Dipyridamole inhibits TGF-β–induced collagen gene expression in human peritoneal mesothelial cells

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Background. Peritoneal matrix accumulation is characteristic of peritoneal fibrosis (PF). Continuous ambulatory peritoneal dialysis (CAPD) patients who had persistent transforming growth factor-β (TGF-β) in their drained effluent had an increased risk of PF. We previously reported that TGF-β stimulates the expression of types I and III collagen mRNA in cultured human peritoneal mesangial cells (HPMCs), which may predispose them to develop PF. Pharmacological interventions to attenuate TGF-β–stimulated matrix accumulation in HPMC may have therapeutic potential for the treatment of PF. The SMAD family and the extracellular signal-regulated protein kinase (ERK1/2, p44/p42) pathways have been shown to participate in TGF-β signaling. Our current study identified these signal pathways in HPMCs and investigated the molecular mechanisms involved in the inhibitory effects of dipyridamole on TGF-β–induced collagen gene expression in HPMCs.

Methods. HPMCs were cultured from human omentum by an enzyme digestion method. Expression of collagen α1(I) mRNA was determined by Northern blotting. The SMAD proteins and the ERK1/2 activity were determined by Western blotting.

Results. TGF-β-stimulated collagen α1(I) mRNA expression of HPMC was inhibited by dipyridamole in a dose-dependent manner. Smad2 and ERK1/2 were activated in response to TGF-β; however, TGF-β had little effect on the protein expression of Smad4. The addition of PD98059, which blocked activation of ERK1/2, suppressed TGF-β–stimulated collagen α1(I) mRNA expression in a dose-dependent manner. At a concentration that inhibited collagen gene expression (17 μg/mL), dipyridamole suppressed ERK1/2 activation by TGF-β. In contrast, the same concentration of dipyridamole had no effect on TGF-β–induced activation of Smad2.

Conclusion. Dipyridamole inhibits TGF-β–induced collagen gene expression in HPMC through modulation of the ERK pathway. Our study of dipyridamole may provide therapeutic basis for clinical applications in the prevention of PF.

Key words: ERK pathway, cell signaling, signal transduction, fibrosis, peritoneal fibrosis, transforming growth factor-β.

Peritoneal fibrosis (PF) is one of the most serious complications after long-term continuous ambulatory peritoneal dialysis (CAPD) [1]. In vitro and in vivo studies have shown that proliferation of human peritoneal mesothelial cells (HPMCs) accompanied by matrix expansion is important in the pathogenic process of PF [2]. Transforming growth factor-β (TGF-β) increases matrix accumulation and has been regarded as the central mediator of the fibrosing process in clinical diseases. CAPD patients who had persistent TGF-β in their drained effluent also had an increased risk of PF [3]. In addition, we previously reported that TGF-β stimulates expression of types I and III collagen mRNA in cultured HPMCs [4]. These observations support the hypothesis that TGF-β may contribute to the development of PF. Pharmacological interventions that can attenuate TGF-β–stimulated matrix accumulation in HPMCs have a therapeutic potential for the prevention or retardation of PF.

Despite the well-known association between TGF-β and matrix accumulation, little information is available regarding the cellular signals TGF-β uses to induce this process. On the cell membrane, TGF-β binds to the type II receptor, which recruits the type I receptor into a complex [5]. The type I receptor, once phosphorylated by the type II receptor, activates Smad2 and allows it to form a heteromultimer with Smad4. This complex then is translocated to the nucleus to regulate transcription of target genes. In a study of human mesangial cells, Poncelet, de Caestecker and Schnaper demonstrated that SMAD proteins are activated by TGF-β1 and then participate in TGF-β1–stimulated type I collagen expression [6]. In addition to the SMAD proteins, the mitogen-activated protein kinase (MAPK) pathways have been proposed to function downstream of the TGF-β signaling pathways [5, 7]. The MAPK pathways actually contain three phosphorylation cascades: the extracellular signal-regulated protein kinase (ERK), the c-Jun N-terminal kinase (JNK), and the p38 MAPK. Recently, in a study of human mesangial cells, Hayshida et al demonstrated...
that ERK are activated by TGF-β, and the blockade of the ERK pathway results in inhibition of TGF-β-induced type I collagen gene expression [8]. These reports suggest that both the SMAD and ERK pathways may be involved in TGF-β–induced collagen synthesis and fibrogenesis. However, there are, to our knowledge, no data available regarding the presence and activation of these intracellular signals of TGF-β in HPMCs.

Dipyridamole [2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido-(5,4-d)-yrimidine] is a widely used antiplatelet agent and acts as a phosphodiesterase inhibitor that increases intracellular cAMP [9]. In addition to its antiplatelet effect, we previously demonstrated that dipyridamole in rat mesangial cells may exert an antifibrogenic effect by reducing the expression of type I collagen mRNA [10]. It has been shown in rat aortic smooth muscle cells (SMC) [11] and mesangial cells [12] that raising intracellular cAMP inhibits activation of ERK. Our previous reports of HPMC using agents that increased intracellular cAMP also demonstrated a reduced expression of collagen α(I) mRNA [4, 13]. These data support our hypothesis that dipyridamole may attenuate TGF-β–induced collagen synthesis in HPMCs. We speculated that, by increasing intracellular cAMP, dipyridamole has an inhibitory effect on TGF-β–induced activation of SMAD proteins and/or the ERK pathways.

We report here an antifibrogenic effect of dipyridamole on TGF-β–treated HPMCs. The intracellular downstream factors, including SMAD proteins and ERK pathways, are explored in TGF-β–stimulated HPMCs. The regulatory effects of dipyridamole on these intracellular signals of HPMCs and the resulting inhibition of collagen gene expression also were investigated. Our results may provide a pharmacological basis for using dipyridamole in the treatment of PF.

METHODOLOGY

Materials

Fetal calf serum (FCS) was obtained from Biochrome KG (Berlin, Germany). Culture flasks and plates were purchased from Corning (Corning, NY, USA) and precoated with 1.6 μg/cm² of Vitrogen 100® (Celtrix Lab, Palo Alto, CA, USA) before cell loading. Trypsin-ethylenediaminetetraacetic acid (EDTA), RPMI-1640 medium, glutamine, and trypsin blue were obtained from GIBCO (Grand Island, NY, USA). Aprotinin, adenine 5′-triphosphate (ATP), leupeptin, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate (Na₃VO₄), bovine serum albumin (BSA), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dibutyryl-cAMP (DBcAMP), 3-isobutyl-1-methylxanthine (IBMX), and other tissue culture reagents were purchased from Sigma (St. Louis, MO, USA). TGF-β1 was obtained from R&D Laboratory. The enzyme immunoassay (EIA) kits for cAMP were obtained from Cayman Chemical (Ann Arbor, MI, USA). Selective cAMP-dependent protein kinase (PKA) inhibitor H-89 was obtained from Calbiochem (La Jolla, CA, USA). BCA reagents were from Pierce (Rockford, IL, USA). Phosphorylated and nonphosphorylated polyclonal antibodies to ERK1/2, Smad2, and Smad4 were all purchased from Biokabs (New England, MA, USA). Human collagen α(I) cDNA was purchased from American Type Culture Collection (Rockville, MD, USA). Agents used to isolate the total RNA and Northern blot analysis were obtained from Boehringer Mannheim (Mannheim, Germany) unless otherwise specified. Dipyridamole used in this study was a generous gift from Boehringer Ingelheim. All other chemicals used were of analytical grade.

Establishment of HPMC culture

Specimens of human omentum were obtained from abdominal surgical procedures for elective gastric cancer resection and the omentum was grossly normal. HPMC culture was carried out as in our previous reports [4, 13]. Briefly, the surgically removed human omentum was washed three times with phosphate-buffered saline (PBS) and then digested with trypsin EDTA (0.125%; GIBCO) for 15 minutes. After centrifugation, the cell pellet was washed with culture medium and then seeded into a gelatin-coated (1 mg/mL) flask. The medium was changed on the third day. RPMI-1640 medium containing 20% FCS, penicillin (100 U/mL), streptomycin (100 μg/mL), and insulin (30 μg/mL) was used. After two to four days, the cells became confluent and were subcultured with medium containing 10% FCS. HPMCs were identified by the presence of vimentin and cytokeratin, but without desmin and factor VIII-related antigen by the immunofluorescence method. All experiments were performed in passage 1 to 3 cells.

Northern blot analysis

To determine the effect of dipyridamole on collagen gene expression, HPMCs were grown in RPMI supplemented with 10% FCS until subconfluence. HPMCs were arrested by 0.5% FCS for 24 hours and then treated with TGF-β (2.5 ng/mL) in the absence or presence of various concentrations of dipyridamole. After 24 hours, cells were harvested for isolation of total RNA, as previously described [4, 13]. The concentration of each sample was determined using spectrophotometry with the absorbance at 260 nm (A₂₆₀). The purity of each sample was determined based on the ratio of A₂₆₀ to A₂₈₀. Ten micrograms of RNA were electrophoresed on a 1% agarose gel containing 1 mol/L formaldehyde in MOPS buffer (0.2 mol/L morpholinopropanesulfonic acid, 0.05 mol/L Na acetate, 0.01 mol/L EDTA). Equivalency of sample loading and lack of degradation was verified by ethidium bromide staining of 28S and 18S rRNA bands.
Then RNA was transferred to nylon membranes by overnight capillary action followed by fixation in an ultraviolet cross-linker.

For Northern blotting, a 1.5 kb EcoRI fragment of collagen α1(I) and a 0.7 kb HindIII/EcoRI fragment of collagen α1(III) were subcloned, respectively, into pBSII/SK (Stratagene, La Jolla, CA, USA) and used as templates for in vitro transcription of antisense digoxigenin-conjugated riboprobes according to the manufacturer’s instructions (Boehringer Mannheim). The blots were developed using CSPD (Boehringer Mannheim) as the substrate for alkaline phosphatase, as described by the supplier. The signal intensity recorded on x-ray film was then quantified with computerized densitometry and was normalized against the signal of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messages.

**Cell preparations and protein extraction**

Human peritoneal mesothelial cells were grown in 10 cm dishes until confluence, growth-arrested for 24 hours with medium containing 0.5% FCS, and then harvested after treatment at the indicated time points with 200 μL ice-cold lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 2 mmol/L EDTA, 2 mmol/L EGTA, 40 mmol/L β-glycerophosphate, 50 mmol/L NaF, 10 mmol/L sodium pyrophosphate, 200 μmol/L sodium orthovanadate, 10 μg leupeptin/mL, 200 units of aprotinin/mL, 1 μmol/L pepstatin A, 1 mmol/L PMSF, 100 mmol/L okadaic acid). The cell lysate was centrifuged at 14,000 rpm for 10 minutes, and the protein concentration of the supernatant was measured by BCA protein assay (Pierce).

**Western blot analysis**

Western blotting analyses of ERK1/2 and Smad2 phosphorylation were performed according to the manufacturer’s protocol using phospho-specific antibodies. Briefly, HPMCs were low serum (0.5% FCS) starved for 24 hours and then stimulated with TGF-β (2.5 ng/mL) for different lengths of time. To evaluate the inhibitory effect of dipyridamole on TGF-β–stimulated activation of ERK and/or SMAD pathways, HPMCs was incubated with 17 μg/mL dipyridamole for 30 minutes before stimulation with TGF-β. Cells were harvested at the indicated time period for Western blotting. In addition, lanes of cells were treated with 3 or 10 μmol/L H89 for 30 minutes before dipyridamole. Cell lysates (20 μg protein) were separated by SDS-PAGE (12%) and then transferred to PVDF membrane (Millipore, Bedford, MA, USA). For immunodetection, membranes were probed with primary antibody followed by incubation with peroxidase-conjugated secondary antibodies. Bands were visualized by the enhanced chemiluminescence (ECL) system (Amersham).

**Statistical analysis**

The results are expressed as mean ± SEM, unless otherwise stated. These statistical analyses were carried out using StatView IV on a personal computer. Statistical significance (P < 0.05) was evaluated using the Student t test or one-way analysis of variance with modified t test performed using the Bonferroni correction.

**RESULTS**

Dipyridamole inhibits TGF-β–induced α1(I) and α1(III) mRNA expression in HPMCs

The effects of TGF-β and dipyridamole on the expression of collagen α1(I) and α1(III) mRNA level were analyzed in HPMC. Similar to our previous report [4], Figure 1 shows that TGF-β induced collagen α1(I) and α1(III) mRNA expression in HPMCs. Compared with basal level (control), TGF-β resulted in a 5.2-fold increment of collagen α1(I) mRNA expression. A dose-dependent reduction of collagen α1(I) mRNA expression by dipyridamole was then determined. As illustrated in Figure 1A, a significant reduction of TGF-β–induced collagen α1(I) mRNA occurred with 17 μg/mL dipyridamole. Dipyridamole had been reported to exert its biological effect through the cAMP-PKA pathway [9]. Accordingly, we examined effect of DBcAMP on TGF-β–induced collagen α1(I) and α1(III) mRNA expression, and tested whether the blockade of cAMP-PKA pathway could overcome the inhibitory effect of dipyridamole in TGF-β–treated HPMC. As expected, the addition of DBcAMP (1 mmol/L), a membrane-permeable analogue of cAMP, decreased the expression of collagen α1(I) and α1(III) mRNA expression. Moreover, in the presence of H-89, a nonspecific PKA inhibitor, the inhibitory effects of dipyridamole on collagen α1(I) and α1(III) mRNA expression were almost completely reversed.

SMAD proteins are present in HPMC and the Smad2 is activated by TGF-β

To investigate the molecular mechanisms involved in TGF-β–induced collagen gene expression in HPMCs, we first examined whether HPMCs express SMAD proteins and then determined their modulations by TGF-β (Fig. 2). Protein levels of Smad2 (58 kD) were detected in quiescent (control) HPMC; they slightly increased from 15 minutes to 4 hours, and then decreased by 16 hours of incubation with TGF-β (Fig. 2A). Protein levels of Smad4 (62 kD) did not change in response to TGF-β. These data are similar to those observed in other cells, demonstrating that SMAD proteins are also present in HPMCs.

To investigate whether TGF-β activates Smad2 in HPMC, lysates were electrophoresed through two parallel polyacrylamide gels, followed by Western blotting.
Fig. 1. Effects of dipyridamole on collagen α1(I) and α1(III) mRNA levels. All human peritoneal mesothelial cells (HPMCs) were grown as described in the Methods section. Some cells were preincubated with dipyridamole (DP), DBcAMP (1 mmol/L), or H-89 (10 μmol/L). After these additions, the cells were then incubated with TGF-β (2.5 ng/mL) for 24 hours in the presence of the same additions. (A) Representative Northern blots of collagen α1(I) mRNA are shown in the top panel. Densitometric analysis of blots from four separate experiments is shown in the bottom panel. Values in the graph are shown as fold increase over control. *P < 0.05 vs. TGF-β alone (lane 3). (B) Northern blots demonstrating inhibitory effect of dipyridamole on TGF-β–induced collagen α1(III) mRNA expression.
As shown in Figure 2B, TGF-β (2.5 ng/mL) induced a rapid phosphorylation of Smad2 that began within 15 minutes, peaked at 15 to 30 minutes, and then returned to baseline values by 8 hours. Results are expressed as the ratio between phosphorylated and nonphosphorylated Smad2.

**Activation of the ERK pathway by TGF-β in HPMCs**

In previous (unpublished) studies we found that ERK1 and ERK2 were present in primary culture of HPMC and were activated after treatment with platelet-derived growth factor (PDGF; 20 ng/mL). We also found that this PDGF-induced ERK1/2 activation could be completely
Fig. 3. Time course of activation of ERK1/2 by TGF-β. HPMCs were treated as described in Figure 2. Representative blots developed with anti-phospho-ERK1/2 antibody (top) or non-phospho-ERK1/2 antibody (middle) are shown. Results of densitometric analysis, expressed as a ratio between phospho- and nonphospho-ERK1/2, from four isolated experiments, are shown in the bottom panel. *P < 0.05, relative to control.

Fig. 4. Effect of PD98059 on TGF-β-induced collagen α1(I) mRNA expression. HPMCs were treated with various concentrations of PD98059 for one hour and then incubated with TGF-β for 24 hours. α1(I) mRNA expression was assessed as described in Figure 1. Representative Northern blots are shown at the top. Results of densitometric analysis from four isolated experiments are shown in the bottom panel. Values in the graph are shown as fold increase over control. *P < 0.05 vs. TGF-β alone (lane 3).

Dipyridamole inhibits activation of ERK1/2, but not Smad2, by TGF-β

To analyze the mechanisms and effect of dipyridamole on TGF-β–mediated collagen gene induction further, the effects of dipyridamole on signaling transduction pathways downstream to TGF-β were examined. As shown, TGF-β induced the activation of ERK1/2, and as expected, pretreatment of HPMCs with PD98059 prevented the TGF-β–stimulated ERK1/2 activation (Fig. 5). Both dipyridamole and DBcAMP abrogated this response in a dose-dependent manner. At a concentration that inhibited collagen gene expression (6 to 17 μg/mL; Fig. 1), dipyridamole suppressed the ERK1/2 activation by TGF-β. In contrast, the same concentration of dipyridamole had no effect on TGF-β–induced activation of Smad2 (Fig. 6). This implied that the inhibitory effect of dipyridamole on TGF-β–induced collagen gene expression in HPMC mainly occurs through modulations of the ERK pathway.

Interaction of the ERK pathway and the SMAD pathway in HPMCs

It has been postulated that the activation of Smad2 by hepatocyte growth factor is downstream of MEK [14]. Although the nature of this interaction is not clear, we performed experiments to evaluate the potential interaction between these two pathways. HPMC pretreated with PD98059 did not influence the activation of Smad2 on TGF-β treatment (Fig. 7). These observations suggest that in HPMC, the TGF-β signal activates two independent pathways: the ERK pathway and the SMAD pathway.
DISCUSSION

We [4] and others [3] have shown that, by inducing the expression of collagen α1(I) and collagen α1(III) mRNA in HPMCs, TGF-β may be associated with an increased risk of PF. Our current study demonstrates that dipyridamole inhibits the TGF-β–induced expression of collagen α1(I) mRNA mainly through a cAMP-dependent mechanism (Fig. 1). Because the molecular link between TGF-β and type I collagen gene expression remains unknown, we evaluated whether the downstream signaling factors of TGF-β, SMAD proteins and/or the ERK pathway, are modulated by dipyridamole. To our knowledge this is the first report investigating the endogenous SMAD pathway in the primary culture of HPMCs. Our study shows that Smad2 and Smad4 are present in HPMC (Fig. 2). In response to TGF-β, Smad2 is acti-
vated (phosphorylated) in a time-dependent manner. These findings indicate that SMAD proteins participate in TGF-β–induced collagen synthesis and the development of PF.

Previous experiments suggest that the Smad2 protein plays a pivotal role in the TGF-β signaling pathway [5–7]. However, our data showed that dipyridamole had no effect on TGF-β–induced Smad2 activation (Fig. 6). In contrast, dipyridamole inhibited TGF-β–induced activation of the ERK pathway (Fig. 5). To verify this, similar experiments were carried out using DBcAMP, which inhibited TGF-β–induced activation of the ERK pathway in a dose-dependent manner (Fig. 5). By using the trypan-blue exclusion method and measuring lactate dehydrogenase (LDH) activity of the supernatant as previously described [4, 13], we found that dipyridamole at a concentration of 17 μg/mL or lower did not result in cytotoxicity of HPMC (data not shown). Therefore, the inhibitory effects of higher concentrations of dipyridamole on TGF-β–induced activation of SMAD proteins were not examined. These data imply that the inhibitory effect of dipyridamole on TGF-β inducibility of collagen α1(I) gene expression mainly operate through modulations of the ERK pathway rather than of the SMAD proteins.

The concept that the ERK pathway may serve as a downstream signaling factor for TGF-β and play a role in fibrogenesis is relatively new [15]. Our preliminary unpublished finding that dipyridamole and DBcAMP inhibited PDGF-stimulated ERK1/2 activation in HPMC led to our current study, which demonstrates that the ERK pathway in HPMC also is activated by TGF-β (Fig. 3). In addition, the blockade of ERK by PD98059 attenuated, but did not completely inhibit, the increase in α1(I) collagen mRNA expression induced by TGF-β (Fig. 4). These data suggest that the ERK pathway plays a role in TGF-β–stimulated collagen gene expression of HPMC. However, because the blockade of the ERK pathway resulted in only a partial suppression of increased α1(I) collagen mRNA expression by TGF-β, other mechanisms may participate in TGF-β–induced α1(I) collagen mRNA expression in HPMC. Furthermore, dipyridamole nearly completely prevented TGF-β–induced α1(I) collagen mRNA expression (Fig. 1), an effect that cannot be fully explained by the complete blockade of the ERK pathway by PD98059 (Fig. 5). These data implied that dipyridamole inhibited the TGF-β–induced α1(I) collagen mRNA expression through another as yet unidentified pathway. A recent study in NRK fibroblasts demonstrated that one protein, called connective tissue growth factor (CTGF), acts as a downstream mediator of TGF-β–induced collagen synthesis and can be down-regulated by elevated intracellular cAMP [16]. The identification of CTGF in TGF-β–treated HPMC and their possible modulation by dipyridamole deserves further study.

Given the involvement of the SMAD pathway and the ERK pathway in TGF-β signaling of HPMC, it is intriguing to speculate that potential cross-talk exists between these two pathways. However, in our current study, PD98059 failed to prevent TGF-β–induced Smad2 activation in HPMC, and this activation of the Smad2 by TGF-β was independent of the ERK signaling pathway. These data suggest that in TGF-β–treated HPMC, the SMAD pathway could be a distinct, parallel pathway from the ERK signaling pathway. Further investigation of these two downstream pathways of TGF-β and their possible interactions may elucidate the pleiotropic effects of TGF-β in HPMCs.

In summary, in HPMCs both the ERK and SMAD pathways are involved in the response to TGF-β. The inhibitory effect of dipyridamole on TGF-β–induced collagen α1(I) gene expression may function mainly through modulating the ERK pathway.

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