Dipyridamole

NSC89   2314   B   002   059

88   8   1   89   7   31

89   10   20

: NSC89-2314-B-002-059

88　8　1　　89　7　31

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(PF)　　　(CAPD)　　　　　　　　PF

(HPMC)　　　　　　　　　　　HPMC

dipyridamole

dipyridamole　　MPMC

HPMC　　　　　　MTT(methyltetra zolium

assay)　　HPMC　　　　ELISA　　　cAMP

dipyridamole　　　PDGF　　HPMC　　　HPMC　　G_1

cAMP　　　　PDGF　cyclin D_1

CDK4　　　　dipyridamole　　PDGF　　　　　PDGF

p27^{kip}　　　pRB　　　　　dipyridamole

dipyridamole　　PDGF　　HPMC

cAMP　　HPMC　　p27^{kip1}　　　pRB

dipyridamole　　PF

(　　　　　　　　　　dipyriamole)

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Proliferation of human peritoneal mesothelial cells (HPMC) accompanied by collagen synthesis is regarded as the main process predisposing to peritoneal fibrosis (PF) in patients of long-term continuous ambulatory peritoneal dialysis (CAPD). However, the precise molecular mechanism regulating HPMC proliferation has never been reported. We previously had reported that dipyridamole (DP) is potential as an antiproliferative and antifibrotic agent. We thus investigated the mechanism and effect of dipyridamole in regulation of HPMC proliferation.

HPMC was cultured from human omentum by an enzyme digestion method. Cell proliferation was measured by methyltetrazolium assay. Intracellular cAMP was measured using an enzyme immunoassay kit. Cell-cycle distribution of HPMC was analyzed by flow cytometry. Expressions of cell-cycle proteins (Cyclin D1, CDK4, pRB and p27^{kip1}) were determined by Western blotting.

Addition of DP suppressed PDGF-stimulated HPMC proliferation by cell-cycle arrest at G_1 phase. The antimitogenic effect of DP was mediated predominantly through the cAMP pathway. PDGF induced elevated protein levels of cyclin D1 but the CDK4 protein level did not change. Dipyridamole and DBcAMP had no effect on levels of cyclin D_1 and CDK4 in PDGF-stimulated HPMC. PDGF decreased p27^{kip1} and induced pRB phosphorylation of HPMC. In contrast, dipyridamole attenuated PDGF-stimulated pRB phosphorylation by preventing p27^{kip1} degradation.

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Dipyridamole appears to inhibit PDGF-stimulated HPMC proliferation through increased cAMP, preservation of p27^{kip1} and decreased pRb phosphorylation. Our study of dipyridamole may provide a therapeutic basis for clinical applications in the prevention of PF.

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The successful use of peritoneal dialysis (PD) as a long-term replacement therapy depends on the sustained integrity of the peritoneal membrane. Peritoneal fibrosis (PF), a serious complication found occasionally in patients on long-term PD, has been demonstrated to compromise the peritoneal membrane to a severe degree [1]. Proliferation of human peritoneal mesothelial cells (HPMC) accompanied by collagen synthesis plays a fundamental role in the pathogenesis of PF. However, the precise cellular mechanism regulating HPMC proliferation has, to our knowledge, never been reported.

Platelet-derived growth factor (PDGF) has been reported to be elevated during peritoneal injury [2]. In rat pleural mesothelial cell culture, PDGF has been shown to stimulate PMC proliferation and collagen synthesis [3]. Dipyridamole [2,6-bis(diethanolamino)-4, 8-dipiperidinopyrimido-(5,4-d)-yrimi-dine] is a popular anti-platelet agent [4]. In addition to its anti-platelet activity, we [5] previously have demonstrated that dipyridamole, through increased intracellular cAMP, may inhibit rat mesangial cell proliferation and reduce their expression of collagen α1(I) mRNA. However, its mechanism remains undetermined.

Cell proliferation is governed by the cell-cycle machinery. In mammalian cells, progression through the cell-cycle is dependent on the balance of positive and negative regulatory cell-cycle proteins [6]. Cyclin D$_1$ when bound to its catalytic partner, the cyclin dependent kinase 4 (CDK4), may result in the formation of active Cyclin D$_1$/CDK4 complex, which is responsible for the phosphorylation of the retinoblastoma gene product (pRB). Unphosphorylated pRB negatively controls G$_1$ progression, and the phosphorylated form of pRB is essential and required for proliferation of normal cells. Cyclin-kinase inhibitors (CKI) negatively regulate the cell-cycle by inhibiting the Cyclin D$_1$/CDK4 complex. p27^{Kip1} is regarded as a major CKI of G$_1$ phase and is most directly involved in cell cycle restriction control [7]. Reduction of p27^{Kip1} expression by PDGF results in cell-cycle re-entry and proliferation of rat mesangial cells. While cyclins and CDKs have been well investigated in a wide range of mammalian cell types, there has been, to our knowledge, no report detailing their induction and regulation in HPMC.

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At first, we performed growth curve experiments to evaluate the effect of dipyridamole on PDGF-stimulated HPMC proliferation (Fig. 1). PDGF (25 ng/ml) resulted in a significant increase of HPMC proliferation. This effect was clearly reduced by pretreating the cells with dipyridamole before the addition of PDGF. In time-response experiments, dipyridamole resulted in a dose-dependent inhibition of HPMC. Dipyridamole, at concentrations of 6 μg/ml and 17 μg/ml, inhibited PDGF-stimulated HPMC proliferation by 40.1% and 57.0%, respectively.

To investigate the cellular mechanism of this inhibitory effect of dipyridamole on HPMC growth, cell cycle distribution was determined by flow cytometry (Fig. 2). After starvation, 79.6 $\pm$ 4.1% of HPMC were arrested in the G$_1$ phase of the cell cycle. After treated with PDGF (25 ng/ml) for 24 hours, 14.3 $\pm$ 5.2% and 26.1 $\pm$ 6.3% of the HPMC were distributed in the S and G$_2$/M phase, respectively. In contrast, 81.5 $\pm$4.5% of the HPMC were still in the G$_1$ phase 24 hours after incubation with PDGF (25 ng/ml) in the presence of dipyridamole (17 μg/ml). Similar cell cycle distribution was observed in cells treated with PDGF (25 ng/ml)

plus IBMX (1 mM).

Dipyridamole is known to function as a phosphodiesterase inhibitor to increase intracellular cAMP levels [4]. Thus, we measured cAMP levels after incubation of HPMC with varying concentrations of dipyridamole (2, 6, and 17 µg/ml). In these experiments, IBMX (1 mM), a known phosphodiesterase inhibitor, was used as a positive control (Fig. 3). Intra-cellular cAMP levels were elevated in a dose-dependent manner by dipyridamole. To elucidate if the growth inhibitory actions of dipyridamole was through the increased cAMP, we next investigated the growth inhibitory effect of DBcAMP in cultured HPMC. The addition of DBcAMP (0.5 mM) resulted in an inhibitory effect on PDGF-stimulated HPMC proliferation. H-89, a cell-permeable PKA inhibitor, could overcome the growth inhibitory effects of dipyridamole and DBcAMP (Fig. 4).

As dipyridamole arrests HPMC in G_1/S transition, we questioned whether this inhibitory effect might be due to alterations of cell-cycle proteins. Western blotting analysis (Fig. 5) indicated that there was a low level of protein expression for cyclin D_1 in quiescent (0 hr) HPMC. During PDGF stimulation, protein levels of cyclin D1 rose between 0-4 hours, and reached maximal levels around 8-12 hours after stimulation. The CDK4 protein level did not oscillate following the addition of PDGF. Dipyridamole had no effect on protein levels of cyclin D_1 or CDK4 in PDGF-stimulated HPMC. However, the addition of dipyridamole resulted in an attenuated pRB phosphorylation (Fig. 6) and prevents PDGF-stimulated degradation of p27[kip1] in HPMC (Fig. 7).

It has been postulated that the relative levels of p27[kip1] versus those of cyclinD_1/CDK4 are crucial in determining whether pRB is activated (phosphorylated) or not [6-7]. A role for cAMP in maintenance of p27[kip1] by inhibiting its depletion has been suggested [10]. The possible mechanism for blockade of pRB phosphorylation may be through the antagonizing of cyclin D_1/CDK4 activity by p27[kip1]. Our data supports the above notion. However, the mechanisms by which dipyridamole prevents the reduction of p27[kip1] in PDGF-treated HPMC deserves further study.

In summary, we found in this work that dipyridamole arrests HPMC in G_1/S transition by preventing the depletion of p27[kip1] and the modulation on pRB phosphorylation.
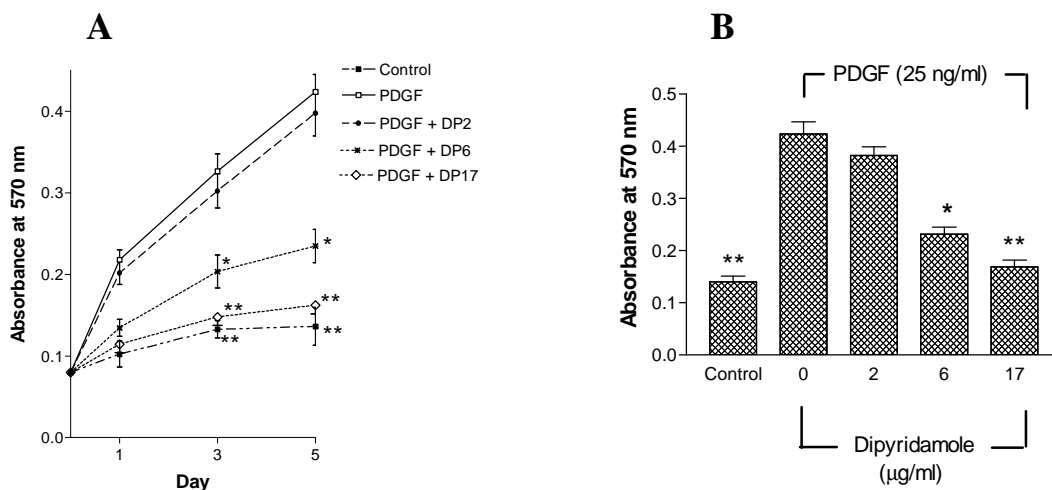

Fig.1. Dipyridamole (DP) inhibits PDGF (25 ng/ml)-stimulated HPMC growth. (A) Time-response curve: Varying concentrations (2, 6, and 17µg/ml) of dipyridamole were added after overnight plating of HPMC and further incubated for 1 to 5 days. (B) Dose-response study: HPMC were incubated for 120 hours after addition of varying concentrations of dipyridamole and a fixed dosage (25 ng/ml) of PDGF. *p<0.05, **p<0.01, compared to PDGF. Each point represents the mean ± SEM of three experiments performed in triplicate.

(A) Arrest, 48 Hr   **(B) PDGF**
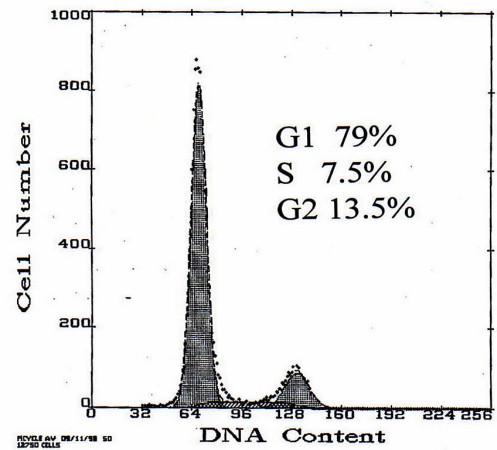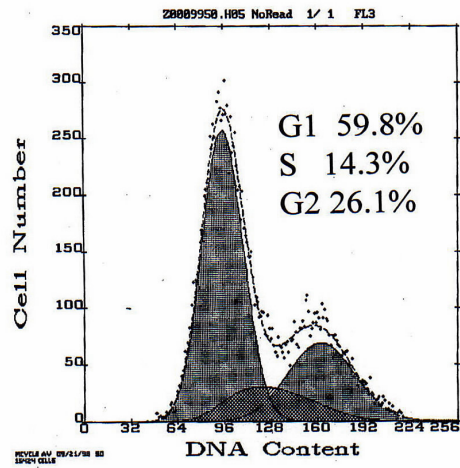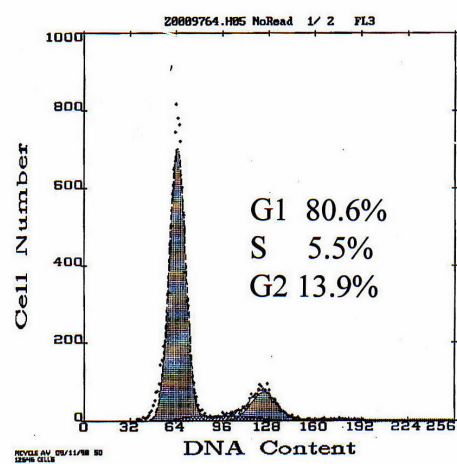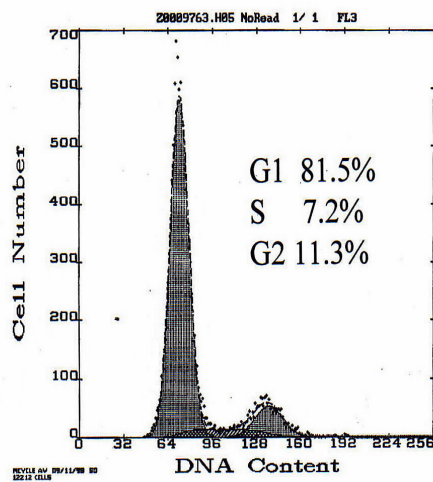
(C) PDGF + Dipyridamole **(D) PDGF + IBMX**

Fig. 2. Flow cytometry analysis for cell-cycle distribution. Subconfluent HPMC were growth arrested by 0.5% FCS for 48 hours (A), then changed into mediums in the presence of PDGF (25 ng/ml) alone (B), or (C) PDGF plus 17 μg/mL dipyridamole, or (D) PDGF plus IBMX (1 mM). The x axis shows DNA content; the y axis shows the number of cells. The experiments were repeated four times with similar results observed.
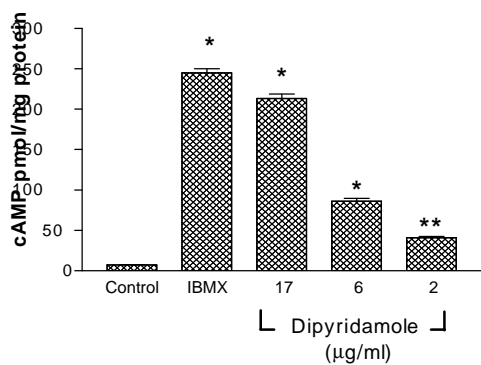
Fig.3. Effect of dipyridamole (unit: $\mu g/ml$) on intra-cellular cAMP levels (pmol/mg protein) as measured by a cAMP kit. Intra-cellular cAMP levels were elevated in a dose-dependent manner by dipyridamole. IBMX (1 mM), a known phosphodiesterase inhibitor, was used as a positive control. *$p<0.01$, **$p<0.05$, relative to control. All experiments were performed four times.
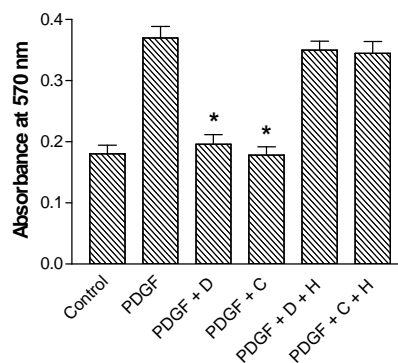
Fig. 4. Effect of dipyridamole(D,17$\mu g/ml$) and DBcAMP (C,0.5mM) on PDGF-stimulated HPMC proliferation. MTT assay was performed on the 5th day. H-89 (H, 3 μM) nearly completely reversed the inhibitory effect of dipyridamole or DBcAMP on PDGF-stimulated HPMC proliferation. *$p<0.01$ *vs* PDGF alone. Data represents the mean $\pm$ SEM of four experiments.

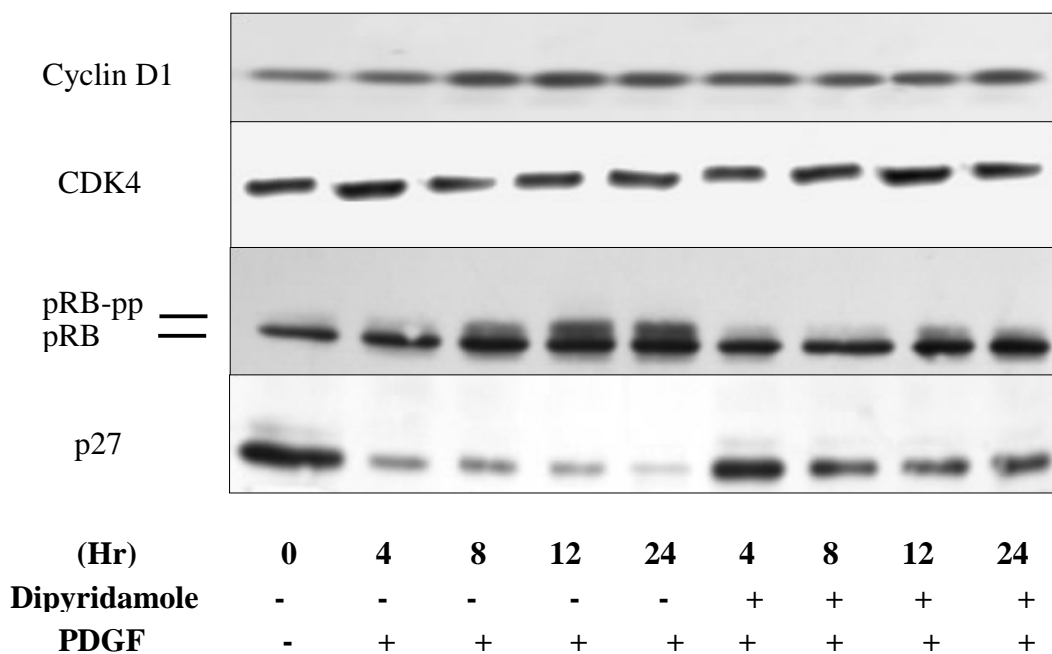| (Hr)	0	4	8	12	24	4	8	12	24
Dipyridamole	-	-	-	-	-	+	+	+	+
PDGF	-	+	+	+	+	+	+	+	+

Fig. 5. Kinetics of expression of cyclin D_1 (34KD), CDK4 (32KD), pRB and p27 [kip1] proteins upon stimulation with PDGF (25 ng/ml) and the effect of dipyridamole (DP, 17 $\mu g/ml$) upon their expression. Lysates (50 μg protein / lane) of HPMC were analyzed by using specific antibodies. Quiescent HPMC (0 hour) were incubated with PDGF (25 ng/ml) alone or in the presence of dipyridamole (17 $\mu g/ml$), and then were harvested at indicated time points for Western blotting analysis. Hyperphosphorylation of pRB slows electrophoretic mobility of the protein, creating the appearance of a double or widened band (gel shift). For evaluation of p27 [kip1], cellular extracts were electrophoresed on 15% SDS-PAGE then immunoblotted with a polyclonal p27 [kip1] antibody. The data is representative of four such studies.

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Our work implicated that dipyridamole may serve as a therapeutic agent for prevention or retardation of PF. The inhibitory effect of dipyridamole demonstrated here can provide basis for future studies. An animal *in vivo* study and possibly a human clinical trail are mandatory. Both studies can be arranged to examine the role of dipyridamole in the treatment of PF.

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