Control of capillary formation by membrane-anchored extracellular inhibitor of phospholipase A_2

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Abstract Secretory phospholipase A_2 ($sPLA_2$) has been reported to be involved in cell proliferation in general and in endothelial cell migration, processes required for capillary formation. Subsequently, we examined the potential control of angiogenesis by $sPLA_2$ inhibition, using a cell-impermeable $sPLA_2$ inhibitor composed of *N*-derivatized phosphatidyl-ethanolamine linked to hyaluronic acid. This inhibitor effectively inhibits the proliferation and migration of human bone marrow endothelial cells in a dose-dependent manner, and suppresses capillary formation induced by growth factors involved in vascularization of tumors and of atherosclerotic plaques. It is proposed that $sPLA_2$ inhibition introduces a novel approach in the control of cancer development and atherosclerosis.

Key words: Angiogenesis; Endothelial cell; Antiangiogenic therapy; Extracellular phospholipase A_2 inhibitor

1. Introduction

The development of primary tumors and metastatic lesions is dependent on angiogenesis, which is initiated by the migration of endothelial cells to the newly formed tumor and their proliferation [1]. The formation of new blood vessels is also involved in the progression of atherosclerotic plaques and its fragility and rupture, which is the main cause of acute ischemic events [2,3]. Accordingly, the control of angiogenesis, by inhibiting endothelial cell proliferation and migration, has been sought for anticancer therapy [4], and for vascular protection [5].

Phospholipase A_2 (PLA_2) constitutes a family of enzymes that in mammalian cells includes the secretory ($sPLA_2$), the intracellular cytosolic ($cPLA_2$) and calcium-independent PLA_2. These enzymes hydrolyze cell membrane phospholipids to produce lyso-phospholipids (lyso-PL) and free fatty acids, among them arachidonic acid (AA). These are potent mediators of diverse pathological processes, including inflammation [6,7], tumor growth and metastasis [8,9]: lyso-PL, in particular lyso-phosphatidic acid, acts as a growth factor and induces cancer cell proliferation [10]. AA is metabolized in mammalian cells to form the eicosanoids, mainly via the cyclooxygenase (COX) pathway, producing prostaglandins and thromboxanes, and the lipoxygenase (LOX) pathway, producing leukotrienes and hydroxyeicosatetraenoic acids [11].

AA and its metabolites are involved in the development of several types of cancer in humans, including colon, breast, gastric and hepatocellular carcinomas [12–15]. Eicosanoids derived from both COX and LOX pathways have been shown to facilitate the invasiveness of tumor cells, and to induce tumor vascularization [16,17]. Indeed, a number of studies have suggested that PLA_2 activity plays an important role in growth factor-induced endothelial cell migration, either directly or via the production of AA derivatives. For instance, the angiogenic activity of the inflammatory cytokine oncostatin M (OSM) is related to COX2 expression, as it can be suppressed by COX2 inhibitors [18]. It has been shown that the COX-2-induced metabolites of AA specifically [thromboxane A2, prostaglandin (PG) PGE2 and PGI2] facilitate endothelial cell migration and growth factor-induced angiogenesis [19]. Although $cPLA_2$ is specific to AA-carrying phospholipids, in activated cells and pathological conditions the majority of AA is produced primarily by $sPLA_2$ [20]. In addition, $sPLA_2$, secreted into plasma and body fluids by activated white cells, induces cell proliferation via a binding-mediated process [21]. It has also been reported that PLA_2 activity, particularly that of $sPLA_2$, is required for basic fibroblast growth factor (bFGF)-induced endothelial cell motility, as it can be suppressed by $sPLA_2$ inhibitors [22,23]. Therefore, it seems that $sPLA_2$ is involved in tumor vascularization in more than one way.

Accordingly, inhibition of $sPLA_2$ activity has been proposed for the control of related pathological states [24–27], and a number of inhibitors have been proposed for this purpose. However, inhibitors that penetrate into the cell might impair the vital phospholipid metabolism and the cell viabil-

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Abbreviations: AA, arachidonic acid; bFGF, basic fibroblast growth factor; COX, cyclooxygenase; ExPLI, extracellular PLA_2 inhibitor; FCS, fetal calf serum; HBMEC, endothelial cells from human bone marrow; HyAc, hyaluronic acid; HyPE, *N*-derivatized phosphatidyl-ethanolamine linked to hyaluronic acid; LOX, lipoxygenase; Lyso-PL, lyso-phospholipids; MVGS, microvascular growth supplement; OSM, oncostatin M; PG, prostaglandin; PLA_2, phospholipase A_2; $sPLA_2$, secretory phospholipase A_2; VEGF, vascular endothelial growth factor

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ity. Consequently, it has been suggested that an inhibitor of PLA_2 activity at the cell membrane, which does not enter the cell, is more suitable for therapeutic purposes [25,27].

Extracellular PLA_2 inhibitors (ExPLIs) which fulfill these requirements have been designed and synthesized in the laboratory of S. Yedgar, by the binding of PLA_2 inhibitors to polymeric carriers of different molecular weights [27,28]. This was done in a way that enables the inhibiting molecule to control the PLA_2 activity at the cell membrane, but its internalization is prevented by the polymeric carrier. The ExPLIs have been found effective in protecting cells from the action of different types of $sPLA_2$ enzymes [28] as well as activation of endogenous $sPLA_2$ by inflammatory mediators [29,30].

In the present study, we have examined the potential use of ExPLIs, as a prototype for cell-impermeable $sPLA_2$ inhibitors, for the control of angiogenic processes [1,4]. For this purpose we determined the ExPLI effect on the proliferation and migration of, and capillary formation by human bone marrow endothelial cells (HBMEC), induced by the angiogenic factors bFGF and vascular endothelial growth factor (VEGF), involved in tumor vascularization, as well as OSM, involved in neovascularization of atherosclerotic plaques [31,32].

2. Materials and methods

2.1. Materials

Endothelial cells from human bone marrow (HBMEC) were established by transfecting human bone marrow endothelial cells with SV40 large T antigen for immortalization. They are kindly provided by Dr. Kenneth J. Pienta (Cancer center, Ann Harbor, MI, USA). Cells were cultured in complete M131 medium (Cascade Biologics) supplemented with 10% fetal calf serum (FCS), 5% microvascular growth supplement (MVGS) which contains 5% FCS, hydrocortisone, human bFGF, heparin, human epidermal growth factor and dibutyryl cAMP (Cascade Biologics), 1% L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin.

The ExPLI employed in the present study was composed of hyaluronic acid-linked *N*-derivatized phosphatidyl-ethanolamine (HyPE), designed and synthesized in the laboratory of S. Yedgar, by truncating hyaluronic acid (Gideon Richter, Budapest, Hungary) to a MW of 50–100 kDa, and linking it to the amino head group of dipalmitoyl-phosphatidyl-ethanolamine (PE) [29].

2.2. Cell proliferation assay

For the proliferation assay, we used the minimal concentration of FCS (7.5%) to allow sufficient viability of endothelial cells. Briefly, after trypsinization, the cells were seeded at a concentration of 5×10^4 cells in each well of 24-well plates (Nunc, Denmark) and then incubated without or with cytokines (25 ng/ml bFGF, 20 ng/ml VEGF or 2.5 ng/ml OSM). HyPE was added at concentrations indicated in Section 3. After incubation for 2 days, cells were detached by trypsin (0.05% w/v; Sigma), resuspended in Isoton II solution (Coulter, France) and counted in a particle counter (CoulterZ1, Coultronics). Hyaluronic acid (HyAc) was used as control.

2.3. Cell migration by wound healing method

Endothelial cells were cultured in 24-well culture plates. After reaching 80% confluence, HBMEC were dislodged by a cell scraper under standard conditions, as previously described [33]. After three times washing the cells with phosphate-buffered saline, the cells were incubated in complete M131 medium in the presence of increasing HyPE concentration (HyAc was used as control). Cell migration was determined by measuring the number of cells migrating into 10 mm^2 'wounding area', 18 h after introducing 'the wound' into the confluent monolayer.

2.4. Formation of capillary tubes in a model of angiogenesis in fibrin gels

HBMEC were embedded in fibrin matrix, which mimics the in vivo situation where fibrin appears to be a common component of the matrix present at sites of inflammation and/or tumor stroma. This model with microcarrier beads was devised according to the method of Nehls et al. [33]. Briefly, HBMEC were allowed to attach to the Cytodex-3 microcarrier beads (Sigma) by incubating cells with beads in M131 containing 10% FCS. The beads were then embedded in a fibrin matrix, obtained by coagulation of a solution of purified fibrinogen at 8 mg/ml in M131 containing 0.2 mM aprotinin, 10% FCS. After addition of thrombin (2 U/ml, final concentration), fibrin gel was formed, and then 500 μl of complete culture medium containing 10% FCS, 5% MVGS was added. Formation of capillary tubes evolving from the surface of the microcarrier beads could be observed after 3 days culture. These capillaries were photographed, the lengths and widths of capillary tubes were measured using the microvision Saisam program (Microvision, Evry, France) on a reverse microscope. Capillary formation was induced by the addition of bFGF (25 ng/ml final concentration), or VEGF (20 ng/ml final concentration), or OSM (2.5 ng/ml final concentration) to the fibrin gel (containing 10% FCS).

The effect of HyPE on the capillary formation was examined in two ways: (1) HyPE (20 μM) was added to the fibrin simultaneously with the HBMEC-coated beads and growth factors; (2) HyPE was added after interaction of the cells with the growth factors, to rule out the possibility that HyPE interferes with the interaction of the growth factors with the cell surface. Specifically, HBMEC-coated beads were incubated with the growth factors for a period of 3 h, after which they were washed and incorporated into the fibrin gel containing HyPE (20 μM).

2.5. Statistical tests

Significance values were determined using the two tailed non-parametric Mann–Whitney and Wilcoxon test using Instat software. All results are expressed as the mean value ± S.E.M. $P<0.05$ was regarded as significant statistical difference.

3. Results

To comprehensively study the possible control of angiogenic processes by inhibition of $sPLA_2$ activity, we determined the effect of HyPE on HBMEC proliferation, migration and capillary formation. As noted in Section 2, the medium used for cultivation of the endothelial cells is already supplemented with growth factors. To examine the effect of HyPE on growth factor-induced endothelial cell activation, we further supplemented the culture medium with bFGF, VEGF and OSM, which further increased HBMEC stimulation by about 50%, as shown below.

3.1. Effect of HyPE on HBMEC proliferation

Fig. 1 shows that HyPE effectively inhibited HBMEC proliferation in a dose-dependent manner, whether the cells were grown in the control culture medium containing standard amount of growth factors, or stimulated with additional amount of VEGF, bFGF or OSM. At the same time, HyAc did not affect the cell proliferation (not shown). It should be

Table 1
Inhibitory effect induced by HyPE on endothelial cell migration by wound healing method

	Number of migrated cells
HBMEC	68 ± 7 (complete wound healing)
HBMEC+HyPE 2.5 μM	64 ± 9 (complete wound healing)
HBMEC+HyPE 5 μM	37 ± 5 (incomplete wound healing)***
HBMEC+HyPE 10 μM	13 ± 6***
HBMEC+HyPE 20 μM	8 ± 3***

After cell scraping, the cells were incubated in complete M131 medium in the absence or presence of HyPE at indicated final concentrations. After 18 h incubation, cells that migrated in 10 mm^2 wounding area were counted. Results of three experiments in duplicate (mean ± S.E.M.). ***$P<0.005$ as compared with control.

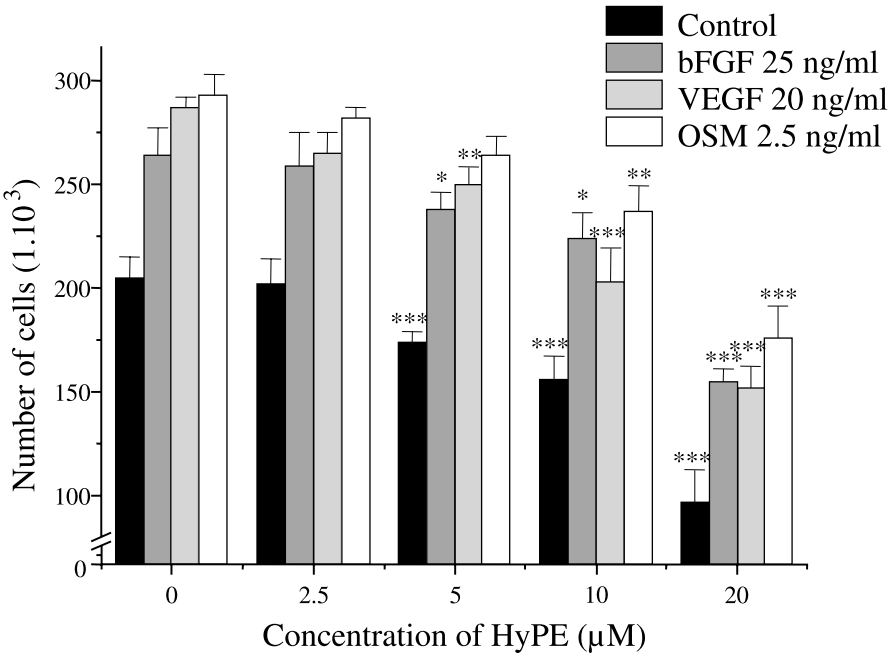

Fig. 1. Inhibitory effect induced by HyPE on bFGF-, VEGF- and OSM-stimulated HBMEC proliferation. 50×10^3 HBMEC-1 were seeded, incubated for 2 days with a minimal concentration of FCS (7.5%) to assure viability of the cells with indicated concentrations of HyPE, and with or without addition of angiogenic factors. Then cells in each well were then counted in a particle counter. Results of four experiments in duplicate, expressed as mean ± S.E.M. per well. ***$P<0.001$, **$P<0.01$, *$P<0.05$, in comparison to cells without HyPE.

noted that HyPE did not induce a toxic effect in the endothelial cells.

3.2. Effect of HyPE on HBMEC migration

HBMEC displacement was monitored following wound formation for 18 h, by determining the migration of the cells in the wounded area. The results, presented in Table 1, show that this period of time was sufficient for complete wound covering in normal conditions, but the addition of HyPE exerted a dose-dependent inhibition of HBMEC migration.

3.3. Effect of HyPE on capillary formation by HBMEC

To study the effect of HyPE on tube formation, HBMEC were cultured on microcarrier beads until they covered the whole bead surface. The beads were embedded in a fibrin matrix and cultivated for 3 days in the control or growth factor-supplemented culture medium. The formation of the HBMEC-derived hollow tube-like structures was visualized and their capillary length was determined as described in Section 2.

Results presented in Fig. 2 show that tube formation in fibrin matrix was enhanced by all angiogenic factors (bFGF, VEGF, and OSM), but this was strongly suppressed by HyPE both in control and in growth factor-supplemented medium. HyAc did not modify the capillary tube formation in both control and stimulated conditions. As shown in Table 2, HyPE clearly decreased the tube dimensions, in particular their length.

As described above, the ExPLIs are composed of an inhibiting lipid moiety (PE) which incorporates into the cell membrane and anchors the polymeric carrier (HyAc) to the cell surface. This raises the possibility that the observed inhibitory effect might be due to interference of the polymeric carrier with the accessibility of the growth factors to the cell surface. To examine this possibility, HBMEC cultured on the microcarrier beads were first stimulated with the growth factors for 3 h (to allow interaction with their receptors at the cell surface), then washed to remove the unbound growth factors and introduced into HyPE-containing fibrin matrix. As shown in Fig. 3, under these conditions, capillary tube formation was

Table 2
Inhibitory effect induced by HyPE on bFGF-, VEGF- and OSM-stimulated capillary tube formation in a three-dimensional fibrin gel

	Length (mean ± S.E.M.) (μm)		Width (mean ± S.E.M.) (μm)	
	−HyPE	+HyPE	−HyPE	+HyPE
Control	232.23 ± 56.13	80.31 ± 30.59***	9.42 ± 1.65	8.32 ± 1.47
bFGF	533.92 ± 65.02	266.73 ± 23.17***	15.83 ± 2.96	11.21 ± 1.52*
VEGF	511.09 ± 72.05	215.68 ± 31.22***	14.86 ± 1.46	9.32 ± 1.18**
OSM	518.82 ± 58.49	234.85 ± 36.32***	16.89 ± 1.89	10.02 ± 1.00***

***$P<0.005$, **$P<0.01$, *$P<0.05$.
Results of three experiments and for each experiment, five beads were examined. Selected beads were those for which the capillary tubes are the best.
Microcarrier beads coated with HBMEC were embedded in fibrin matrix in the presence or absence of HyPE (20 μM, final concentration) and growth factors (25 ng/ml bFGF, 20 ng/ml VEGF or 2.5 ng/ml OSM), as indicated in Section 2. After 3 days culture, in the presence or absence of HyPE the lengths and widths of capillary tubes were measured using a microvision program.

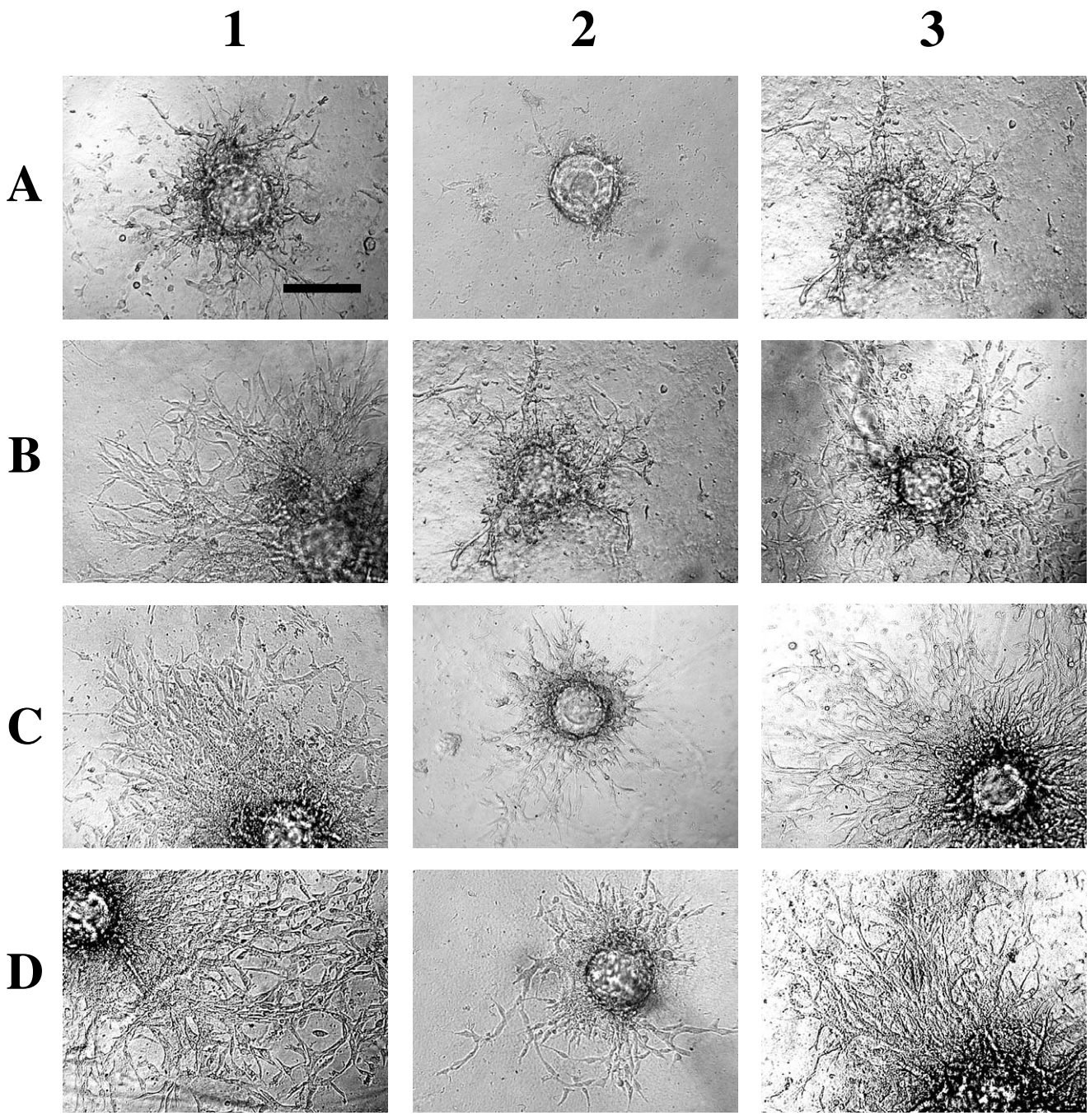

Fig. 2. Inhibitory effect induced by HyPE on capillary tube formation in a three-dimensional fibrin gel in conditions where growth factors, HyPE (20 μM) or HyAc were added to fibrin simultaneously with HBMEC-1-coated beads. Row A: control; row B: bFGF (25 ng/ml); row C: VEGF (20 ng/ml); row D: OSM (2.5 ng/ml). Column 1: without HyPE; column 2: HyPE 20 μM; column 3: HyAc 20 μM.

effectively suppressed by HyPE, suggesting that the HyPE effect is not due to a defective growth factor accessibility due to steric hindrance by the polymer at the cell surface of the endothelial cells.

4. Discussion

Previous studies have suggested a role for $sPLA_2$ in cell motility. Rizzo et al. [23], testing the effect of several $sPLA_2$s on bovine aortic endothelial cells, found that an $sPLA_2$ inhibitor markedly decreased this endothelial cell migration, and proposed that $sPLA_2$ plays a physiological role in this process. Similarly, Sa et al. [22] showed that the stimulating effect of bFGF on wound-induced movement of bovine aortic endothelial cells was markedly reduced by pharmacological inhibition of PLA_2, suggesting that bFGF-induced cell migration involves PLA_2 activity.

The results of the present study demonstrate that the extracellular $sPLA_2$ inhibitor, HyPE, is a potent regulator of several essential processes required for angiogenesis, specifically, proliferation and migration of human endothelial cells and capillary formation induced by growth factor which are involved in vascularization of tumors (bFGF, VEGF) and atherosclerotic plaque (OSM) [31,32]. The suppression of capil-

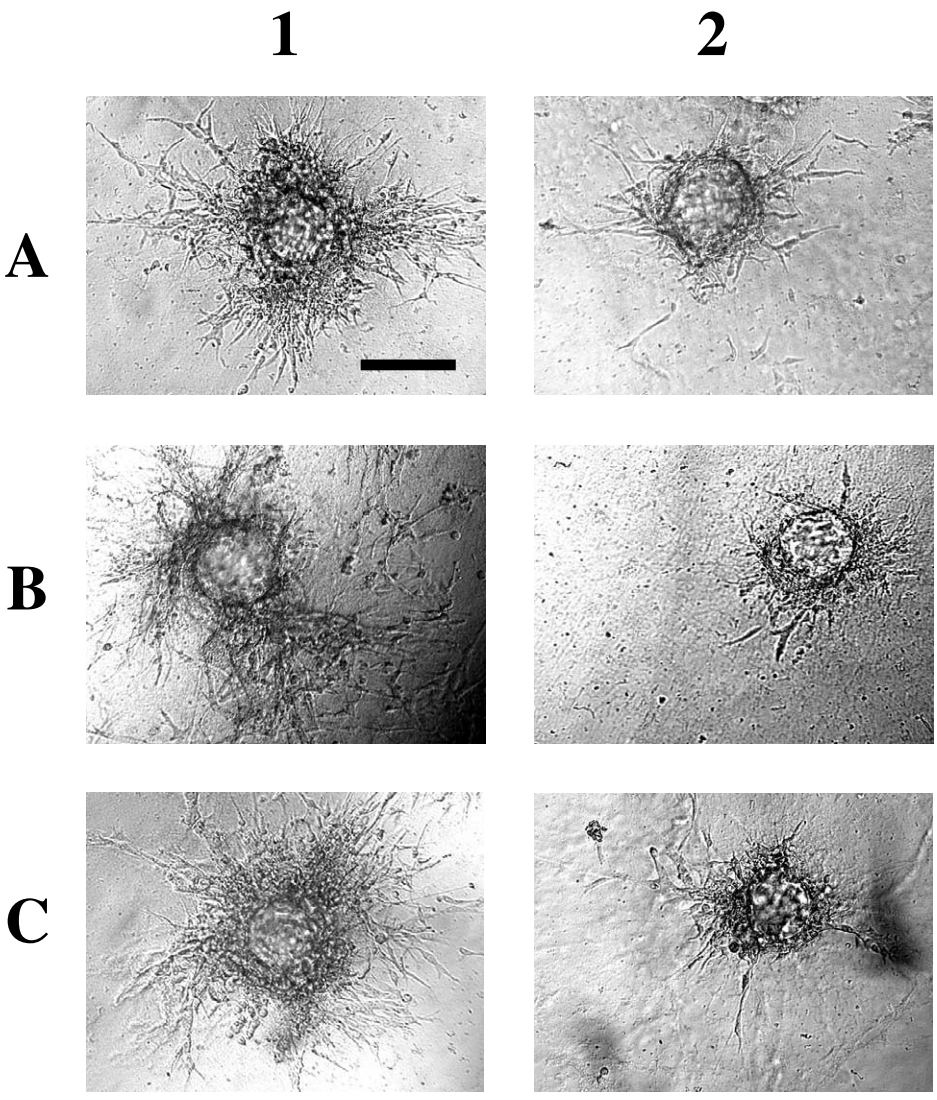

Fig. 3. Inhibitory effect induced by HyPE on capillary tube formation in a three-dimensional fibrin gel in conditions where HBMEC-coated beads were firstly incubated with the growth factors for a period of 3 h, and washed and then incorporated into the fibrin gel without or with HyPE. Row A: bFGF (25 ng/ml); row B: VEGF (20 ng/ml); row C: OSM (2.5 ng/ml). Column A: without HyPE; column B: with HyPE (20 μM).

lary formation was obtained whether HyPE was added prior to or after stimulation of the cells with the growth factors. Together with the finding that HyAc alone did not affect capillary formation, this suggests that HyPE exerts its inhibitory effect not by hindering the accessibility of the growth factors to the cell surface, but rather by interfering in the signaling process initiated by the growth factors, although the exact mechanism(s) of their effect remains to be elaborated. Taken together, these findings provide support for the key role of $sPLA_2$ in the pathophysiology relating to stimulated angiogenesis and organ vascularization.

Endothelial cell proliferation and angiogenesis involved the participation of various lipid mediators, including lyso-LPs and different eicosanoids, the production of which is initiated by PLA_2. Therefore, by suppressing membrane lipid hydrolysis [28,30], the ExPLIs have the potential to control angiogenesis, by regulating the production of more than one mediator.

All in all, the results and considerations presented in the present study support the regulating role of involvement of $sPLA_2$ in processes, present the ExPLIs as cell-impermeable PLA_2 inhibitors for the control of angiogenesis, thus introducing a novel approach in the research and possibly therapy of cancer and other pathological conditions involving organ vascularization.

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