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Sanguinarine-induced G_1-phase arrest of the cell cycle results from increased p27KIP1 expression mediated via activation of the Ras/ERK signaling pathway in vascular smooth muscle cells

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Abstract

The present study identified a novel mechanism for the effects of sanguinarine in vascular smooth muscle cells (VSMC). Sanguinarine treatment of VSMC resulted in significant growth inhibition as a result of G_1-phase cell-cycle arrest mediated by induction of p27KIP1 expression, and resulted in a down-regulation of the expression of cyclins and CDKs in VSMC. Moreover, sanguinarine-induced inhibition of cell growth appeared to be linked to activation of Ras/ERK through p27KIP1-mediated G_1-phase cell-cycle arrest. Overall, the unexpected effects of sanguinarine treatment in VSMC provide a theoretical basis for clinical use of therapeutic agents in the treatment of atherosclerosis.

Keywords: Sanguinarine; VSMC; ERK; G_1-phase cell-cycle arrest; p27; Ras

The increased proliferative capacity of vascular smooth muscle cells (VSMC)[1] is a key pathological process in the development of atherosclerosis and restenosis [1]. Cellular proliferation is governed primarily by regulation of the cell cycle [2], which consists of four distinct sequential phases (G_0/G_1, S, G_2, and M). Cyclin-dependent kinase (CDK) is a major regulator of the transition between the phases of the cell cycle [3]. Cyclin/CDK complexes are composed of a regulatory subunit, cyclin, and an active kinase subunit, CDK. The cyclin/CDK complexes are controlled by both positive and negative regulators [4]. p21WAF1 (p21) and p27KIP1 (p27) are two primary negative regulators of CDK in VSMC and play an important role in the inhibition of CDK activity [5]. Both p21 and p27 inhibit the phosphorylation of cyclin D/CDK4 and cyclin E/CDK2 complexes, thereby inhibiting activity of this complex and causing cell-cycle arrest in the G_1 phase [3]. The effects of sanguinarine on the activities of these proteins and, thus, on the cell cycle, remain to be determined in VSMC.

Mitogen-activated protein kinase (MAPK) activation is typically associated with cell survival, proliferation, and differentiation, as the enzyme is activated by

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[1] *Abbreviations used:* VSMC, vascular smooth muscle cells; CDK, cyclin-dependent kinase; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase; RBD, human Ras-binding domain; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EV, empty vector.

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mitogens and several cell survival factors [6–8]. Extracellular signal-regulated kinase 1/2 (ERK1/2) plays a major role in regulating cell growth and differentiation, as it is highly induced in response to growth factors and cytokines [9,10]. Ras, a small G protein, is activated by various stimuli of cell growth and differentiation. In its active form, Ras stimulates the phosphorylation cascade of protein kinases, such as the ERK signaling pathway [11,12]. Recently, however, it was determined that activation of ERK had led to cell death in several cell lines [13–15].

Sanguinarine (13-methyl-[l,3]benzodioxolo[5,6-c]-l,3-dioxolo[4,5-I] phen-anthrdinium) is a benzophenanthridine alkaloid present in the roots of plants, such as *Sanguinaria canadensis* and other *Poppy fumaria* species [16]. Sanguinarine reportedly has antimicrobial, antioxidant, as well as anti-inflammatory properties [17]. Sanguinarine also exhibits anti-platelet effects in experimental animals and inhibits angiogenesis *in vitro* [18,19]. Recent studies have shown that sanguinarine inhibits growth in various cancer cell lines by regulating the cell cycle and by signaling apoptosis [20–22]. Although many studies have analyzed the effects of sanguinarine on growth inhibition in several cell lines [20–22], the relevant pathway integrating cell-cycle regulation and the signaling pathways involved in growth inhibition in VSMC remains to be identified.

The purpose of the present study was to examine the roles of MAPK signaling pathways in regulating sanguinarine-induced inhibition of cell growth in VSMC. A novel mechanism of growth inhibition was identified; the Ras/ERK pathway is important in mediating sanguinarine-induced cell growth inhibition through p27-mediated cell-cycle arrest.

Materials and methods

Materials

Sanguinarine and the monoclonal antibody to SM-α-actin (clone 1A4) were obtained from Sigma (St. Louis, MO). Polyclonal antibodies against cyclin E, cyclin A, CDK2, CDK4 and CDK6 were obtained from Santa Cruz (Santa Cruz, CA). Polyclonal antibodies against cyclin D1, p21, p53, p27, ERK, phosphor-ERK, p38 MAP kinase, phosphor-p38 MAP kinase, JNK and phosphor-JNK were obtained from New England Biolabs. U0126, SP600125 and SB203580 were obtained from Calbiochem (San Diego, CA). Anti-Ras antibody was obtained from Transduction Laboratories. The pCMV vector encoding dominant negative Ras (RasN17) and constitutively active form of Ras (RasV12) were purchased from Clontech.

Cell cultures

Human aortic smooth muscle cells (VSMC) were purchased from Bio-Whittaker (San Diego, CA) and cultured in smooth muscle cell growth medium containing 10% FBS, 2 ng/ml human basic fibroblast growth factor, 0.5 ng/ml human epidermal growth factor, 50 μg/ml gentamycin, 50 μl/ml amphotericin-B, and 5 μg/ml bovine insulin. Especially, the inhibitors for the MAP kinases (U0126, SP600125 and SB203580) were maintained in the culture medium throughout the 12 h period of treatment with sanguinarine.

Cell viability assay

Subconfluent VSMC in the exponential growth phase were cultured with sanguinarine for various incubation periods in 24-well plates. Cell viability was determined using a modification of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the conversion of tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-tetrazolium to a formazan product [23] by mitochondrial dehydrogenase. The formazan product was quantified by measuring the absorbance at 490 nm.

[^{3}H]thymidine incorporation

VSMC, grown to near confluence in 24-well tissue culture plates, were treated with various concentrations of sanguinarine (0–400 nM in DMSO). The [^{3}H]thymidine incorporation experiments were performed as described previously [24].

Cell cycle analysis (FACS)

Cells were harvested, fixed in 70% ethanol, and stored at −20 °C. Cells were then washed twice with ice-cold PBS and incubated with RNase and the DNA intercalating dye, propidium iodide. Cell cycle phase analysis was performed using flow cytometry with a Becton Dickinson Facstar flow cytometer and Becton Dickinson cell fit software.

Immunoprecipitation, immunoblotting, and immune complex kinase assays

Growth-arrested cells were treated with sanguinarine in the presence of 10% FBS for various treatment time periods at 37 °C. Cell lysates were prepared and immunoprecipitation, immunoblotting, and immune complex kinase assays were performed as described previously [24].

Transient transfection

Cells were transfected with plasmid DNA using the Superfect reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Affinity precipitation of the active form of Ras (Ras-GTP)

Subconfluent cells (5×10^4 cells/cm^2) were seeded on 100-mm dishes, serum-starved, and treated with TNF-α (100 ng/ml) for various treatment times. After washing with ice-cold PBS once, the cells were lysed by adding 500 μl lysis buffer comprised of 25 mM HEPES, 10 mM EDTA, 1% Igepal CA630, complete protease inhibitor cocktail (Roche Diagnostics), 1 mM sodium orthovanadate, and 10% glycerol. The cell lysate was clarified by centrifugation for 15 min at 14,000*g* and the protein concentration of the lysate was determined using a BCA assay (Pierce, Rockford, IL). Equal amounts of cell lysate (500 μg) were subjected to affinity precipitation for Ras-GTP using 10 μl agarose suspension conjugated with a GST fusion protein that corresponded to the human Ras-binding domain of c-Raf (GST-RBD) (Upstate Biotechnology, Lake Placid, NY). After a 1-h incubation at 4 °C, the agarose was washed three times with lysis buffer and was boiled with 30 μl SDS sample buffer. The product was resolved by 15% SDS–PAGE, followed by immunoblotting with anti-Ras antibody [25].

Statistical analysis

Where appropriate data are expressed as means ± SE and were analyzed using factorial ANOVA and Fisher's least significant difference test. Statistical significance was set at $P < 0.05$.