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Sanguinarine inhibits VEGF-induced angiogenesis in a fibrin gel matrix

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Abstract. *Background:* The identification of possible ways to block blood vessels formation has become a major scientific objective of the last decade and several phytochemicals are currently being exploited to target tumour angiogenesis.

Aim: The effects of Sanguinarine (SA), an alkaloid from the root of Sanguinaria Canadensis, were evaluated in an *in vitro* angiogenesis model; moreover the effects on Akt phosphorylation in porcine aortic endothelial cell line (AOC) were also examined.

Methods: SA (300 nM) was tested in the presence or absence of VEGF (100 ng/ml) in a three dimensional angiogenesis bioassay obtained pipetting a suspension of AOC on microcarrier beads in a fibrinogen solution before the addition of thrombine. Endothelial cell proliferation was measured at 48, 96, 144, 192 h. The phosphorylation of Akt was measured by ELISA in 2×10^5 AOC treated as described above.

Results: The addition of SA abolished ($p < 0.001$) VEGF stimulatory effect on AOC growth at all the examined times. In addition, the stimulatory effect induced by VEGF on Akt phosphorylation was significantly ($p < 0.001$) inhibited by SA.

Conclusion: SA appear to be an antiangiogenic natural product by directly suppressing the proliferative effect of VEGF on endothelial cell line: this effect could be mediated by blocking the VEGF-induced Akt activation.

Keywords: Angiogenesis assay, Sanguinarine, VEGF, Akt

1. Introduction

The requirement for blood vessels arises from the need to maintain oxygen homeostasis, as well as to guarantee the delivery of nutrients and the removal of waste products. Nutrients and oxygen to the growing embryo are provided by the earliest vessels as a result of vasculogenesis, during which precursor cells commit to form endothelial cells. Subsequently, angiogenesis extends and remodels these structures into a highly organized and stereotyped vascular network of larger vessels ramifying into smaller ones. During adulthood, in contrast, most blood vessels remain quiescent and angiogenesis occurs only in the cycling ovary and in the placenta during pregnancy. However, endothelial cells retain their remarkable ability to rapidly divide in response to particular stimula, such as hypoxia, i.e. during wound healing and repair [18]. In many disorders these stimula become excessive and the balance between stimulators and

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inhibitors is tilted, resulting in angiogenic switch. The best-known conditions in which angiogenesis is switched on are malignant, ocular and inflammmatory disorders, but many additional processes are involved, such as obesity, asthma, diabetes, cirrhosis, multiple sclerosis, endometriosis, AIDS, bacterial infections and autoimmune diseases. Therefore, the molecular and cellular mechanisms leading to the angiogenic response in the endothelium have been extensively studied in the past two decades due to the resulting therapeutic potentials and clinical implications [7]. It is now well established that angiogenesis is orchestrated by a variety of activators and inhibitors that coordinates sequentially the complex series of events of new vessel growth. The Vascular Endothelial Growth Factor (VEGF) by engagement with its receptor appears to be a master regulator of this process [16]. The signal transduction pathways between its receptors and the final effectors are only fragmentarily known. However, the knowledge of these pathways and their interrelationships is extremely important since it may provide new targets of antiangiogenic therapies. It has been demonstrated that a major pathway through which VEGF promotes endothelial cell survival is PI 3-kinase-dependent activation of the anti-apoptotic kinase Akt/protein kinase B. A large body of literature has documented hyperactivation of Akt during tumor angiogenesis [17]. Therefore, the idea of blocking angiogenesis as a therapeutic target for fighting cancer has an enormous intellectual appeal. It should be noted that out of 22 angiogenesis inhibitors currently under active clinical trials, 11 are natural products or natural-based compounds [13]. Among the phytochemicals, an antiangiogenic role has been hypothesized for sanguinarine (SA; 13-methyl [16, 18] benzodioxolo [5,6-c]-1,3-dioxolo [4,5-i] phenantridinium), a benzophenantridine alkaloid derived from the plant Sanguinaria canadensis [14]. Therefore, the present study was aimed to evaluate the effect of SA, in presence or absence of VEGF, first on porcine aortic endothelial cell (AOC) growth in a three-dimensional fibrin gel matrix [4] and then on Akt phosphorylation.

2. Material and methods

All reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise specified.

2.1. Endothelial cell culture

An immortalized porcine aortic endothelial cell line (AOC) [8] was generously provided by José Yelamos (Hospital Universitario Virgen de la Arrixaca, El Palmar, 30120 Murcia, Spain). In all experiments, AOC at 19^{th} passage were used and seeded in culture medium (CM) composed of M199 supplemented with sodium bicarbonate (2.2 mg/ml), penicillin (100 UI/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml).

2.2. Angiogenesis bioassay

2.2.1. Three-dimensional endothelial cell culture on a fibrin gel support

The microcarrier-based fibrin gel angiogenesis assay was performed as described by Grasselli et al. [15] with some modifications. Briefly, 12.5 mg gelatin-coated cytodex-3 microcarriers in 1 ml PBS were incubated for 3 h to hydrate. After two washings in PBS and one in CM, the microcarriers were put in flasks containg 5 ml CM; AOC (5×10^5) were added and cultured for 24 h in order to let the endothelial cells coat the microcarriers. For the fibrin gel preparation, 40 μl microcarriers covered by AOC were pipetted into 6 well plates containing a solution of fibrinogen (1 mg/ml PBS, pH 7.6), added with 1250 IU thrombine (250 μl). Fibrin gels were allowed to polymerize for 30 min at 37°C, then they were

equilibrated for 60 min with 2 ml M199. After a change of the medium, AOC were treated with VEGF (100 ng/ml; PeproTech EC Ltd, London, UK) in the presence or absence of sanguinarine (300 nM). This concentration has been chosen after preliminary experiments [16] documenting that this is the most effective concentration among those previously tested. Plates were incubated at 37 °C under humidified atmosphere (5% CO_2). AOC were cultured for 192 h, renewing treatment totally every 48 h as described above.

2.2.2. Quantification of AOC growth on fibrin gel matrix

Endothelial cell proliferation in the fibrin gel matrix was evaluated by means of the public domain NIH Program Scion Image Beta 4.02 (Scion Corporation, MA, USA, http://rsb.info.nih.gov/nih-image/). Ten pictures were taken for each gel at 48, 96, 144 and 192 h; images were converted into gray scale, resized to 50% (Paintbrush Software, MS Office) and saved as Bitmap 24bit format compatible with Scion. The modified images were then imported into the program and measurements were made drawing the perimeter of the area occupied by AOC expressed as number of pixel. In order to validate the measurement of the area covered by AOC in fibrin gels as a reliable method to evaluate cell proliferation, fibrin gels were stained by the nuclear dye bis-benzimide (Hoechst 33258, 20 μg/ml in PBS for 60 min) and examined by the fluorescence microscope. This procedure was performed 20 times; for each experiment the number of nuclei were counted under fluorescence and pictures of the area covered by AOC were taken in order to measure the surface covered in the fibrin gel. A strong correlation was observed between the area covered by AOC and the number of nuclei found in the same area ($r = 0.96$).

2.3. Akt activity

2.3.1. Cell treatment

2×10^5 cells were seeded in eppendorf tubes in 1 ml CM and treated for 10 minutes at 21 °C with VEGF (100 ng/ml) in the presence or absence of SA (300 nM). After treatment, cells were centrifuged for 10 min. at 400 $\times$ g. The surnatants were then discarded and cells were lysed adding 1 ml of lysis buffer (20 mM MOPS, 50 mM β-glycerolphosphate, 50 mM sodium fluoride, 1mM sodium vanadate, 5 mM EGTA, 2mM EDTA, 1% NP40, 1 mM dithiothreitol (DTT), 1 mM benzamidine, 1 mM PMSF and 10 μg/ ml leupeptin e aprotinin) for 10 min. on ice. Cell lysates were centrifuged at 13000 $\times$ g for 15 min. Clear supernatants were collected and stored at -70 °C until assays were performed.

2.3.2. Akt activity assay

Akt activity was evaluated by Akt/PKB Kinase Activity Assay Kit (Stressgene, Victoria, Canada). This assay is based on a solid phase enzyme-linked immuno-adsorbent assay (ELISA) that utilizes a synthetic peptide as a substrate for PKB and a polyclonal antibody that recognizes the phosphorylated form of the substrate. The assay is designed for the analysis of PKB activity in the solution phase. The substrate, which is readily phosphorylated by PKB, is pre-coated on the wells of the provided PKB substrate microtiter plate. The samples to be assayed are added to the appropriate wells, followed by the addition of ATP to initiate the reaction. The kinase reaction is terminated and a phosphospecific substrate antibody, which binds specifically to the phosphorylated peptide substrate, is added to the wells. The phosphospecific antibody is subsequently bound by a peroxidase conjugated secondary antibody. The assay is developed with tetramethylbenzidine substrate (TMB) and a color develops in proportion to PKB phosphotransferase activity. The color development is stopped with acid stop solution and the intensity of the color is measured in a Spectra Shell microplate reader at 450 nm.

Table 1
Effect of SA, in the presence or absence of VEGF, on AOC growth

	C	SA	VEGF	VEGF+SA
48 h	7891 ± 407 a, A	7021 ± 503 a, A	12510 ± 812 a, B	8159 ± 332 a, A
96 h	13752 ± 699 b, A	11658 ± 345 b, A	19469 ± 1085 b, B	13385 ± 7694 b, A
144 h	25401 ± 1314 c, A	28815 ± 2234 c, A	33677 ± 1754 c, B	26652 ± 2073 c, A
192 h	34398 ± 1399 d, A	35472 ± 2321 d, A	48995 ± 835 d, B	38714 ± 1576 d, A

Values in the same column with different superscripts (a, b, c, d) are significantly ($p < 0.001$) different. Data represent the area covered by AOC in the fibrin gel (number of pixel).
Values in the same row with different superscripts (A, B, C, D) are significantly ($p < 0.001$) different. Data represent the area covered by AOC in the fibrin gel (number of pixel).

2.3.3. Statistical analysis

Data are expressed as means ± SEM of six replicates/treatment repeated in five independent experiments. Statistical analysis was performed by means of multifactorial ANOVA using Statgraphics package (STSC Inc., Rockville, MD, USA). When significant differences were found, means were compared by Scheffé F test; p values < 0.05 were considered statistically significant.

3. Results

3.1. Effect of SA on AOC growth

AOC proliferation significantly ($p < 0.001$) increased with time in all groups; as shown in Table 1, the area covered by AOC almost doubled during each 48 h incubation. AOC incubated with SA showed a growth rate similar to controls at each evaluation time. As expected, the VEGF treatment resulted always significantly ($p < 0.001$) effective in stimulating AOC proliferation. The addition of SA abolished ($p < 0.001$) VEGF stimulatory effect on cell growth at all the examined times (Table 1, Fig. 1).

3.2. Effect of SA on Akt activity

Akt activity in AOC was significantly ($p < 0.001$) increased by VEGF treatment. The stimulatory effect induced by VEGF was significantly ($p < 0.001$) inhibited by SA. On the contrary, this substance was unable to modify basal Akt activity (Fig. 1).

4. Discussion

Angiogenesis is a strictly controlled process in the healthy, adult human body. It is regulated by a variety of endogenous angiogenic and angiostatic factors. In pathological conditions such as cancer, chronic inflammation or atherosclerosis angiogenesis occurrs [12]. Biosignaling involved in angiogenic activation of endothelium is relatively well known. Extracellular signals involved in this process are mainly represented by paracrine factors, the majority of which are ligands of surface transmembrane receptors and extracellular matrix components that usually bind to integrins and to specialized receptors. Among the increasing list of angiogenesis activators lies the key molecule in this process, the Vascular Endothelial Growth Factor (VEGF, also referred to as VEGF-A) which, by binding to its receptors, is thought to play an essential role in postnatal physiological and pathological angiogenesis. VEGF by itself can initiate the angiogenic cascade of events, acting as a proliferation, migration and survival factor

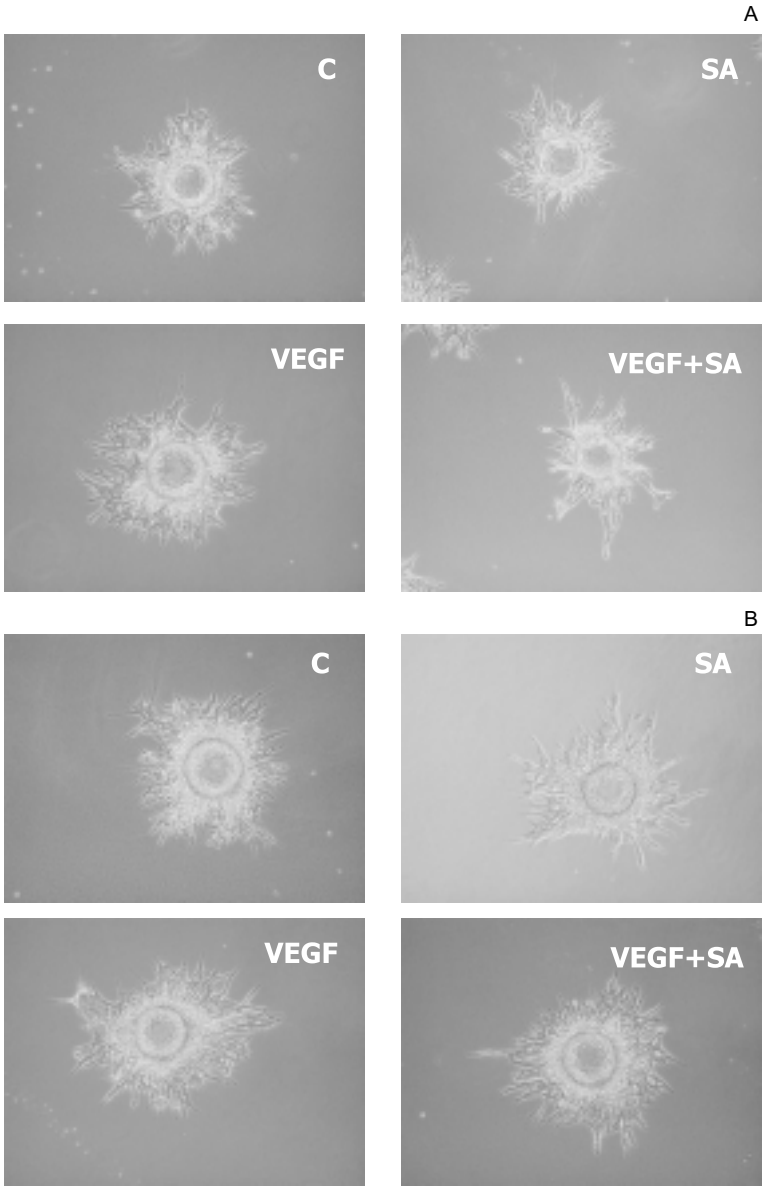

Fig. 1. Phase contrast micrographs showing AOC growth at 48 (A), 96 (B), 144 (C) and 192 h (D) in fibrin gel matrix. Cells were cultured in CM, treated with SA (300 nM) or with VEGF (100 ng/ml) in the presence or absence of SA.

for endothelial cells both in vitro and in vivo [6]. Our present data confirm that VEGF treatment, at all the examined time points, induces a marked enhancement of AOC growth in a three dimensional fibrin gel matrix. During angiogenesis process, endothelial cells proliferate, produce factors able to degrade the extracellular matrix, change their adhesive properties, migrate, avoid apoptosis and, finally, differentiate in new vascular tubes. All these events are controlled by the environmental signals whose transduction pathways form cascades leading to gene transcription and networks of cross-talk determining the final behavior of the cell. Unfortunately, the signal transduction mechanisms are not completely clarified yet.

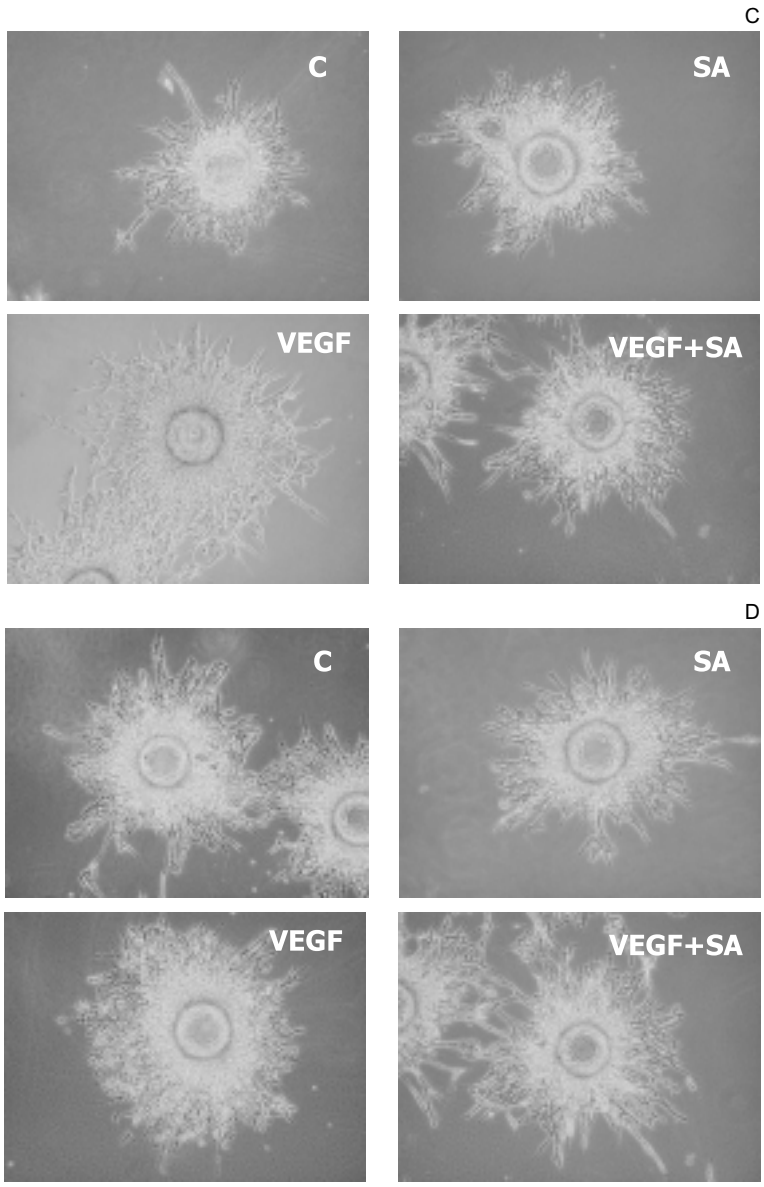

Fig. 1, continued.

However, unraveling these pathways would offer therapeutic options to ameliorate or perhaps even cure disorders that are now leading causes of mortality. It has been demonstrated that a major pathway through which VEGF promotes endothelial cell survival is PI 3-kinase (phosphoinositide 3'-kinase)-dependent activation of the anti-apoptotic kinase Akt/protein kinase B. Akt appears to be the key point of integration between VEGFR signalling and other endothelial functions [9]. Evidence supporting the role of Akt in such pathways in endothelial cells includes blockade of PI3K with inhibitors and overexpression of dominant-negative constructs for Akt [1]. In agreement with previous studies [6,10,17], present data confirm that VEGF treatment stimulates Akt activity in endothelial cells. Since the involvement of Akt activation in endothelial cell growth has been demonstrated [2], it is likely that the VEGF-induced AOC

proliferation, evidenced in present work, can be triggered by the VEGF-induced Akt phosphorylation.

It is well known that angiogenesis is crucial for malignant tumour growth and metastases and therefore it has become an attractive target for anticancer therapy. Theoretically applicable to most solid tumours, this therapy may be advantageous over existing cytotoxic therapy, since it is directed at genetically stable endothelium growing with tumours rather than at malignant cells, which acquire resistance to treatment [11]. Nowadays, angiogenesis inhibition represents an active area of cancer drug discover, with several agents and approaches now entering late stages of clinical development. Interestingly, out of 22 angiogenesis inhibitors currently under active clinical trials there are 11 natural products or natural-based compounds. This clearly shows the potential of natural products for the discovery of new lead entites as angiogenesis modulators [13]. Among the plant-derived substances, the effect of Sanguinarine (SA) from Sanguinaria Canadensis was studied in an in vitro angiogenesis bioassay. Our results, in agreement with those of Eun and Koh [14], show that this alkaloid is ineffective alone but can abolish VEGF-induced endothelial cell proliferation. Furthermore, our study evidence that the inhibitory effect of SA lasts throughout all the examined incubation times till to 192 h of culture. This is an important finding since it rules that the effect of SA is limited to the initial treatment. In addition, from our data we can argue that the antiangiogenetic effect of SA could be mediated by blocking the VEGF-induced Akt phosphorylation, a pivotal event in angiogenesis signalling. The inhibition of VEGF-induced endothelial cell proliferation by SA suggests that this substance can potentially suppress blood vessel formation in vivo since VEGF is known to be a strong migration, sprouting, survival and proliferation factor during both physiological and pathological angiogenesis [3,5]. The inhibitory effect of SA on signaling pathway could be due to a disruption of the interaction between VEGF and its receptors. Additional elucidation of detailed molecular mechanisms and the precise molecular target(s) associated with SA antiangiogenic activities of SA is the subject of ongoing investigation. Taken together, since antiangiogenic therapy represents one of the most promising new approaches to anticancer therapy, further study will be needed to better elucidate the potential antiangiogenetic effect of SA.

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