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Tricin Inhibits Proliferation of Human Hepatic Stellate Cells In Vitro by Blocking Tyrosine Phosphorylation of PDGF Receptor and its Signaling Pathways

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ABSTRACT

4′,5,7-Trihydroxy-3′,5′-dimethoxyflavone (Tricin), a naturally occurring flavone, has anti-inflammatory potential and exhibits diverse biological activities including antigrowth activity in several human cancer cell lines and cancer chemopreventive effects in the gastrointestinal tract of mice. The present study aimed to investigate the biological actions of tricin on hepatic stellate cells (HSCs) in vitro, exploring its potential as a treatment of liver fibrosis, since HSC proliferation is closely related to the progression of hepatic fibrogenesis in chronic liver diseases leading to irreversible liver cirrhosis and hepatocellular carcinoma. Tricin inhibited platelet-derived growth factor (PDGF)-BB-induced cell proliferation by blocking cell cycle progression and cell migration in the human HSC line LI90 and culture-activated HSCs. It also reduced the phosphorylation of PDGF receptor β and the downstream signaling molecules ERK1/2 and Akt, which might be due to its tyrosine kinase inhibitor properties rather than inhibition of the direct binding between PDGF-BB and its receptor. Our findings suggest that tricin might be beneficial in HSC-targeting therapeutic or chemopreventive applications for hepatic fibrosis. J. Cell. Biochem. 113: 2346–2355, 2012.

KEY WORDS: HEPATIC STELLATE CELL; TRICIN; PDGF; SIGNAL TRANSDUCTION; LIVER FIBROSIS

Hepatic fibrosis is a major complication resulting from the excessive accumulation of extracellular matrix (ECM) during the wound-healing process after chronic liver injuries, such as viral infection, alcohol abuse, autoimmune liver diseases, and metal overload [Rockey et al., 2006; Friedman, 2008b]. Most importantly, hepatic fibrosis has been implicated as a contributing

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factor in the development of hepatocellular carcinoma (HCC) that commonly occurs in the presence of liver cirrhosis [Rockey et al., 2006; Friedman, 2008b].

Hepatic stellate cells (HSCs) are the cell type most responsible for the excessive production and deposition of ECM during the development of hepatic fibrosis, and the process is also mediated by various inflammatory cytokines [Rockey et al., 2006; Friedman, 2008ab]. HSCs are mesenchymal cells located in the space of Disse, are normally quiescent, and are mainly responsible for the uptake, storage, and delivery of retinoids [Friedman, 2008a]. During liver fibrogenesis, they become activated and acquire a myofibroblast-like phenotype—a series of phenotypic changes that collectively lead to the accumulation of EMC. These include increased proliferation, chemotaxis, contractility, retinoid loss, proinflammatory responses and cytokine release, excessive ECM synthesis, and altered matrix degradation [Friedman, 2008a]. According to recent studies, the degree of fibrogenesis in liver diseases is most likely to be affected by the increased proliferation of HSCs [Rockey et al., 2006; Friedman, 2008ab]. Additionally, activated HSCs promote HCC progression via their cross-talk in the cancer microenvironment [Amann et al., 2009; Yang et al., 2011; Zhao et al., 2011]. Therefore, suppression of HSC activation and proliferation has been proposed as a therapeutic strategy for the treatment and prevention of the hepatic fibrosis that leads to irreversible liver cirrhosis and HCC.

Numerous compounds have previously been investigated for their anti-fibrotic potential, including botanicals, such as flavonoids and other phenolic derivates with anti-oxidative properties [Godichaud et al., 2000; Stickel et al., 2002]. These compounds belong to a larger family group known as the phytochemicals. Flavonoids play important metabolic roles in plants and animals and their biological activities include hepatoprotection and the inhibition of fibrosis [Harborne and Williams, 2000; Middleton et al., 2000; Havsteen, 2002]. However, of more than 6,500 flavonoids identified to date, few have been investigated in depth. 4′,5,7-Trihydroxy-3′, 5′-dimethoxyflavone (tricin; Fig. 1) [Zhou and Ibrahim, 2010] is a member of the flavone subgroup of flavonoids, found in rice, oats, barley, and wheat [Saleh et al., 1988; Kong et al., 2007], that possesses anti-oxidant activity [Yong et al., 2002]. Although its physiological function in plants is not well defined, it is thought to be produced during times of environmental stress or following pathogenic attack [Park et al., 2007] in order to exert potential allelopathic effects [Fiorentino et al., 2008].

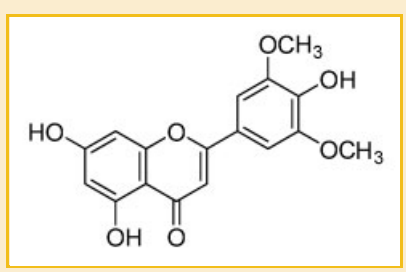

Fig. 1. Chemical structure of tricin.

Recent research on tricin in vitro has revealed anti-inflammatory [Moscatelli et al., 2006], anti-viral [Sakai et al., 2008], anti-histamic [Kuwabara et al., 2003], and anti-cancer [Cai et al., 2004] activities. Moreover, the cancer chemopreventive effect of dietary tricin has also been intensively studied in the gastrointestinal tract of mice [Cai et al., 2005, 2009; Oyama et al., 2009]. Tricin interferes with heritable or inflammation-associated carcinogenesis in the Apc^{Min} mouse or in the chemical-induced colon cancer model, which might be in part due to the inhibition of cyclo-oxygenase-2-prostaglandin E-2 axis [Cai et al., 2005; Al-Fayez et al., 2006] or the reduced expression of tumor necrosis factor-α [Oyama et al., 2009] at the local sites, respectively. However, neither therapeutic nor chemopreventive properties of tricin on hepatic fibrogenesis have been examined in vitro or in vivo. The objective of this investigation, therefore, was to evaluate the biological in vitro effect of tricin on HSCs, exploring the possibility of its future clinical application for liver diseases.

MATERIALS AND METHODS

CHEMICALS

Tricin (>99% pure confirmed by reverse phase high-performance liquid chromatography) was synthesized by M.N. and M.K. at Gifu University (Gifu, Japan) as previously described [Ninomiya et al., 2011]. Tricin obtained as a yellow amorphous powder was dissolved in DMSO to a final concentration of 100 mM. Epigallocatechin-3-gallate (EGCG) from Sigma–Aldrich (St Louis, MO), and platelet-derived growth factor (PDGF-BB) and transforming growth factor (TGF)-β1 from Peprotech (London, UK) were purchased.

CELL CULTURE AND TREATMENTS

The HSC cell line LI90 [Murakami et al., 1995], established from a human hepatic mesenchymal tumor and with characteristics compatible to activated HSCs [Ueki et al., 1998] was provided by the Health Science Research Resources Bank (HSRRB; Japan Health Sciences Foundation, Tokyo, Japan). Cells were cultured in Dulbecco's minimal essential medium (DMEM; Gibco, Invitrogen Co., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Gibco, Invitrogen Co.) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were used between total passage numbers 18–22 for experiments. HSCs freshly isolated from human liver were purchased from ScienCell Research Laboratories (Carlsbad, CA), and characterized as CD90-, desmin-, smooth muscle α-actin (α-SMA; Dako Cytomation, Carpinteria, CA)−, and fibronectin+. Cells were expanded in the company-supplied medium for the first and second passages, and cultured in DMEM supplemented with 10% FBS thereafter. Cells were used for primary HSC experiments between passages 4–6 when immunofluorescence staining demonstrated α-SMA expression (data not shown).

Cells were seeded at $4–5 \times 10^3$ or 1×10^5 cells/well in 96- or 6-well culture plates, respectively, cultured for 24 h to allow settling, then serum-starved for 24–48 h in DMEM without FBS. They were then exposed to the relevant growth factors and tricin as indicated for each assay. The tricin stock solution was diluted with medium to the desired concentration at 0.2% (v/v) of the final DMSO

content in culture medium, which on its own did not affect cell growth [less than 5% decrease in 5-bromo-2′-deoxy-uridine (BrdU) incorporation].

CELL GROWTH AND BrdU INCORPORATION ASSAY

Asynchronously growing cells in 96-well plates were fixed in formaldehyde [4% in phosphate-buffered saline (PBS)], and subjected to nuclear staining with DAPI (Dojon, Kumamoto, Japan). Images were acquired with an IN Cell Analyzer 1000 (GE Healthcare, Piscataway, NJ), and cell numbers were analyzed using the Developer Toolbox software (GE Healthcare).

For the BrdU incorporation assay cells were incubated with medium containing 10 μM BrdU (Roche Diagnostics, Mannheim, Germany) for 24 h. BrdU incorporated during DNA synthesis was detected by the Cell Proliferation ELISA BrdU (colorimetric) kit (Roche Diagnostics) according to the manufacturer's instructions.

CELL MIGRATION ASSAY

Cell migration was assessed by a Transwell chemotaxis filter assay as previously reported [Masamune et al., 2005] with some modifications, using BioCoat™ growth factor reduced (GFR) matrigel™ invasion chambers (BD Biosciences, Mountain View, CA). Serum-starved LI90 cells were trypsinized, and resuspended at 4×10^4 cells/ml in serum-free medium containing 0.1% bovine serum albumin (BSA), in which tricin was added at the indicated concentrations. PDGF-BB (750 μl at 10 ng/ml) was added to the lower chamber, and 500 μl of cell suspension was added to the upper chamber. The chambers were incubated at 37°C for 24 h, after which the cells that had migrated to the underside of the membrane were stained with Diff-quick (Baxter, Miami, FL), and counted in 10 random high-power fields (HPF, ×100); the mean count was determined from triplicate wells.

DETECTION OF INTRACELLULAR REDOX STATE

Reactive oxygen species (ROS) sensitive fluorescent probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2-DCFDA; Molecular Probes, Eugene, OR) was added to the culture at a final concentration of 10 μM. After 30-min incubation at 37°C, fluorescent signal of the cells was analyzed by flow cytometry using the FACSCalibur system and the CellQuest program (BD Biosciences).

RNA ISOLATION, cDNA SYNTHESIS, AND REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION (PCR)

cDNA synthesis from RNA was performed directly from cultured cell lysate using the TaqMan Gene Expression Cells-to-Ct Kit (Ambion Inc., Austin, TX), following the manufacturer's instructions. Preoptimized TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) for collagen-α1(I) (COL1A1, Hs00164004_m1) and -α1(IV) (COL4A1, Hs01007469_m1) were used to measure mRNA expression levels relative to the endogenous reference gene, GAPDH (4326317E) or 18S ribosomal RNA (rRNA; 4319413E), on StepOne™ Real-Time PCR System (Applied Biosystems). The $2-\Delta\Delta$comparative threshold cycle (C_t) method was used to analyze the relative changes in gene expression.

CELL CYCLE ANALYSIS

Cell cycle analysis was assessed by flow cytometry based on DNA content. Following the standard procedure of staining with propidium iodide (PI, 50 μg/ml, Sigma–Aldrich), flow-cytometric analysis was performed using the FACSCalibur system (BD Biosciences), and cell cycle compartments were integrated using ModFit LT software (Verity Software House, Topsham, ME).

PROTEIN EXTRACTION AND WESTERN BLOTTING

Cell lysates were prepared in RIPA buffer or M-PER mammalian protein extraction reagent containing cocktails of protease and phosphatase inhibitors (Pierce, Woburn, MA), and the protein content measured using the bicinchoninic acid protein assay (Pierce). Western blotting was performed as described previously [Seki et al., 2010]. Monoclonal antibodies (Abs) for retinoblastoma gene product (Rb), cleaved caspase-3, phospho (p)-Akt (Ser473), p38 (Cell Signaling Technology, Beverly, MA), p-Rb (Ser780), PDGF receptor β (PDGF-Rβ; BD Transduction Laboratories, San Diego, CA), α-SMA (Dako Cytomation), and β-actin (Sigma–Aldrich) or polyclonal Abs for p-Rb (Ser807/811), poly (ADP-ribose) polymerase (PARP), Akt, ERK1/2, p-ERK1/2 (Thr202/Tyr204), p-p38 (Thr180/Tyr182; Cell Signaling Technology), p-PDGF-Rβ (Tyr716) (Upstate, Lake Placid, NY), p-PDGF-Rβ (Tyr751), and procollagen-α(I) (Santa Cruz Biotechnology, Santa Cruz, CA) were used as primary Abs. These were followed by appropriate horseradish peroxidase (HRP)-conjugated secondary Abs (anti-mouse and anti-rabbit, GE Healthcare; and anti-donkey, Santa Cruz Biotechnology).

The Ab–protein complexes were detected using the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). Signal intensities were analyzed using a LAS1000 image analyzer and Multi Gauge software (Fuji Film, Tokyo, Japan). For the detection of phosphorylated PDGF-Rβ, standard immunoprecipitation (IP) was performed with anti-phospho-tyrosine (p-Tyr) mouse mAb (Cell Signaling Technology) and agarose beads coupled to goat anti-mouse IgG (TrueBlot, eBioscience, San Diego, CA), precipitated samples were subjected to Western blot analysis as described above with anti-PDGF-Rβ Ab (Cell Signaling Technology) and HRP-conjugated anti-rabbit secondary Ab.

PDGF RECEPTOR BINDING ASSAY

To examine whether tricin could affect PDGF-BB binding to its receptors in HSCs, a radioligand receptor-binding assay using [^{125}I]-PDGF-BB was performed as previously described with slight modifications [Chen and Zhang, 2003; Sakata et al., 2004; Weber et al., 2004]. Briefly, sub-confluent, serum-starved HSCs in 24-well culture plates were used with or without tricin treatment. Cells were incubated with 0.06 nM [^{125}I]-PDGF-BB (>800 Ci/mmol; Perkin Elmer Japan, Kanagawa, Japan) with or without unlabeled PDGF-BB (1,000 ng/ml) in 250 μl DMEM containing 0.1% BSA at 4°C for 3 h or 37°C for 30 min. After washing four times with ice-cold DMEM, cells were dissolved by adding 250 μl 0.1 M NaOH buffer containing 1% Triton X-100 and 0.1% BSA, and cell-associated radioactivity was measured in a gamma counter (ARC-2000; Aloka, Tokyo, Japan). The difference in radioactivity between cells incubated with (i.e., non-specific binding) and without (i.e., total binding) excessive

unlabeled PDGF-BB was considered to reflect specific binding. For determination of saturation binding, the assay was performed with various concentrations of [^{125}I]-PDGF-BB (0.03, 0.06, 0.12, and 0.24 nM). A specific binding curve and Scatchard plot were obtained to estimate the dissociation constant (K_d) and maximum binding (B_{max}) of [^{125}I]-PDGF-BB using GraphPad Prism 5 software (GraphPad software, Inc., San Diego, CA).

IN VITRO KINASE ASSAY

Recombinant PDGF-Rβ tyrosine kinase (cytoplasmic domain aa 557–1,106 of NP_002600.1; Carna Biosciences, Kobe, Japan; 0, 100, or 200 ng) was preincubated with various concentrations of tricin in 20 μl of kinase buffer [25 mM Tris-HCl (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na_3VO_4, 10 mM $MgCl_2$] at 4°C for 30 min. The kinase reaction was initiated by the addition of 10 μM ATP (Promega, Madison, WI) and 250 ng/ml polyGlu$_4$Tyr (Sigma–Aldrich) as substrate into a final volume of 25 μl, and incubated at room temperature for 30 min. Kinase activity was detected as a luminescence signal using the ADP-Glo Kinase assay kit (Promega) and ARVO MX 1420 multilabel counter (PerkinElmer), and the IC_{50} value of tricin was determined using GraphPad Prism 5 software.

STATISTICAL ANALYSIS

All values were expressed as means ± standard deviation (SD). The results shown are representative of three independent experiments in triplicates or quadruplicates. Statistical analysis was performed by the two-tailed unpaired *t*-test. Differences between experimental groups with a *P*-value of <0.05 were considered statistically significant.

RESULTS

TRICIN INHIBITS THE PROLIFERATION OF LI90 CELLS IN VITRO: INHIBITION OF PDGF-BB-INDUCED EFFECTS

We first examined the effect of various concentrations of tricin on the proliferation of LI90 cells over a 3-day period. Tricin significantly decreased serum-induced growth in a dose-dependent manner (Fig. 2A). Furthermore, tricin fully inhibited the cell growth induced with PDGF-BB, which has been reported as the most important mitogen of HSCs [Rockey et al., 2006; Friedman, 2008ab], at a dose of 12.5 μM (Fig. 2A). Interestingly, the PDGF-BB-induced increase of ROS levels in LI90 cells, which is compatible with the previous notion that activation and proliferation by PDGF produces ROS in HSCs [Adachi et al., 2005; De Minicis and Brenner, 2007],

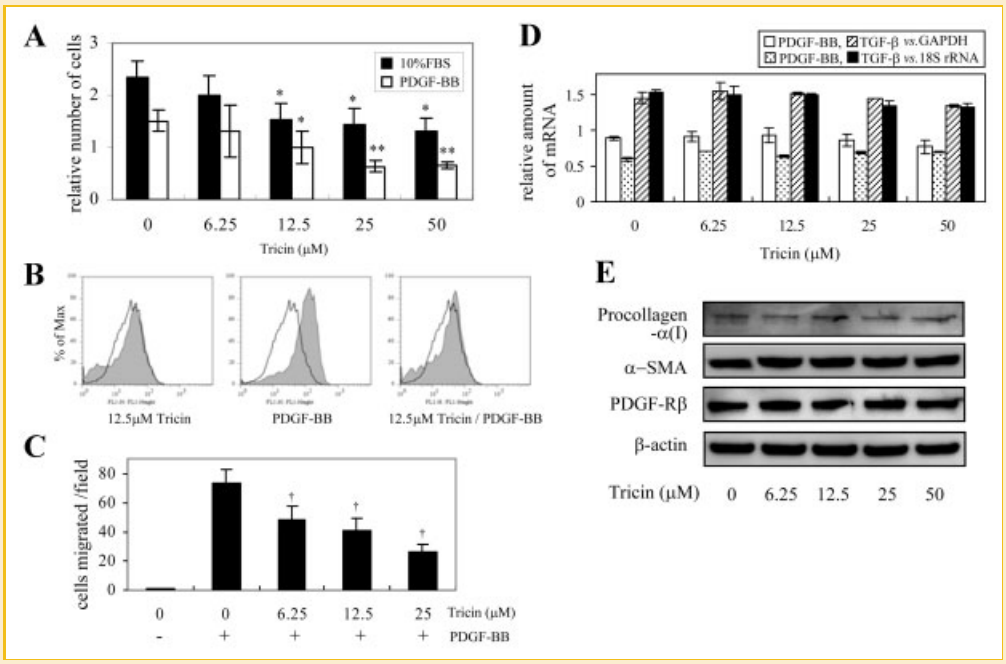

Fig. 2. Effects of tricin on IL90 cell line. A,B: Serum-starved LI90 cells were stimulated with 10% FBS or PDGF-BB (10 ng/ml) in the presence or absence of tricin at indicated concentrations for 3 days. Values indicate the relative number of cells (±SD) compared to the control well (vehicle supplemented with 0.2% FBS, set at 1.0; A). Shaded histogram in flow cytometric analysis represents intracellular ROS levels of each sample compared with control (vehicle alone, open histogram). Representative data from three independent experiments are shown (B). C: PDGF-BB-induced migration ability of LI90 cells evaluated in the presence or absence of tricin using matrigel-coated transwell. Mean numbers (±SD) of cells migrated after 24 h are shown. $^{*}P<0.05$, $^{**}P<0.01$, †$P<0.005$ versus cells without tricin treatment. D: Serum-starved LI90 cells stimulated with PDGF-BB (10 ng/ml) or TGF-β1 (8 ng/ml) in the presence or absence of tricin for 24 h. Relative expression of collagen-α1(I) mRNA compared to control unstimulated well (vehicle alone, set at 1.0) after normalization to GAPDH or 18S rRNA expression is shown. E: LI90 cell extracts prepared after 3-day incubation with or without tricin. Indicated proteins were detected using Western blotting. Densitometric analysis of the protein bands is presented in Supplementary Figure 1A.

was almost completely inhibited after 3 days of co-incubation with 12.5 μM tricin (Fig. 2B). It was also shown previously that migration might lead to the accumulation of HSCs in hepatic fibrogenesis and that PDGF-BB could work as an important chemoattractant of HSCs [Rockey et al., 2006; Friedman, 2008ab]. The transwell cell migration matrigel assay in the present study indicated that tricin could significantly reduce PDGF-induced migration of LI90 cells (Fig. 2C).

TGF-β1, not PDGF-BB, stimulation increased collagen-α1(I) mRNA levels in LI90 cells (Fig. 2D), which is consistent with previous reports that TGF-β is the most potent inducer of ECM synthesis in HSCs [Rockey et al., 2006; Friedman, 2008ab], while tricin did not influence collagen-α1(I) mRNA levels in the culture with PDGF-BB and TGF-β1. mRNA quantification of collagen-α1(IV) showed the same tendency (data not shown). Furthermore, procollagen-α(I) protein and α-SMA expression was unaffected by tricin treatment in Western blot analysis (Fig. 2E). Taken together, our data suggest that tricin could inhibit PDGF-induced activities in LI90 cells, including the cell proliferation and migration important in hepatic fibrogenesis. The property of tricin would be beneficial for therapeutic intervention in hepatic fibrosis, even if it did not seem to impair the fibrogenic ability per cell in our experimental setting.

TRICIN INDUCES GROWTH ARREST, BUT NOT APOPTOSIS, OF LI90 CELLS

Flow cytometry using PI was performed to analyze the effects of tricin on cell cycle distribution (Fig. 3A). Following the addition of tricin, we observed a larger proportion of G0/G1 phase cells (with tricin vs. without tricin: 71.1% vs. 60.6%) and a smaller proportion of S phase (15.0% vs. 18.6%) and G2/M phase cells (13.9% vs. 20.8%) 24 h after PDGF-BB stimulation, indicating that tricin inhibited PDGF-BB-induced cell cycle progression beyond G1 phase.

The BrdU assay, in which tricin concentrations above 6.25 μM significantly inhibited PDGF-BB-induced BrdU incorporation by LI90 cells (Fig. 3B), also indicated that tricin reduced PDGF-BB-stimulated cell proliferation. Western blot analysis showed that PDGF-BB-stimulated phosphorylation of the cell cycle-related transcriptional factor Rb protein [Giacinti and Giordano, 2006]

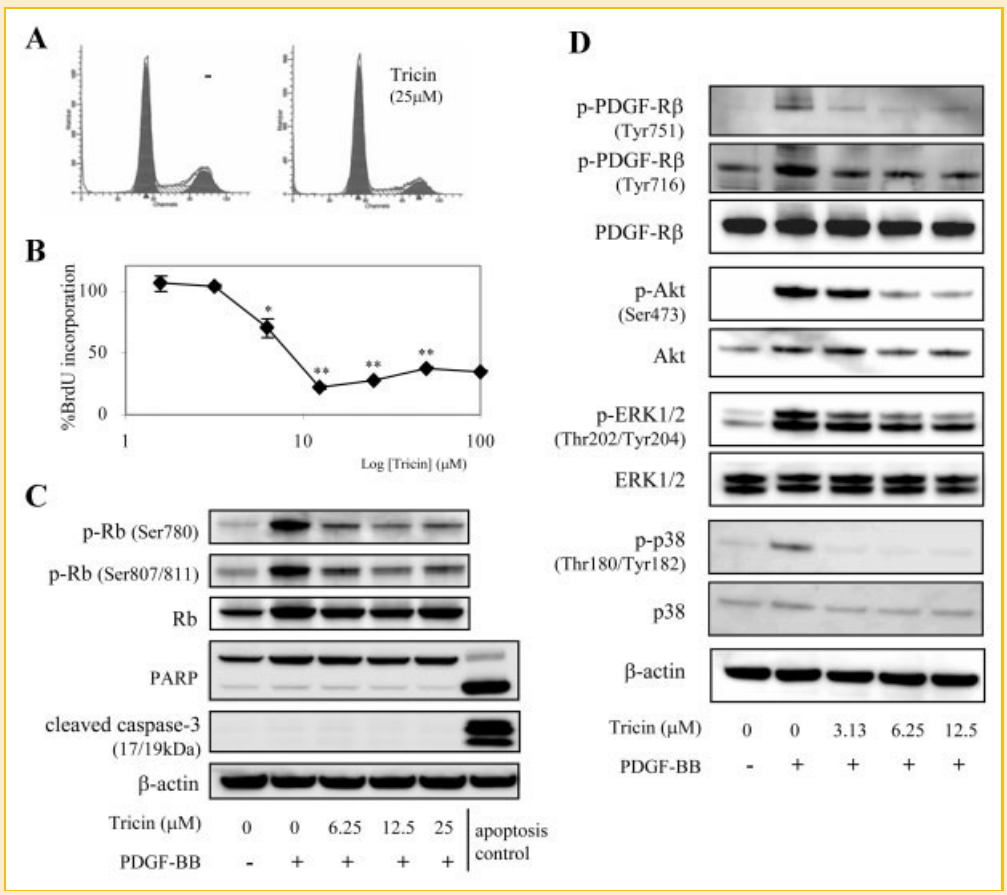

Fig. 3. Inhibitory effects of tricin on PDGF-BB-induced cell proliferation and PDGF-Rβ signaling pathways. Serum-starved LI90 cells were stimulated with PDGF-BB (10 ng/ml) in the presence or absence of tricin at indicated concentrations. A: Representative histograms of cell cycle profiles at 24-h incubation following PI DNA staining and flow cytometry are shown from three independent experiments with similar results. B: ELISA analysis of BrdU incorporation after 24-h incubation. % Ratio of BrdU incorporation compared to cells without tricin. Mean values (±SD) of triplicate wells are shown. $^{*}P < 0.05$, $^{**}P < 0.005$ versus cells without tricin. C,D: After 48 h (C) or 10 min (D) incubation, cell extracts were prepared and analyzed using Western blotting. Positive control for apoptosis in (C) prepared from LI90 cells treated using CPT-11 and ultraviolet radiation. Western blots are quantified in Supplementary Figure 1B,C.

was remarkably inhibited following addition of tricin to the culture together with PDGF-BB (Fig. 3C), whereas tricin could not induce cleaved forms of caspase-3 and PARP protein in the apoptosis pathway. Taken together, these results suggest that, at the doses examined, tricin could inhibit PDGF-BB-stimulated proliferation of LI90 cells inducing cell cycle arrest, but not apoptosis.

EFFECTS OF TRICIN ON PDGF β RECEPTOR SIGNALING PATHWAYS IN LI90 CELLS

As demonstrated in Figure 2E, tricin had no effect on PDGF-Rβ expression in LI90 cells at least under our experimental setting, indicating that it might inhibit LI90 cell proliferation without reducing the level of membrane PDGF-Rβ. We therefore further investigated the effects of tricin on PDGF-R signaling pathways. Serum-starved LI90 cells were stimulated for 10 min with 10 ng/ml PDGF-BB in the presence or absence of the indicated concentrations of tricin; cell lysates were then subjected to Western blot analysis with Abs against molecules in the PDGF-R signaling pathway. As shown in Figure 3D, tricin inhibited PDGF-induced phosphorylation of PDGF-Rβ without affecting PDGF-Rβ protein expression.

Tyrosine phosphorylation of PDGF-Rβ serves as a critical link between extracellular PDGF stimulation and intracellular signaling, and previous studies showed that activation of c-Raf/MEK/ERK and PI3-kinase/Akt pathways are key to PDGF-induced proliferation, the latter also for migration [Rockey et al., 2006; Friedman, 2008ab]. Tricin significantly inhibited PDGF-BB-induced phosphorylation of ERK and Akt in a dose-dependent manner, thus inhibiting PDGF-induced tyrosine phosphorylation of PDGF-Rβ and downstream activation of ERK and PI3-kinase/Akt pathways in LI90 cells. In addition, tricin markedly inhibited PDGF-BB-induced phosphorylation of p38 MAPK.

EFFECT OF TRICIN ON CULTURE-ACTIVATED HSCs

LI90 cells have been studied because their features are compatible with activated HSCs [Ueki et al., 1998]. However, to confirm the inhibitory effects of tricin on HSC proliferation, we used primary HSCs isolated from fresh human liver in later experiments. Tricin significantly reduced PDGF-BB-stimulated cell proliferation at doses above 12.5 μM, as assessed by a BrdU incorporation assay (Fig. 4A). Further, tricin inhibited PDGF-BB-induced phosphorylation of PDGF-Rβ, ERK, and Akt in HSCs (Fig. 4B), suggesting that its inhibition of cell proliferation is largely due to suppression of the PDGF-R signaling pathway as consistent with LI90 cells.

MECHANISMS OF TRICIN INHIBITION ON PDGF RECEPTOR PHOSPHORYLATION

Our experiments showed that tricin inhibited the very early events of PDGF signaling (i.e., receptor autophosphorylation). The phosphorylation status of PDGF-Rβ was also assessed by IP of p-Tyr followed by PDGF-Rβ immunodetection in HSCs. PDGF-BB caused a marked increase in the tyrosine phosphorylation of PDGF-Rβ in serum-starved HSCs (Fig. 4C), which was inhibited by co-treatment with PDGF-BB and 25 μM tricin; the total amount of PDGF-Rβ in each sample was unaffected. Notably, the decrease in receptor phosphorylation by tricin was not significantly dependent on pre-incubation time before the addition of PDGF-BB, at least between 10 min and 2 h.

We therefore further examined the kinetics of tricin pretreatment by removing tricin from the culture prior to PDGF stimulation. After the pretreatment with 25 μM tricin for 10 min, 2 h, and 18 h, HSCs were washed twice, stimulated with PDGF-BB in fresh medium, then subjected to Western immunoblot detection of p-PDGF-Rβ. As shown in Figure 4D, PDGF-BB-induced phosphorylation of PDGF-Rβ was inhibited with tricin pretreatment in a time-dependent manner. Interestingly, an 18-h pretreatment was necessary for the marked decrease of phosphorylation comparable to that in the co-culture of tricin and PDGF-BB, indicating that tricin is required during PDGF stimulation for the rapid kinetics of PDGF-Rβ phosphorylation inhibition.

Consequently, a direct interaction between tricin and PDGF or PDGFR was conceivable to study the possible mechanisms of PDGFR phosphorylation inhibition. We performed a radioligand receptor-binding assay using [^{125}I]-PDGF-BB as shown in Figure 5. The binding of [^{125}I]-PDGF-BB to cell surface receptors was tested with various concentrations of tricin for 3 h at 4°C and for 30 min at 37°C. EGCG was used as a comparison with tricin as it is known to inhibit the phosphorylation of receptor tyrosine kinases (RTKs) [Liang et al., 1997] including PDGF-R on HSCs [Chen and Zhang, 2003; Sakata et al., 2004], as well as to inhibit RTK-ligand binding [Liang et al., 1997; Chen and Zhang, 2003; Sakata et al., 2004; Weber et al., 2004; Adachi et al., 2007, 2008]. As shown in Figure 5A, specific binding was not inhibited with tricin at either temperature, whereas EGCG induced a significant decrease of PDGF receptor-ligand binding in a dose-dependent manner at 4°C (Fig. 5B).

In subsequent experiments, HSCs were pretreated overnight with 25 μM tricin, washed twice, then subjected to the binding assay with various concentrations of [^{125}I]-PDGF-BB at 4°C. As shown in Figure 5C, no real difference in the specific binding curves was observed in either the presence or absence of tricin pretreatment. Accordingly, the Scatchard analysis of these data showed no significant difference in B_{max} (with tricin vs. without tricin; 50.0 fmol/10^6 cells vs. 42.4 fmol/10^6 cells) and K_d (0.172 nM vs. 0.150 nM) values. These data indicate that inhibition of receptor-ligand binding might not account for the inhibitory effect of tricin on PDGF-R phosphorylation, in marked contrast to EGCG, at least under our experimental settings.

Microscopic observation of hematoxylin and eosin (HE)-stained HSCs showed morphological changes following overnight tricin treatment, in which cell cytoplasms contained numerous inclusion bodies or granules (Fig. 6A). Furthermore, cell pellets treated with tricin were yellow in color (data not shown), indicating that tricin is incorporated into cellular compartments. To gain insights into the mechanisms involved in the inhibitory action on PDGF signaling in HSCs, we investigated the effect of tricin on PDGF-R kinase activity in vitro. As shown in Figure 6B, tricin significantly inhibited tyrosine kinase activity of the recombinant active PDGF-Rβ kinase domain ($IC_{50} = 1.27–1.56$ μM), suggesting that it might act directly as a tyrosine kinase inhibitor. The effect of tricin on PDGF-R might be due, in part, to the inhibition of receptor kinase activities on downstream signaling molecules.

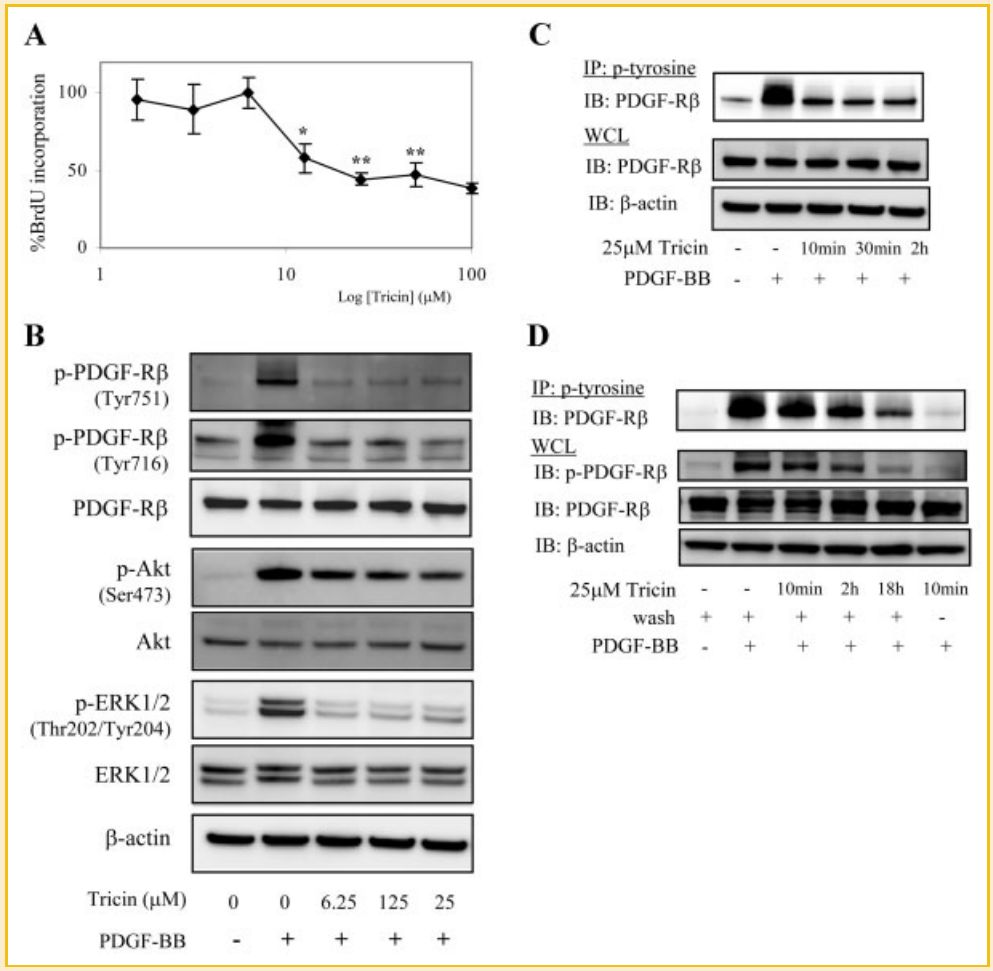

Fig. 4. Effects of tricin on culture-activated HSCs. A: Serum-starved HSCs were stimulated with PDGF-BB (10 ng/ml) in the presence or absence of various doses of tricin. ELISA analysis of BrdU incorporation after 24-h incubation. % Ratio of BrdU incorporation compared to cells without tricin. Mean values (±SD) of triplicate wells are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ versus cells without tricin. B,C: Serum-starved HSCs untreated or treated with tricin at indicated concentrations. After 2-h incubation (B) or the indicated times (C), PDGF-BB (final concentration, 10 ng/ml) was added except for the control well. Cell lysates were prepared after 10-min stimulation, and given proteins were detected using Western blotting (B). p-PDGF-Rβ was detected with IP-Western blot using anti-p-tyrosine and anti-PDGF-Rβ antibodies (C). D: Serum-starved HSCs were exposed to 25 μM tricin for 10 min, 2 h, or 18 h, washed (lanes 3–5) or left exposed (lane 6), then stimulated with PBGF-BB for 10 min. Cell lysates were used to detect p-PDGF-Rβ by Western blotting with anti-p-PDGF-Rβ (Tyr751) Ab or by IP-Western blot as for (C). Densitometric analysis of the protein bands is presented in Supplementary Figure 1D–F. IB, immunoblotting; WCL, whole cell lysate.

DISCUSSION

HSCs are increasingly being recognized as key mediators in the progression of hepatic fibrosis. Previous in vitro and in vivo studies suggest that PDGF is the most potent mitogen of HSCs and is therefore likely to be an important mediator of increased HSC proliferation during hepatic fibrogenesis in chronic liver diseases [Rockey et al., 2006; Friedman, 2008ab]. Of the three PDGF isoforms, PDGF-BB has the most potent activity [Pinzani et al., 1991], and the onset of quiescent HSC proliferation coincides with an increase in the expression of PDGF-Rβ with no change in PDGF-Rα [Wong et al., 1994]. Our current study indicates that tricin significantly inhibits PDGF-BB-induced cell proliferation by blocking cell cycle progression, largely through inhibition of PDGF-Rβ phosphorylation, in both LI90 cell lines and activated HSCs.

Once PDGF binds to PDGF-R molecules on the cell surface, the receptor undergoes dimerization and autophosphorylation at tyrosine residues, which triggers PDGF-related downstream signaling, including Ras, Raf-1, MEK, and ERK. PDGF-Rβ recruits PI3-kinase which activates Akt and is necessary for the mitogenesis and chemotaxis induced by PDGF-BB in HSCs [Rockey et al., 2006; Friedman, 2008ab]. Our data clearly indicate that tricin inhibits phosphorylation of these downstream signaling molecules, which might result in an inhibitory action on PDGF-BB activities in HSCs.

Previous reports indicate that many flavonoids possess protein-tyrosine kinase inhibitory activities [Hagiwara et al., 1988; Geahlen et al., 1989], but not all have been fully investigated. EGCG is well-studied, and shows inhibitor properties of RTKs, such as PDGFR, epidermal growth factor receptor (EGFR), and vascular endothelial growth factor receptor (VEGFR) [Liang et al., 1997; Lamy et al.,

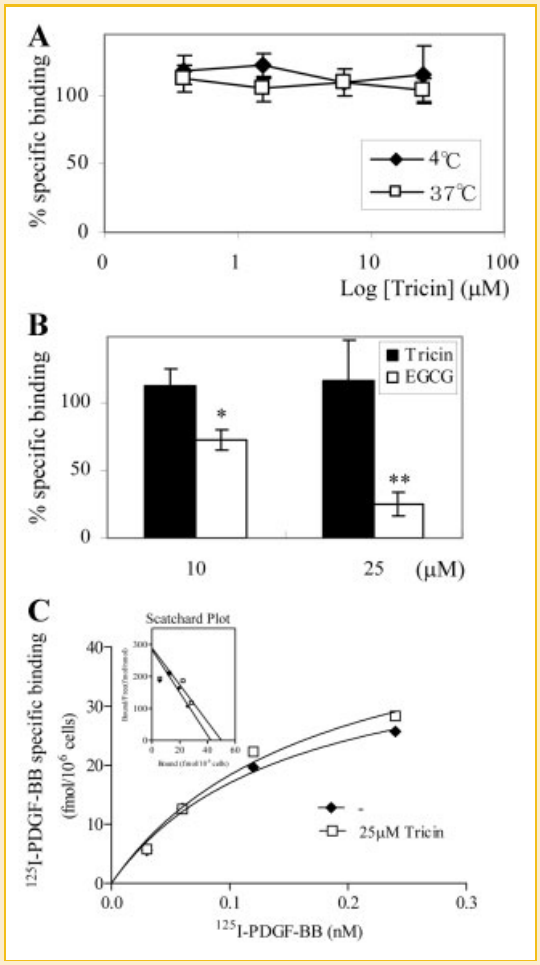

Fig. 5. Effects of tricin on PDGF-BB-receptor binding in culture-activated HSCs. A,B: Serum-starved HSCs treated with or without tricin or EGCG at various concentrations. After 10 min, [^{125}I]-PDGF-BB (0.06 nM) was added in the presence or absence of unlabeled PDGF-BB (1,000 ng/ml). % Ratio of [^{125}I]-PDGF-BB-specific binding at 4°C (A,B) or 37°C (A) compared to cells without tricin. Mean values (±SD) of triplicate wells are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ versus without EGCG treatment. C: Serum-starved HSCs were exposed to 25 μM tricin for 24 h, then washed in fresh serum-starving medium. [^{125}I]-PDGF-BB specific binding examined using various concentrations of [^{125}I]-PDGF-BB at 4°C. Results are presented as specific binding curves with mean blots and Scatchard analysis format.

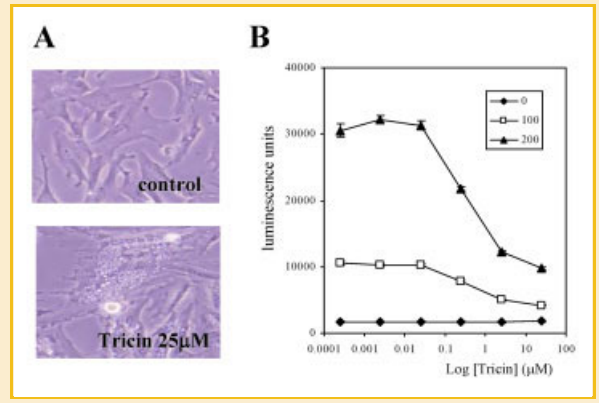

Fig. 6. Effect of tricin on PDGF-Rβ kinase activity. A: HSCs were incubated in the presence or absence of 25 μM tricin for 24 h, then HE stained. Representative photographs from microscopy are shown. B: In vitro kinase assay using recombinant PDGF-Rβ tyrosine kinase (100 or 200 ng/well) performed in the presence or absence of various concentrations of tricin. Mean kinase activity (±SD) detected as luminescence signal shown from triplicate wells. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

2002; Chen and Zhang, 2003; Sakata et al., 2004; Weber et al., 2004; Masamune et al., 2005; Adachi et al., 2007, 2008; Teillet et al., 2008]. The mechanisms of this inhibitory action remain unclear, but may in part be due to inhibition of receptor-ligand binding [Liang et al., 1997; Chen and Zhang, 2003; Sakata et al., 2004; Weber et al., 2004; Adachi et al., 2007, 2008]. In previous HSC studies, EGCG blocked PDGF-BB binding to its receptor in a non-competitive manner [Chen and Zhang, 2003; Sakata et al., 2004]. EGCG may also inhibit PDGF-Rβ gene expression by blocking the activation of transcription factors activator protein-1 and nuclear factor-kappa B, required for gene transcription [Chen and Zhang, 2003].

Our study found no significant effect of tricin on either PDGF receptor binding or PDGF-Rβ expression, suggesting that the inhibitory mechanisms of PRGF-R activation differ between tricin and EGCG. Recently, a crucial role for the nicotinamide adenine (dinucleotide) phosphate (NAD(P)H) oxidase in PDGF-induced proliferation of HSCs has been reported, involving the production of ROS via NAD(P)H oxidase activation in response to PDGF-BB. ROS further induce HSC proliferation through the phosphorylation of p38 MAPK [Adachi et al., 2005; De Minicis and Brenner, 2007]. In our study, tricin inhibited the PDGF-induced phosphorylation of p38 MAPK, so further investigations into the inhibitory mechanisms of PDGF-R phosphorylation should include the action of tricin on the NAD(P)H oxidase system. Moreover, it is of interest that tricin inhibited the in vitro VEGF-induced proliferation of human umbilical vein endothelial cells (HUVECs) and the phosphorylation of VEGFR2 in HUVECs (our unpublished data), suggesting a role as a multiple RTKs inhibitor. In addition, VEGF–VEGFR axis activity plays an important role in angiogenesis which is linked to progression to cirrhosis and HCC in chronic liver disease. On the other hand, growth inhibitory and cell cycle arresting properties of tricin in cultured cancer cell lines have been reported, although the mechanisms underlying these actions have not been clarified [Cai et al., 2004].

Targeting of anti-fibrotic drugs to HSCs is a promising strategy to block the fibrotic processes that lead to liver cirrhosis and HCC. Dietary tricin would be considered relatively safe in a preliminary evaluation for clinical development [Verschoyle et al., 2006; Ninomiya et al., 2011], and its biological features would be beneficial in the therapy of human liver fibrosis. Therefore, an in vivo study of tricin would be an important experiment in an appropriate mouse model of hepatic fibrogenesis.

In recent studies, the cancer chemopreventive efficacy of dietary tricin has been shown in the gastrointestinal tract of mice [Cai et al., 2005, 2009; Oyama et al., 2009], but dietary tricin failed to impede tumor development in nude mice implanted with breast cancer cells, possibly because of its poor bioavailability in the tumor site [Cai et al., 2004]. Indeed, Cai et al. reported that steady-state tricin levels

in the plasma (0.45 ± 0.12 nmol/ml) and liver (2.0 ± 0.5 nmol/g) were substantially lower than in the small intestine (174 ± 97 nmol/g) in mice receiving dietary tricin (0.2%) for a week [Cai et al., 2007]. These results indicate a need for the pharmaceutical optimization of tricin formulation or suitable prodrug development to increase its bioavailability, as is typically the case for other flavonoids [Hirpara et al., 2009]. We recently reported that the tricin–alanine–glutamic (T-Ala-Glu) acid conjugate exhibited excellent bioavailability and more than 40 times higher plasma tricin concentrations than intact tricin alone after oral administration to rats, which was clearly confirmed by an increase in cellular permeability [Ninomiya et al., 2011]. This approach could be used in the clinical development of treatment or chemoprevention for chronic liver disease.

In summary, tricin inhibits the in vitro PDGF-induced proliferation of HSCs as a result of the inhibition of PDGF-R phosphorylation and downstream signaling pathways rather than PDGF receptor binding. Further investigations into the mechanisms of these inhibitory actions and their application for an in vivo model are needed for therapeutic developments for hepatic fibrogenesis.

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