 xenografts (by 2.6-fold, $P < 0.05$) and C2D-HuH7 xenografts (by 57%, $P < 0.01$) in association with reduced mean xenograft weight. Compared with the control groups, celecoxib treatment did not alter the body weight in the nude mice bearing either HuH7 or C2D-HuH7 xenografts (Fig. 1b).

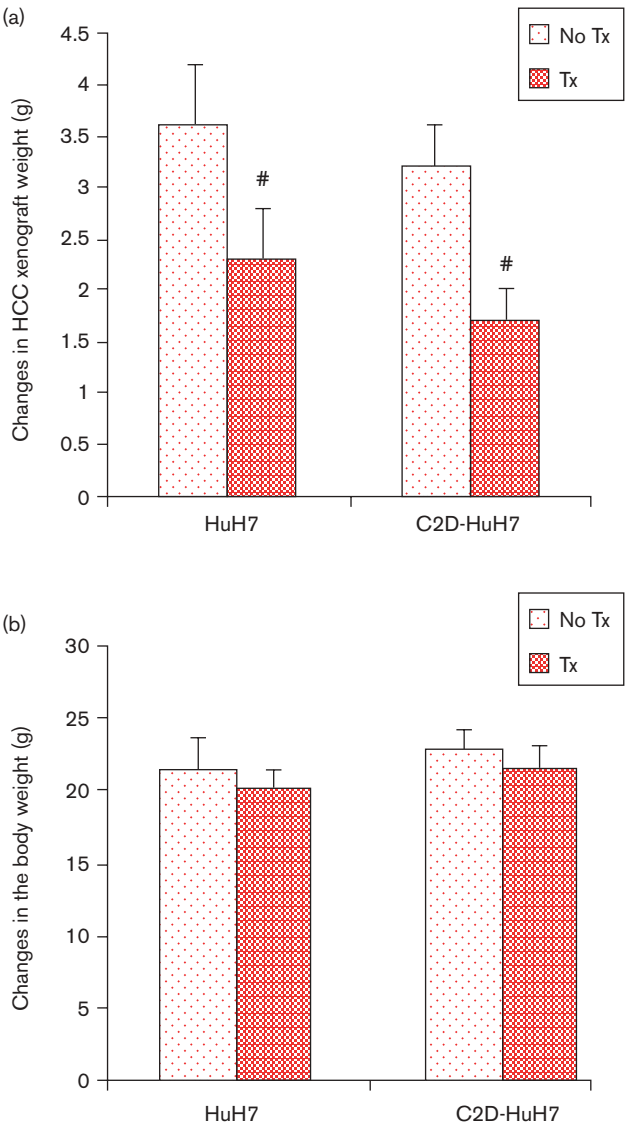
Celecoxib-reduced hepatocellular carcinoma xenografts is associated with decreased plasma levels of α-fetoprotein

As shown in Fig. 3a, celecoxib treatment resulted in significant decrease in plasma AFP levels in HuH7 group by 43% ($P < 0.05$) and in C2D-HuH7 group by 28% ($P < 0.05$). Celecoxib-reduced mean HCC xenograft weight was significantly correlated with the degree of decreased plasma AFP in both HuH7 ($r = 0.86$, $P = 0.02$) and C2D-HuH7 ($r = 0.82$, $P = 0.01$) groups (data not shown).

Celecoxib mediates alteration in cell cycle-related modulators

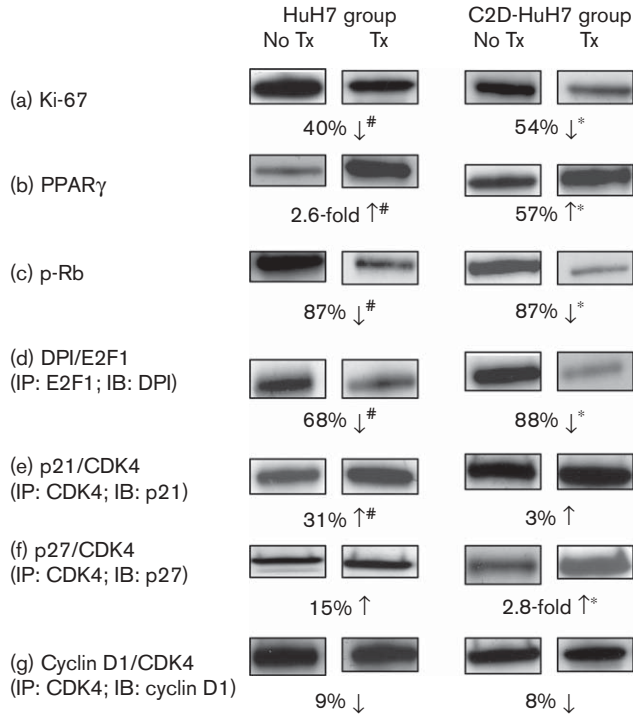
To further determine the molecular mechanisms of celecoxib-reduced growth of human HCC xenografts, we investigated the effects of celecoxib on the expression of a series of cell cycle-related modulators in COX-2-expressing HuH7 and C2D-HuH7 xenograft tissue specimens. As shown in Fig. 2c and d, celecoxib treatment suppressed p-Rb by 87% in both HuH7 ($P < 0.05$) and C2D-HuH7 ($P < 0.01$) groups, which was associated with 68% ($P < 0.05$) and 88% ($P < 0.01$) reduction of DP1/E2F1 complex in HuH7 and C2D-HuH7 groups, respectively. These indicated that cel-

Fig. 1



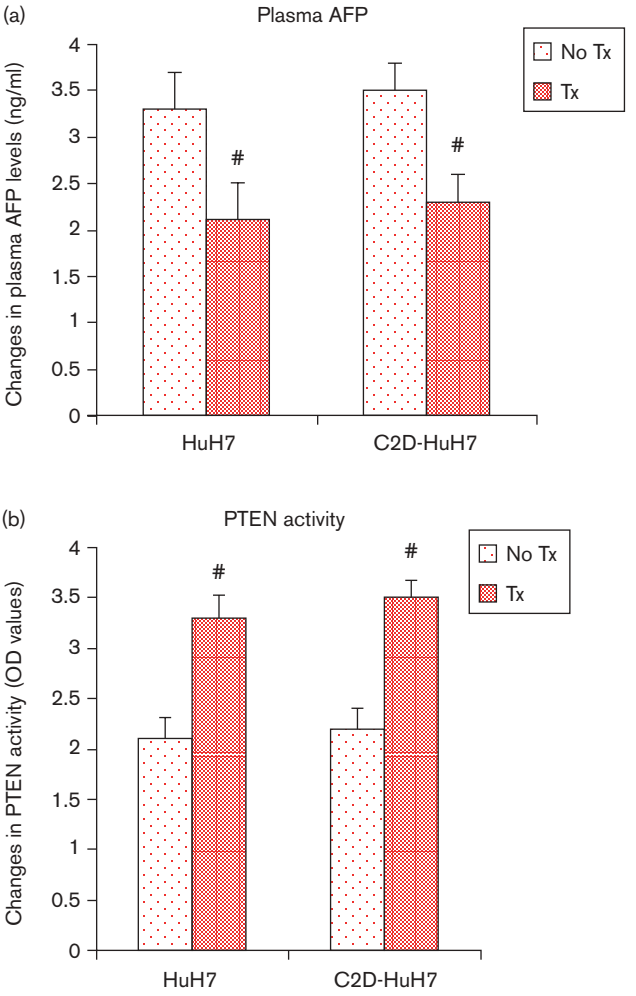
Effects of celecoxib on HuH7 xenograft and body weight of the nude mice. (a) Celecoxib-reduced mean weight of the HuH7 and C2D-HuH7 xenografts in the nude mice regardless of COX-2 expression. (b) Celecoxib did not significantly affect the body weight of the nude mice bearing HuH7 or C2D-HuH7 xenografts. $^{\#}P < 0.05$ as compared with the respective control group; COX-2, cyclooxygenase-2; HCC, hepatocellular carcinoma; Tx, treatment.

Fig. 2



Effects of celecoxib treatment on expression of Ki-67, PPARγ, and cell cycle modulators. (a) Celecoxib significantly reduced Ki-67 expression in both HuH7 and C2D-HuH7 xenografts. (b) Celecoxib increased PPARγ in both HuH7 and C2D-HuH7 xenografts, but it is significantly more in HuH7 xenografts. (c) Celecoxib significantly reduced p-Rb expression. (d) Celecoxib significantly reduced DP1 binding with E2F1, (e) p21 binding with CDK4, (f) p27 binding with CDK4, and (g) CD1 binding with CDK4. The signal bands were quantified, and expressed as mean ± SD from three tissue specimens in each group. $^{\#}P<0.05$ and $^{*}P<0.01$ as compared with the respective control group. PPARγ, peroxisome proliferator-activated receptor γ; Tx, treatment.

Fig. 3



Effects of celecoxib treatment on production of α-fetoprotein (AFP), cell apoptosis, and PTEN activity. (a) Plasma AFP was quantified by an enzymatic immunoassay (EIA) and expressed as ng/ml. (b) PTEN activity was quantified by an EIA and expressed as optical density (OD) value. $^{\#}P<0.05$ as compared with control. PTEN, phosphatase and tensin homolog deleted on chromosome ten; Tx, treatment.

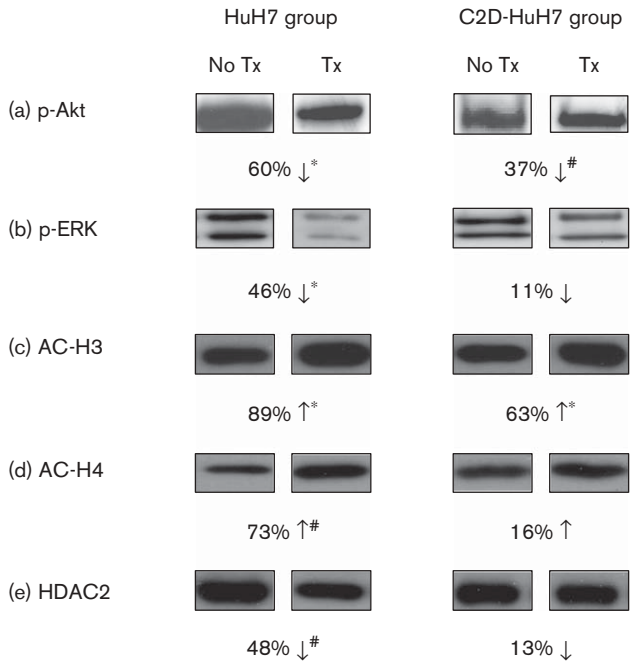
ecoxib-reduced p-Rb and DP1/E2F1 complex seems COX-2 independent.

We then examined whether celecoxib alters the formation of p21/CDK4, p27/CDK4, and CD1/CDK4 complexes. As shown in Fig. 2e, celecoxib treatment resulted in a 31% increase of p21/CDK4 complex in HuH7 xenograft ($P < 0.05$), but it did not significantly alter the formation of p21/CDK4 complex in C2D-HuH7 xenografts ($P = 0.08$). In contrast, celecoxib treatment did not alter formation of p27/CDK4 complex in HuH7 xenograft ($P = 0.09$,), but it resulted in a 2.8-fold increase of p27/CDK4 complex in C2D-HuH7 xenografts (Fig. 2f, $P < 0.01$). As shown in Fig. 2g, celecoxib treatment did not significantly alter the formation of CD1/CDK4 complex in both HuH7 and C2D-HuH7 xenografts. These indicated that the effects of celecoxib on formation of p21/CDK4, p27/CDK4 complexes depend on COX-2 expression in these HCC xenografts.

Celecoxib mediates alteration in PTEN/PI3K/Akt, ERK signaling

We reported that COX-2 deletion resulted in decreased phosphorylation of Akt, but not ERK in cultured HuH7 cells [15]. To further determine the role of PTEN/PI3K/Akt and ERK signaling in celecoxib-reduced growth of HCC xenografts, we examined the changes of PTEN activity, p-Akt, and p-ERK in the xenograft tissue specimens after celecoxib treatment. As shown in Fig. 4a, celecoxib inhibited p-Akt formation by 60% in HuH7 xenografts ($P < 0.01$) and 37% in C2D-HuH7 xenografts ($P < 0.05$). Consistent with these, celecoxib increased PTEN activity by 45% in HuH7 xenografts ($P < 0.05$) and 35% in C2D-HuH7 xenografts (Fig. 3b,

Fig. 4



Effects of celecoxib treatment on expression of p-Akt, p-Erk, AC-H3, AC-H4, and HDAC2. The western blots were used. (a) p-Akt, (b) p-Erk, (c) AC-H3, (d) AC-H4, and (e) HDAC2 expression altered by celecoxib treatment. $^{\#}P<0.05$ and $^{*}P<0.01$ as compared with the respective control group. HDAC2, histone deacetylase 2; p-ERK, phosphorylated extracellular-regulated kinase; Tx, treatment.

$P < 0.05$), respectively. As shown in Fig. 4b, celecoxib inhibited expression of p-ERK by 46% in HuH7 xenografts ($P < 0.01$), but only 25% in C2D-HuH7 xenografts ($P = 0.06$). These suggested that celecoxib-increased PTEN activity and reduced production of p-Akt seems COX-2 independent, but celecoxib-reduced production of p-ERK might be more COX-2 dependent.

Celecoxib mediates alteration in acetylation of histone

Several studies indicated that acetylation of histone is involved in inhibition of tumor growth [29–31]. To determine whether acetylation of histone mediates celecoxib-reduced growth of HuH7 xenografts, we examined the expression of AC-3 and AC-H4 in HuH7 xenografts after celecoxib treatment. As shown in Fig. 4c, celecoxib treatment resulted in a significant and comparable increase of AC-H3 production in both HuH7 and C2D-HuH7 xenograft tissues ($P < 0.01$). However, as shown in Fig. 4d, celecoxib resulted in a more significant increase of AC-H4 production in HuH7 xenografts (73%, $P < 0.05$) than in C2D-HuH7 xenografts (16%, $P = 0.07$). HDAC2 has been reported to inhibit acetylation of histone and promote tumor growth [29]. Our result showed that celecoxib significantly reduced HDAC2 only in HuH7 (48%, $P < 0.05$), but not in C2D-HuH7 xenograft tissues (13%, $P = 0.07$, Fig. 4e).

Discussion

It is known that aberrant COX-2 expression plays an important role in carcinogenesis, including HCC [5–7]. Recently, we demonstrated that celecoxib significantly reduces growth of human HCC cells *in vitro* and the mean weight of HCC xenograft *in vivo* [14]. Although our in-vitro data indicated that celecoxib-reduced HCC growth appears both COX-2 dependent and independent, the results were derived from comparison of two different human HCC cell lines [14]. HuH7 cells are human HCC cells that highly express COX-2 [14]. We recently established a pool of C2D-HuH7 cells with complete deletion of COX-2 expression and were able to develop C2D HuH7 xenografts in the nude mice [15]. This model provides a unique way to assess anti-HCC effects and mechanisms of celecoxib in relation to COX-2 expression.

First, we assessed how celecoxib affects the growth of HuH7 and C2D-HuH7 xenografts in nude mice. Celecoxib at 50 mg/kg of body weight in mice provides a total absorption equivalent to human exposure of 200 mg twice daily [34]. According to the above calculation, celecoxib was used at the dose of 50 mg/kg/day to provide the equivalent of 200 mg human twice-daily exposure. Our results revealed that celecoxib treatment for 5 weeks caused effective growth inhibition of both HCC xenografts regardless of COX-2 expression. Interestingly, such inhibition was slightly more effective in C2D-HuH7 xenografts (47%) than in HuH7 xenografts (36%), which was consistent with degree of suppressed expression of Ki-67 expression in these xenografts. We reported that COX-2-independent mechanisms might be involved in celecoxib-reduced in-vitro HCC cell proliferation [14]. Our data extended these findings and demonstrated that celecoxib can effectively inhibit in-vivo growth of HCC xenografts in the absence of COX-2 expression. These indicated that a COX-2-independent pharmacological mechanism is involved in celecoxib-reduced growth of HCC xenografts. Indeed, a low dose of celecoxib (equivalent to human dose of 200 mg/day) seems enough to achieve the comparable anti-HCC effects regardless of COX-2 expression. As not all HCCs overexpress COX-2 [6], our findings provide a rationale that celecoxib might serve as a HCC chemopreventive agent regardless of COX-2 expression. These findings are of particular clinical value to support further clinical trial on testing a low dose of celecoxib for HCC chemoprevention.

AFP has been widely used for diagnosing HCC and assessing treatment effects or recurrence of HCC in humans. Our result showed that celecoxib decreased plasma AFP levels in both groups, although it was more significant in COX-2-expressing HuH7 xenografts. These further confirm the potent anti-HCC effects of celecoxib

in both groups. Our findings also suggested a potential value of using plasma AFP as a noninvasive biomarker to predict the effect of celecoxib or other candidate agents on HCC chemoprevention.

PPARγ is a member of the nuclear hormone receptors. Recent data showed that after activation by its ligands, PPARγ inhibits cell growth and induces apoptosis in human liver cancer cells [35,36]. Studies have reported that PPARγ is a target of the COX-2-independent pathway mediated by nonsteroidal anti-inflammatory drugs [37,38]. Our previous work suggested that celecoxib alters expression of PPARγ in HuH7 cells *in vitro* [14]. In this study, we found that celecoxib upregulated PPARγ expression in both HuH7 and C2D-HuH7 xenografts. As PPARγ expression was more significantly upregulated in HuH7 xenografts, this indicated a possibly more important signaling pathway in celecoxib-mediated COX-2-dependent mechanisms.

We and other research groups reported that celecoxib-reduced cancer cell growth is associated with suppressed cell cycle progression and promoted apoptosis [12–14]. Effective inhibition of G1-S transition suppresses tumor growth. The G1-S checkpoint is mainly controlled by the complexes of cyclin D1 with CDKs (i.e. CDK2, CDK4, and CDK6), Rb phosphorylation, and CDK inhibitors, including $p21^{waf1/cip1}$ and $p27^{kip1}$. We first demonstrated that celecoxib significantly inhibited production of p-Rb and binding of DP1with E2F1 in both C2D-HuH7 and HuH7 xenografts. These results reconfirmed our in-vitro reports that celecoxib suppresses HCC cell growth by inhibiting G1-S transition [14]. In addition, our results also suggest that celecoxib-reduced G1-S transition is COX-2 independent. It is known that p21 and p27 regulate cell cycle progression through binding with CDK4 and inhibiting its activity. We found that celecoxib tended to increase the p21/CDK4 complex in HuH7 xenografts, but the p27/CDK4 complex in C2D-HuH7 xenografts. This discrepancy in cell cycle signaling indicated that different mechanisms of celecoxib reduced growth of HCC xenografts in G1-S checkpoint in relation to COX-2 expression. A significantly greater upregulation of p27/CDK4 complex in C2D-HuH7 xenografts suggested its important role in celecoxib-mediated COX-2-independent anti-HCC mechanisms. Taken together, our data suggested that celecoxib-reduced cell cycle progression might involve different levels of signaling that could be either COX-2 dependent or independent.

Studies have indicated important roles of PTEN/PI3K/Akt signaling in carcinogenesis and cancer progression by stimulating cell proliferation and inhibiting apoptosis [21,39–42]. Akt is composed of an N-terminal pleckstrin homology domain and a C-terminal kinase catalytic domain. Phosphorylation at Thr^{308} and Ser^{437} results in Akt activation, which further phosphorylates several key proteins resulting in promoting cell cycle progression and inhibiting apoptosis [13]. We reported that COX-2 deletion reduces p-Akt in HuH7 xenografts [15]. In this study, we found that celecoxib significantly reduced p-Akt in association with increased PTEN activity in both C2D-HuH7 and HuH7 xenografts, although these effects were more profound in COX-2-expressing HuH7 xenografts. These further support the potential role of altered PTEN/PI3K/Akt signaling in celecoxib-reduced HCC growth of HuH7 xenografts regardless of the status of COX-2 expression.

Increased p-ERK activates transcription of the mitogenic and cell regulatory genes that promote oncogenesis by altering cell cycle progression and apoptosis [25–27, 40,43]. It has been reported that p-ERK is increased in HCC [28], suggesting its signaling is involved in HCC development. Our previous results indicate that COX-2 deletion does not significantly alter p-ERK in HuH7 xenografts [15]. In this study, we found that celecoxib reduced p-ERK more significantly in HuH7 xenografts than in C2D-HuH7 xenografts. These indicated that altered ERK signaling in celecoxib-reduced HCC growth of HuH7 xenografts is possibly COX-2 dependent.

Acetylation of histone-3 and -4 is involved in gene transcription [29–31] and cell growth. We reported that COX-2 deletion results in increased AC-H3 and AC-H4, but decreased HDAC2 expression in HuH7 xenografts [15]. In this study, we found that celecoxib significantly increased AC-H3 in both HuH7 and C2D-HuH7 xenografts. However, celecoxib-increased AC-H4 and reduced HDAC2 were seen only in COX-2-expressing HuH7 xenografts, but not C2D-HuH7 xenografts. These indicated that celecoxib-altered AC-H3 and AC-H4 could be COX-2 dependent and independent. Further studies will be needed to elucidate the detailed mechanisms.

Acknowledgements

The authors are indebted to Namgyal Kyulo, MPH, for his contribution to statistical analysis. This study is supported by grant CA095975 from the National Cancer Institute (K.-Q. H.) and UCI institutional research grants from GI Division and Chao Family Comprehensive Cancer Center (K.-Q. H.).

References

1 Di Bisceglie AM. Malignant neoplasms of the liver. In: Schiff ER, Sorrel MF, Maddrey WC. *Schiff's disease of the liver*. 8th ed. **Vol. 2**. Philadelphia: Lippincott-Raven Publishers; 1999, pp. 1281–1304.
2 Kew MC. Hepatic tumors and cysts. In: Feldman M, Sleisenger MH, Scharschmidt BF. *Gastrointestinal and liver disease*. 6th ed. Philadelphia: WB Saunders; 1998, pp. 1364–1387.
3 El-Serag HB, Mason A. Rising incidence of hepatocellular carcinoma in the United States. *N Engl J Med* 1999; **340**:745–750.
4 El-Serag HB, Mason A, Key C. Trends in survival of patients with hepatocellular carcinoma between 1997 and 1996 in the United States. *Hepatology* 2001; **33**:62–65.

5 Willams CS, Mann M, DuBois RN. The role of cyclooxygenase in inflammation, cancer and development. *Oncology* 1999; **18**:7908–7916.
6 Hu K-Q. Rationale and feasibility of chemoprevention of hepatocellular carcinoma by cyclooxygenase-2 inhibitors. *J Lab Clin Med* 2002; **139**: 234–243.
7 Fosslien E. Biochemistry of cyclooxygenase (COX-2) inhibitors and molecular pathology of COX-2 in neoplasia. *Crit Rev Clin Lab Sci* 2000; **37**:431–502.
8 Hu K-Q, Yu C-H, Mineyama Y, McCrachen JD, Hillebrand DJ, Hasan M. Inhibited proliferation of cyclooxygenase-2 expressing human hepatoma cells by NS-398, a selective COX-2 inhibitor. *Int J Oncol* 2003; **22**: 757–763.
9 Bae SH, Jung ES, Park YM, Kim BS, Kim BK, Kim DG, *et al*. Expression of cyclooxygenase-2 (COX-2) in hepatocellular carcinoma and growth inhibition of hepatoma cell lines by a COX-2 inhibitor, NS-398. *Clin Cancer Res* 2001; **7**:1410–1418.
10 Kern MA, Schubert D, Sahi D, Schöneweiss MM, Moll I, Haugg AM, *et al*. Proapoptotic and antiproliferative potential of selective cyclooxygenase-2 inhibitors in human liver tumor cells. *Hepatology* 2002; **36**:885–894.
11 Cheng ASL, Chan HY, Leung W, Wong N, Johnson PJ, Sung JY. Specific COX-2 inhibitor, NS-398, suppresses cellular proliferation and induces apoptosis in human hepatocellular carcinoma cells. *Int J Oncol* 2003; **23**:113–119.
12 Kern MA, Schöneweiss MM, Sahi D, Bahlo M, Haugg AM, Kasper HU, *et al*. Cyclooxygenase-2 inhibitors suppress the growth of human hepatocellular carcinoma implants in nude mice. *Carcinogenesis* 2004; **25**:1193–1199.
13 Leng J, Han C, Demetris AJ, Michalopoulos GK, Wu T. Cyclooxygenase-2 promotes hepatocellular carcinoma cell growth through AKT activation: evidence for AKT inhibition in celecoxib-induced apoptosis. *Hepatology* 2003; **38**:756–768.
14 Cui W, Yu CH, Hu KQ. *In vitro* and *in vivo* effects and mechanisms of celecoxib-induced growth inhibition of human HCC cells. *Clin Cancer Res* 2005; **11**:8213–8221.
15 Cui W, Hu SX, Tang ZY, Hu KQ. *In vivo* effects of cyclooxygenase-2 deletion on cellular signaling in hepatocellular carcinoma xenografts in nude mice. *J Cancer Mol* 2007; **3**:49–54.
16 Johnsen SP, Larsson H, Tarone RE, McLaughlin JK, Nørgård B, Friis S, *et al*. Risk of hospitalization for myocardial infarction among users of rofecoxib, celecoxib, and NSAIDs: a population-based case-control study. *Arch Intern Med* 2005; **165**:978–984.
17 Johnson PJ. The role of serum alpha-fetoprote in estimation in the diagnosis and management of hepatocellular carcinoma. *Clin Liver Dis* 2001; **5**: 145–159.
18 Shirabe K, Takenaka K, Gion T, Shimada M, Fujiwara Y, Sugimachi K. Significance of alpha-fetoprotein levels for detection of early recurrence of hepatocellular carcinoma after hepatic resection. *J Surg Oncol* 1997; **64**:143–146.
19 Peng SY, Chen WJ, Lai PL, Jeng YM, Sheu JC, Hsu HC. High α-fetoprotein level correlates with high stage, early recurrence and poor prognosis of hepatocellular carcinoma: significance of hepatitis virus infection, age, p53 and β-catenin mutations. *Int J Cancer* 2004; **112**:44–50.
20 Osaki M, Oshimura M, Ito H. PI3K-Akt pathway: its function and alterations in human cancer. *Apoptosis* 2004; **9**:667–676.
21 Lawlor Ma, Alessi DR. PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *J Cell Sci* 2001; **114**:2903–2910.
22 Yao R, Cooper GM. Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science* 1995; **267**: 2003–2006.
23 Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, *et al*. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* 1997; **275**:1943–1947.
24 Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, *et al*. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet* 1997; **15**:356–362.
25 Fang JY, Richardson BC. The MAPK signaling pathways and colorectal cancer. *Lancet Oncol* 2005; **6**:322–327.
26 Gysin S, Lee SH, Dean NM, McMahon M. Pharmacologic inhibition of RAF/MEK/ERK signaling elicits pancreatic cancer cell cycle arrest through induced expression of p27Kipi. *Cancer Res* 2005; **65**:4870–4880.
27 Wiesenauer CA, Yip-Schneider MT, Wang Y, Schmidt CM. Multiple anticancer effects of blocking MEK-ERK signaling in hepatocellular carcinoma. *J Am Coll Surg* 2004; **198**:410–421.
28 Schmidt CM, McKillop IH, Cahill PA, Sitzmann JV. Increased MAPK expression and activity in primary human hepatocellular carcinoma. *Biochem Biophys Res Commun* 1997; **236**:54–58.
29 Liu T, Kuljaca S, Tee A, Marshal GM. Histone deacetylase inhibitors: multifunctional anticancer agents. *Cancer Treat Rev* 2006; **32**:157–165.
30 Wade PA. Transcriptional control at regulatory checkpoints by histone deacetylase: molecular connections between cancer and chromatin. *Hum Mol Genet* 2001; **10**:693–698.
31 Cress WD, Seto E. Histone deacetylases, transcriptional control, and cancer. *J Cell Physiol* 2002; **184**:1–16.
32 Song J, Noh JH, Lee JH, Eun JW, Ahn YM, Kim SY, *et al*. Increased expression of histone deacetylase 2 is found in human gastric cancer. *APMIS* 2005; **113**:264–268.
33 Meuillet EJ, Mahadevan D, Berggren M, Coon A, Powis G. Thioredoxin-1 binds to the C2 domain of PTEN inhibiting PTEN's lipid phosphatase activity and membrane binding: a mechanism for the functional loss of PTEN's tumor suppressor activity. *Arch Biochem Biophys* 2004; **429**:123–133.
34 Orengo IF, Gergois J, Phillpis R, Guevara A, Lewis AT, Black HS. Celecoxib, a cycloxygenase-2 inhibitor as a potential chemopreventive to UV-induced skin cancer. *Arch Dermatol* 2002; **138**:751–755.
35 Li MY, Deng H, Zhao JM, Dai D, Tan XY. PPARγ pathway activation results in apoptosis and COX-2 inhibition in HepG2 cells. *World J Gastroenterol* 2003; **9**:1220–1225.
36 Toyoda M, Takagi H, Horiguchi N, Kakizaki S, Sato K, Takayama H, *et al*. A ligand for peroxisome proliferator activated receptor γ inhibits cell growth and induces apoptosis in human liver cancer cells. *Gut* 2002; **50**:563–567.
37 Wick M, Hurteau G, Dessev C, Chan D, Geraci MW, Winn RA, *et al*. Peroxisome proliferator-activated receptor-γ is a target of nonsteroidal anti-inflammatory drugs mediating cyclooxygenase-independent inhibition of lung cancer cell growth. *Mol Pharmacol* 2002; **62**:1207–1214.
38 Lehmann JM, Lenhard JM, Oliver BB, Ringold GM, Kliewer SA. Peroxisome proliferatorsactivated receptors alpha and gamma are activated by indomethacin and other non-steroidal anti-inflammatory drugs. *J Biol Chem* 1997; **272**:3406–3410.
39 Roy HK, Olusola BF, Clemens DL, Karolski WJ, Ratashak A, Lynch HT, *et al*. AKT proto-oncogene overexpression is an early event during sporadic colon carcinogenesis. *Carcinogenesis* 2002; **23**:201–205.
40 Altomare DA, Tanno S, De Rienzo A, Klein-Szanto AJ, Tanno S, Skele KL, *et al*. Serine/threonone kinase AKT is frequently activated in human bile duct cancer and is associated with increased radioresistance. *Cancer Res* 2004; **64**:3486–3490.
41 Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat Rev Cancer* 2002; **2**:489–501.
42 Testa JR, Bellacosa A. AKT plays a central role in tumorigenesis. *Proc Natl Acad Sci U S A* 2001; **98**:10983–10985.
43 Lenormand P, Sardet C, Pages G, L'Allemain G, Brunet A, Pouyssegur J. Growth factors induce nuclear translocation of MAP kinase (p42 mapk and p44 mapk) but not of their activator MAPK kinase kinase (p45 mapk) in fibroblasts. *J Cell Biol* 1993; **122**:1079–1088.