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Regulation of tumor suppressor PDCD4 by novel protein kinase C isoforms

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ABSTRACT

Transforming growth factor-β1 (TGF-β1) induces apoptosis in normal hepatocytes and hepatoma cells. PDCD4 is involved in TGF-β1-induced apoptosis via the Smad pathway. The tumor promoter 12-O-tetradecanoylphorbor-13-acetate (TPA), a protein kinase C stimulator, inhibits TGF-β1-induced apoptosis. However, the mechanisms of TPA action on PDCD4 expression remain to be elucidated. Therefore. the regulatory mechanism of PDCD4 expression by PKC was investigated. The treatment of the human hepatoma cell line, Huh7 with TPA suppressed PDCD4 protein expression and TGF-β1 failed to increase the PDCD4 protein expression. PKC inhibitors Ro-31-8425 or bisindolylmaleimide-1-hydrocholoride (pan-PKC inhibitors) and rottlerin (PKCδ inhibitor), but not Go6976 (PKCα inhibitor), enhanced the induction of PDCD4 protein by TGF-β1. Furthermore, siRNA-mediated knockdown of PKCδ and ε, but not PKCα, augmented the TGF-β1-stimulated PDCD4 protein expression. However, TPA or pan-PKC inhibitor did not alter the PDCD4 mRNA expression either under basal- and TGF-β1-treated conditions. The down-regulation of PDCD4 by TPA was restored by treatment with the proteasome inhibitor MG132. These data suggest that two isoforms of PKCs are involved in the regulation of the PDCD4 protein expression related to the proteasomal degradation pathway.

1. Introduction

The programmed cell death 4 (PDCD4) gene shows an increased expression when apoptosis is induced. It was first reported as MA3, a gene associated with apoptosis in mice [1]. It was also identified as a gene whose expression is inhibited by topoisomerase inhibitor [2], as well as a gene involved in the cell cycle in human [3,4], and the gene was mapped at 10q24 [5]. The overexpression of this gene in cancer cells has been demonstrated to induce apoptosis [6]. Azzoni et al. [7] reported the expression of this gene to be regulated by interleukins. Subsequently, it has similarly been identified as a cancer-related gene in chickens [8,9] and rats [10]. In addition, this gene has also been reported to inhibit skin carcinogenesis and is thought to be a tumor suppressor [11–14].

PDCD4 has a nuclear localization signal (NLS) at both the N- and C-termini, and in the central region contains two MA3 domains homologous to the M1 domain of the protein synthesis initiation factor eIF4G. PDCD4 inhibits the cap-dependent translation through the binding of eIF4A at the MA3 domain, and inhibits transcription of certain genes through the inhibition of AP-1, a heterodimeric transcription factor that promotes cell proliferation [12,15,16]. TGF-β1, which is known to induce apoptosis in primary hepatocytes and hepatocellular carcinoma (HCC) cells, increases PDCD4 expression and PDCD4 accumulates in the nucleus during apoptosis [6]. The ability to avoid apoptosis is one of the important cellular mechanisms in carcinogenesis [17]. The phorbol ester,12-O-tetradecanoylphorbor-13-acetate (TPA) is a protein kinase C (PKC) stimulator and a well-established tumor promoter. Phorbol esters and growth factors that stimulate PKCs are known to antagonize TGF-β1-induced apoptosis and Smad signaling [18–20], however, the mechanisms by which TPA suppress TGF-β1-induced apoptosis has not been fully elucidated. The present study demonstrates that TPA reduces basal and TGF-β1-induced PDCD4 protein expression in a PKC isoform-specific manner.

2. Materials and methods

2.1. Cells and reagents

The human hepatoma cell line Huh7 was obtained from the Japanese Cancer Research Resources Bank (Osaka, Japan). The cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum in 5% CO^2 at 37 °C. TGF-β1 and epidermal growth factor (EGF) were purchased from R&D systems (Minneapolis, MN, USA),

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12-O-tetradecanoylphorbor-13-acetate (TPA) from Sigma-Aldrich. The PKC inhibitors Ro-31-8425, bisindolylmaleimide-1-hydrocholoride (Bis-1), Go6976, and rottlerin, the PI3K/Akt inhibitor LY294002, the mTOR inhibitor rapamycin, and the proteasome inhibitor MG132 were from Calbiochem (San Diego, CA, USA).

2.2. Cell proliferation assay

1×10^4 cells/well were plated onto a 24-well plate with 0.5 ml of culture medium. TPA was added 24 h after cell placement, and 30 min later the medium was replaced with one containing TGF-β1 at the indicated concentration and the cells were cultured for an additional 48 h. The cells were then rinsed twice with phosphate-buffered saline (PBS) and incubated with 0.5 ml of DMEM containing 50 μl Premix WST-1 (Takara, Shiga, Japan) for 2 h according to the manufacturer's protocol. The absorbance of formazan products at 450 nm was measured with a CS-9300 microplate reader (Shimadzu, Tokyo, Japan).

2.3. DNA ladder

Cells grown on 90 mm dishes (2×10^6 cells/dish) were treated with or without TPA in the presence or absence of TGF-β1 in DMEM containing 10% FBS for 48 h. Fragmented DNA was extracted and separated on a 2% agarose gel as previously described [6]. DNA ladders were visualized with Et-Br staining under UV light.

2.4. Western blotting

The protein expression of PDCD4 was investigated by Western blotting. The cells cultured under various conditions were collected and lysed by sonication with SDS buffer containing 50 mM Tris (pH 6.8), 2.3% SDS and 1 mM PMSF. The cell debris was eliminated by centrifugation at 10,000 rpm for 10 min and the supernatant was collected. After measuring the protein concentration using a protein assay kit (Bio-Rad, Hercules, CA), 30 or 40 μg of protein was mixed with buffer, separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Bio-Rad), and blocked with 0.1% Tween and 1% skim milk in PBS overnight. The membranes were incubated with anti-PDCD4 antibody [6] in PBS with 0.1% Tween, 1% skim milk for 1 hour. Anti-human β-actin antibody was used as a control. The membranes were washed five times with 0.1% Tween 20 in PBS and stained with horseradish peroxidase-conjugated secondary antibodies. All immunoblots were detected by the enhanced chemiluminescence (ECL) system (Amersham, Buckinghamshire, England) according to the manufacturer's instructions. Anti-PKCα, PKCδ, and PKCε antibodies were obtained from Santa-Cruz Biotechnology (Santa Cruz, CA), anti-Akt, phopho-Akt (Ser473), S6 kinase, phosphor-S6 kinase (T389), Erk(p44/42), phosphor-Erk (Thr202/Tyr204) from Cell Signaling Technology (Beverly, MA) and β-actin from Sigma (St. Louis, MO). All experiments were independently repeated at least thrice.

2.5. Real-time PCR

Total RNA of cells was extracted using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Real-time PCR was performed by the TaqMan Gene Expression Assay system (Applied Biosystems, Foster City, CA) using exons 2 and 4 of PDCD4 (No HS00205438-m1) and GAPDH (No HS99999905-m1) as a control according to the manufacturer's protocol.

2.6. Transfection of Huh7 cells with siRNAs

siRNAs against PKCα and δ isoforms (validated siRNA AM51331) are purchased from Ambion (Austin, TX, USA). PKCε-siRNA (HP validated, S100587784) and Allstar negative control siRNA 1027281 were from Qiagen (Heiden, Germany). The cells were transfected with siRNA using Lipofectamine RNAi max (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, the cells were seeded on 35 or 60 mm dish and cultured in DMEM supplemented with 10% FCS at 37 °C. After culturing for 2 to 3 days, the medium was changed to antibiotics-free DMEM containing 10% FCS before adding OptiMEM containing Lipofectamine and siRNA mixture and incubated at 37 °C for 24 or 48 h.

3. Results

3.1. TPA inhibits TGF-β1-induced growth suppression and PDCD4 expression

The effects of TPA on TGF-β1-induced growth suppression were examined in Huh7 cells. As shown in Fig. 1A, TPA antagonized TGF-β1-induced growth suppression in Huh7 cells, confirming previous reports [18]. In addition, the cells treated with TPA showed a decreased DNA ladder formation (Fig. 1B). TGF-β1 induces PDCD4 expression and apoptosis in Huh7 cells [6]. Therefore, the effect of TPA on PDCD4 expression was examined in Huh7 cells. The addition of 10, 100, 500, and 1000 nM TPA suppressed PDCD4 protein expression in a dose-dependent fashion (Fig. 2A). TPA also inhibited PDCD4 protein expression induced by TGF-β1 as shown in Fig. 2B.

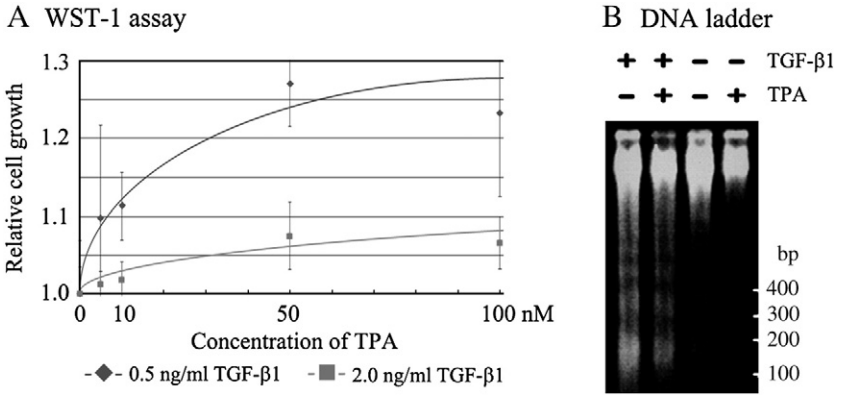

Fig. 1. TPA restored TGF-β1-induced cell growth inhibition and apoptosis in Huh7 cells. (A) TPA was added to Huh7 cell culture at the indicated concentration for 30 min, followed by treatment with 0.5 or 2.0 ng/ml of TGF-β1 for 48 h. Cell growth was determined by a WST-1 assay. The values were expressed as the ratio of OD with TPA and without TPA obtained from three independent experiments. (B) Huh7 cells grown on 90 mm dishes (2×106 cells/dish) were treated for 48 h with or without TPA (50 nM) in the presence and absence of TGF-β1 (4 ng/ml), and fragmented DNA was separated on an agarose gel under UV.