CADPE Suppresses Cyclin D1 Expression in Hepatocellular Carcinoma by Blocking IL-6-induced STAT3 Activation

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Abstract. *The initiation and growth of hepatocellular carcinoma (HCC) are closely linked to chronic inflammation. Not only is cyclin D1 overexpressed, but it is also related to aggressive progression in HCC. However, the mechanism of expression cyclin D1, a cell-cycle regulator of paramount importance, in the tumor microenvironment remains unknown. Here, we investigated the mechanism of cyclin D1 expression induced by interleukin-6 (IL-6) and whether 3-[3,4-dihydroxy-phenyl]-acrylic acid 2-[3,4-dihydroxy-phenyl]-ethyl ester (CADPE), a derivate of caffeic acid, suppresses cyclin D1 expression. CADPE significantly inhibited IL-6-induced signal transducer and activator of transcription 3 (STAT3) activity in the Huh7 HCC cell line and attenuated IL-6-induced cyclin D1 transcription. Moreover, overexpression of constitutively active STAT3 increased cyclin D1 transcriptional activity and protein expression, whereas overexpression of a dominant-negative STAT3 deletion mutant (STAT3 (1-588)) reduced cyclin D1 transcriptional activity. In addition, CADPE effectively deacetylated histone 4 and prevented STAT3 recruitment to the cyclin D1 promoter, consistent with a role for the CADPE target, STAT3, in the regulation of cyclin D1 transcription. Collectively, these results indicate that CADPE suppresses cyclin D1 expression in HCC cells by blocking both IL-6-mediated STAT3 activation and recruitment of STAT3 to the cyclin D1 promoter.*

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Hepatocellular carcinoma (HCC), the most common type of liver cancer in humans, is caused by chronic inflammation in many cases. Interleukin-6 (IL-6), released in response to viral infection and systemic inflammation in the liver, is the prototype cytokine activator of signal transducer and activator of transcription 3 (STAT3), a transcription factor involved in regulating cell-cycle progression and apoptosis. The concentration of IL-6 in serum is greater in HCC patients than in normal individuals (1), and there is a gender disparity in liver cancer that reflects differences in IL-6 production by liver macrophage-like Kupffer cells (2). IL-6 has various effects on tumor formation and progression, and is also involved in hepatic regeneration.

Cyclin D1 is an important cell-cycle regulatory protein that is required for completion of the G_1/S-phase transition in normal mammalian cells (3). Cyclin D1 associates with specific cyclin-dependent kinases (Cdks), such as Cdk4/Cdk6, to form a cyclin D1/Cdk complex capable of phosphorylating the retinoblastoma tumor suppressor (pRB). Phosphorylation of pRB abrogates pRB-mediated suppression of the transcription factor E2F, allowing E2F to stimulate the expression of target genes required for G_1/S-phase transition and DNA replication (4). Overexpression of cyclin D1 mRNA and protein has been found in several solid tumor types, including human parathyroid adenoma, and breast, colon, lung and liver cancer (5-7), and is associated with early onset of cancer and aggressive tumor progression (8-11). Cyclin D1 is also intimately involved in abnormal cell growth processes, angiogenesis, and resistance to apoptosis (12), making it an attractive therapeutic target for controlling tumor growth (13, 14).

3-[3,4-Dihydroxy-phenyl]-acrylic acid 2-[3,4-dihydroxy-phenyl]-ethyl ester (CADPE) is a derivate of caffeic acid (CA) that is endowed with antioxidant and anti-inflammatory properties (15-19). We previously reported that CADPE inhibits tumor angiogenesis by blocking Src-dependent activation of STAT3 in renal carcinoma cells, and was more effective in inhibiting Src tyrosine kinase and STAT3 activity than was CA. Moreover, CADPE visibly impeded the growth of xenografted tumors in a nude mouse model (20).

In the present study, we investigated CADPE suppression of cyclin D1 expression in HCC cells.

Materials and Methods

Materials. CADPE (Imagene, Seoul, Korea) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 500 mg/ml and stored at –20˚C. An antibody that recognizes a phosphorylated/activated form of STAT3, as well as anti-STAT3, anti-rabbit immunoglobulin G (IgG), anti-acetylated-histone 3 (AcH3), and anti-acetylated-histone 4 (AcH4) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). An anti-cyclin D1 antibody was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Recombinant human IL-6 was purchased from R&D Systems, Inc (Minneapolis, MN, USA).

Cell culture. Huh-7 (Hepatocellular carcinoma; ATCC, Manassas, VA, USA) and Cos-7 (African green monkey kidney cells; ATCC, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics at 37˚C in a humidified 95% air, 5% CO_2 environment.

Measurement of cell viability. Cell viability was determined using a conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reduction assay. In this assay, the mitochondrial respiratory chain enzyme, succinate dehydrogenase, of viable cells converts MTT to insoluble blue formazan crystals. Cells were plated at a density of 1×10^4 cells/well in 24-well plates and maintained in DMEM containing 10% FBS. The cells were made quiescent at confluence by incubation in serum-free DMEM for 24 hours, followed by treatment with should be state here concentrations of CADPE for the desired time. After incubation, cells were washed three times with phosphate-buffered saline (PBS), and treated with MTT solution (final concentration, 5 mg/ml) for 4 hours at 37˚C. The supernatant was then removed, and formazan crystals were dissolved with DMSO. Absorbance at 570 nm was measured with a microplate reader (Molecular device, US/V Max).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Huh7 cells were cultured under serum-free conditions for 3 hours and pretreated with 50 μM CADPE before adding 20 ng/ml IL-6. Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was quantified by measuring absorbance at 260 nm, and RNA purity was assessed by determining the A260/A280 ratio (1.8 or higher). The integrity of RNA was checked by visual inspection of 28S and 18S ribosomal RNA on an agarose gel. cDNA was prepared by incubating 1 μg of total RNA in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol and RNase inhibitors with 250 units of avian myeloblastosis virus-reverse transcriptase (Promega, Madison, WI, USA), 1 μM of each dNTP, and random primers (0.05 μM) for 60 minutes at 37˚C. Targets were amplified from cDNA by PCR using the following specific primers: cyclin D1, 5'-GAA CAA ACA GAT CAT CCG CAA ACA-3' (sense) and 5'-TGC TCC TGG CAG GCC CGG AGG CAG-3' (antisense); β-actin, 5' ATC TGG CAC CAC ACC TTC TA-3' (sense) and 5' CTC GGT GAG GAT CTT CAT GA-3' (antisense). PCR was performed in a thermal cycler using the following parameters: 95˚C for 5 minutes, followed by 25 cycles of 94˚C for 1 minute, 55˚C (for β-actin) or 58˚C (for cyclin D1) for 1 minute, and 72˚C for 1 minute. The amplified products were visualized on 2% agarose gels.

Western blot analysis. Huh7 cells were cultured under serum-free conditions for 3 hours and pretreated with 0 100 μM CADPE before adding 20 ng/ml IL-6. Cos7 cells were seeded onto 60-mm dishes at 2×10^5 cells/dish in serum-free medium, and then transfected with WT STAT3 using Effectene. After 24 hours, cells were pretreated with 50 μM CADPE for 1 hour, and then treated with 20 ng/ml IL-6. Cells were washed twice with cold PBS, and then lysed in ice-cold modified radioimmuno-precipitation assay (RIPA) buffer (50 mM Tris-HCl pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM Na_3VO_4, and 1 mM NaF) containing protease inhibitors (2 mM phenylmethylsulfonylfluoride, 100 μg/ml leupeptin, 1 μg/ml aprotinin, and 2 mM EDTA). The lysates were centrifuged for 20 minutes at 13,000 × g at 4˚C, and the supernatants were collected. Proteins in lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with primary antibodies, washed, then incubated with peroxidase-conjugated secondary antibodies, washed, and visualized using an ECL system (Amersham, Piscataway, NJ, USA).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were based on a protocol described elsewhere (21). Huh7 cells were cultured under serum-free conditions for 6 hours and pretreated with 50 μM CADPE before adding 20 ng/ml IL-6. Cells were fixed with 10 % formaldehyde. After fixing cells with formaldehyde, soluble chromatin samples were immunoprecipitated with anti-normal rabbit IgG antibody (control), or with antibodies against phospho-Tyr705-STAT3 (pY^{705}STAT3), $pY^{694/699}$STAT5, p300, AcH3, or AcH4 at 4˚C overnight. DNA isolated from immunoprecipitated material following reversal of formaldehyde crosslinking was amplified by PCR. The STAT3-specific primers used were 5'-AGG TCG CTG GCG AAA GGT-3' (sense) and 5'-TCT TGG TGA CCA TTT GGA GAC-3' (antisense), and the nonspecific (control) primers used for STAT3 were 5' CCA GTT TCC TCT GTC TT-3' (sense) and 5'-TGC TCA CAG CAA GAT GCG A-3' (antisense).

Transient transfection and luciferase assay. A cyclin D1 promoter-reporter construct (pGL3-cyclin D1) was prepared by inserting a 2.9-kb region of the human cyclin D1 promoter into the pGL3-basic luciferase reporter plasmid. Huh7 or Cos7 cells were seeded on 24-well plates at a density of 4×10^4 cells/well in serum-free DMEM. Cells were co-transfected with the promoter-luciferase reporter construct (250 ng) and various combinations of the following constructs (250 ng of each plasmid): wild-type STAT3 (WT STAT3),

constitutively active STAT3 (CA STAT3), and a deletion mutant of STAT3 (STAT3 (1-588)). Cells were transfected using Effectene reagent as described by the manufacturer (Qiagen, Valencia, CA, USA). After 24 hours, cells were pretreated for 1 hour with 50 μM CADPE, and then 20 ng/ml IL-6 were added to each well. Luciferase assays were performed as described elsewhere (20).

Statistical analysis. Data are presented as mean±S.E.M. of *n* separate experiments. Differences between groups were analyzed using Student's *t*-tests; *p*-values <0.05 were considered statistically significant.

Results

CADPE induces a gradual reduction in HCC cell viability. Recent studies in our laboratory have demonstrated that CADPE (Figure 1A) inactivates STAT3 in Caki-I renal carcinoma cells and significantly inhibits the growth of xenografted tumors in nude mice (20). Here, we found that CADPE reduced the viability of Huh7 cells in a dose- and time-dependent manner using MTT assays. Although 84% of Huh7 cells were viable after serum-starvation for 24 hours, only 65% of serum-starved cells treated with CADPE (50 μM) for 6 hours were viable (Figure 1B). Cyclin D1, which is an important cell-cycle regulator required for completion of the G_1/S transition, has a central role in cell growth, including that of cancer cells. Given this, we next sought to determine whether CADPE suppresses cyclin D1 expression.

CADPE suppresses transcription of cyclin D1. To assess whether CADPE affects the transcription of cyclin D1 induced by IL-6, we performed RT-PCR analysis. In order to remove other contaminants from conditioned medium, Huh7 cells were starved for 6 hours prior to treatment with IL-6. When IL-6 (20 ng/ml) was given for 6 hours, the mRNA level of cyclin D1 was increased to 5 times that of the control. In contrast, pretreatment with CADPE (50 uM) significantly abrogated the IL-6 effect, reducing the cyclin D1 mRNA level to that of the control group (Figure 2A).

CADPE significantly inhibits IL-6-induced STAT3 activity in HCC cells. The phosphorylation of STAT3 at tyrosine 705 residue (Y705) is required for nuclear translocation and binding to specific promoter sequences on target genes. Thus, we sought to determine whether CADPE inhibits IL-6-induced phosphorylation of STAT3 at Y705 in HCC cells. IL-6 (20 ng/ml) induced an increase in STAT3 phosphorylation, which was reduced by CADPE (50-100 μM) in a dose-dependent manner (Figure 2B). To confirm this inhibitory effect of CADPE, we transfected STAT3-deficient Cos7 cells with WT STAT3 and assessed the effects of IL-6 on STAT3 phosphorylation status. The exogenously expressed WT STAT3 was activated in Cos7 cells by IL-6 and was somewhat inactivated by pretreatment with 50 μM

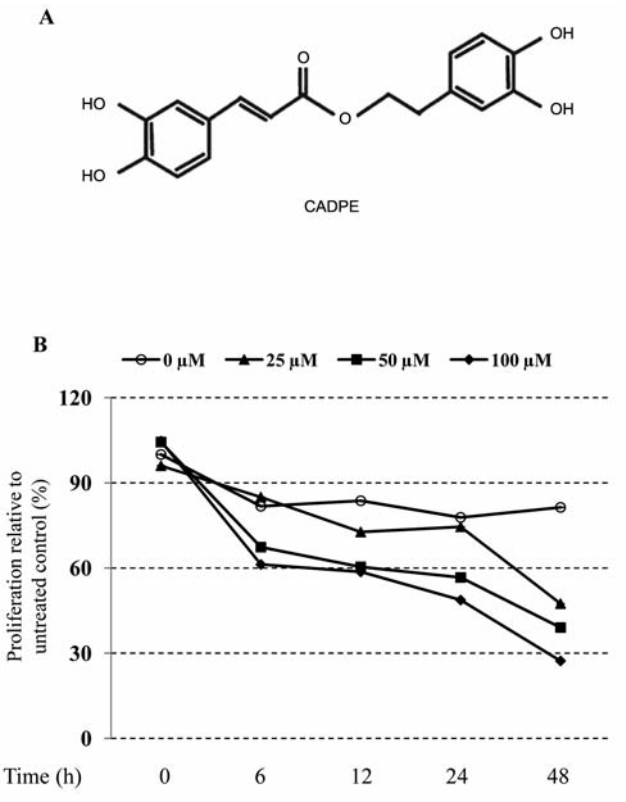

Figure 1. *A: Chemical structure of CADPE and viability of CADPE-treated Huh7 cells. B: Viability of each group was assessed by MTT assay. Data are shown as the means±SDs of three independent experiments performed in triplicate.*

CADPE (Figure 2C). These results suggest that the effect of CADPE was specific for STAT3 phosphorylation and did not affect total STAT3 protein levels in Huh7 or Cos7 cells.

The expression level of cyclin D1 is regulated in a STAT3-dependent manner. To determine whether cyclin D1 was regulated in a STAT3-dependent manner, we evaluated cyclin D1 protein expression in Huh7 cells transfected with WT STAT3, CA STAT3, and STAT3 (1-588), *C*-terminal deleted mutant. In preliminary experiments, we confirmed that total protein levels of immunodetectable STAT3 were increased in Huh7 cells transfected with WT STAT3, and CA STAT3 compared with mock-transfected controls, and endogenous STAT3 levels were lower in cells transfected with STAT3 (1-588). In cells overexpressing CA STAT3, the levels of phosphorylated STAT3 were greatly increased in association with an increase in the levels of cyclin D1 protein (Figure 3). Cyclin D1 levels were not increased in cells transfected with WT STAT3 (in which STAT3 phosphorylation was only slightly increased) or in cells transfected with STAT3 (1-588). Collectively, these results indicate that cyclin D1 expression is under the control of phosphorylated/activated STAT3 in Huh7 cells.

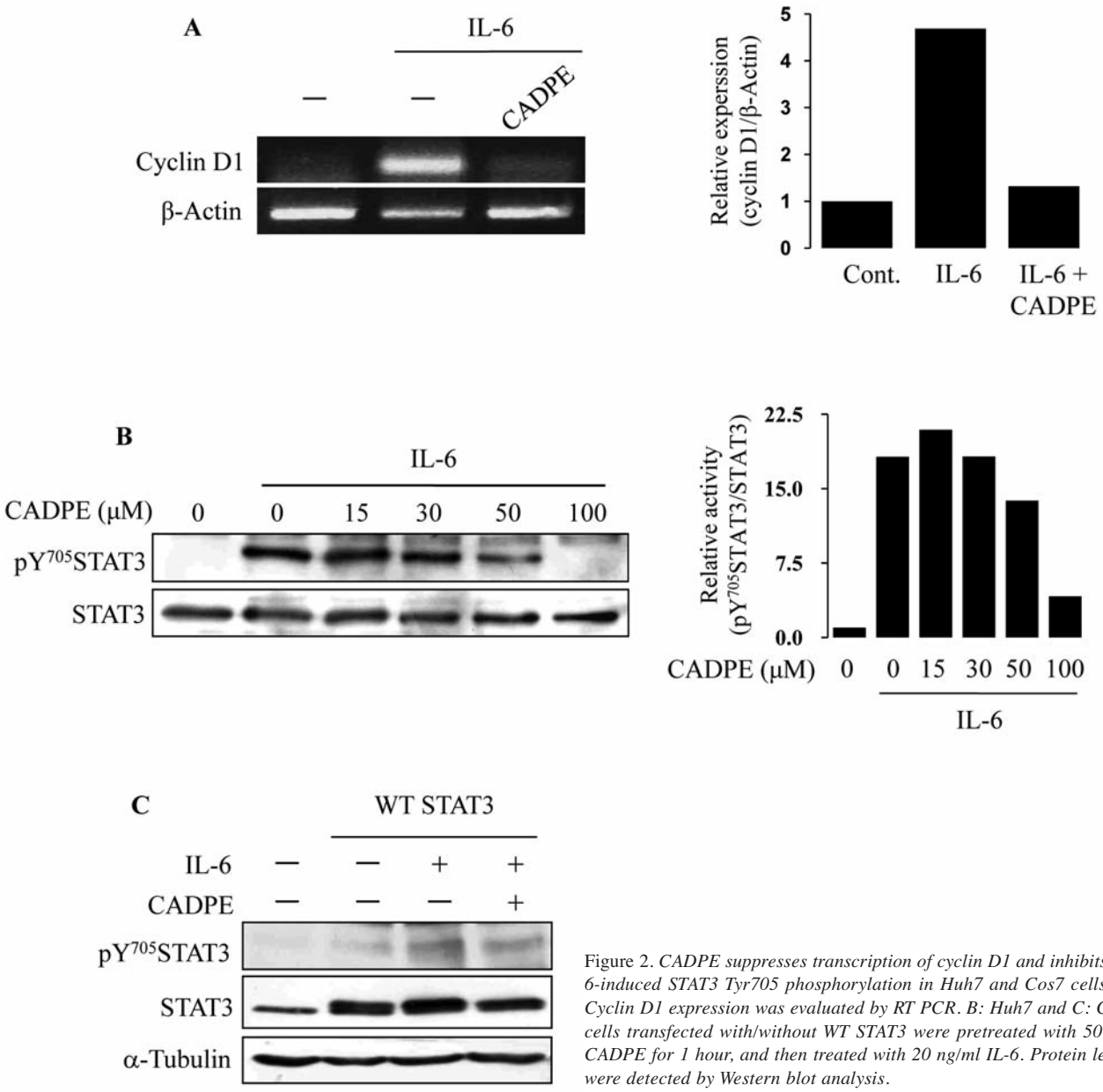

Figure 2. *CADPE suppresses transcription of cyclin D1 and inhibits IL-6-induced STAT3 Tyr705 phosphorylation in Huh7 and Cos7 cells. A: Cyclin D1 expression was evaluated by RT PCR. B: Huh7 and C: Cos7 cells transfected with/without WT STAT3 were pretreated with 50 μM CADPE for 1 hour, and then treated with 20 ng/ml IL-6. Protein levels were detected by Western blot analysis.*

To further assess the mechanism by which CADPE-induced inhibition of STAT3 suppresses the transcriptional activity of cyclin D1, we analyzed the effects of CADPE on IL-6-induced cyclin D1 transcriptional activity using a cyclin D1 promoter-luciferase reporter construct (pGL3-cyclin D1). In Huh7 cells transfected with pGL3-cyclin D1, IL-6 increased cyclin D1 transcription by up to approximately 2.3-fold. This increase was largely abrogated by pretreatment with 50 μM CADPE. In the absence of IL-6, co-transfection of Huh7 cells with CA STAT3 increased the relative activity of cyclin D1 transcription by ~3.5-fold, consistent with a functional role for STAT3 in regulating cyclin D1 expression. Nuclear translocation of STAT3 requires both phosphorylation at tyrosine 705 within the transactivation domain (TAD) and dimerization of the Src homology 2 domain (SH2D). To verify whether nuclear translocation of STAT3 is crucial for transcriptional regulation of cyclin D1, we used the STAT3 (1-588) mutant form in which both SH2D and TAD are deleted. Overexpression of STAT3 (1-588) in Huh7 cells significantly reduced the activity of the cyclin D1 promoter compared with cells transfected with the CA STAT3

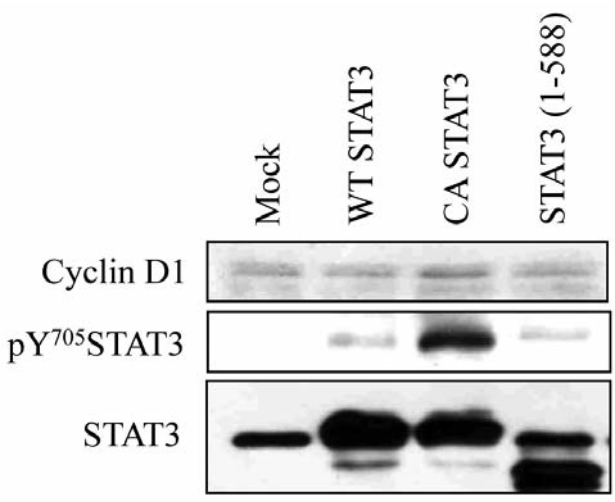

Figure 3. *Cyclin D1 is expressed in a STAT3-dependent manner. Huh7 cells transfected with WT STAT3, CA STAT3, and STAT3 (1-588). Cyclin D1, pY^{705}STAT3, total STAT3, and α-tubulin were analyzed by immunoblotting.*

construct (Figure 4A). We further assessed the STAT3-dependence of CADPE-mediated inhibition of cyclin D1 transcriptional activity using the STAT3-deficient cell line Cos7. In untransfected parental Cos7 cells, IL-6 had no significant effect on cyclin D1 promoter activity. However, in Cos7 cells transfected with WT STAT3, IL-6 induced a significant (~2.5-fold) increase in cyclin D1 reporter activity. Importantly, pretreatment of WT STAT3-transfected Cos7 cells with 50 μM CADPE eliminated the effect of IL-6 (Figure 4B).

CADPE inhibits IL-6-induced cyclin D1 expression by preventing recruitment of STAT3 to the cyclin D1 promoter. CADPE not only inactivated STAT3 but also reduced cyclin D1 expression at the transcriptional and translational level. To clarify the mechanism of cyclin D1 suppression caused by CADPE, we next examined whether CADPE acted by reducing the recruitment of endogenous STAT3 to the cyclin D1 promoter. Using ChIP assays, we found that treatment of Huh7 cells with IL-6 increased the recruitment of both phosphorylated/activated STAT3 and p300, which is known to form a co-regulatory complex with STAT3. Pretreatment of cells with CADPE substantially reduced IL-6-induced recruitment of STAT3 to the cyclin D1 promoter. In parallel experiments, we found no evidence for STAT5 binding to the cyclin D1 promoter, consistent with the observed inability of IL-6 to activate STAT5 (Figure 5). CADPE treatment also resulted in deacetylation of histone 4 in the cyclin D1 promoter, but had no effect on histone 3 acetylation (data not shown).

Discussion

Tumors frequently occur at regions of continuous inflammation, and a variety of inflammatory cells are known to infiltrate malignant tumors (22). Several studies have linked chronic inflammation with the development of cancer, and shown that treatment with non-steroidal anti-inflammatory compounds reduces the incidence of tumors (23-25). HCC is caused mainly by either hepatitis B-type virus (HBV) or hepatitis C-type virus (HCV), both of which are accompanied by chronic inflammation. HCC is thus a typical example of an inflammation-mediated cancer. Serum levels of IL-6 are substantially increased in chronic inflammatory liver conditions, including alcoholic hepatitis, HBV and HCV infection, and steatohepatitis. Consistent with a role for IL-6 in the pathogenesis of liver cancer, gender-specific differences in IL-6 produced by liver-resident Kupffer cells are associated with a difference in the incidence of HCC between males and females (2). IL-6 also has a central role in hepatocyte priming and proliferation during liver regeneration (26). Although IL-6 is known to induce STAT3 activation through the cytokine-activated Janus kinase (JAK) signaling pathway in the immune system (27), the mechanism by which IL-6 mediates cyclin D1 expression in HCC is unknown. Our results demonstrate that IL-6 significantly activated STAT3 and induced cyclin D1 expression through recruitment of activated STAT3 to the cyclin D1 promoter.

STATs have a prominent role in regulating cell-cycle progression and apoptosis, and STAT3 in particular promotes both tumorigenesis and tumor maintenance (28). Consistent with this observation, several studies have found that STAT3 is constitutively activated in a variety of solid tumors, including liver, breast, lung, head and neck, gut, and pancreatic tumors (29-34). Initial reports have demonstrated that inhibition of aberrantly activated STAT3 exerts an antitumor effect in HCC (35). Thus, blocking aberrant STAT3 activation is regarded as an attractive pharmacological strategy for targeting malignant tumor growth.

CADPE is a derivative of CA, which is a phenolic ring-containing compound with known antioxidant properties (36-38). In our previous study demonstrated that CADPE inactivates STAT3 in Caki-I renal carcinoma cells and significantly inhibits the angiogenesis of xenografted Caki-I tumors in nude mice (20). Taken together, our data show that CADPE opposes the action of the prototypical pro-inflammatory liver cytokine IL-6, strongly suppressing the activating phosphorylation of STAT3 and inhibiting cyclin D1 transcription by preventing recruitment of STAT3 to the cyclin D1 promoter (Figure 6). As such, the data demonstrate the therapeutic potential of CADPE in targeting STAT3 in the HCC microenvironment.

Cyclin D1, a target of CADPE, has properties that enhance several processes relevant to malignant cell

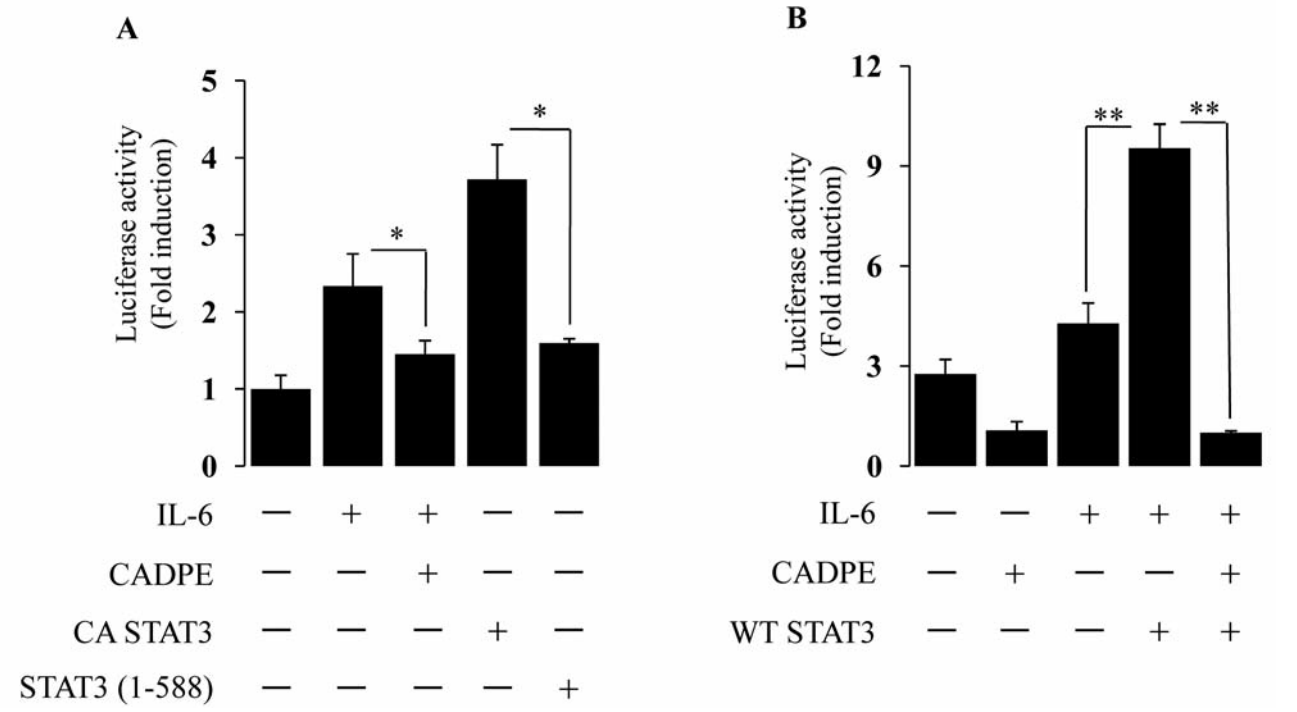

Figure 4. *CADPE inhibited transcriptional activity of cyclin D1. A: Huh7 and B: Cos7 cells were co-transfected with a cyclin promoter-reporter construct and WT STAT, CA STAT3, and STAT3 (1-588). Data are shown as the mean±SD of three independent experiments. Significantly different at *p<0.05 and **p<0.01.*

transformation, including abnormal growth, angiogenesis, and resistance to apoptosis (4). Overexpressed cyclin D1 cooperated with an activated *Ras* oncogene to transform primary rodent kidney cells (39) and with a *Myc* oncogene in lymphomagenesis in transgenic mice (40, 41). The rearrangement and overexpression of cyclin D1 have been reported in liver cancer (6). Integration of antisense plasmids against cyclin D1 inhibited the growth and tumorigenecity in human colon cancer and esophageal cancer cells (42, 43). Most interestingly, our finding reveals CADPE suppressed the expression of cyclin D1 induced by IL-6, one of the major cytokines expressed in HCC, which suggests CADPE is very likely to inhibit the progress of HCC under conditions of chronic inflammation.

In these studies, we showed that STAT3, which is activated by IL-6, regulated cyclin D1 expression, using RT-PCR analysis, Western-blot analysis, ChIP assay and promoter-reporter assays. CADPE effectively dephosphorylated STAT3 at tyrosine 705 in HCC cells and prevented IL-6-induced recruitment of a STAT3-containing complex to the cyclin D1 promoter. Our results demonstrate that CADPE has therapeutic potential, showing that the expression of IL-6-induced cyclin D1 protein is inhibited *via* STAT3 inactivation in the tumor microenvironment of HCC.

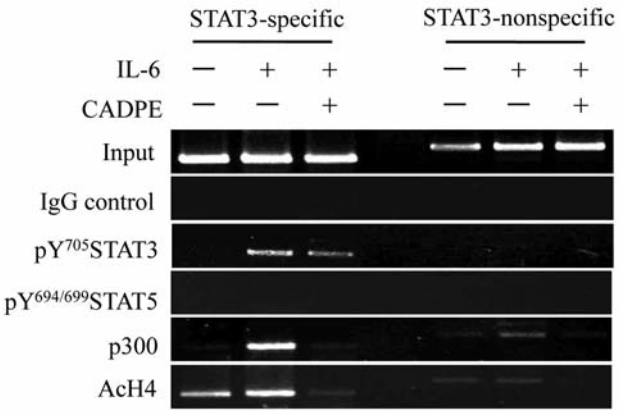

Figure 5. *CADPE blocked recruitment of STAT3 to the cyclin D1 promoter. Sonicated chromatin samples were immunoprecipitated with anti-normal rabbit IgG, anti-pY^{705}STAT3, anti-pY$^{694/699}$STAT5, anti-p300, anti-AcH3, and anti-AcH4 antibodies. Isolated DNA was amplified by PCR.*

Acknowledgements

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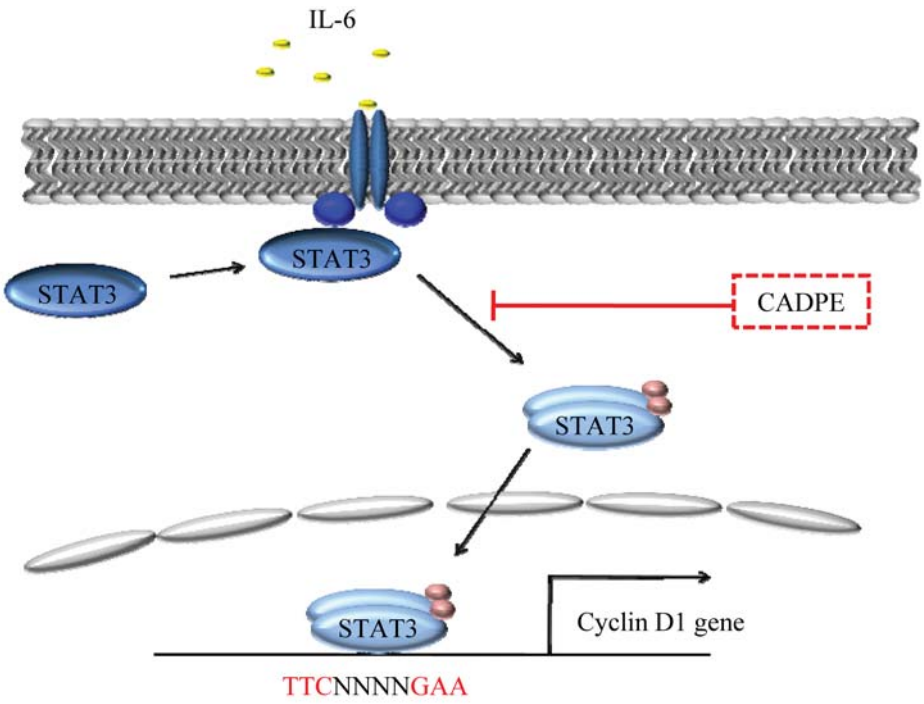

Figure 6. *Schematic representation of the action of CADPE in IL-6-induced cyclin D1 expression. STAT3 is activated by IL-6 signaling and binds to the human cyclin D1 promoter. CADPE inhibits IL-6-induced phosphorylation of STAT3, and in turn suppresses expression of cyclin D1.*

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