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Activation of Telomerase and Cyclooxygenase-2 in PDGF and FGF Inhibition of C_2-Ceramide-Induced Apoptosis

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In the present study, the roles of telomerase and prostaglandin E_2 (PGE_2) in platelet-derived growth factor (PDGF's) and fibroblast growth factor-2 (FGF-2's) effects against C_2-ceramide-induced cell death were investigated. C_2-ceramide reduced the viability of NIH3T3 cells in a condition without calf serum (CS) in accordance with decreasing telomerase activity according to the TRAP assay. The addition of CS significantly protected cells from C_2-ceramide-induced apoptosis through increased telomerase activity, and the phosphorylations of PDGF and the FGF-2-like receptor in NIH3T3 cells were detected. Adding PDGF and FGF-2 decreased the cytotoxic effect elicited by C_2-ceramide through stimulating telomerase activity, which was blocked by adding a telomerase inhibitor (TI). Activations of ERKs and JNKs were detected in PDGF- and FGF-2-treated NIH3T3 cells, and the telomerase activities induced by PDGF and FGF were respectively inhibited by the addition of the ERK inhibitor, PD98059, and the JNK inhibitor, SP600125. Accordingly, induction of cyclooxygenase-2 (COX-2) protein expression and PGE_2 production was detected in PDGF- and FGF-2-treated NIH3T3 cells, and the telomerase activities stimulated by PDGF and FGF were reduced by adding a specific COX-2 inhibitor, NS398, through a decrease in PGE_2 production. Incubation of cells with PGE_2 or the EP1 agonist, 17-PT, but not the EP2 agonist, sulprostone, the EP3 agonist, butaprost, or the EP4 agonist, PGE_1 alcohol, significantly enhanced the telomerase activity of NIH3T3 cells. PGE_2 protection of NIH3T3 cells against C_2-ceramide-induced cell death was identified by the MTT and LDH-release assays, and it was inhibited by adding the EP1 antagonist, SC-19220. Ceramide metabolites including ceramide-1-phosphate (C1P) and sphingosine-1-phosphate (S1P), and a standard control of exogenous ceramide C_2-dihydroceramide show no effect on the telomerase activity and viability of NIH3T3 cells. The involvement of COX-2/PGE_2-mediated telomerase activation by PDGF and FGF-2 against C_2-ceramide-induced cell death is first demonstrated herein.
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Ceramide is an important lipid second messenger in response to cell stress, and has been shown to modulate cell proliferation, differentiation, and apoptosis. A variety of extracellular stimuli, including oxidative stress, UV radiation, and various cytokines, induce the generation of ceramide through activating sphingomyelinase (Komatsu et al., 2001; Clarke et al., 2007; Zeidan et al., 2008). Sanvicens and Cotter (2006) indicated that ceramide might be a key mediator in oxidative stress-induced apoptosis. The relevance of ceramide as a potential therapeutic target for treating oxidative stress-induced human diseases such as stroke, cancer, and retinal pathologies has been identified (Acharya et al., 2003; Ayasolla et al., 2004).

Growing evidence shows that accelerated telomeric shortening with aberrant telomerase activity contributes to the pathogenesis of several human diseases. The DNA–protein complexes at the end of chromosomes are comprised of telomerases, which protect chromosomal termini from degradation. It has been shown that a progressive reduction in telomere length in somatic cells leads to cellular senescence, and the immortality of tumor cells and stem cells is associated with their ability to maintain the length of the telomeres (Smith et al., 2003; Dalerba et al., 2005). Three main subunits, hTR (the RNA template for adding the TTAGGG repeat sequence), TEP1 (telomerase-associated protein), and telomerase reverse transcriptase (hTERT) (the catalytic subunit of telomerase), have been identified. Both hTR and TEP1 are ubiquitously

Abbreviations: JNKs, c-Jun NH_2-terminal kinases; ERKs, extracellular signal-regulated protein kinases; PDGF, platelet-derived growth factor; FGF-2, fibroblast growth factor-2; PGE_2, prostaglandin E_2; COX-2, cyclooxygenase-2; COX-1, cyclooxygnease-1; LDH, lactate dehydrogenase; CS, calf serum; hTERT, telomerase reverse transcriptase; TEP1, telomerase-associated protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ActD, actinomycin D; CHX, cycloheximide; TRAP, telomeric repeat amplification protocol; C1P, ceramide-1-phosphate; S1P, sphigosine-1-phosphate.

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expressed in several types of cells, whereas activation of hTERT has been detected in about 80% of cancers, and is correlated with the immortalization and proliferation of cancer cells (Püttmann et al., 2005; Chapman et al., 2006). The inhibitory effects of ceramide on telomerase activity have been investigated. Ogretmen et al. (2001) indicated that ceramide is a candidate for an upstream regulator to inhibit telomerase activity in A549 human lung adenocarcinoma cells. Sundararaj et al. (2004) provided evidence to support ceramide inducing the rapid degradation of telomeres through blocking the telomere binding activity of nuclear glyceraldehyde-3-phosphate dehydrogenase (GAPDH). However, the role of telomerase reduction in ceramide-induced cell death is still unclear.

Growth factors are important bioactive molecules, and several biological functions of growth factors including proliferation, antiapoptosis, and inflammation have been reported. Basic fibroblast growth factor-2 (FGF-2) is a potent stimulator of proliferation via activation of FGF receptors (FGFRs) (Raballo et al., 2000; Garmy-Susini et al., 2004). In addition, platelet-derived growth factor–BB (PDGF–BB) is considered a mitogenic and chemotactic factor because it stimulates the phosphorylation of PDGF receptors (PDGFRs). A diverse array of cellular responses induced by PDGF including cell proliferation, migration, and survival of mesenchymal cells has been reported (Gruber et al., 2004; Kang et al., 2005; Ng et al., 2008). Mitogen-activated protein kinase (MAPK) family members are critical signaling molecules for FGF-2 and PDGF responses. Activation of extracellular regulated kinases (ERKs) has been shown in growth factor-mediated mitogenic responses in various cell types. PDGF–BB and FGF-2 also activate ERKs, protein kinase-1/c-Jun NH_2-terminal kinase (JNK), and stress-activated protein kinase-2 (p38). Evidence indicates that activation of receptor tyrosine kinases, such as PDGFRs and FGFRs, regulate both cell death and cell growth. However, the constitutive activation of PDGFR and FGFR has been shown to cause growth arrest and apoptosis in some cell lines (Mansukhani et al., 2000). Therefore, it is unclear how activation of PDGFRs and FGFRs regulates two such diverse cellular responses as proliferation and apoptosis.

Prostaglandins (PGs) are produced from cells in response to stimuli such as cytokines, lipopolysaccharide, and growth factors (Chen et al., 2002; Shen et al., 2004). PG synthesis is regulated by cyclooxygenases (COXs), and COX-2 is inducibly expressed by a variety of mitogens and inflammatory inducers to catalyze the production of PGs from arachidonic acid. FGF-2 and PDGF are known to induce PG production via stimulating COX-2 gene expression. Tessner et al. (2003) indicated that FGF-2 upregulates COX-2 expression through p38 MAPKs in I407 cells. Goppelt-Struebe et al. (2000) reported that COX-2 induced by PDGF is dependent on NF-κB activation in mesangial cells. However, the role of COX-2/PGs in FGF2's and PDGF's inhibition of apoptosis is still unclear. NIH3T3 cells have been shown to be sensitive to PDGF and FGF stimulation (Bromann et al., 2005; Soulet et al., 2005). In the present study, it was determined that PDGF and FGF-2 possess the ability to protect NIH3T3 cells from C_2-ceramide-induced apoptosis. Roles of telomerase and COX-2/prostaglandin E_2 (PGE_2) in PDGF and FGF-2 against C_2-ceramide-induced apoptosis were investigated.

Materials and Methods

Cell culture

NIH3T3 cells were obtained from American type culture collection (ATCC; Manassas, VA), and incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, antibiotics (100 U/ml penicillin A and 100 U/ml streptomycin), and 10% heat-inactivated calf serum (CS), and maintained at 37°C in a humidified incubator containing 5% CO_2.

Chemicals

C_2-ceramide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), PDGF–BB, FGF-2, proteinase K, RNase H, actinomycin D (ActD), cycloheximide (CHX), sulprostone, butaprost, sphingosine-1-phosphate (S1P), ceramide-1-phosphate (C1P), C_2-dihydroceramide, and PGE_1 alcohol were obtained from Sigma Chemical (St. Louis, MO). Antibodies of ERKs, p38, JNKs, and Akt were obtained from Cell Signaling (Beverly, MA). Antibodies of phosphotyrosine (PY20), COX-2, COX-1, and α-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). TI, PD98059, and SP600125 were obtained from Calbiochem (La Jolla, CA).

Cell viability

MTT was used as an indicator of cell viability as determined by its mitochondrial-dependent reduction to formazone. Cells were plated at a density of 4×10^5 cells/well in 24-well plates for 12 h, followed by treatment with the indicated compounds for a further 12 h. Cells were washed with phosphate-buffered saline (PBS) three times, and MTT (50 mg/ml) was added to the medium for 4 h. Then, the supernatant was removed, and the formazone crystals were dissolved using 0.04 N HCl in isopropanol. The absorbance was read at 600 nm with an enzyme-linked immunosorbent assay (ELISA) analyzer (Dynatech MR-7000; Dynatech Laboratories, Chantilly, VA).

LDH release assay

The percentage of LDH release was expressed as the proportion of LDH released into the medium compared to the total amount of LDH present in cells treated with 2% Triton X-100. The activity was monitored by the oxidation of the reduced form of nicotinamide-adenine dinucleotide (NADH) at 530 nm by an LDH assay kit (Roche, Indianapolis, IN). Cytotoxicity was determined by the equation: [(OD530 of the treated group − OD530 of the control group)/(OD530 of the Triton X-100-treated group − OD530 of the control group)] × 100%.

DNA gel electrophoresis

NIH3T3 cells were treated with C_2-ceramide in the presence or absence of CS for 24 h, and the cell pellet was washed with PBS followed by adding 80 μl of lysis buffer (50 mM Tris (pH 8.0), 10 mM ethylenediamine tetraacetic acid (EDTA), 0.5% sodium sarkosinate, and 1 mg/ml proteinase K) at 56°C for an additional 3 h. At the end of the reaction, 0.5 mg/ml RNase A was added to each sample and incubated at 56°C for another hour. The DNA in each sample was extracted with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) before loading. Samples were mixed with loading buffer (50 mM Tris, 10 mM EDTA, 1% (w/w) low-melting-point agarose, and 0.025% (w/w) bromophenol blue) and loaded onto pre-solidified 2% agarose gels containing 0.1 mg/ml Ethidium bromide. The agarose gels were run in TBE buffer and observed and photographed under UV illumination.

Western blotting

The protocol for Western blotting was described in our previous study (Lin et al., 2007). Briefly, equal amounts of total proteins from each sample were loaded and separated on 8–12% sodium dodecylsulfate (SDS)–polyacrylamide minigels, followed by transfer to immobilon polyvinylidenedifluoride (PVDF) membranes (Millipore, Bedford, MA). The membrane was incubated with 1% bovine serum albumin (BSA) at room

temperature for 1 h, followed by incubation with specific antibodies overnight at 4°C. Expressions of the indicated proteins were detected by staining with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma).

Telomeric repeat amplification protocol (TRAP)

NIH3T3 cells were treated with different components for 24 h, and total cell extracts were prepared as described in our previous article (Shen et al., 2008). The telomerase activity in cells was examined by the methods as described in the previous article (Kim et al., 1994). Briefly, ice-cold lysis buffer (10 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM ethyleneglycol tetraacetic acid (EGTA), 10% glycerol, 0.5% 3-[3-cholamidopropyl-dimethylammonio]-1-propane-sulfonate (CHAPS), 0.1 mM 4-(2-amino-ethyl)-benzenesulphonyl fluoride hydrochlorine (PMSF), and 5 mM β-mercapto-ethanol) was added to the cell pellets, and stored at −20°C. Protein (0.5 μg) was taken from the cell lysate, and 0.1 μg TS primer (AATCCGTCGAGCAGAGTT), 5 μl of 10× reaction buffer (200 mM Tris–HCl (pH 8.0), 15 mM $MgCl_2$, 630 mM KCl, 0.05% Tween-20, and 10 mM EGTA), and 46 μM dNTP were added at 30°C for 30 min and then 85°C for 5 min to stop the reaction. The CX primer (0.1 μg; CCCTTACCCTTACCCTTACCCTAA), 0.1 μg of the TSNT primer (AATCCGTCGAGCAGAGTTAAAA-GGCCGAGAAGC), and 0.1 μg of the NT primer (ATCGCTTCTCGGCCTTTT) were added to the mixture, and then a polymerase chain reaction (PCR) was run with 15 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. At the end of the reaction, 15 μl of the reaction products was analyzed on 12% non-denatured polyacrylamide minigels in 1× TBE, and the telomeric fragments were visualized by silver staining (GE Healthcare Bio-sciences AB, Uppsala, Sweden).

Measurement of PGE_2 production

NIH3T3 cells were sub-cultured in 24-well plates, and were incubated with indicated compounds for 24 h. One hundred microliters of supernatant of culture medium was collected for the determination of PGE_2 concentrations by ELISA (Cayman Enzyme Immunoassay kit).

Statistical analysis

Values are expressed as the mean ± SE. The significance of the difference from the respective controls for each experimental test condition was assayed using Student's *t*-test for each paired experiment. A *P*-value <0.05 or 0.01 was regarded as indicating a significant difference.

Results

C_2-ceramide induction of apoptosis with a reduction in telomerase activity in NIH3T3 cells

The cytotoxic effect of C_2-ceramide in NIH3T3 cells in the presence or absence of CS was investigated. As illustrated in Figure 1A, chromosome-condensed cells induced by C_2-ceramide were detected by Giemsa staining under microscopic observation in a condition without CS, and those cells were completely abolished by adding 10% CS. Data of the MTT assay showed that C_2-ceramide dose-dependently reduced the viability of NIH3T3 cells in a serum-free (SF) condition, which was inhibited by adding 10% CS (Fig. 1B; left panel). C_2-ceramide-induced DNA ladders were reduced by the addition of CS via a DNA integrity assay (Fig. 1B; right panel). These results suggest that the addition of CS can protect cells from C_2-ceramide-induced apoptosis. Furthermore, analysis of telomerase activity in NIH3T3 cells in response to C_2-ceramide was performed by a TRAP assay. As illustrated in Figure 1C, the telomerase activity of NIH3T3 cells increased with the loading amount of cell lysate, and the activity was abolished by heat (H; 95°C for 10 min), RNase, or proteinase (PK) treatment. In the present of C_2-ceramide treatment without CS, a dose-dependent decrease in telomerase activity was identified (Fig. 1D). Incubation of NIH3T3 cells with CS dose-dependently induced telomerase activity according to the TRAP assay (Fig. 1E). Anti-phospho tyrosine antibody PY-20 has been extensively used to examine the activated receptors containing tyrosine phosphorylation. We further examined the tyrosine phosphorylation status in total proteins of NIH3T3 cells in the presence of CS stimulation by Western blotting using a specific phosphotyrosine antibody (PY20). As shown in Figure 1F, increases in the levels of phosphorylated PDGFR-(190 kDa) and FGFR (120 ∼ 145 kDa)-like protein were observed (Fig. 1F). This suggests that activation of PDGFR and FGFR may be involved in CS's protection of NIH3T3 cells from C_2-ceramide-induced cytotoxicity.

PDGF and FGF-2 protects NIH3T3 cells from C_2-ceramide-induced apoptosis by stimulating telomerase activity

In order to elucidate if activation of PDGFR and FGFR can protect cells from C_2-ceramide-induced cell death, the exogenous addition of PDGF and FGF followed by C_2-ceramide treatment was carried out. As illustrated in Figure 2A, C_2-ceramide-induced chromatin-condensed cells were inhibited by the addition of PDGF or FGF-2 in NIH3T3 cells. Data of the MTT assay showed that the addition of PDGF or FGF-2 attenuated the cytotoxic effect elicited by C_2-ceramide in NIH3T3 cells (Fig. 2B). Furthermore, an increase in the telomerase activity was observed in PDGF- or FGF-2-treated NIH3T3 cells, and that increase was inhibited by incubating cells with the translation inhibitor, cycloheximide (CHX), or the transcription inhibitor, actinomycin D (Act D) (Fig. 2C). A protective effect of PDGF and FGF-2 against C_2-ceramide-induced cell death with an increase in the telomerase activity was identified.

The telomerase inhibitor (TI) reverses the protective effects of PDGF and FGF against C_2-ceramide-induced cell death

In order to identify the role of telomerase activation in PDGF's and FGF's protection of NIH3T3 cells from C_2-ceramide-induced cell death, a pharmacological study using a chemical TI was performed. As illustrated in Figure 3A, an increase in telomerase activity elicited by PDGF or FGF-2 was significantly suppressed by the addition of a TI. In the same part of the experiment, the effect of TI on PDGF or FGF-2 protection against cell death elicited by C_2-ceramide was examined by the MTT and LDH release assays. Data of the MTT assay showed that the addition of the TI significantly attenuated the protective effect of PDGF or FGF-2 against the cytotoxicity of NIH3T3 cells elicited by C_2-ceramide (Fig. 3B). Moreover, the addition of PDGF or FGF-2 reduced the increase in the LDH level in the medium elicited by C_2-ceramide, which was blocked by the addition of the TI (Fig. 3C). These results suggest that telomerase activation participates in PDGF's and FGF's protection of NIH3T3 cells against C_2-ceramide-induced cell death.

Activation of ERK and JNK in PDGF and FGF protect NIH3T3 cells from C_2-ceramide-induced cell death

The alternative activation of MAPKs including ERKs, p38, and JNKs by PDGF and FGF-2 has been reported; therefore the role of MAPK activation in PDGF's and FGF's protection of NIH3T3 cells against C_2-ceramide-induced cell death was examined.

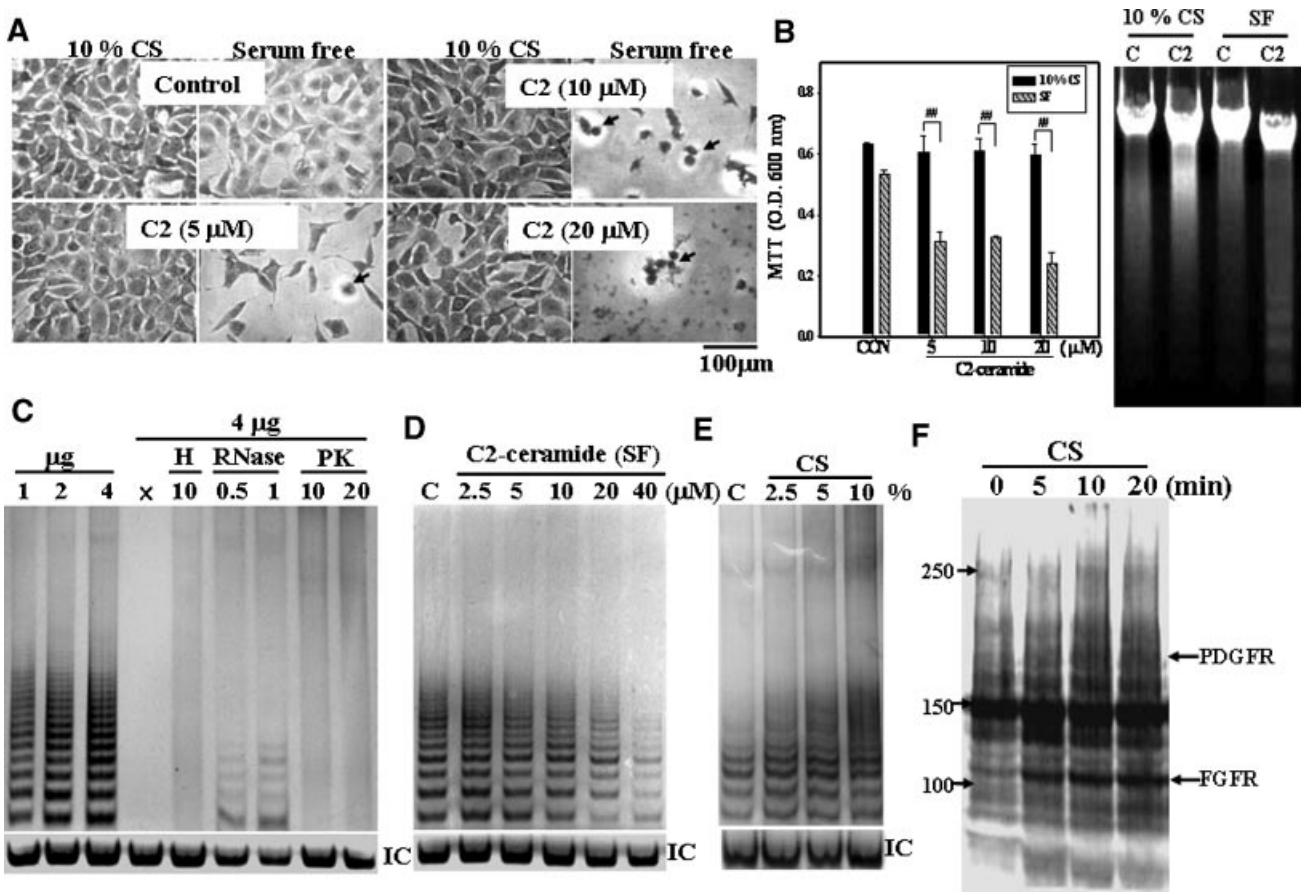

Fig. 1. CS protection of NIH3T3 cells against C_2-ceramide-induced apoptosis through inducing telomerase activity, and PDGF and FGF receptor phosphorylation. A: C_2-ceramide induced chromatin-condensed cells in a condition without CS, but not with CS. NIH3T3 cells were treated with different doses (5, 10, and 20 μM) of C_2-ceramide (C_2) for 24 h in conditions with or without (serum free) 10% CS. The morphology of NIH3T3 cells under different treatments was observed microscopically via Giemsa staining. B: CS protection of NIH3T3 cells from C_2-ceramide-induced cell death by the MTT assay (Left panel) and DNA integrity assay (Right panel). As described in (A), the viability and DNA integrity of cells under different treatments were detected by an MTT assay and DNA integrity assay as described in Materials and Methods Section. C: Detection of intracellular telomerase activity by a TRAP assay. Different amounts (1, 2, and 4 μg) of cell lysates were applied in the TRAP assay as described in Materials and Methods Section. In heat- (H), RNase-, and proteinase K (PK)-treated groups, the lysate (4 μg) was incubated at 95°C for 10 min (H), or with RNase A (0.5 and 1 U/ml), or PK (10 and 20 U/ml) at 37°C for 10 min, followed by the TRAP assay. The intensity of telomerase activity was examined by silver staining. "x" indicated no protein added in the TRAP reaction as a negative control. D: C_2-ceramide inhibition of telomerase activity occurred in a dose-dependent manner. Cells were treated with different doses of C_2-ceramide, and the telomerase activity was analyzed by a TRAP assay. E: CS addition induced telomerase activity in NIH3T3 cells. NIH3T3 cells were treated with or without different percentages (2.5%, 5%, and 10%) of CS for 24 h, and the telomerase activity was examined by a TRAP assay. F: CS induced PDGF and FGF receptor activation via stimulating protein phosphorylation in NIH3T3 cells. Cells were treated with CS (10%) for different times (0, 5, 10, and 20 min), and the expression of tyrosine-phosphorylated proteins was examined by Western blotting using anti-phosphotyrosine antibody PY-20. All data have been repeated at least three times, and similar results were obtained.

Data of the Western blot analysis showed that the respective activation of the PDGFR and FGFR via inducing PDGFR and FGFR protein phosphorylation in accordance with increasing ERK and JNK, but not p38 or AKT, protein phosphorylation with PDGF and FGF-2 treatment was identified in NIH3T3 cells (Fig. 4A). Two specific chemical inhibitors, PD98059 (PD) for ERKs and SP600125 (SP) for JNKs, were applied to investigate the role of ERK and JNK activations in PDGF- and FGF-2-induced telomerase activity. As illustrated in Figure 4B,C, phosphorylated ERK and JNK proteins induced by PDGF and FGF-2 were respectively significantly inhibited by the addition of PD and SP. Data of the TRAP assay showed that the incubation of cells with PD or SP attenuated the telomerase activity stimulated by PDGF or FGF-2 (Fig. 4D,E). Results suggest that ERK and JNK activations are involved in PDGF- and FGF-2-induced telomerase activation.

PDGF and FGF induced COX-2 protein expression and PGE_2 production in NIH3T3 cells

We further examined the effects of PDGF and FGF-2 on COX-2 protein expression in NIH3T3 cells. Data of the Western blot analysis indicated that the addition of PDGF or FGF-2 time-dependently induced COX-2 protein expression in NIH3T3 cells (Fig. 5A). The increase in COX-2 protein by PDGF or FGF-2 was significantly attenuated by adding the transcriptional inhibitor, ActD, or the translational inhibitor, CHX (Fig. 5B). In the presence of the ERK inhibitor, PD98059, or the JNK inhibitor, SP600125, the induction of COX-2 protein was suppressed (Fig. 5C). A similar amount of α-tubulin protein (α-TUB) in each lane is described as an internal control. Additionally, the amount of PGE_2 in the medium was detected as representative of COX-2 enzyme activity in NIH3T3 cells

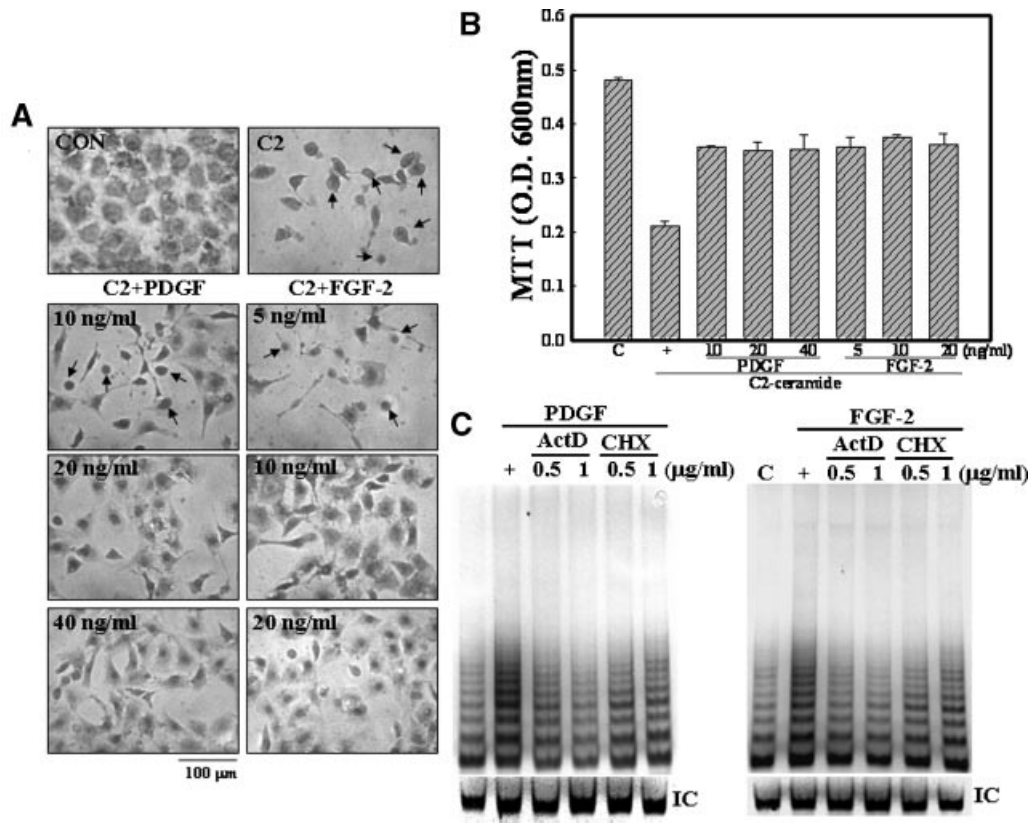

Fig. 2. PDGF and FGF-2 protect NIH3T3 cells from C_2-ceramide-induced cell death by stimulating telomerase activity in the condition without CS. A: C_2-ceramide-induced morphological changes were inhibited by the addition of PDGF and FGF-2 to NIH3T3 cells. Cells were treated with different doses of PDGF and FGF-2 for 10 min, followed by C_2-ceramide (20 μM) treatment for an additional 24 h. Cell morphology was detected by Giemsa staining and observed microscopically. B: As described in (A), the viability of NIH3T3 cells under different treatments was examined by an MTT assay. C: PDGF and FGF-2 induced the enzyme activity of telomerase in NIH3T3 cells, which was blocked by adding CHX and ActD. Cells were treated with different doses (0.5 and 1 μg/ml) of CHX and ActD for 10 min, followed by incubation with PDGF (10 ng/ml) or FGF-2 (5 ng/ml) for 24 h. Telomerase activity was detected by the TRAP assay. All data have been repeated at least three times, and similar results were obtained.

under different treatments. As shown in Figure 5D, significant increases in PGE_2 levels in PDGF- and FGF-2-treated NIH3T3 cells were observed, and those were inhibited by the addition of PD98059 or SP600125.

Induction of COX-2 protein and PGE_2 production participates in PDGF- and FGF-stimulated telomerase activity

The relationship between COX-2 induction and telomerase activation in PDGF's and FGF's protection of NIH3T3 cells against C_2-ceramide-induced cell death was investigated. Incubation of cells with the specific COX-2 inhibitor, NS398 alone shows no effect on the endogenous telomerase activity, and adding NS398 reduces the telomerase activity elicited by PDGF or FGF in NIH3T3 cells (data not shown; Fig. 6A). The addition of PGE_2 (2 μg/ml) significantly induced telomerase activity in NIH3T3 cells without the addition of PDGF or FGF (Fig. 6B). Four EP agonists, including the EP1 agonist, 17-PT, the EP2 agonist, sulprostone (SUL), the EP3 agonist, butaprost (BUT), and the EP4 agonist, PGE1 alcohol (PGE1), were used to investigate their effects on telomerase activity by the TRAP assay. As shown in Figure 6C, 17-PT, but not SUL, BUT, or PGE1, significantly stimulated telomerase activity in NIH3T3 cells. Accordingly, PGE_2 induction of telomerase activity was dose-dependently blocked by the EP1 antagonist, SC-19220 (Fig. 6D). Additionally, the effect of PGE_2 on C_2-ceramide-induced cell death was examined by the MTT assay (Fig. 6E) and LDH release assay (Fig. 6F). A significant reduction in C_2-ceramide-induced cell death by PGE_2 was detected, and it was blocked by adding the EP1 antagonist, SC-19220. SC-19220 alone shows no effect on the viability and endogenous telomerase activity in NIH3T3 cells (data not shown).

Ceramide-1-phosphate (C1P), sphingosine-1-phosphate (S1P) and C_2-dihydroceramide (DiOH-C_2) show no effect on the telomerase activity and viability of NIH3T3 cells

In order to identify if ceramide metabolites C1P and S1P affect the cytotoxic effect and telomerase inhibition elicited by C_2-ceramide. As described in Figure 7A,B, neither C1P nor S1P affects the endogenous telomerase activity and viability of NIH3T3 cells. DiOH-C_2 is an inactive form of C_2-ceramide as a control, and data of TRAP assay and MTT assay indicated that DiOH-C_2 addition showed no effect on telomerase activity and cellular viability. As the same part of experiments, C1P and S1P did not affect the cytotoxicity elicited by C_2-ceramide in NIH3T3 cells (Fig. 7C). It suggests that ceramide metabolites C1P and S1P may not be involved in the present study.

Discussion

The roles of telomerase and COX-2/PGE_2 in PDGF's and FGF-2's protection against cell death elicited by C_2-ceramide were investigated. Data of the present study showed that the

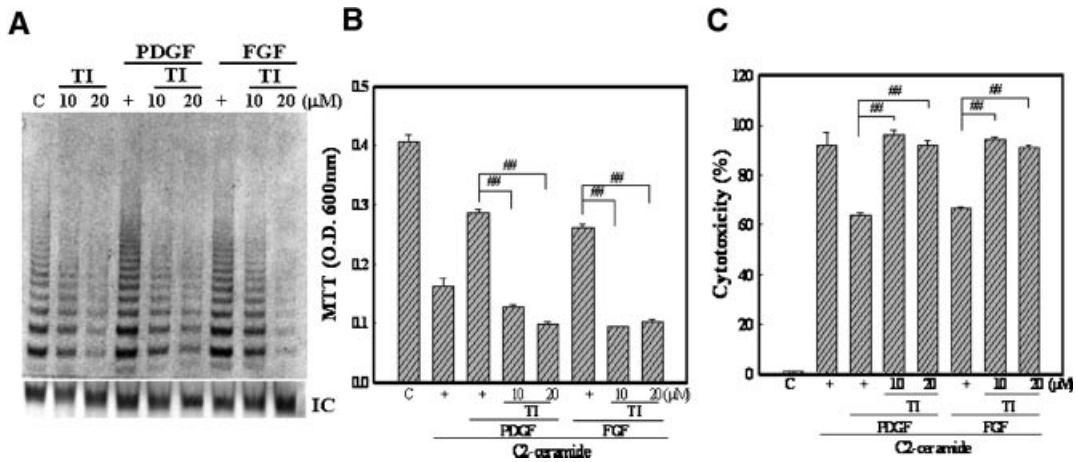

Fig. 3. Telomerase inhibitor (TI) attenuates the cytoprotective effects of PDGF and FGF-2 against C_2-ceramide-induced cell death accompanied by reducing telomerase activity. A: The addition of a TI inhibited telomerase stimulated by PDGF (10 ng/ml) and FGF-2 (5 ng/ml) in NIH3T3 cells. Cells were treated with the TI (10 and 20 μM) for 30 min, followed by adding PDGF or FGF-2 for 24 h, and the telomerase activity in cells was detected by a TRAP assay. B,C: The TI inhibited PDGF and FGF protection of NIH3T3 cells against C_2-ceramide-induced cell death by the MTT (B) and LDH (C) release assays. Cells were treated with TI (10 and 20 μM) in the presence of PDGF (10 ng/ml) or FGF-2 (5 ng/ml) for 30 min, followed by C_2-ceramide treatment for an additional 24 h. The viability of cells under different treatments was examined by the MTT assay. In addition, LDH released into the medium was detected by the LDH release assay. Data of TRAP assay have been repeated at least three times, and similar results were obtained. Each value is presented as the mean ± SE of three independent experiments. $^{\#\#}P < 0.01$ indicates a significant difference between indicated groups as analyzed by Student's *t*-test.

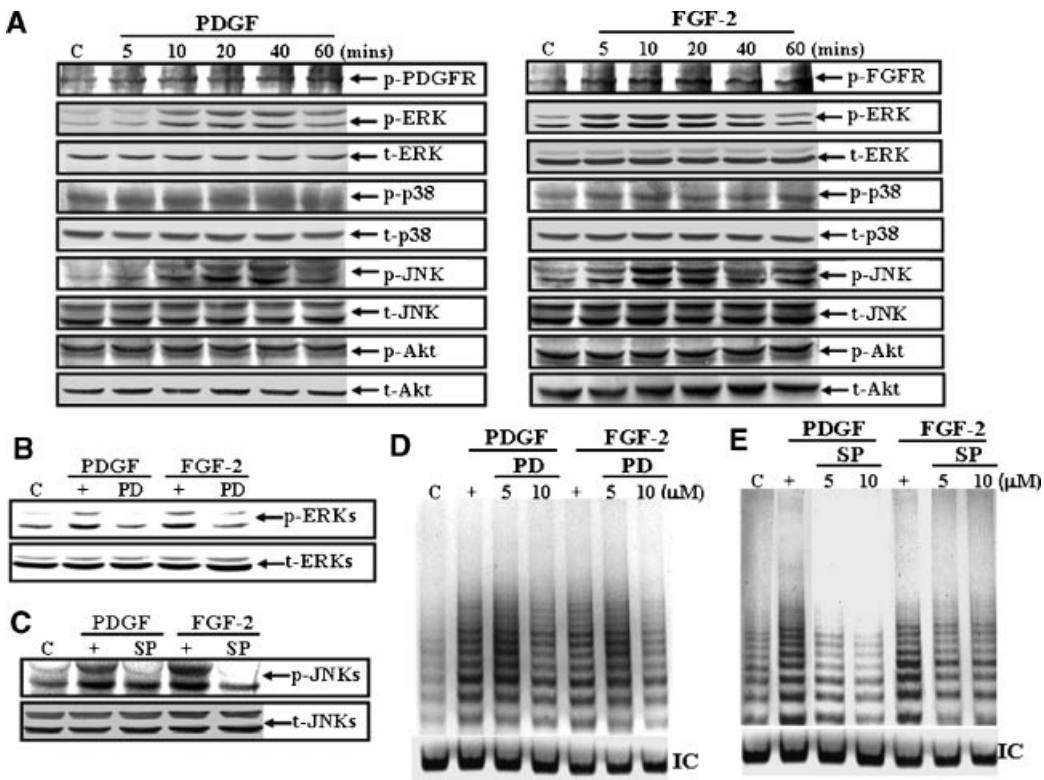

Fig. 4. Induction of ERK and JNK protein phosphorylations in PDGF- and FGF-2-induced telomerase activation. A: PDGF's and FGF-2's induction of ERK and JNK, but not p38 or Akt, protein phosphorylations in NIH3T3 cells. Cells were treated with PDGF (10 ng/ml; left panel) or FGF-2 (5 ng/ml; right panel) for different times (5, 10, 20, 40, and 60 min). The expressions of the indicated proteins were examined by Western blotting using specific antibodies, and the expression of the phosphorylated PDGFR and FGFR was detected by an anti-phospho-tyrosine antibody (PY20). B,C: PD98059 (PD) and SP600125 (SP) inhibited PDGF and FGF-2-induced ERK and JNK protein phosphorylations in NIH3T3 cells. Cells were treated with PD or SP (10 μM) for 30 min followed by PDGF (10 ng/ml) or FGF-2 (5 ng/ml) treatment for an additional 40 min. Expressions of the indicated proteins was examined by Western blotting. D,E: PD and SP inhibited the telomerase activity elicited by PDGF and FGF-2. As described in (B), the telomerase activity under different treatments was examined by the TRAP assay.

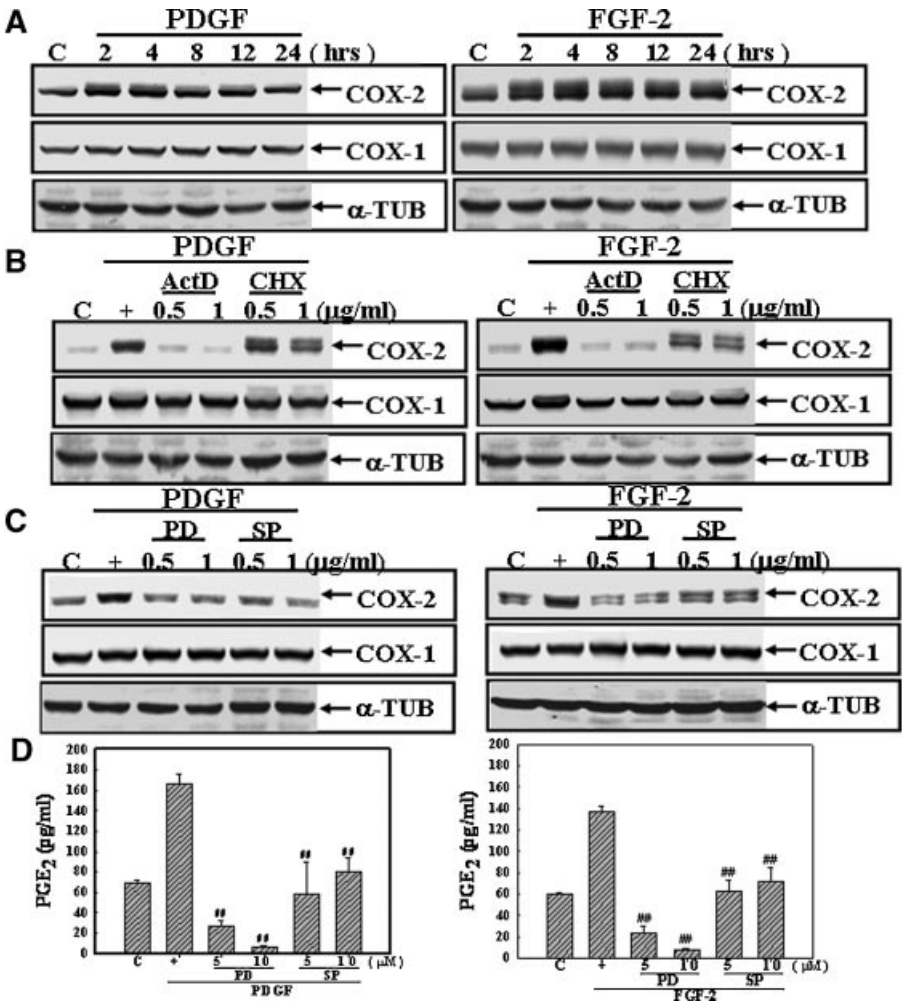

Fig. 5. Induction of COX-2, but not COX-1, protein expression by PDGF and FGF-2 with an increase in PGE2 production in NIH3T3 cells. A: PDGF and FGF-2 time-dependently induced COX-2, but not COX-1, protein expression. Cells were treated with PDGF (10 ng/ml) or FGF-2 (5 ng/ml) for different times (2, 4. 8, 12, and 24 h), and the expressions of COX-2 and COX-1 protein were detected by Western blotting. B: ActD and CHX inhibited PDGF- and FGF-2-induced COX-2 protein expression in NIH3T3 cells. Cells were treated with Act D or CHX (0.5 and 1 μg/ml) for 30 min followed by PDGF (10 ng/ml) or FGF-2 (5 ng/ml) treatment for an additional 24 h. The expressions of COX-2 and COX-1 protein were examined. C: PD and SP inhibited PDGF- and FGF-induced COX-2 protein expression in cells. Cells were treated with PD or SP (5 and 10 μM) for 30 min, followed by incubation with PDGF or FGF-2 for an additional 24 h, and the expressions of COX-2 and COX-1 proteins were examined. D: The addition of PD and SP inhibited PDGF- and FGF-2-induced COX-2 activity. As described in (C), the amount of PGE_2 in the medium was examined by a PGE_2-detecting kit. An expression of α-tubulin protein (α-TUB) is described as an internal control for similar amount of protein loaded in each lane. Data of Western blotting have been repeated at least three times, and similar results were obtained. Each value is presented as the mean ± SE of three independent experiments. $^{\#\#}P < 0.01$ indicates a significant difference from PDGF or FGF-2-treated groups.

activation of telomerase and COX-2/PGE_2 may contribute to PDGF's and FGF-2's protection of NIH3T3 cells against C_2-ceramide-induced cytotoxic effects, and both events are located downstream of ERK and JNK activation. Telomerase induced by PDGF and FGF-2 was inhibited by the COX-2 inhibitor, NS398, and the addition of PGE_2 protected NIH3T3 cells from C_2-ceramide-induced cell death by elevating telomerase activity, which was blocked by treatment with the EP2 antagonist, SC19220. These findings supported the notion that the protective effects of FGF-2 and PDGF on the viability of NIH3T3 cells in response to C_2-ceramide stimulation, at least in part, are through COX-2/PGE_2-dependent telomerase activation.

Telomerase is an enzyme, which maintains the length of telomeres found at the end of chromosomes, and aberrant activation of telomerase is associated with the immortality and malignancy of cancer cells (Püttmann et al., 2005; Chapman et al., 2006). The progressive shortening of telomere length in somatic cells leads to cellular senescence (Smith et al., 2003; Dalerba et al., 2005); therefore, maintenance of telomeres may be beneficial in delaying the aging process. Growth factors have been shown to prevent the aging process, and are extensively applied in cosmetic products, whereas associations between telomerase and growth factors remain unclear. NIH3T3 cells are non-transformed immortalized fibroblasts, and the expressions of PDGFR and FGFR in NIH3T3 cells have been reported. Kondo et al. (2001) indicated the association of telomerase activity with PDGF or FGF-2 in retinoblastoma cell lines. Data of the present study provided evidence to support PDGF and FGF-2 possessing the ability to induce telomerase

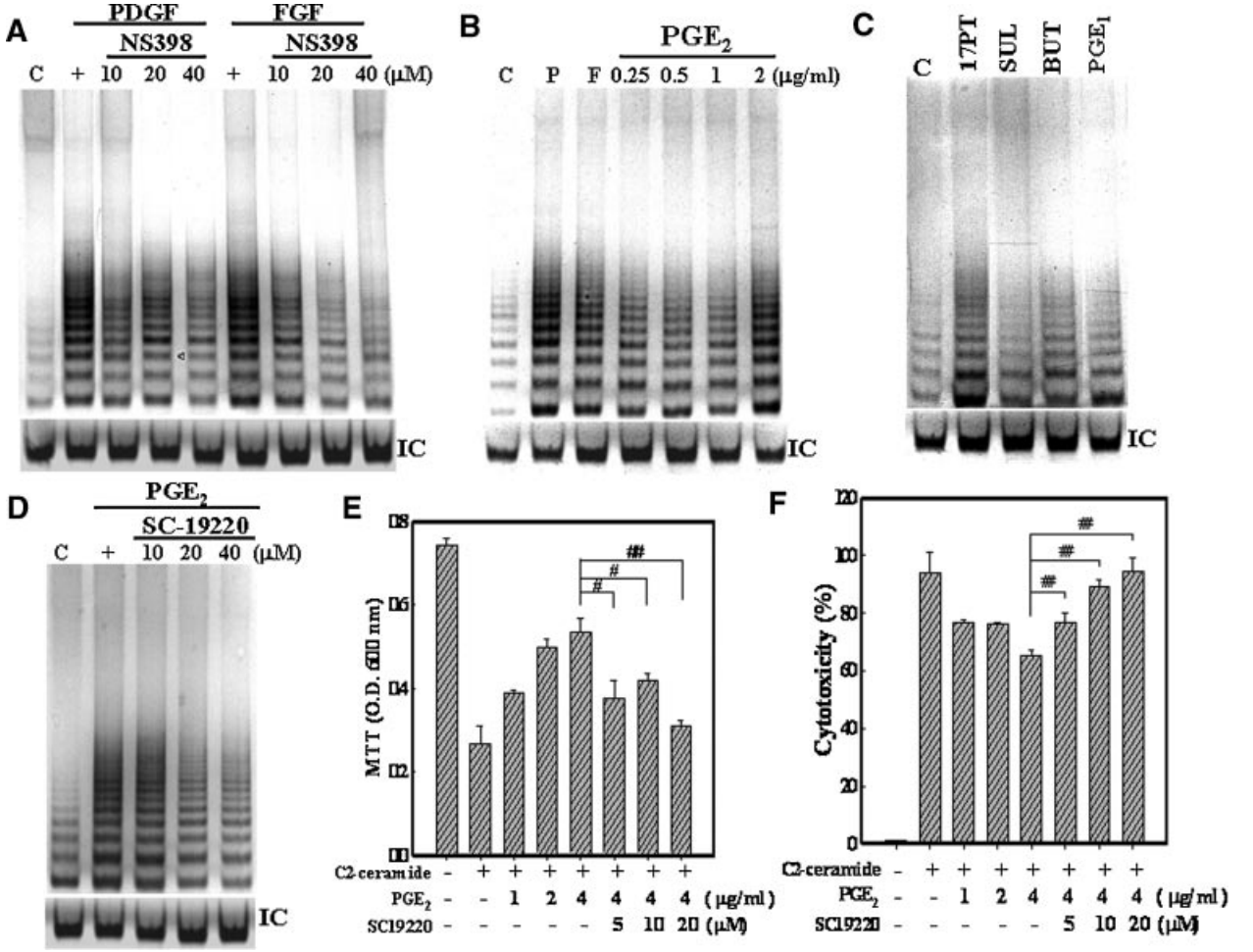

Fig. 6. PGE_2 protection of NIH3T3 cells against C_2-ceramide-induced cytotoxicity through EP1-dependent telomerase activation. A: Telomerase induced by PDGF and FGF-2 was inhibited by adding the COX-2 inhibitor, NS398. Cells were treated with different doses (10, 20, and 40 μM) of NS398 for 30 min followed by PDGF (10 ng/ml) or FGF-2 (5 ng/ml) treatment for an additional 24 h. Telomerase activity was analyzed by a TRAP assay. B: PGE_2 stimulated telomerase activity in NIH3T3 cells. Cells were treated with PDGF (10 ng/ml; P), FGF-2 (5 ng/ml; F), or different doses (0.25, 0.5, 1, and 2 μg/ml) of PGE_2 for 24 h, and the telomerase activity was analyzed by a TRAP assay. C: The EP1 agonist, 17-PT, but not the EP_2 agonist, sulprostone, the EP_3 agonist, butaprost, or the EP_4 agonist, PGE_1 alcohol, enhanced telomerase activity in NIH3T3 cells. Cells were treated with the indicated EP agonists for 24 h, and the telomerase activity was analyzed. D: The EP_1 antagonist, SC-19220, attenuated PGE_2-induced telomerase activity in NIH3T3 cells. Cells were treated with different doses (10, 20, and 40 μM) of SC-19220 for 30 min, followed by PGE_2 (2 μg/ml) treatment for 24 h. Telomerase activity was analyzed by the TRAP assay. E,F: PGE_2 protected against C_2-ceramide-induced cell death, which was blocked by the EP1 antagonist, SC-19220. NIH3T3 cells were treated with PGE_2 with or without prior SC-19220 treatment, followed by C_2-ceramide treatment for 24 h. The viability of cells under different treatments was examined by the MTT assay (E) and LDH release assay (F). Each value is presented as the mean ± SE of three independent experiments. Data of TRAP assay have been repeated at least three times, and similar results have been obtained. $^{\#\#}P < 0.01$ indicates a significant difference between indicated groups.

activity, and protect NIH3T3 cells from C_2-ceramide-induced apoptosis. The protective effects elicited by PDGF or FGF-2 is attenuated by adding a specific commercial TI. Results suggest that telomerase activation may contribute to the cytoprotective or anti-aging effects of PDGF and FGF-2.

Overexpression of growth factors or their receptors is a common event in the proliferation of cells and protects cells from apoptosis. However, the precise role of growth factors is not fully understood. PDGF and FGF-2 exist extensively in all tissues, and the overexpression of PDGF and FGF-2 in tumors has been shown to be poor diagnostic markers (Eggert et al., 2000). In bovine corneal endothelial (BCE) cells, FGF-2 stimulates cell proliferation by activating the AKT pathway (Lu et al., 2006). Gu et al. (2004) indicated that activation of the PI3K/AKT pathway plays an important role in FGF-2-induced cell survival. Agas et al. (2008) indicated that FGF-2 induction is involved in $PGF_{2\alpha}$ protection of osteoblast survival by inducing the Bcl-2 protein. PDGF induced proliferation in human LI90 hepatic stellate cells through inducing COX-2 and PGE_2 production (Hui et al., 2004). Chaudhary and Hruska (2001) indicated that PDGF but not FGF-2 elicited a survival signal for the survival of osteoblasts. The effects of PDGF and FGF-2 on the C_2-ceramide-induced cytotoxic effect are still undefined. Data of the present study indicated that PDGF and FGF-2 inhibited C_2-ceramide-induced cell death in NIH3T3 cells, and the inhibitory effect was blocked by the ERK inhibitor, PD98059, and the JNK inhibitor, SP600125 (data not shown). Involvement of ERKs and JNKs in the cytoprotective effect of PDGF and FGF-2 against C_2-ceramide-induced cell death was elucidated.

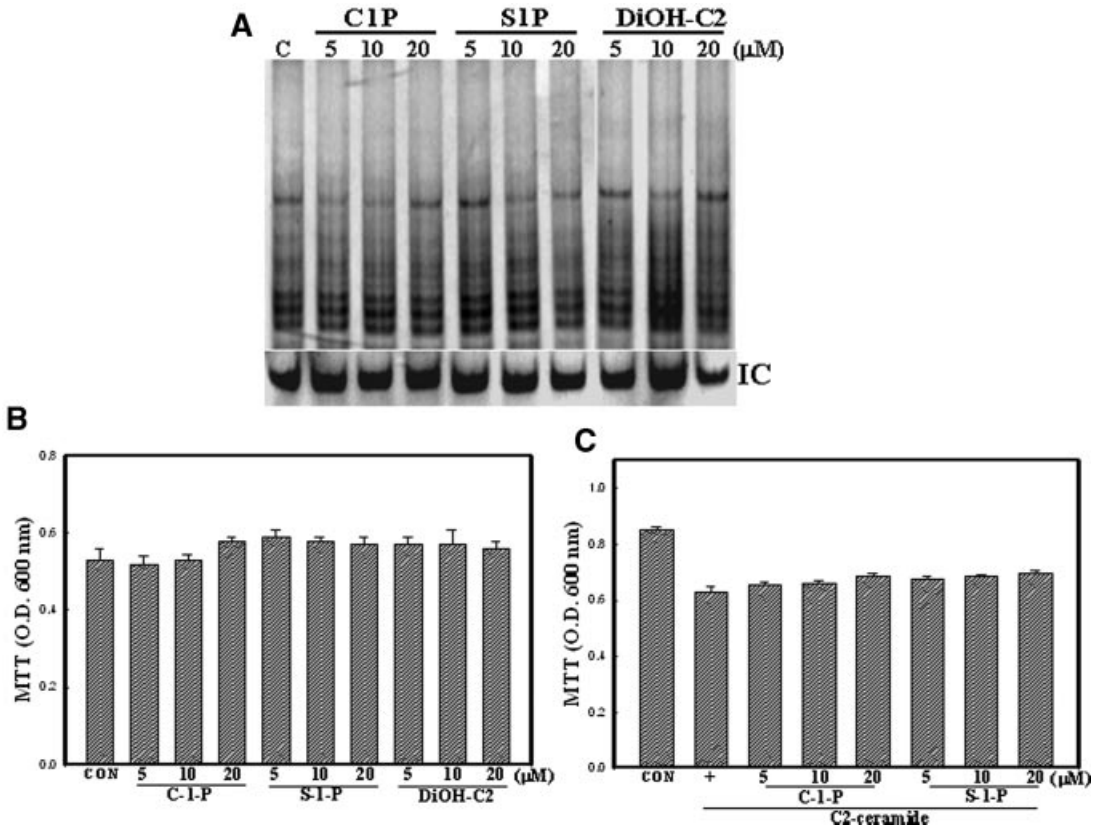

Fig. 7. Sphingosin-1-phosphate (S1P), ceramide-1-phosphate (C1P), and C_2-dihydroceramide (DiOH-C_2) show no effect on the telomerase activity and viability of NIH3T3 cells. NIH3T3 cells were treated with different doses of S1P, C1P, and DiOH-C_2 for 24 h in the condition without CS. A: Telomerase activity in each group was examined by TRAP assay. B: The viability of NIH3T3 cells under different treatments was examined by MTT assay. C: Neither S1P nor C1P affect the cytotoxic effect stimulated by C_2-ceramide in NIH3T3 cells. Cells were treated with different doses of C1P and S1P for 30 min, followed by adding C_2-ceramide (20 μM) for an additional 24 h. The viability of cells in each group was detected by MTT assay. Data of TRAP assay have been repeated at least three times. Each value of MTT assay is presented as the mean ± SE of three independent experiments.

Regulation of COX-2 is mediated by a variety of cytokines or growth factors. The binding of PDGF and FGF-2 to their respective receptors has been demonstrated to induce COX-2 expression via activating a number of signaling pathways. Tessner et al. (2003) showed FGF-2 induction of COX-2 through p38 MAPK in I407 cells. FGF-2-induced COX-2 protein was inhibited by PD98059, an ERK-pathway inhibitor, in aortic smooth muscle cells (Karim et al., 1997). In rat renal mesangial cells, PDGF induced a rapid increase in COX-2 gene expression through activation of NF-κB/IkB transcriptional factors (Goppelt-Struebe et al., 2000). We demonstrated herein that the activation of ERKs and JNKs in PDGF- and FGF-2-treated NIH3T3 cells which stimulated PDGFR and FGFR phosphorylation was identified using the indicated antibodies, and PDGF- or FGF-2-induced COX-2 protein and telomerase activity were inhibited by PD98059 and SP600125. These results suggest that COX-2 activated by PDGF and FGF-2 is mediated by the activation of ERKs and JNKs.

COX-2 activation and PGE_2 production have been linked to stimulation of cellular proliferation and to inhibition of apoptosis. NS398 suppression of the proliferation of tumor cells such as hepatoma and esophageal squamous cell carcinoma (Hu et al., 2003; Zhi et al., 2006). PGE_2's effects on proliferation and apoptosis are mediated by activating or inhibiting four G protein-coupled PGE receptors including EP1, EP2, EP3, and EP4. PGE_2-induced proliferation in colon carcinoma cells and

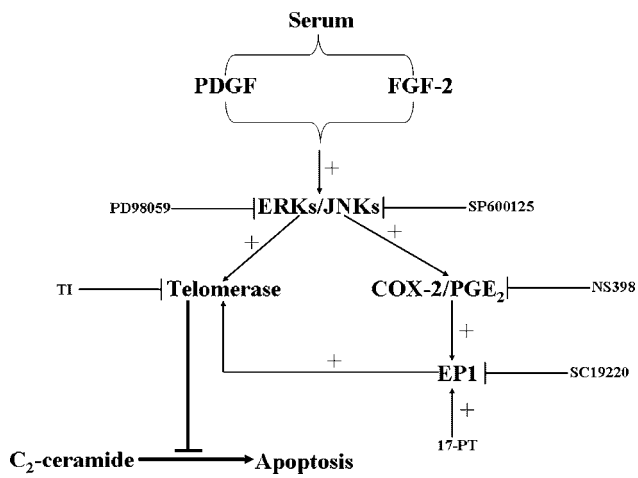

Fig. 8. A tentative cytoprotective mechanism elicited by serum addition against C_2-ceramide-induced cell death is proposed. PDGF, platelet derived growth factor; FGF-2, fibroblast derived growth factor-2; JNKs, c-Jun NH_2-terminal kinases; ERKs, extracellular signal-regulated protein kinases.

metastasis in breast carcinoma cells were blocked by an EP4 receptor antagonist (Cherukuri et al., 2007; Pan et al., 2008). EP1 and EP4 antagonists inhibit the proliferation of adenocarcinoma cells (Han and Wu, 2005; Ma et al., 2006; Hawcroft et al., 2007). In the present study, we demonstrated an elevation in PGE_2 production via stimulating COX-2 protein expression by PDGF and FGF-2, and protection of NIH3T3 cells from C_2-ceramide-induced cytotoxicity by PGE_2 in accordance with an induction in telomerase activity, which was blocked by adding the EP1 antagonist, SC-19220. Contributions of PGE_2 to the cytoprotective effects of PDGF and FGF-2 via the EP1 receptor are first reported herein. The mechanism by which PGE_2/EP1 receptor/telomerase regulates this response elicited by PDGF and FGF-2 warrants further investigation.

Albumin is a highly soluble protein in the plasma at normal concentrations between 35 and 50 g/L. Albumin has been shown to bind and transport several materials including metals, fatty acids, cholesterol, and drugs. However, an interaction between albumin and ceramide is still unclear. Siskind et al. (2002) indicated that the permeability increase by C_2-ceramide was reversed by BSA addition. In our study, we found that the amount of BSA in the 10% CS is around 0.5%, and C_2-ceramide-induced cytotoxic effect is blocked by adding different doses (0.1%, 0.2%, and 0.5%) of BSA (data not shown). It suggests that BSA may contribute to CS prevention of C_2-ceramide-induced cell death in NIH3T3 cells.

In conclusion, this study demonstrated that telomerase activation might play a critical role in the cytoprotective effects of PDGF and FGF-2. The protective effects elicited by PDGF and FGF-2 were inhibited by the COX-2 inhibitor, NS398, and both PGE_2 and 17-PT, an EP1 receptor-selective agonist, stimulated telomerase activity accompanied by a reduction in the cytotoxic effect of C_2-ceramide. Additionally, PGE_2 inhibition of C_2-ceramide-induced cell death was blocked by the EP1 receptor-selective antagonist, SC-19220. We demonstrated that the induction of PGE_2 production via the EP1 receptor to stimulate telomerase activity contributes to the antiapoptotic effect of PDGF and FGF-2. A tentative cytoprotective mechanism elicited by serum addition against C_2-ceramide-induced cell death is proposed in the present study (Fig. 8).

Acknowledgments

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