Pigment Epithelial-derived Factor Inhibits c-FLIP Expression and Assists Ciglitazone-induced Apoptosis in Hepatocellular Carcinoma

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Abstract. *Background: Pigment epithelial-derived factor (PEDF) displays its antiangiogenicity by mechanisms partly involving suppression of the cellular FADD-like IL-1β-converting enzyme (FLICE)/caspase-8-inhibitory protein (c-FLIP) expression in endothelial cells. c-Jun NH_2-terminal kinases (JNKs) regulate c-FLIP expression in endothelial cells. The effect of PEDF on other cells remains unclear. Materials and Methods: c-FLIP expression was assessed by semi-quantitative reverse transcriptase (RT)-PCR and immunoblotting. Pharmacological inhibitors were used to examine the involvement of PEDF signaling. Results: PEDF can also down-regulate c-FLIP expression in hepatoma cell line SK-Hep-1. PEDF induced p38 kinase phosphorylation in SK-Hep-1 cells. The effect of PEDF on c-FLIP expression was attenuated by p38 kinase inhibitor, but not JNK inhibitor. In addition, PEDF pretreatment significantly increased the sensitivity of SK-Hep-1 cells to procaspase-8 cleavage and apoptosis induced by ciglitazone. Conclusion: PEDF-induced p38 signaling causes c-FLIP down-regulation in SK-Hep-1. We postulate PEDF has a novel effect on apoptotic inducible activity in hepatoma cells.*

Pigment epithelial-derived factor (PEDF) is a potent antiangiogenic factor (1). Human PEDF is expressed in various tissues in the body (1, 2) and is crucial to vasculogenesis, including in development of the liver (3). In addition, the liver is the major organ that synthesizes PEDF and controls serum PEDF levels (4, 5). In this regard, serum PEDF levels are decreased in patients with liver cirrhosis or hepatocellular carcinoma (HCC) complicated by cirrhosis (5, 6).

PEDF has recently been found to inhibit growth of HCC in nude mice though its anti-angiogenic effects on tumor endothelial cells (5, 7). The anti-angiogenic property of PEDF is partly related to its being able to induce apoptosis of endothelial cells (8, 9). c-Jun NH_2-terminal kinase (JNK) activation by PEDF is required for the inhibition of vascular endothelial growth factor (VEGF)-induced cellular/caspase-8-inhibitory protein (c-FLIP) expression (8) (FLICE). *c-FLIP* mRNA exists as multiple splice variants, but at the protein level, only two forms, c-$FLIP_L$ (55 kDa) and c-$FLIP_S$ (28 kDa), have been detected so far (10-12). It is proposed that PEDF mediates endothelial cell apoptosis though JNK activation which then suppresses c-FLIP and propels cells into a proapoptotic state (8).

Overexpression of *c-FLIP* gene was found in many malignancies including HCC and cell lines (13, 14). Overexpression of *c-FLIP* also renders HCC resistant to apoptosis-inducing members of the tumor necrosis factor (TNF) family (15, 16). These findings suggest c-FLIP as a target of HCC therapy. In the present study, we investigated the effect of PEDF on c-FLIP expression in a HCC cell line, SK-Hep-1.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin, were purchased from Gibco BRL (Grand Island, NY, USA). p38 mitogen-activated protein kinase inhibitor (SB203580), JNK inhibitor (SP600125), caspase-8 inhibitor (z-IETD-fmk), caspase-9 inhibitor (z-LEHD-fmk), pan-caspase-inhibitor (z-VAD-fmk; benzyloxy-carbonyl-Val-Ala-Asp (OMe)-fluoromethylketone) and PPARγ agonist (ciglitazone; CGZ) were purchased from Calbiochem (San Diego, CA, USA). Recombinant PEDF derived from *Escherichia coli* was

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prepared as described previously (17), and the endotoxin content detected by E-TOXATE kit (Sigma, St. Louis, MO, USA) was less than 0.03 endotoxin units (EU)/ml.

Cell lines and culture conditions. SK-Hep-1 cells were cultured in DMEM supplemented with 10% FBS and 50 units/ml penicillin/streptomycin in an incubator at 37˚C with 5% CO_2.

Western blot analysis. Following PEDF treatment (200 ng/ml), SK-Hep-1 cells (~5×10^5 cells on a 35 mm well) were scraped into 150 μl lysis buffer (20 mM HEPES pH 7.4, 1% SDS, 150 mM NaCl, 1 mM EGTA, 5 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 0.1 mM sodium orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). The lysate was incubated on ice for 15 min. Cell debris was removed by centrifugation at 4˚C. Samples were then resolved on a sodium dodecyl sulfate (SDS)-polyacrylamide gel and then electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Samples were probed with antibodies to activated p38 (Promega, Madison, WI, USA), activated JNK (Promega), FLIPS/L (H-202; Santa Cruz Biotech Inc., Santa Cruz, CA, USA), caspase-8 (clone 1-1-37; Upstate Biotechnology, Lake Placid, NY, USA), and cleaved caspase-3 (Abcam Ltd, Cambridge, UK), according to the manufacturer's instructions, and then washed thee times in PBS with 0.1% Tween 20 (PBST). Antibodies against p38/SAPK2 or β-actin (Sigma-Aldrich Corp, St. Louis, MO, USA) were used to verify equal loading of protein. The blots were then incubated with horseradish peroxidase-labeled anti-rabbit or anti-mouse (Amersham Biosciences, Piscataway, NJ, USA) secondary antibody diluted in PBST for 1 h, and then washed thee times in PBST prior to visualization using an enhanced chemiluminescent technique. X-ray films were scanned on a GS-700 Imaging Densitometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and analyzed using Labworks 4.0 software (Ultra-Violet Products Ltd, Cambridge, UK).

Semi-quantitative reverse transcriptional (RT)-PCR. Total RNA of was extracted from treated SK-Hep-1 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Synthesis of cDNA was performed with 1 μg of total RNA at 50˚C for 50 min, using oligo (dT) primers (Invitrogen) and reverse transcriptase (Superscript III; Invitrogen). Semi-quantitative RT-PCR analysis of *c-FLIP* and *GAPDH* levels were performed as previous described (18). The PCR products were subjected to electrophoresis on 2% agarose gel and the DNA was visualized by staining with ethidium bromide under ultraviolet irradiation. The intensities of the PCR products were quantified by densitometrically using a FUJI LAS-3000 system and Multi Gauge Ver. 1.01 software (FUJIFILM, Tokyo, Japan).

Cell viability analysis. SK-Hep-1 cells were seeded in 10% FBS-DMEM in 96-well microculture plates (10,000 cells/well) (Nunclon, Roskilde, Denmark) for 24 h. The culture medium was then replaced by 1% FBS-DMEM in the presence of PEDF (200 ng/ml) or solvent control (PEDF preserved in a solvent containing 1 M imidazole, 0.5 M NaCl, and 20 mM Tris–HCl, pH 6.4) for 24 h. The cells were then incubated with 1, 5, or 10 μM CGZ. In a second series of experiments, PEDF-treated cells were further co-treated with different concentrations of caspase inhibitors and 10 μM CGZ for 24 h. Cell viability was then determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and data are presented as a percentage of control viability.

Statistical analysis. Data are expressed as the mean±SD. The Mann-Whitney *U*-test was used to determine the statistically significant differences and $p<0.05$ were considered significant.

Results

PEDF down-regulates c-FLIP expression in hepatoma SK-Hep-1 cell. We investigated whether expression of *c-FLIP* mRNA in the SK-Hep-1 cells is regulated by PEDF using a semi-quantitative RT-PCR assay. As shown in Figure 1A, the levels of *c-FLIP* mRNA were significantly lower at 12-24 h after treatment as compared with untreated control (0 h). To further determine whether the levels of c-FLIP protein were also reduced by PEDF, Western blot analyses were performed and results revealed that the levels of both c-FLIP isoforms were lower 12-24 hours after PEDF treatment (Figure 1B). These results indicated that c-FLIP expression is down-regulated by PEDF in SK-Hep-1 cells at both the message and protein levels.

PEDF induces p38 kinase but not JNK phosphorylation in SK-Hep-1 cells. In searching for the signaling mechanism of PEDF-induced *c-FLIP* down-regulation, we analyzed whether JNK and p38 kinase phosphorylation can be induced by PEDF exposure. SK-Hep-1 cells were treated with 200 ng/ml PEDF for intervals ranging between 0.5 to 7 h. Whole cell extracts were prepared and analyzed using antibodies against the active phosphorylated forms of JNK and p38 kinase. As shown in Figure 2A, p38 kinase was significantly phosphorylated at 2 h after PEDF treatment. PEDF-induced p38 kinase phosphorylation reached a maximum 3 h after treatment (increased by 3.4±0.9-fold as compared to cells without treatment) and returned to the basal level by 7 h (Figure 2B). There were no obvious changes in levels of phosphorylated JNK (p-JNK1/2) during the observation period (Figure 2A). Equal loading in all experiments was confirmed by re-exposure of the membranes to p38 kinase antibody. Control experiments revealed that the basal levels of p-p38 were not affected by the solvent (data not shown).

PEDF-induced p38 kinase phosphorylation may be essential for the inhibition of c-FLIP expression. The crucial role of p38 kinase signaling on PEDF down-regulation of *c-FLIP* expression was examined by employing p38 kinase inhibitor SB203580. SK-Hep-1 cells were pretreated with either SB203580 or SP600125 (JNK inhibitor) for 1 h before PEDF exposure and *c-FLIP* expression was monitored by RT-PCR and Western blot analysis. As shown in Figure 3A, RT-PCR analysis revealed that pretreatment with SB203580 completely prevented the reduction of *c-FLIP* mRNA induced by PEDF (lane 3 compared to lane 2), whereas pretreatment of cells with SP600125 or the inhibitor solvent, DMSO, had no such effect (lanes 4 and 5). This finding

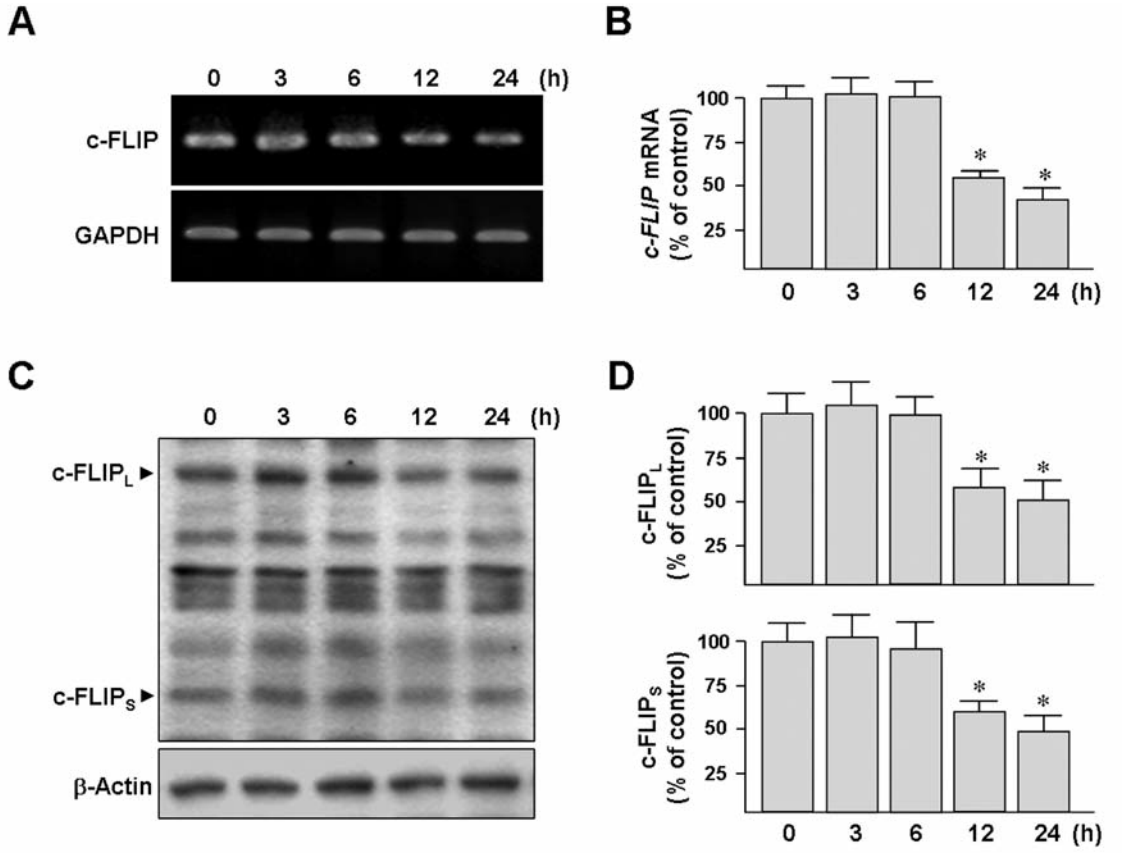

Figure 1. *PEDF suppresses c-FLIP mRNA expression in SK-Hep-1 cells. A and B: Cells were stimulated with 200 ng/ml PEDF for the indicated time periods. Total RNA was extracted, and RT-PCR analysis for c-FLIP was performed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was examined for normalization purposes. Results expressed as the mean ±SD of three independent experiments are shown. *p<0.05 versus untreated cells (0 h). PEDF suppresses c-FLIP protein expression in SK-Hep-1 cells. Cells were treated with 200 ng/ml PEDF for the indicated time periods and c-FLIP was detected by Western blot analysis. β-Actin was examined as loading control. Representative blots (C) and densitometric analysis with SD (D) are shown. *p<0.02 versus untreated cells.*

suggests that p38 kinase activation is required for PEDF-down-regulation of *c-FLIP* expression in SK-Hep-1 cells. Western blot analysis of c-FLIP protein also revealed that SB203580 completely blocked reduction of c-FLIP protein induced by PEDF in SK-Hep-1 cells (Figure 3B, lane 3 compared to lane 2). Taken together, these results imply that activation of the p38 kinase cascade by PEDF is essential for its down-regulation of *c-FLIP*.

PEDF sensitizes SK-Hep-1 cells for CGZ-induced cell death. It has been reported that peroxisome proliferator-activated receptor gamma (PPARγ) agonists induce apoptosis of human HCC (19, 20). We tested whether PEDF-down-regulation of c-FLIP expression can enhance apoptosis induced by CGZ, a PPARγ agonist. As shown in Figure 4A, MTT cell viability assay revealed that treatment of cells with different concentrations of CGZ (5 and 10 μM) resulted in slight growth inhibition compared to control cells (98±5% and 88±6%, respectively). Interestingly, the growth inhibitory effect of CGZ was markedly enhanced when cells were pretreated with PEDF for 24 h (90±6% and 42±11% compared with cells treated with 5 and 10 μM CGZ, respectively).

We are interested in the nature of this growth inhibition activity, particularly if apoptosis is induced by PEDF pretreatment. Pretreatment with a pan-caspase-inhibitor (z-VAD-fmk) almost completely suppressed PEDF/CGZ-induced cell death (Figure 5B, compare column 2 with 5; $p<0.001$). Cells treated with CGZ or PEDF/CGZ and stained with 4'6-diamidino-2-phenylindole (DAPI) showed typical apoptotic features, including nuclear condensation and nuclear fragmentation (data not shown). These results indicate that the PEDF-assisted cell death is *via* caspase-dependent apoptosis.

Since p38 kinase mediated c-FLIP induction (Figure 3), we tested whether p38 MAPK inhibitor abolishes the apoptosis-enhancing effect of PEDF. As shown in Figure 4B,

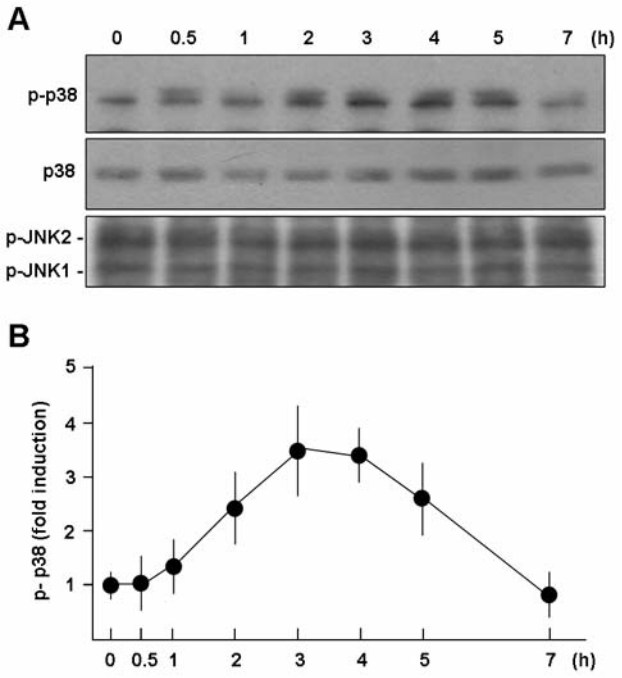

Figure 2. *PEDF time-dependently induces p38 kinase phosphorylation. A: SK-Hep-1 cells were exposed to 200 ng/ml PEDF for the indicated time periods. Western blotting was performed to detect the active phosphorylated forms of p38 kinase (p-p38) and JNK (p-JNK). Antibodies were then stripped and re-incubated with anti-p38 kinase antibody (lower panel) to detect the levels of total p38 kinase as a loading control. B: Intensities of p-p38 MAPK determined by densitometry. The value for cells without PEDF treatment (0 h) was set to 1. For quantification, blots of at least three independent experiments were used.*

after pretreatment with SB203580 for 1 h, PEDF/CGZ-induced cell viability was 93±7%, compared to PEDF/CGZ sequential treatment (compare column 5 with 4; $p<0.05$). However, pretreatment with JNK inhibitor SP600125, did not affect PEDF/CGZ-induced cell death (data not shown). Taken together, these results indicate that PEDF sensitizes SK-Hep-1 cells for CGZ-induced apoptosis. These results confirm the pivotal role of p38 kinase signaling in the effect of PEDF. These results also suggest that the level of c-FLIP is crucial for PEDF function.

Enhanced caspase-8 and caspase-3 activation after PEDF and CGZ sequential treatments. Caspase-8 activation was shown to be inhibited by c-FLIP$_L$ and/or c-FLIP$_S$ (21). We stimulated SK-Hep-1 cells by PEDF, CGZ and PEDF/CGZ and subsequently analyzed cell lysates for procaspase-8 cleavage. Our results revealed that the procaspase-8 cleavage product (p42 subunit) was slightly but noticeably increased in cells treated with CGZ alone for 24 h (Figure 5A). Importantly, PEDF/CGZ sequential treatments induced a ~2.5-fold increase of the activated p42 subunit.

CGZ has been shown to stimulate apoptosis with the activation of caspase-3 (22). Samples were harvested at 24 h, the time frame for apoptosis to occur. Western blot showed that the procaspase-3 cleavage product (p17 subunit) was detected (Figure 5A; CGZ). No noticeable increase in p17 occurred in cells treated by PEDF alone. In contrast, PEDF/CGZ sequential treatments induced substantial increase of p17 expression by ~3.1-fold compared to CGZ treatment. In addition, we investigated PPARγ levels by Western blotting and the results showed that there was no change in the PPARγ protein levels with exposure to PEDF or CGZ (Figure 5A), suggesting that PEDF/CGZ-induced procaspase cleavages are through a PPARγ-independent mechanism.

We further examined the involvement of cleavage of procaspase-8 in PEDF/CGZ-mediated SK-Hep-1 apoptosis. After PEDF pretreatment, the cells were treated with CGZ and caspase-8 inhibitor (z-IETD-fmk) or caspase-9 inhibitor (z-LEHD-fmk). MTT assay revealed that the PEDF/CGZ-induced apoptosis was almost completely blocked by pretreatment with caspase-8 inhibitor (Figure 5B; viability of 93±5% *vs*. 42±11%). Caspase-9 inhibitor had no such protective effect. Taken together, these results suggest that the down-regulation of *c-FLIP* by PEDF leads to sensitization of SK-Hep-1 cells to CGZ-induced apoptosis.

Discussion

Human HCC displays a striking resistance to apoptosis mediated by several death receptors, such as FAS and TNF-related apoptosis-inducing ligand receptors (TRAIL-Rs) (13, 15, 23). One possible reason is the higher levels of caspase-8-inhibitory protein, c-FLIP, in HCCs than in non-tumor liver tissues (13, 14). *c-FLIP* down-regulation induced by c-FLIP antisense oligodeoxynucleotides has been demonstrated to dramatically render HCC cells sensitive to death receptor-mediated apoptosis (13). This makes c-FLIP as a potential target for hepatoma treatment. In the present study, we demonstrated that PEDF down-regulates the mRNA and protein expression of *c-FLIP* in the hepatoma cell line SK-Hep-1. Therefore, in addition to its antiangiogenic properties on endothelial cells, PEDF may have additional effect on hepatoma cell survival.

The physiological serum concentration of PEDF is still lacking consensus, ranging from 5 μg/ml to 1-9 ng/ml depending on analyzed methods (4, 5). The PEDF concentration (200 ng/ml) used in this study is widely used to study angiogenesis inhibition *in vitro* (8, 24). We found that PEDF at this level can down-regulate *c-FLIP* expression, as well as enhance CGZ-induced procaspase-8/-3 cleavage, and apoptosis of SK-Hep-1 cells. Therefore, the same levels of PEDF shown to have growth inhibitory effect on cultured endothelial cells could also regulate hepatoma gene expression and affect hepatoma cell survival. This indicates

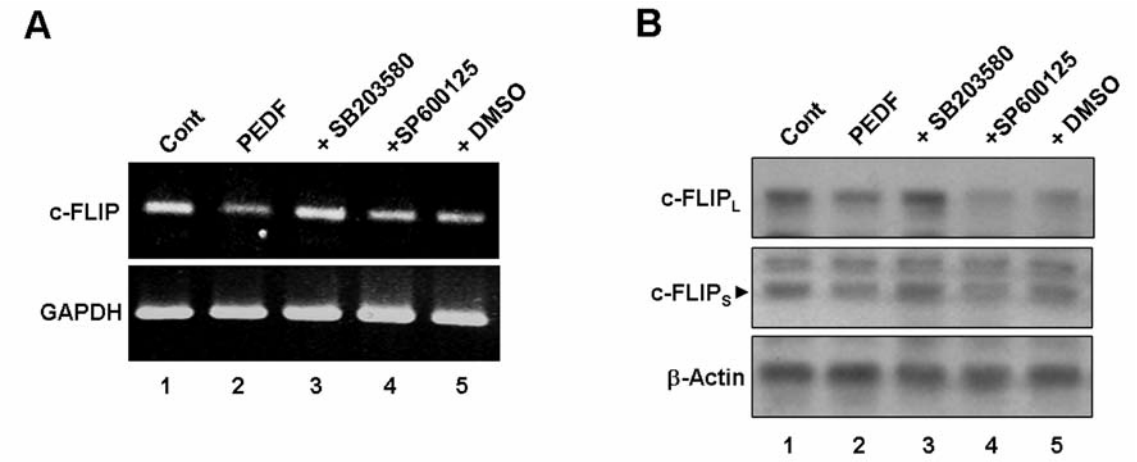

Figure 3. *p38 kinase inhibitor prevents PEDF-induced c-FLIP down-regulation. A: SK-Hep-1 cells were treated with 200 ng/ml PEDF or PEDF solvent (Cont) for 24 h, or were pretreated with 10 μM p38 inhibitor (SB203580), 10 μM JNK inhibitor (SP600125), or DMSO (0.05%) for 1 h then exposed to 200 ng/ml PEDF for 24 h. Total RNA was extracted, and RT-PCR analysis for c-FLIP was performed. Experiments were repeated twice and the results were reproducible. B: Western blot analysis of c-FLIP and β-actin. The data are representative of similar results in two separate experiments.*

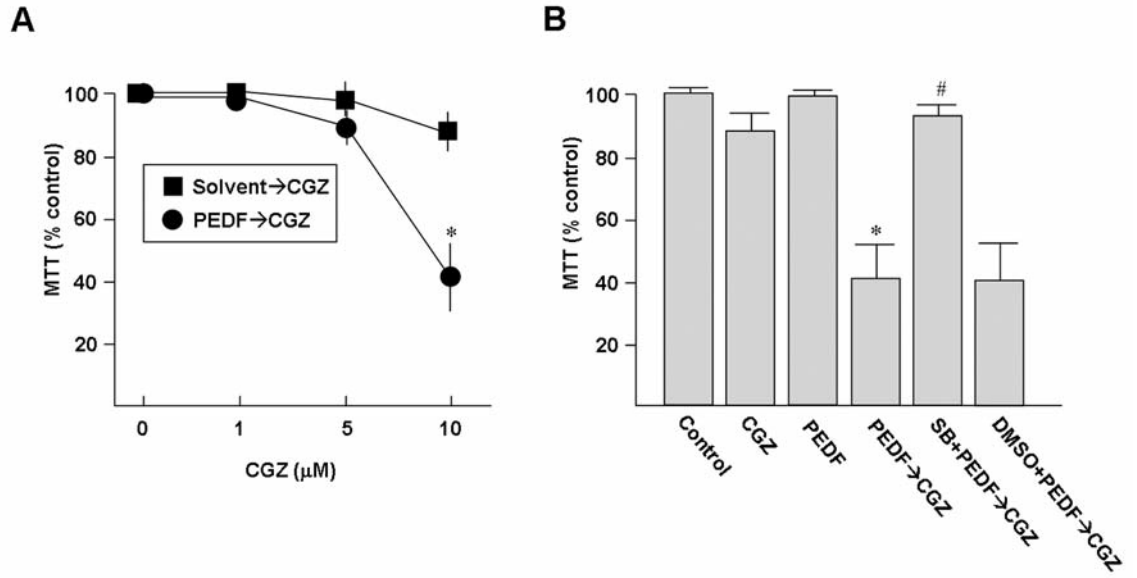

Figure 4. *Sensitization of SK-Hep-1 cells for ciglitazone (CGZ)-induced growth inhibition with PEDF. A: SK-Hep-1 cells were pretreated with 200 ng/ml PEDF or PEDF solvent for 24 h and then exposed to either 1, 5, or 10 μM of CGZ for a further 24 h. Subsequently, MTT cell viability assay was carried out. Variations shown represent SD from three independent experiments (n=4 dishes). *p<0.05 versus solvent-pretreated cells+10 μM CGZ. B: Pharmacologic inhibition of p38 kinase protects SK-Hep-1 cells from PEDF/CGZ-induced apoptosis. Cells were either left untreated or were treated with 10 μM p38 inhibitor (SB203580; SB) or DMSO for 1 h and then treated with different agents as indicated. Cell survival was analyzed by MTT assay. Data are shown as the mean±SD of three independent experiments. *p<0.05 versus CGZ-treated cells. #p<0.05 versus PEDF/CGZ-sequentially treated cells.*

studying the effect of PEDF on hepatoma cells is an authentic approach to improving HCC therapy.

CGZ a synthetic thiazolidinedione derivative, is a PPARγ agonist that is used as antidiabetic agent (25). Recently, increasing evidence shows the potential application of PPARγ agonists as anticancer agents for various types of cancer, including HCC (19, 20, 26). The anticancer effect of PPARγ agonists may involve mechanisms such as induction of cell-cycle arrest, and increase of cancer cell necrosis and apoptosis (27). It has been reported that PPARγ agonist-induced apoptosis is related to the induction of the caspase cascade such as caspase-8 and -9, the reduction of

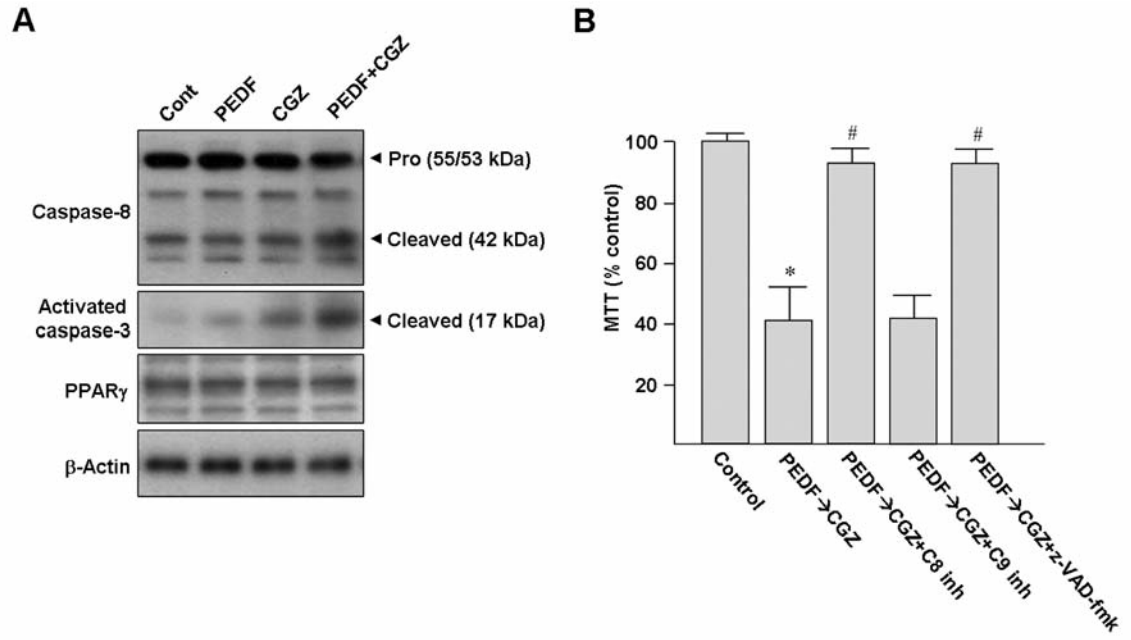

Figure 5. *A: CGZ induces cleavage of procaspase-8 and -3 in SK-Hep-1 cells with or without PEDF pretreatment. Cells were left untreated or were treated with 200 ng/ml PEDF for 24 h and then exposed to 10 μM CGZ for a further 24 h. Subsequently, cells were lysed and analyzed by Western blotting with the indicated antibodies. Representative blots from three independent experiments are shown. B: Effects of caspase inhibitors on PEDF/CGZ-induced apoptosis. SK-Hep-1 cells were pretreated with 200 ng/ml PEDF for 24 h and then exposed to 10 μM CGZ or CGZ combined with 20 μM z-IETD-fmk (caspase-8 inhibitor; C8 inh) or 20 μM z-LEHD-fmk (caspase-9 inhibitor; C9 inh) or 20 μM zVAD-fmk (pan-caspase inhibitor; Pan) for further 24 h before MTT assay. *p<0.05 versus untreated control cells. #p<0.001 versus PEDF/CGZ- sequentially treated cells.*

antiapoptotic proteins, such as c-FLIP, BCL-2 and inhibitor of apoptosis proteins, and the increase of apoptotic inducer, such as BAX and p53 in various cell types (28). However, although a PPARγ agonist alone is cytotoxic and might serve as a promising agent against malignancy, the levels required to induce cell death are higher than clinically achievable (1-10 μM) (29, 30). In the present study, we observed that with PEDF pretreatment, CGZ can induce apoptosis at a lower concentration (Figure 4A). This notion is further strengthened by the greater activation of caspase-3 with PEDF/CGZ sequential treatments than in cells treated with PEDF or CGZ alone (Figure 5A). These results suggest that PEDF lowers the thresholds of CGZ-induced apoptosis in hepatoma cells and might provide a solution for the application of PPARγ agonists in hepatoma therapy.

Our study suggests a possible model in which PEDF induces SK-Hep-1 cells to enter a proapoptotic state by down-regulation of c-FLIP expression. However the actual signaling pathway may be more complicated. For examples, CGZ may down-regulate caspase-3 inhibitors, such as inhibitor of apoptosis proteins or suppress cyclin D1 expression to arrest the cell-cycle as reported with other PPARγ agonists (28, 31). Our observation merely gives another example to indicate the complexity of CGZ-induced apoptosis.

The present study is the first to report the involvement of p38 kinase in the signaling pathway of PEDF-mediated down-regulation of *c-FLIP* gene expression. PEDF activates p38 kinase to induce apoptosis of human umbilical vein endothelial cells (9). Our recent study demonstrated the involvement of p38 kinase in PEDF-induced interleukin-10 expression in human monocyte-derived macrophages (32). In addition, PEDF induction of p38 kinase phosphorylation has been demonstrated in microglial cells, but the biological significance is still unclear (33). Previously, JNK activation by PEDF has been reported to be responsible for attenuation of VEGF-induced c-FLIP expression in endothelial cells (8). JNK seems not to be involved in c-FLIP expression in HCC cells since PEDF was unable to induce JNK phosphorylation (Figure 2A) and JNK inhibitor (SP600125) was unable to reverse the effect of PEDF-mediated *c-FLIP* down-regulation (Figure 3). The mechanism linking p38 kinase and c-FLIP expression remains unclear in HCC. The activation of NF-κB and phosphatidylinostiol-3 kinase/AKT pathways has been shown to be essential for constitutive c-FLIP expression in HCC (13). Whether p38 signaling can suppress NF-κB and Akt pathway and lead to down-regulate c-FLIP expression remains to be determined.

In conclusion, the present study demonstrated that PEDF suppresses c-FLIP expression in SK-Hep-1 cells by p38 kinase signaling. Furthermore, cell apoptosis induced by sublethal doses of CGZ was augmented by PEDF pretreatment. This shows the capability of PEDF to induce SK-Hep-1 cells into a proapoptotic state. PEDF expression has been implicated to involve in the progression of HCC (5, 7). The studies of the direct effect of PEDF on HCC and combinatorial effects with anticancer agents may be an important focus of investigation to establish novel therapeutic strategies for the improvement of HCC therapy.

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