Celecoxib Inhibits Interleukin-6/Interleukin-6 Receptor–Induced JAK2/STAT3 Phosphorylation in Human Hepatocellular Carcinoma Cells

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Abstract
Growing evidence shows an association between chronic liver inflammation and hepatocellular carcinoma (HCC) development. STAT3, which is associated with inflammation and cellular transformation, is constitutively activated in human HCC tissues but not in normal human liver tissues. Although interleukin-6 (IL-6) is elevated in the serum of patients with HCC, it is not fully understood whether STAT3 constitutive activation is positively correlated with autocrine IL-6 secreted by HCC cells. Here, we reported that in HCC cells, the elevated levels of both IL-6 and IL-6 receptor (IL-6R, gp80), not IL-6 alone, correlated with STAT3 activation. We also explored whether the anticancer effects of celecoxib, an anti-inflammatory drug, may be due to the inhibition of the IL-6/STAT3 pathway in HCC cells. Our results showed that celecoxib decreased STAT3 phosphorylation by reducing Janus-activated kinase (JAK2) phosphorylation and caused apoptosis in HCC cells. Celecoxib could also block exogenous IL-6–induced STAT3 phosphorylation and nuclear translocation. Moreover, we observed more significant inhibition of cell viability when celecoxib was combined with doxorubicin or sorafenib. We conclude that the elevated levels of IL-6/IL-6R may be correlated with STAT3 activation in HCC cells. Celecoxib may be a candidate for HCC therapy through blocking IL-6/STAT3 pathway and can be combined with other anticancer drugs to reduce drug resistance caused by IL-6/STAT3 signals.

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Introduction
Primary liver cancer is the sixth most common cancer of all human cancers worldwide and is a major health problem due to the poor prognosis (1). Hepatocellular carcinoma (HCC) accounts for more than 85% of all primary liver cancers, with a 5-year survival rate of 9% and a median survival time of less than 1 year (2–4). Epidemiologic studies have established that HCC occurs in association with hepatitis B virus infection and ethanol intake, but the molecular mechanisms have not been fully known. Over the past few years, growing evidence has shown that cytokines, such as interleukin-6 (IL-6), and transcription factors, such as NF-kB and STAT3, play an important role in HCC development (2, 5–9). Patients with HCC show elevated levels of IL-6 in their serum as compared with those with liver cirrhosis or with healthy individuals. In addition, the level of IL-6 is remarkably high in HCC stage III patients (5). STAT3, a major transducer to mediate the signal from IL-6 to the nucleus, is found significantly correlated with the prognosis of HCC patients (9), indicating that IL-6/STAT3 signaling pathway might be a therapeutic target.

Constitutively activated STAT3 may induce tumor formation in nude mice and is frequently detected in various human cancers (10–12). STAT3 may be involved in oncogenesis, cell proliferation, angiogenesis, immune evasion, and apoptotic resistance (13–15). Growing evidence has shown that a blockade of constitutively activated STAT3 can cause apoptosis in vitro, inhibit tumor growth in vivo, and enhance the sensitivity to chemotherapy and radiotherapy (11, 16–21).

IL-6 induces STAT3 phosphorylation at tyrosine residue 705 through IL-6 receptors (IL-6R and gp130) and Janus family kinases [Janus-activated kinase (JAK); refs. 22, 23]. Phosphorylated STAT3 molecules dimerize and translocate from the cytoplasm to the nucleus, where they bind to specific DNA elements to regulate the downstream genes,
including Bcl-X<sub>L</sub>, CyclinD1, HIF-1α, Mcl-1, p53, and VEGF. These genes are involved in cell proliferation, angiogenesis, and apoptosis (24, 25).

In this study, we reported that the levels of IL-6/IL-6R, not IL-6 alone, correlated with the activation of STAT3 in HCC cells. In addition, celecoxib, a COX-2 inhibitor, blocked IL-6/IL-6R-induced STAT3 phosphorylation in HCC cells by suppressing JAK2 phosphorylation and caused cell death. Although many STAT3 small molecule inhibitors have been described, none of them is currently selected for clinical trials. Celecoxib may be a candidate for HCC therapy or may be combined with other anticancer drugs.

Materials and Methods

Cell culture

HCC cell lines Hep3B, HepG2, Huh-7, SNU-387, and SNU-449 were obtained from American Type Culture Collection (ATCC). The cell lines have been characterized by ATCC, and we have not done any further characterization. Hep3B (HBV positive) and HepG2 (HBV negative) cells were cultured in Minimum essential medium, Eagle (MEM; ATCC) supplemented with 10% FBS and 1% penicillin/streptomycin. Huh-7 (HBV negative) cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. SNU-449 (HBV positive), SNU-387 (HBV positive), and SNU-398 (HBV positive) cells were cultured in RPMI 1640 medium (ATCC) supplemented with 10% FBS and 1% penicillin/streptomycin. Human hepatocytes (HH) were purchased from ScienCell Research Laboratories and were cultured in Hepatocyte Medium (HM; ScienCell) supplemented with 5% FBS and 1% penicillin/streptomycin. IL-6 was purchased from Cell Sciences.

Western blot

Cells were washed by ice-cold PBS buffer and lysed by ice-cold radioimmunoprecipitation assay buffer containing protease inhibitor cocktail and phosphatase inhibitor cocktail. The lysates were then spun at 13,200 rpm for 10 minutes at 4°C and the supernatant was collected. Protein samples were separated by SDS-PAGE, transferred onto polyvinylidene difluoride membrane, and immunoblotted with appropriate antibody. Antibodies to p-STAT3<sup>Y705</sup>, p-STAT3<sup>S727</sup>, STAT3, cleaved caspase-3, p-JAK2<sup>Y1007/1008</sup>, JAK2, p-JAK1<sup>Y1022/1023</sup>, JAK1, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and horseradish peroxidase-conjugated secondary antibody were from Cell Signaling Technology. The target protein was examined by chemiluminescence (Cell Signaling Technology).

MTT assay

Cells were seeded in a 96-well plate and were treated as indicated for 24 hours. After the treatment, 25 μL of MTT (Sigma) was added to each well. After 3-hour incubation at 37°C, 100 μL of N,N-dimethylformamide (Sigma) solubilization solution was added to each well. Absorbance was read at 595 nm.

Immunohistochemistry

Liver tumor tissue assay was purchased from BioChain Institute, Inc. Immunohistochemical labeling was conducted according to the manual of the tissue assay. Sections were labeled using primary antibodies against p-STAT3<sup>Y705</sup> (Cell Signaling Technology) and IL-6 (ab6672; Abcam Inc.). The staining was carried out with Histostain-Plus Kit (Invitrogen). The frequency of IL-6 and p-STAT3<sup>Y705</sup> positive was analyzed as described by Lin and colleagues (26).

Immunofluorescence

Cells were seeded on a glass slide and were treated as indicated. After the treatment, the cells were washed with cold PBS buffer and fixed with cold methanol for 15 minutes at −20°C. After 2 quick washes with cold PBS, the slide was blocked with 5% normal goat serum and 0.3% Triton X-100 in PBS for 1 hour at room temperature. Then the slide was incubated with STAT3 antibody (Cell Signaling Technology) at 4°C. After overnight incubation, the slide was washed with PBS-T solution (PBS plus 0.1% Tween-20) and then incubated with Alexa Fluor 594 secondary antibody (Molecule Probe, Invitrogen) for 1 hour at room temperature. The cells were mounted with Vectashield HardSet mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Photographs were captured by a Leica camera (Leica Microsystems).

STAT3 DNA binding assay

STAT3 DNA binding assay was conducted according to the manual of STAT3 DNA binding ELISA kit (Active Motif). Briefly, cells were seeded in a 10-cm plate. After the treatment, nuclear protein was extracted and mixed with STAT3 DNA probe. The protein–DNA complex was then transferred into an ELISA assay plate. After the incubation with primary and secondary antibodies, the developing solution was added. The absorbance was read at 450 nm.

Reverse transcription PCR

RNA was extracted using RNase Kit (Qiagen). Reverse transcription was carried out using Omniscript reverse transcription kit (Qiagen). PCR conditions were as follows: 94°C for 5 minutes followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute with a final extension of 72°C for 10 minutes.

Apoptosis assay

Apoptosis was measured with caspase-3/7 assay (Promega) according to the manufacture’s protocol. Briefly, cells were seeded into a 96-well plate. After the treatment, 100 μL of Apo-One Caspase-3/7 reagent was added to each well and was incubated at 37°C for 30 minutes. The fluorescence was measured at an excitation wavelength range of 485 nm and an emission wavelength range of 530 nm.

Colony formation

Cells were treated for specific time point as indicated. Then the treated cells were trypsinized, stained with trypan
blue, and counted. The same number of cells were then seeded in 10-cm plates and allowed to grow. After 3 weeks, the cells were fixed with cold methanol and stained with 1% crystal violet in 25% methanol.

**Statistical analysis**

Data were presented as the mean ± SEM in which the mean values were compared. Statistical analyses were conducted by Student’s t test. The value of $P < 0.05$ was considered statistically significant and was indicated with an asterisk.

**Results**

**STAT3 activation is positively correlated with the levels of IL-6/IL-6R in HCC cells**

To investigate whether STAT3 activation would positively correlate with the levels of IL-6 secreted by HCC cells, we first analyzed the levels of phosphorylated STAT3 in 6 HCC cell lines and in primary HH. HepG2, Huh-7, SNU-387, and SNU-449 cells showed an increase in phosphorylated STAT3 as compared with HH cells (Fig. 1A). To explore whether these 4 cell lines with higher levels of phosphorylated STAT3 may have higher levels of IL-6, we examined the levels of IL-6 in these cells. Because most IL-6 protein was secreted into the medium, IL-6 protein in these cells was not detectable by Western blotting. Therefore, we evaluated IL-6 mRNA in these cells by reverse transcriptase PCR (RT-PCR). The result was beyond our expectations. SNU-387 and SNU-449 cells had higher levels of IL-6 than HH cells (Fig. 1B), whereas HepG2 and Huh-7 cells had higher levels of phosphorylated STAT3 but lower levels of IL-6. To explore whether neutralizing IL-6 would decrease STAT3 phosphorylation, we treated Hep3B, SNU-449, and SNU-387 cells with 500 ng/mL of anti-IL-6 antibody for 72 hours. The treatment decreased the levels of phosphorylated STAT3 in SNU-449 and SNU-387 cells and induced caspase-3 activation, whereas the same treatment did not increase caspase-3 activation in Hep3B cells (Supplementary Fig. S1).

To confirm these results, immunohistochemistry for IL-6 and p-STAT3*Y705* was also carried out on normal liver tissues (3 cases) and on tissues from patients with viral subfulminant hepatitis (2 cases) or HCC (25 cases). Two normal tissues expressed moderate (+++) IL-6.

![Image](image_url)
immunostaining and 1 weak (+) IL-6 immunostaining. One intense (+++) IL-6 immunostaining and 1 moderate (+) IL-6 immunostaining were observed on 2 viral subfulminant hepatitis samples. Seven of 25 HCC samples were intense (+++), and the other 16 HCC samples were moderate (11 cases) and weak (7 cases). As for p-STAT3705, all of the 3 normal samples were negative. One viral subfulminant hepatitis sample was negative, and 1 was weak. Three HCC samples were intense, 8 samples were moderate, 8 samples were weak, and 7 samples were negative (Fig. 1C). Overall, both benign and malignant tissues were IL-6 positive compared with their connective tissues, such as blood vessels, whereas 20% of benign samples and 76% of HCC samples were p-STAT3705 positive. Given the results from cell lines and tissues, these data suggest that IL-6 is a factor for STAT3 activation in HCC tissues and cancer cell lines but not in normal cells and benign tissues. Even in HCC samples, other risk factors rather than IL-6 may exist to activate STAT3 in HCC cells.

Because IL-6Rs including both IL-6R (gp80) and gp130 can also mediate STAT3 activation during tumorigenesis (22, 27, 28), we analyzed the mRNA of IL-6R and gp130 in HCC cells by RT-PCR. The levels of IL-6R were higher in all 6 cancer cell lines than in HH cells. In addition, IL-6R was remarkably overexpressed in HepG2, Huh-7, and SNU-449 cells (Fig. 1D). The results of IL-6R were quantified with GAPDH, using Image J software. However, no significant overexpression of gp130 was observed in HCC cell lines (Fig. 1D). On the basis of the results of IL-6 and IL-6R (Fig. 1A, B, and D), our data suggest a trend toward a positive correlation between the levels of pSTAT3705 and the levels of IL-6/IL-6R, but not those of IL-6 alone, in HCC cells.
Celecoxib inhibits IL-6/IL-6R–induced STAT3 phosphorylation in HCC cells

Some anti-inflammatory drugs, such as celecoxib, a COX-2 inhibitor, have shown inhibitory effects on tumor growth in vitro and in vivo. To examine whether the antitumor effects may be due to the inhibition of some inflammatory pathways, such as IL-6/STAT3 pathway, we examined celecoxib. Four HCC cell lines with higher IL-6/IL-6R and phosphorylated STAT3 were treated with celecoxib for 24 hours. We observed that celecoxib effectively reduced the levels of p-STAT3Y705, whereas it had no effects on p-STAT3S727 and total STAT3 (Fig. 2A). To explore the mechanism of its inhibitory effects on p-STAT3Y705, JAK1 and JAK2 were evaluated. P-JAK2Y1007/1008 was suppressed in all 4 HCC cell lines with celecoxib treatment, whereas p-JAK1Y1022/1023 was not detectable. Neither JAK1 nor JAK2 changed upon celecoxib treatment (Fig. 2A).

We next examined the effects of celecoxib on STAT3 DNA binding ability, using Huh-7 and SNU-449 cell lines. As illustrated in Figure 2B, celecoxib reduced STAT3 DNA binding ability in a dose-dependent manner. To further investigate whether celecoxib would affect STAT3 downstream genes, we used RT-PCR to examine the mRNA expression of Bcl-2, Bcl-XL, CyclinD1, HIF-1α, Survivin, and VEGF. As shown in Figure 2C, celecoxib treatment downregulated the mRNA expression of these genes.

Celecoxib induces apoptosis in HCC cells and reduces cell viability

To investigate whether blocking p-STAT3Y705 with celecoxib would cause apoptosis, we treated HCC cells (Huh-7, HepG2, SNU-387, and SNU-449) with different concentrations of celecoxib and analyzed the levels of cleaved caspase-3 and the activity of caspase-3/7. The results showed that both the levels of cleaved caspase-3 and the activity of caspase-3/7 were enhanced by celecoxib treatment (Fig. 3A and B). In addition, the treatment significantly decreased cell viability of all 4 cell lines (Fig. 3C), which was consistent with the apoptotic results. Human

Figure 3. Celecoxib induces apoptosis and reduces cell viability. A and B, HCC cell lines were treated with celecoxib. The levels of cleaved caspase-3 (A) and the activity of caspase-3/7 were analyzed (B). C, cell viability was measured in celecoxib-treated HCC cell lines by MTT assay. D, SNU-449 cells were treated with celecoxib. After the overnight treatment, the same number of living cells with either dimethyl sulfoxide treatment or celecoxib treatment was reseeded. The cells were allowed to grow in fresh medium without celecoxib for 2 weeks. After 2 weeks, the cells were fixed and stained. Data from B–D represent 3 independent results.
primary hepatocytes and HCC cells (HeP3B and SNU-398) with lower levels of phosphorylated STAT3 were less sensitive to celecoxib treatment (Supplementary Fig. S2). Moreover, neutralization of IL-6 in SNU-449 and SNU-387 cells with anti-IL-6 antibody desensitized the cells to further celecoxib-induced apoptosis (Supplementary Fig. S3).

Clonogenic assay was also conducted to investigate the colony-forming ability after celecoxib treatment (Fig. 3D). Our results showed that the colony-forming ability was significantly reduced by celecoxib treatment in a dose-dependent fashion.

Celecoxib inhibits exogenous IL-6–induced p-STAT3Y705

To investigate whether celecoxib may block exogenous IL-6–induced STAT3 activation, HeP3B cells with low levels of IL-6 and p-STAT3Y705 were pretreated with 25 and 50 µmol/L of celecoxib for 2 hours, followed by 25 ng/mL of IL-6 for 30 minutes. After the treatment, p-STAT3Y705 and STAT3 were analyzed by Western blotting. The pretreatment with 25 µmol/L celecoxib inhibited IL-6–induced p-STAT3Y705, and the pretreatment with 50 µmol/L celecoxib effectively blocked p-STAT3Y705 (Fig. 4A). To further explore whether celecoxib inhibited IL-6–induced p-STAT3Y705 by repressing JAK2 phosphorylation, we examined the effect of celecoxib on the phosphorylation of JAK2 caused by IL-6 stimulation. Figure 4B clearly showed that IL-6 induced JAK2 phosphorylation whereas celecoxib pretreatment prevented its phosphorylation. To confirm whether IL-6 would induce phosphorylated STAT3 nuclear accumulation and total STAT3 nuclear translocation and whether celecoxib pretreatment would block this process, HeP3B cells were pretreated with 50 µmol/L celecoxib for 2 hours, followed by 25 ng/mL IL-6 for 30 minutes. Phosphorylated STAT3 accumulated in the nucleus in response to IL-6 treatment, whereas celecoxib pretreatment blocked this process (Fig. 5A). STAT3 was present in the cytoplasm in the absence of IL-6. Following IL-6 treatment, STAT3 translocated to the nucleus whereas celecoxib pretreatment blocked this process (Fig. 5B).

Inhibition of STAT3 by celecoxib reduces cell viability more significantly when combined with other anticancer drugs

We previously reported that the blockade of IL-6/STAT3 pathway could overcome IL-6–induced drug resistance in HCC cells (21). We also showed in Figure 2B that celecoxib could block the IL-6/STAT3 pathway. To investigate whether inhibition of STAT3 by celecoxib would lead to more cell death when combined with other anticancer drugs, we treated SNU-449 cells with doxorubicin (Fig. 6A) or sorafenib (Fig. 6B) with or without celecoxib. Doxorubicin or sorafenib treatment reduced viable cell numbers more significantly in the presence of celecoxib (Fig. 6A and B). In addition, cells treated with celecoxib and sorafenib significantly lost colony-forming ability as compared with the effect of either agent (Fig. 6C).

Discussion

STAT3 is activated in response to infection, hormones, growth factors, and cytokines, as well as by the activation of intracellular kinases (16). Constitutive STAT3 activation plays a critical role in tumor formation and development in a variety of primary human cancers and cell lines. Cellular transformation by various factors, such as virus infection or protein tyrosine kinase activation, accompanies aberrant activation of STAT3 (29, 30), which may promote tumor cell proliferation through the upregulation of CyclinD1, Myc, and Pim-1 (30–33) and may suppress apoptosis through the upregulation of Bel-XL and Survivin (34, 35). It may also mediate invasion through the upregulation of matrix metalloproteinase (MMP) 1 and MMP2 (36, 37) and increase angiogenesis and metastasis through the upregulation of VEGF and Twist (13, 38). In addition, activated STAT3 may suppress antitumor immunity through the inhibition of Th1 immunostimulatory molecules (39). Downregulation of constitutively activated STAT3 not only induces apoptosis in cancer cells but also overcomes chemoresistance and radioresistance (30).

Because no gain-of-function STAT3 mutants have been found, it seems important to investigate the factors for STAT3 activation in different types of human cancer. IL-6 is
involved in STAT3 activation in a variety of human cancers. In the liver, short-term treatment with IL-6 provides hepatoprotection against various liver injuries by stimulating liver regeneration (40–43). However, a murine model of HCC showed that when mice were treated with diethyl-nitrosamine, which can induce hepatocellular carcinogenesis, males were more likely to develop HCC than females. The gender disparity was due to the higher levels of IL-6 in males and was not present in IL-6 knockout mice, indicating an association between IL-6 and HCC.
Figure 6. Celecoxib induces more cell death when combined with other anticancer drugs. A and B, SNU-449 cells were treated with different concentrations of doxorubicin (A) and sorafenib (B) with and without celecoxib. After 24 hours, cell viability was measured by MTT assay. Data represent 3 independent results. C, SNU-449 cells were treated as indicated in A. After the treatment, living cells were counted and the same number of cells was reseeded and cultured for 3 and 4 weeks, respectively. Colonies were fixed by cold methanol and stained by 1% crystal violet.
progression (44). Another mouse study shows that inflammatory cytokines, including IL-6, promote liver cancer metastasis (45).

Although the serum levels of IL-6 are elevated in patients with HCC, and STAT3 is constitutively activated in HCC cells (5, 26), no direct evidence shows that IL-6 secreted by HCC cells is correlated with STAT3 constitutive activation. In this study, we showed that IL-6 was a critical factor that contributed to STAT3 constitutive activation in HCC cells. Some HCC cells and tissues had higher p-STAT3\textsuperscript{705} and higher levels of IL-6. However, some HCC cells had higher p-STAT3\textsuperscript{705} but lower levels of IL-6, and some had lower p-STAT3\textsuperscript{705} but higher levels of IL-6. Moreover, IL-6 was detected in normal human liver cells and tissues but their p-STAT3\textsuperscript{705} was not detectable or was very weak. These results made us hypothesize that IL-6R or gp130 may be another factor for STAT3 constitutive activation in HCC because IL-6R has been found overexpressed in human ovarian cancer cells and the elevated IL-6R level activates STAT3 in these cancer cells. (28), gp130 can also activate STAT3 during colitis-associated tumorigenesis (22). Our results showed that it was IL-6R, but not gp130, that was overexpressed and activated STAT3 in some HCC cells. gp130 was not found overexpressed in ovarian cancer cells either. Taken together, the elevated levels of IL-6/IL-6R, not IL-6 alone, led to STAT3 constitutive activation in HCC cells.

In the second part, we hypothesized that whether the anticancer effects of some anti-inflammatory drugs may be due to the blockage of some inflammatory pathways, such as IL-6/STAT3. Celecoxib, a COX-2 inhibitor, has shown anticancer effects in colon carcinoma, lung carcinoma, and prostate cancer in vitro and in vivo through different mechanisms, such as blocking p-ERK or phosphorylated extracellular signal–regulated kinase (p-ERK; refs. 46–48). Here, we showed that celecoxib could inhibit IL-6/IL-6R-induced STAT3 activation in HCC cells by blocking JAK2 phosphorylation. Moreover, the anti-inflammatory effects of celecoxib may be also due to the blockage of JAK2/STAT3 because COX-2 is upregulated by STAT3 (25). Finally, we showed that celecoxib could be combined with other anticancer agents to overcome the drug resistance. IL-6/STAT3 targets some genes involved in antiapoptosis such as Bcl-2, Bcl-X\textsubscript{L}, and Survivin. They are thought to be involved in resistance to conventional cancer treatment. Celecoxib treatment decreased the expression of these STAT3 downstream genes, which inhibited the resistance of cancer cells to anticancer drugs. Therefore, when other anticancer drugs were combined with celecoxib, the treatment caused more cell death. In summary, our results suggest that celecoxib may be a candidate for HCC therapy by inhibiting IL-6/STAT3 pathway and may be combined with other anticancer drugs to reduce drug resistance caused by STAT3.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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