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Fangchinoline inhibits rat aortic vascular smooth muscle cell proliferation and cell cycle progression through inhibition of ERK1/2 activation and c-*fos* expression

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Abstract

Fangchinoline (FAN; a plant alkaloid isolated from *Stephania tetrandrae*) is a nonspecific Ca^{2+} channel blocker. The objective of the present study was to investigate the effect of FAN on the growth factor-induced proliferation of primary cultured rat aortic smooth muscle cells (RASMCs). FAN significantly inhibited both 5% fetal bovine serum (FBS)- and 50 ng/mL platelet-derived growth factor (PDGF)-BB-induced proliferation, [^{3}H]thymidine incorporation into DNA and phosphorylation of extracellular signal-regulated kinase 1/2. In accordance with these findings, FAN revealed blocking of the FBS-inducible progression through G_0/G_1 to S phase of the cell cycle in synchronized cells and caused a 62% decrease in the early elevation of c-*fos* expression induced after 5% FBS addition. Furthermore, significant antiproliferative activity of FAN is observed at concentrations below those required to achieve significant inhibition of Ca^{2+} channels by FAN. These results suggest that FAN reduced both FBS- and PDGF-BB-induced RASMCs proliferation by perturbing cell cycle progression. This antiproliferative effect of FAN is dependent on the MAP kinase pathway, but cannot be limited to its Ca^{2+} modulation.

Keywords: Fangchinoline; Aortic smooth muscle cells; MAP kinase; Cell cycle; Proliferation; c-*fos*

1. Introduction

Abnormal vascular smooth muscle cells (VSMCs) proliferation has a fundamental role in the pathogenesis of vascular diseases, such as atherosclerosis, hypertension and restenosis [1,2]. An alternative approach to preventing the proliferation and migration of VSMCs may be to influence the downstream intracellular signaling events responsible for transducing the signals from the various growth factor receptors. Possible candidates for such manipulation are the MAP kinases. MAP kinase, also known as ERK, is a critical enzyme used by many growth factors and substances to regulate various cellular functions including proliferation [3]. These serine–threonine kinases are a group of highly conserved and ubiquitously expressed proteins that have been shown to become activated by phosphorylation in response to numerous different stimuli [4]. Once activated, they then translocate to the nucleus where they initiate the transcription of several immediate early genes involved in cellular proliferation and growth, such as c-*fos*, c-*jun* and c-*myc* [5]. Therefore, it is of great interest to investigate antiproliferating agents for use as a potential preventive or therapeutic agent of atherosclerosis, the major etiology of cardiovascular disease.

It is well known that the creeper *Stephania tetrandrae* S. Moore (or fenfangji) has been used for hundreds of years as antirheumatic, antihypertensive, analgesic and antipyretic agents [6]. FAN ($C_{37}H_{40}O_6N_2$: 608.2, see Fig. 1), a bis-benzylisoquinoline alkaloid, is one of the major ingredients

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; FAN, fangchinoline; FACS, fluorescence activated cell sorter; MAP, mitogen-activated protein; PDGF, platelet-derived growth factor; RASMC, rat aortic smooth muscle cell; TET, tetrandrine.

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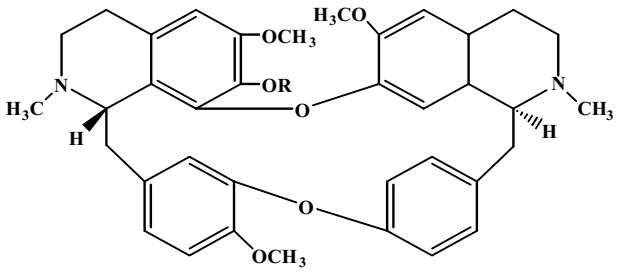

Fangchinoline R=H
Tetrandrine R= CH_3

Fig. 1. Structures of FAN and TET.

of fenfangji. FAN is known to inhibit histamine release on in vitro assay [7] and also known to lower the blood pressure as a non-specific calcium channel antagonist [8]. Its derivative, TET (7-*O*-methyl-FAN) is also reported to show dual action of cytotoxicity and cytoprotection in Neuro 2a mouse neuroblastoma cell [9] and antiproliferative effect in rat pulmonary artery smooth muscle cells [10]. In the present study, we investigated the antiproliferative effects of FAN on primary cultured RASMCs stimulated by the administration of FBS and PDGF-BB. The influence of FAN on the FBS-inducible cell cycle progression was also examined by flow cytometry analysis. Finally, we looked into inhibition of the MAP kinase pathway and the mRNA expression of the transcription factor, c-*fos*, as part of the early G_0/G_1 transition induced in quiescent RASMCs by mitogenic stimulation.

2. Materials and methods

2.1. Chemicals and reagents

The cell culture materials and FBS were obtained from Gibco-BRL. [^{3}H]Thymidine was from Amersham Pharmacia Biotech. PDGF-BB was from Upstate Biotechnology. Anti-phospho-ERK1/2 was purchased from New England Biolabs. FAN and TET were isolated from the roots of *S. tetrandrae* S. Moore (Menispermaceae) as previously described [11]. The roots of *S. tetrandrae* were collected in Hebei Province and identified by Prof. Wen-Han Lin, Department of Pharmacognosy, College of Pharmacy, Peking University. A voucher specimen was deposited in the same university. Other chemicals were of analytical grade.

2.2. Cell culture

RASMCs were isolated by enzymatic dispersion using the modified method of Chamley *et al.* [12] as described previously [13]. The rat thoracic aorta was from 8-week-old male Sprague–Dawley rats (Experimental Animal Center of Peking University) and incubated in DMEM containing 2 mg/mL collagenase (Type I) for 20 min at 37°. After removal of the adventitia, small fragments of outer membrane and endothelial cells, the aorta was minced and incubated in DMEM containing 2 mg/mL collagenase and 125 μg/mL elastase (Type II, Sigma Chemical Co.) for 1.5 hr at 37°. The freshly isolated cells were resuspended in DMEM supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 8 mM HEPES, 2 mM L-glutamine at 37° in a humidified atmosphere of 95% air and 5% CO_2 incubator. Most experiments were performed with primary cultured RASMCs after serum starvation. The purity of RASMCs cultures was confirmed by immunocytochemical localization of α-smooth-muscle actin. The passages of RASMCs used in these experiments were 6–9.

2.3. Measurement of cell proliferation and DNA synthesis

RASMCs proliferation was measured by determining cell number. Cells were seeded at a concentration of 4×10^4 cells/well in 12-well plates and grown in DMEM containing 10% FBS for 24 hr. The cells were then cultured with serum free (0.4% FBS) medium containing FAN, TET or vehicle for another 24 hr. The cells were counted by hemocytometer at 24, 48 and 72 hr after treatment with 5% FBS or 50 ng/mL PDGF-BB.

DNA synthesis as assayed by the incorporation of [^{3}H]thymidine into cell DNA [13] was measured in order to study the effects of FAN on cell growth. RASMCs were seeded in 24-well culture plates at a density of 7000 cells/well and grown to 70% confluence. The medium was then replaced by serum-free medium (0.4% FBS) consisting of DMEM and Ham's F-10 medium (1:1) containing FAN or vehicle. Twenty-four hours later, cultures were then exposed to 5% FBS or 50 ng/mL PDGF-BB for 20 hr before 1 μCi/mL [^{3}H]thymidine was added to the medium. Four hours later, labeling reaction were terminated by aspirating the medium and subjecting the cultures to sequential washes on ice with PBS containing 10% trichloroacetic acid and ethanol/ether (1:1 v/v). Acid-insoluble [^{3}H]thymidine was extracted into 250 μL of 0.5 M NaOH/well, and 100 μL of this solution were mixed with 5 mL scintillation cocktail (Packard, Ultima Gold) and quantified with a liquid scintillation counter (model LS 3801, Beckman). Fifty microliters of the residual solution were prepared for the determination of protein with Bradford [14] protein assay (Bio-Rad).

2.4. Cell cycle analysis

To estimate the proportions of cells in various phases of the cell cycle, cellular DNA contents were measured by flow cytometry (FACS). Cells were plated, allowed to attach overnight, and placed in DMEM plus 0.4% FBS and added FAN for 24 hr as described above. Twenty-four

hours after serum addition, the cells were harvested by trypsinization. The pellets were suspended in PBS and washed twice. The cells were then resuspended in the same volume of PBS. The cell number was adjusted to $(1-3) \times 10^6$ using PBS and centrifuged at 1200 rpm for another 10 min. The centrifuged pellet was suspended by 70% ethanol and fixed overnight at 4°. The fixed cells were briefly vortexed and centrifuged at 2000 rpm for 5 min. The ethanol was discarded and the pellets were stained with 0.5 mL of propidium iodide (PI) solution (50 μg/mL PI in sample buffer containing 100 μg/mL of RNase A). Before analysis by FACS, each sample was incubated for 1 hr at room temperature. The PI–DNA complex in each cell nucleus was measured with FACS Calibur (Becton & Dickinson Co.). The rate of the cell cycle within G_0/G_1, S and G_2/M phases was determined by analysis with the computer program Modfir LT (Verity Software House, Inc.).

2.5. Immunoblotting

Western blotting analysis was performed to establish whether FAN affects ERK1/2 MAP kinase phosphorylation. RASMCs in 12-well plates were pretreated with FAN or with vehicle for 24 hr in 0.4% FBS-containing medium, then 4.6% FBS or 50 ng/mL PDGF-BB was added for 1, 5, 15 and 30 min in the presence or absence of FAN or vehicle. At the time of harvest, cells were lysed with SDS sample buffer containing 62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, and 50 mM dithiothreitol. Equal amounts of protein (10 μg) were run on 10% SDS–polyacrylamide gel (SDS–PAGE) electrophoresis. Proteins were then transferred to polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech). The PVDF memberane was then blocked for 1 hr at room temperature in 5% non-fat dry milk (Amersham Pharmacia Biotech). For analysis of phospho-ERK1/2 MAP kinase, blots were incubated overnight with phospho-specific ERK1/2 rabbit polyclonal primary antibody at 1:2000 dilution in bovine serum albumin (BSA)/Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBS-T) buffer. After washing in TBS-T solution, blots were incubated further with horseradish peroxidase-conjugated IgG secondary antibody (New England Biolabs) for 3 hr at 4°. The blots were then washed five times in TBS-T, and antibody-bound protein was visualized with an Enhanced Chemiluminescense (ECL) kit (Amersham Pharmacia Biotech). The intensities of phospho-ERK1/2 and total ERK1/2 band were quantified by Scion-Image for Windows Program (Scion Corporation).

2.6. c-fos expression

Total RNA from approximately 3×10^6 RASMCs was extracted by the single-step method using TRIzol Reagent (Gibco-BRL). One microgram of total RNA was reverse-transcribed (RT) to synthesize singe strand cDNA by first-strand synthesis system kit (Gibco-BRL). Subsequently, 2 μL of the RT reaction were subjected to polymerase chain reaction (PCR) in order to co-amplify a fragment of c-*fos* and GAPDH gene, which was used as an internal control [15]. The sequences of the oligonucleotide primers used for cDNA amplification were 5′-CCC CTG TCA ACA CAC AGG AC-3′ (sense) and 5′-GCA ATC TCG GTC TGC AAC GC-3′ (antisense) for c-*fos*, and 5′-CCT GCA CCA CCA ACT GCT TA-3′ (sense) and 5′-GAT GCC AGT GAG CTT CCC GT-3′ (antisense) for GAPDH [14]. After an initial denaturation step at 94° for 5 min, The PCR reaction was performed for 24 cycles. The cycle profiles for c-*fos* and GAPDH genes amplification were 94° for 30 s; 58° for 30 s and 72° for 1 min. A final extension was performed at 72° for 5 min. The c-*fos* gene expression was normalized to GAPDH values. The RT–PCR products were electrophoresed on 1.5% agarose gels containing 0.5 μg/mL ethidium bromide. After electrophoresis, cDNA bands were visualized and photographed using the GDS 7500 Gel Documentation System (UVP). The band analysis was performed by Scion Corporation Imaging System.

2.7. Statistical analysis

All values are expressed as means ± SEM, one-way ANOVA followed by Dunnett post-test were performed using GraphPad. Differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of FAN on RASMCs proliferation

The number of RASMCs in culture was determined after stimulation with FBS or PDGF-BB. The cell number was significantly increased by treatment with 5% FBS or 50 ng/mL PDGF-BB for 24 hr, and decreased significantly by 24 hr pre-treatment of FAN (Fig. 2A and B). The percentages inhibition exerted by FAN 0.1, 1.0 and 3.0 μM were 15.1 ± 0.9% ($P < 0.05$, N = 3, quadruplicated), 22.4 ± 0.7% ($P < 0.01$, N = 3, quadruplicated) and 57.3 ± 5.4% ($P < 0.01$, N = 3, quadruplicated) on FBS-stimulated cells (Fig. 2A), and 10.9 ± 0.4% ($P < 0.05$, N = 3, quadruplicated), 28.1 ± 0.6% ($P < 0.01$, N = 3, quadruplicated) and 57.6 ± 5.2% ($P < 0.01$, N = 3, quadruplicated) on PDGF-BB-stimulated cells (Fig. 2B), respectively. After 3 days of continuous treatment, a significant inhibition of cell growth was revealed (Fig. 2C and D). The number of RASMCs in culture was determined after 24, 48 and 72 hr stimulation with FBS and PDGF-BB. The results were shown in Fig. 2C and D. Treatment of cells with 5% FBS or PDGF-BB increased the cell number in a time-dependent fashion. The addition of 3.0 μM FAN or 5.0 μM TET together with stimuli significantly inhibited the increase in cell number ($P < 0.01$, N = 4, duplicated, Fig. 2C and D) and FAN

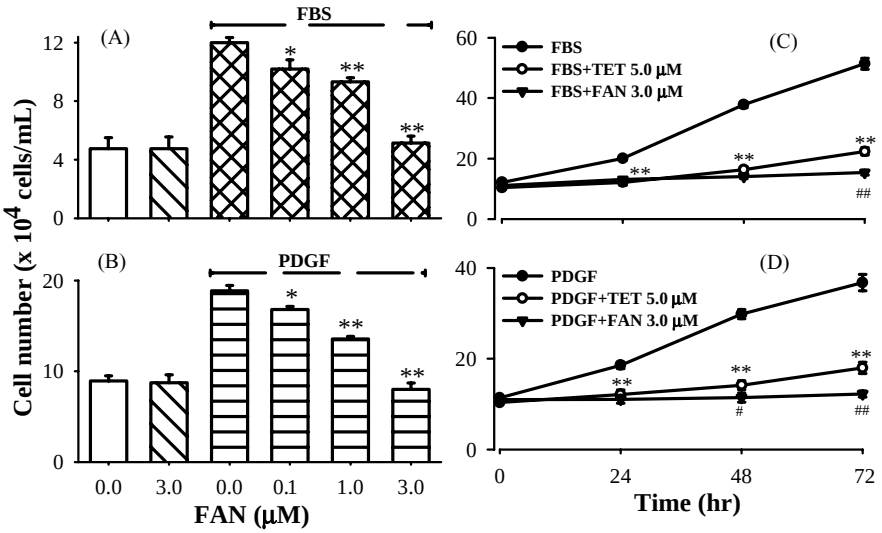

Fig. 2. Effects of TET on the proliferation of RASMCs. Quiescent RASMCs were stimulated with 5% serum and 50 ng/mL PDGF. After 0, 24, 48 and 72 hr incubation in the absence or presence of FAN and TET, cells were harvested and the viability was examined by trypan blue dye exclusion test. The number of viable cells was estimated using a hemocytometer. Results are shown as the representive of four different experiments. Data are expressed as mean ± SEM. (A, B) Effects of different concentrations of FAN on the FBS- or PDGF-BB-induced proliferation. (C, D) Effects of FAN and TET on the proliferation for the different incubation period. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. corresponding stimulus control, respectively. $^{\#}P < 0.01$ and $^{\#\#}P < 0.01$ vs. TET.

showed higher potency than TET ($P < 0.05$ after 48 hr and $P < 0.01$ after 72 hr, Fig. 2C and D). To confirm that the above inhibitory effects were not due to toxicity or damage to the cells, trypan blue viability tests were carried out in cells treated in parallel with growth studies. There was no loss in viability of cells treated with FAN 6.0 μM, less than 3.0% of the cells took up the dye. Furthermore, no floating cells were observed on any particular day of the treatment. Thus, detachment and loss of cells did not account for the inhibition of cell proliferation.

3.2. Effect of FAN on DNA synthesis

The [^{3}H]thymidine incorporation in control quiescent cells was 97.8 ± 17.6 cpm/μg protein, while in FBS- and PDGF-BB-stimulated cells, the incorporation increased up to 1269.4 ± 105.0 and 1423.5 ± 120.0 cpm/μg protein, respectively. FAN treatment significantly inhibited FBS- or PDGF-BB-induced DNA synthesis in a concentration-dependent manner (Fig. 3), the percentages inhibition being 22.3 ± 2.0% ($P < 0.01$, N = 4, duplicated) and 59.7 ± 6.1% ($P < 0.01$, N = 4, duplicated) in FBS-stimulated cells, and 43.1 ± 2.9% ($P < 0.01$, N = 4, duplicated) and 90.0 ± 6.3% ($P < 0.01$, N = 4, duplicated) in PDGF-BB-stimulated cells with 1.0 and 3.0 μM FAN, respectively.

3.3. Effects of FAN on cell cycle in synchronized RASMCs

Effects of the FAN on cell cycle progression were also analyzed (Fig. 4). The serum-deprivation of VSMCs in primary culture for 24 hr resulted in an approximately 95.7% synchronization of the cell cycle in the G_0 phase. The percentage of cells in S phase increased from 1.5 ± 0.2 to 13.1 ± 1.1% 24 hr after serum repletion. In contrast, FAN-treated cells showed a significant block of cell cycle progression. FAN 0.1, 1.0 and 3.0 μM decreased the percentage of cells in S phase to 11.5 ± 1.0, 6.9 ± 0.2% ($P < 0.05$, N = 3, duplicate) and 1.6 ± 0.3% ($P < 0.01$, N = 3, duplicate), respectively (Fig. 4). This finding indicated that FAN must act at the early events of the cell cycle to be effective against DNA synthesis.

3.4. Effects of FAN on ERK1/2 MAP kinase phosphorylation

To further examine the underlying mechanisms of the antiproliferative effect exerted by FAN, RASMCs were stimulated with 5% FBS or 50 ng/mL PDGF-BB in the

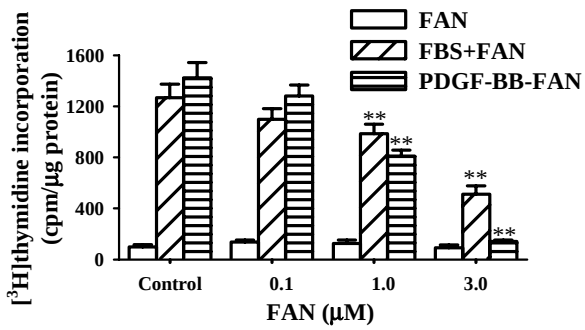

Fig. 3. Effects of FAN on [^{3}H]thymidine uptake into primary cultured RASMCs stimulated by FBS and PDGF-BB. After confluence, the cells were then cultured with serum free (0.4% FBS) medium containing FAN or vehicle for 24 hr and then exposed to 5% FBS and 50 ng/mL PDGF-BB for 20 hr before 1 μCi/mL [^{3}H]thymidine was added to the medium. Four hours later, labeling reaction were terminated and quantified with a liquid scintillation counter. Results are shown as the representative of four different experiments. Data are expressed as mean ± SEM. $^{**}P < 0.01$ vs. control.

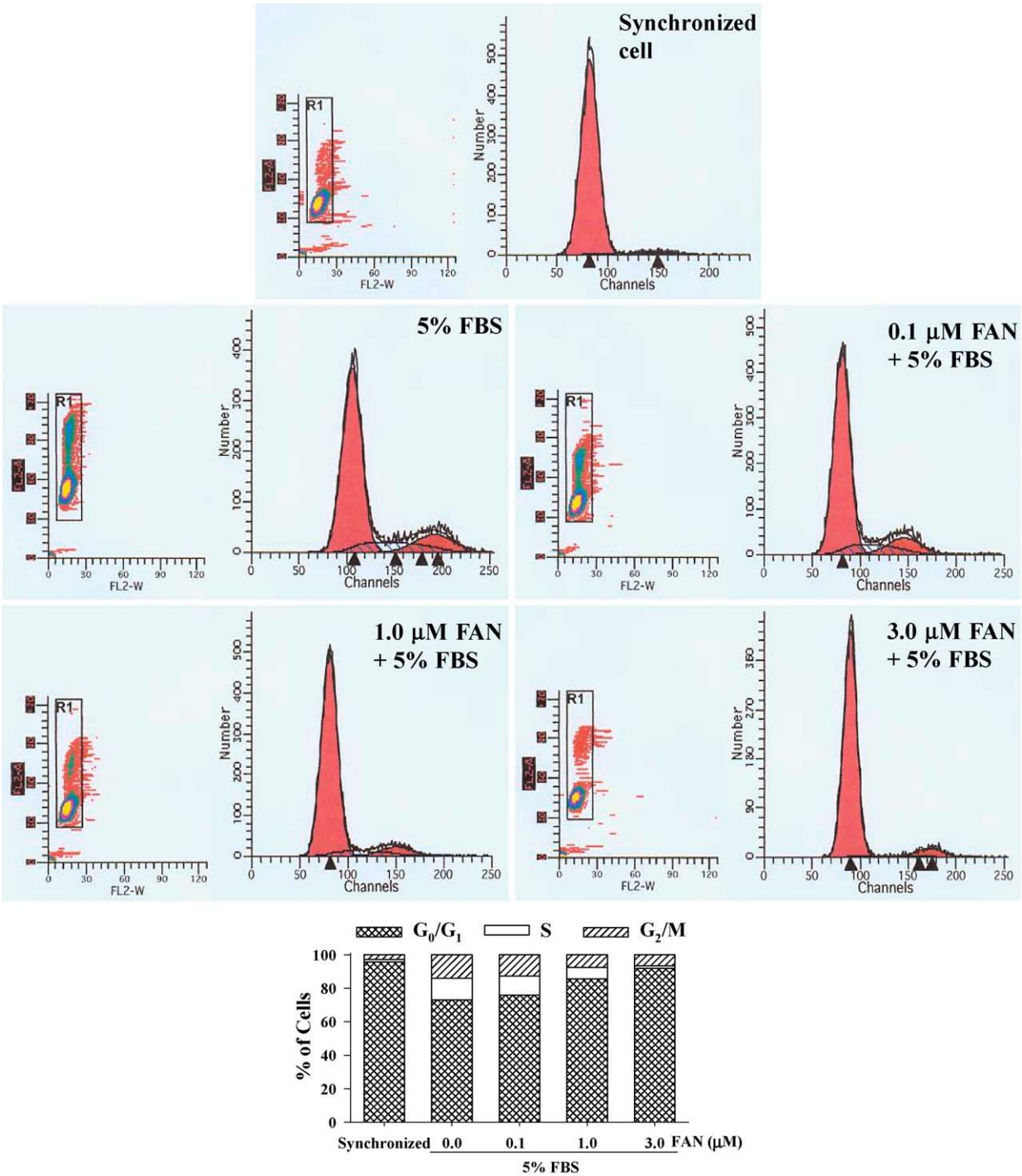

Fig. 4. FAN impairs cell cycle progression of RASMCs. Cells were exposed to either vehicle and 0.1, 1.0 and 3.0 mM FAN during 24 hr and treated as indicated in Section 2.4. Individual nuclear DNA content is as reflected by fluorescence intensity of incorporated propidium iodide. Each item is derived from a representative experiment, where data from at least 11,000 events were obtained.

presence or absence of FAN and the phosphorylation of ERK1/2 MAP kinase was assayed. Figure 5 shows a typical experiment. ERK1/2 phosphorylation was rapidly induced and high at 5 min after FBS and PDGF-BB addition, and was reduced by 30 min after growth factor addition (Fig. 5A and B). Pretreatment with FAN 1.0 and 3.0 μM significantly inhibited either FBS (Fig. 5C)- or PDGF-BB (Fig. 5D)-induced ERK1/2 MAP kinase phosphorylation. The percentage inhibition were $43.0 \pm 8.8\%$ ($P < 0.05$, $N = 3$, FAN 1.0 μM) and $63.9 \pm 10.3\%$ ($P < 0.01$, $N = 3$, FAN 3.0 μM) against FBS-stimulation (Fig. 5C), and $45.1 \pm 10.3\%$ ($P < 0.05$, $N = 3$, FAN 1.0 μM) and $74.2 \pm 7.3\%$ ($P < 0.01$, $N = 3$, FAN 3.0 μM) against PDGF-BB-stimulation (Fig. 5D), respectively.

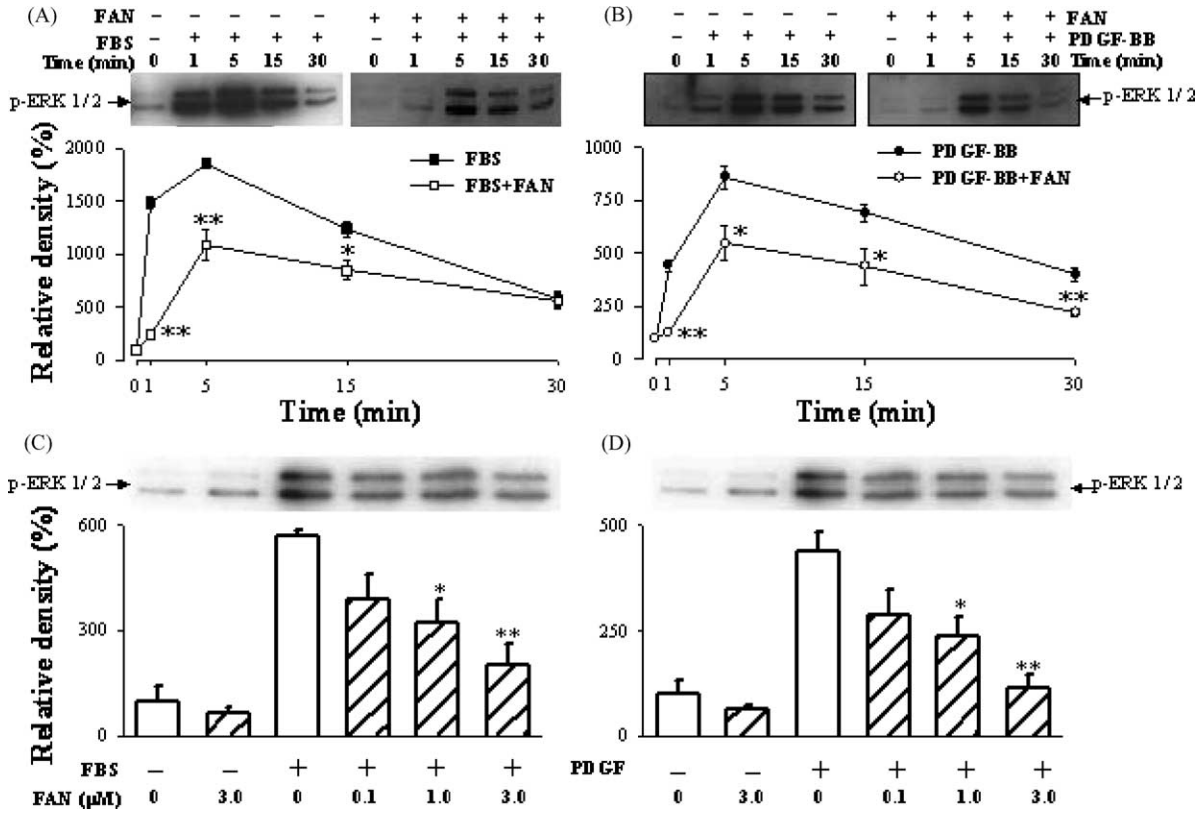

Fig. 5. Time course of the phosphorylation of ERK1/2 and effect of FAN on the phosphorylation of ERK1/2 in RASMCs treated with 5% FBS (A and C) and 50 ng/mL PDGF-BB (B and D) in the presence or absence of FAN. Cells were cultured in 12-well plates until confluence and replaced the medium with serum-free medium in the presence or absence of FAN. After 24 hr cultivation, the RASMCs were stimulated with 5% FBS or 50 ng/mL PDGF-BB for different time periods (5 min for C and D). Cells were lysed, and 20 μg protein was analyzed with SDS–PAGE. Relative activities were quantified by scanning densitometry and showed the levels of each activity as relative value of the blank, respectively. Western blot was repeated three times. Data are expressed as mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. corresponding value that treated with stimuli only, respectively.

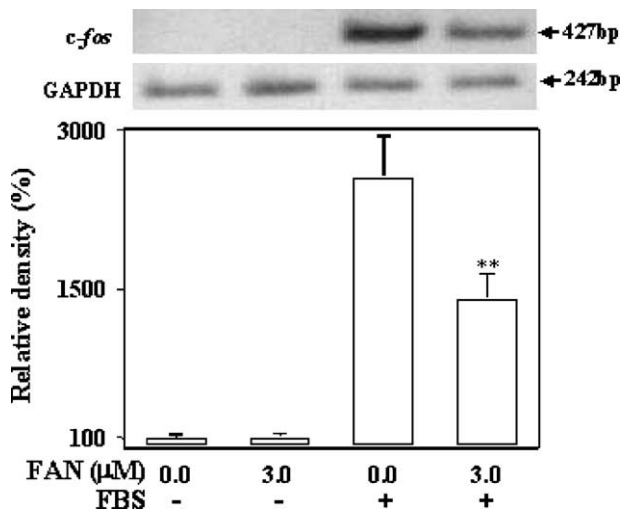

Fig. 6. Effect of FAN on c-*fos* expression stimulated by 5% FBS in primary cultured RASMCs. Confluent cells were cultured in the presence or absence of 3.0 μM FAN in serum-free medium for 24 hr, and were then stimulated with 5% FBS for 30 min. Total RNA isolated from RASMCs was reverse-transcribed into cDNA followed by 24 cycles of PCR amplification with c-*fos* primer. GAPDH gene was used for normalization. Results represent three different experiments. Data are expressed as mean ± SEM. $^{**}P < 0.01$ vs. stimulus control.

3.5. Effect of FAN on c-fos expression

In view of the effects of FAN on early cell cycle entry, the influence of the drug on the expression of c-*fos*, a gene expressed in the G_0/G_1 transitional phase as part of the early response to mitogenic stimulation, was also studied. After 30 min FBS stimulation, cells that were previously starved for 24 hr, showed expression of c-*fos* (Fig. 6). However, when the stimulation was carried out in the presence of FAN 3.0 μM, activation was inhibited significantly. Densitometric analysis of the blots revealed that, in cells that were stimulated in the presence of FAN, c-*fos* expression was reduced by 44.8 ± 4.4% ($P < 0.01$, N = 3, duplicate). Relative density of this gene in quiescent cultures was taken as 100% (Fig. 6).

4. Discussion

VSMCs proliferation is a key process underlying the formation of atherosclerotic plaques [1]. Moreover, it is also the primary factor in reocclusion of arteries after angioplasty [16]. In the present study, we found that

FAN reduces either FBS- or PDGF-BB-induced proliferation of RASMCs (Fig. 2), acting on early events in DNA synthesis as established both by a decrease in [^{3}H]thymidine incorporation (Fig. 3) and by cell cycle progression arrest in G_0/G_1 phase as shown by FACS (Fig. 4). In addition, FAN decreases FBS- and PDGF-BB-induced ERK1/2 MAP kinase phosphorylation (Fig. 5). These results suggest that FAN could be useful in preventing the progression of vascular complications, such as restenosis after percutaneous transluminal coronary angioplasty.

As revealed by flow cytometry, the antiproliferative effect of FAN is associated with an accumulation of cells in G_0/G_1 phase of the cycle. Because there was no evidence of apoptosis induction during treatment of RASMCs by FAN (data not shown), the observed accumulation in G_0/G_1 reflected a specific effect of FAN on cell cycle progression rather than a decrease of cell number due to apoptosis. We therefore hypothesized that the antiprolierative properties of FAN are causally related to the modulation of signaling cascade involved in cell cycle progression and proliferation.

Recent studies have emphasized the role of G–S events in the regulation of cell proliferation through complex stimulant and inhibitory signals driven by cyclin-dependent kinases and their inhibitors, respectively. At the G_0/G_1 transition, the expression of several transcription factors like c-*myc*, c-*myb* and c-*fos* appears to be fundamental [17]. Accordingly, FAN also inhibited the serum-induced expression of the early-immediate gene, c-*fos* (Fig. 6). It is, therefore, possible that the antiproliferative effect of FAN results from its ability to block entry of the cells in S-phase due to interference in the early G_0/G_1 transition phase.

MAP kinase participate in both growth factor- and integrin-associated signaling pathways [18]. PDGF-BB activates the ERK1/2 pathway by triggering RAS–RAF activation, MEK1 phosphorylation, and ERK1/2 phosphorylations [19]. An increasing body of evidence shows that a wide range of growth factors can activate ERK1/2 MAP kinase signal transduction pathways leading to cellular growth by stimulating transcription factors that induce the expression of c-*fos* and others [20]. As shown in Fig. 5, FAN inhibited the levels of phosphorylated ERK1/2 MAP kinse stimulated by FBS and PDGF-BB, in the same concentration range that inhibits RASMCs proliferation and DNA synthesis.

Ca^{2+} channels appear to contribute to DNA synthesis in smooth muscle cells [21]. This is of relevance to TET, which exhibits non-specific Ca^{2+} channel antagonism [22,23]. TET, a natural analogue of FAN, exhibits higher potency than FAN at inhibition of Ca^{2+} channels [8], but exhibited lower potency than FAN in inhibition of RASMCs proliferation (Fig. 2C and D). In addition, significant antiproliferative activity of FAN is observed at concentrations below those required to achieve significant inhibition of Ca^{2+} channels by FAN. Furthermore, it is possible that the inhibitory action of FAN on RASMCs mitogenesis cannot be limited to blockade of stimulant-induced calcium uptake and release, since FAN exhibits antimitogenic potential even when added up to 72 hr after stimulation, i.e. when intracellular calcium has returned to baseline [24]. Graf *et al.* [25] reported that PDGF-BB-induced activation of ERK1/2 in primary cultured RASMCs was independent on its activation of intracellular calcium release. Previous reports and our results when taken together, suggest that the inhibitory effects of FAN on the cell proliferation induced by FBS and PDGF-BB are not simply due to their inhibitory action on the Ca^{2+} elevations but instead are also due to more complex factors.

In summary, our study showed that FAN is able to inhibit the proliferation and DNA synthesis induced in RASMCs by the complex mixture of the mitogens of serum. Even if the precise mechanisms behind the growth inhibitory effect of FAN are not known, flow cytometry studies and evaluation of proto-oncogene expression suggest that a discrete event of the cell cycle as early as the G_0/G_1 transitional phase can be identified as a site of action for FAN. This beneficial property of FAN may be of importance in atherosclerosis in which proliferation is an important determinant of atherosclerotic plaque development and stability.

Acknowledgments

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