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Effect of an oversulfated exopolysaccharide on angiogenesis induced by fibroblast growth factor-2 or vascular endothelial growth factor in vitro

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Abstract

The aim of this study was to determine the angiogenic properties of an oversulfated exopolysaccharide (OS-EPS) derived from a polysaccharide secreted by the mesophilic bacterium *Alteromonas infernus.* We compared the effect of this OS-EPS with that of a non-oversulfated exopolysaccharide (EPS) on human umbilical vein endothelial cell (HUVEC) proliferation, migration and differentiation induced by basic fibroblast growth factor (FGF-2) or vascular endothelial growth factor (VEGF). OS-EPS enhanced HUVEC proliferation by 58% when used alone, and by respectively 30% and 70% in the presence of FGF-2 and VEGF. OS-EPS also increased the density of tubular structures on Matrigel in the presence of FGF-2 or VEGF. Vascular tube formation was related to α_6 integrin subunit expression, which was enhanced by 50% in the presence of the growth factors. Indeed, a monoclonal anti-α_6 blocking antibody abolished this vascular tube formation. EPS had no effect in any of the experimental conditions, underlying the importance of sulfation in the angiogenic effects of exopolysaccharide. By potentiating the angiogenic activity of FGF-2 and/or VEGF, OS-EPS, which possesses low anticoagulant activity and thus a low hemorrhagic risk, could potentially be used to accelerate vascular wound healing or to promote the growth of collateral blood vessels in ischemic tissues.

Keywords: Angiogenesis; Polysaccharide; Angiogenic growth factor; α_6 Integrin subunit; Collagen gel; Matrigel

INTRODUCTION

Angiogenesis – the formation of new blood vessels from a preexisting vascular network – is a complex and tightly regulated process [1]. Abnormal angiogenesis participates in tumor development and angioproliferative diseases [2]. However, pharmacological stimulation of angiogenesis could be used to accelerate vascular wound healing and to promote the growth of collateral blood vessels in ischemic tissues [3, 4].

Angiogenesis is stimulated by a large number of growth factors, notably including FGF-2 and VEGF. FGF-2 is mainly released by damaged cells at wound edges [5]. VEGF production is upregulated in hypoxic conditions such as those found in ischemic tissues [6]. In response to angiogenic growth factors, endothelial cells degrade the basement membrane of the parent vessel, migrate into the surrounding interstitial matrix towards the angiogenic stimulus, proliferate to prolong new blood vessels, and differentiate into contiguous tubular sprouts which subsequently anastomose to form functional capillary loops [7]. FGF-2 and VEGF show angiogenic activity in animal models [8]. Some extracellular matrix components, such as laminin, collagen and fibronectin, also regulate endothelial cell functions (invasion, migration, proliferation and differentiation) via integrins -- cell surface proteins whose expression can be induced by angiogenic growth factors [9].

The angiogenic effects of FGF-2 and VEGF, which belong to the heparin-binding growth factor family, can be modulated by polysaccharides [10, 11]. These growth factors interact with HSPG (low-affinity receptors), thereby inducing an angiogenic signal through tyrosine-kinase receptors (high-affinity receptors) [12, 13]. Polysaccharides can potentiate the angiogenic activities of growth factors by protecting them from enzymatic degradation and by binding to them, thereby promoting tyrosine-kinase receptor dimerization [14, 15]. They can also interfere with the binding of angiogenic growth factors to tyrosine-kinase receptors or HSPG by charge competition with HSPG [16].

A newly discovered exopolysaccharide -- an acidic hetero-polysaccharide -- is synthesized and secreted by a new species of mesophilic bacteria named *Alteromonas infernus*, isolated from a fluid sample collected among a dense population of *Riftia pachyptila* tubeworms in the vicinity of an active hydrothermal vent [17]. This bacterium is classified as non-pathogenic by the Pasteur Institute. The native exopolysaccharide has a high molecular weight (10^6 g/mol) with low sulfate content (≤10%). Structural characterization has shown that this exopolysaccharide is a highly branched acidic heteropolysaccharide composed of neutral sugars (glucose, galactose) and uronic acids (glucuronic acid and galacturonic acid) [18]. The native exopolysaccharide was chemically sulfated and depolymerized with free radicals, with a view to obtaining a bioactive compound compatible with a therapeutic use. We have previously shown that this oversulfated LMW exopolysaccharide derivative (40% sulfate groups) exhibits anticoagulant properties. It is less anticoagulant than heparin (a widely used antithrombotic drug), and should therefore carry a lower risk of bleeding [19]. To explore its capacity to promote angiogenesis in ischemic tissues, we studied the effects of this oversulfated LMW exopolysaccharide (OS-EPS) in comparison with a non-oversulfated species (EPS), on human umbilical vein endothelial cell proliferation, migration and differentiation in the presence of FGF-2 or VEGF. We have previously shown that the potentiation of FGF-2 angiogenic properties by fucoidan, a polysaccharide extracted from brown marine algae, is related to overexpression of integrin α_6 subunit on the endothelial cell surface [20, 21]. We therefore also studied the effect of the two LMW exopolysaccharide derivatives on α_6 integrin subunit expression. OS-EPS was compared with EPS in order to study the effect of sulfate content on the biological properties of the exopolysaccharide.

MATERIALS AND METHODS

Materials

Medium M199 (containing Hank's salts, L-glutamine and 25 mM HEPES), RPMI 1640 culture medium, antibiotics (penicillin and streptomycin), L-glutamine, Hank's balanced salt solution with or without phenol red, PBS, HEPES, 0.05% trypsin/0.02% EDTA, and Versene were from Gibco BRL; FBS was from ATGC; collagenase A was from Roche Diagnostics; and gelatin was from Sigma Chemical Co. Growth factor-reduced Matrigel™ (without detectable FGF-2 or VEGF) was from BD Biosciences. Rat tail tendon type I collagen was from Biogenesis. Human recombinant FGF-2 and human recombinant VEGF of the 165 amino-acid form were from AbCys. Rat anti-human α_6 integrin subunit mAb (CD49f) conjugated to phycoerythrin was from BD Biosciences. Anti-human α_6 subunit blocking antibody and its corresponding irrelevant isotype matched antibody were from Chemicon International.

The two LMW exopolysaccharides were obtained from HMW exopolysaccharide (GY 785) by previously described procedures [19, 22, 23]. EPS and OS-EPS are homogenous fractions with average molecular mass of 13 kDa and 24 kDa respectively and 10% and 40% sulfate groups, respectively, as determined by analytical high-performance size-exclusion chromatography [19].

Cell culture

Endothelial cells were isolated from human umbilical cord veins by enzymatic digestion with 0.1% collagenase as previously described [24]. HUVEC were cultured in M199 and RPMI medium (v/v) supplemented with 20% FBS, 2 mM L-glutamine, 10 mM HEPES, 2.5 µg/ml Fungizone, 80 units/ml penicillin and 80 µg/ml streptomycin. They were seeded into flasks pre-coated with 0.5% gelatin and incubated in humidified 5% CO2-air at 37°C. Endothelial

cells were identified by their typical cobblestone morphology. All experiments were performed with third-passage HUVEC.

Endothelial cell proliferation

HUVEC were seeded in 0.5% gelatin-covered wells. After 24 h of incubation, the culture medium was replaced with medium supplemented with 5% FBS and containing OS-EPS or EPS, each tested at 10 µg/ml, with or without FGF-2 (5 ng/ml) or VEGF (10 ng/ml). The medium was renewed every 2 days. Cells were detached with Versene-0.01% collagenase and counted with a hemocytometer after 3 days (FGF-2 conditions) or 6 days (VEGF conditions).

Endothelial cell migration assay

HUVEC (1.5×10^4 cells) in culture medium with or without 1 ng/ml FGF-2 or VEGF and 10 µg/ml OS-EPS or EPS were seeded in 0.5% gelatin-coated Transwell chambers (BD Biosciences). The chambers were inserted into 24-well plates containing culture medium with or without 10 ng/ml FGF-2 or VEGF and 10 µg/ml OS-EPS or EPS. Following incubation for 6 h, cells that remained on the upper side of the Transwell membrane were removed with cotton swabs. The cells on the filter were fixed with 1.1% glutaraldehyde and stained with Giemsa. Total cell counts per filter were determined by light microscopy.

***In vitro* angiogenesis assay**

Endothelial cell treatment: for FGF-2 experiments, HUVEC (3×10^5 cells/well) were seeded in 0.5% gelatin-covered 6-well plates. After 24 h of incubation, the medium was replaced by medium containing OS-EPS or EPS (10 µg/ml), with or without FGF-2 (5 ng/ml). The medium was renewed after 2 days of treatment. After 3 days of incubation the cells were detached with Versene-0.01% collagenase. For VEGF experiments, HUVEC (1.2×10^5

cells/well) were seeded in type I collagen (1.5 mg/ml)-covered 24-well plates. After 24 h of incubation, the medium was replaced every 2 days by medium containing OS-EPS or EPS (10 µg/ml), with or without VEGF (10 ng/ml). In the presence of VEGF, HUVEC formed tubular structures. After 6 days, HUVEC were detached by digestion with Versene-0.1% collagenase. After detachment, HUVEC were used for the *in vitro* Matrigel angiogenesis assay and for quantification of integrin α_6 subunit expression by means of flow cytometry (see below).

In vitro Matrigel angiogenesis assay: after treatment (see above), HUVEC were resuspended in culture medium supplemented with 5% FBS and seeded onto Matrigel-covered 48-well plates (3×10^4 cells/well). Before seeding onto Matrigel, part of HUVEC was resuspended in medium containing 10 µg/ml anti-α_6 blocking antibody or irrelevant isotype-matched antibody. After 18 h of incubation, the cells were fixed with 1.1% glutaraldehyde. Matrigel was dehydrated with 75% ice-cold ethanol then 96% ethanol before staining with Giemsa. Tube formation was examined by phase-contrast microscopy and was quantified with Mesurim software (http:/www.ac-amiens.fr/svt/outilprat/Mesurim/Index.htm).

Flow cytometry

After treatment, HUVEC were centrifuged (200 *g* for 8 min at 4°C) and re-suspended in Hanks-2% FBS. HUVEC (10^5 cells) were then incubated with an anti-α_6 integrin subunit antibody for 30 minutes at 4°C, and analyzed in a FACSCan flow cytometer (BD Biosciences).

Statistical analysis

The effects of OS-EPS and EPS were analyzed using ANOVA and Fisher's protected least-significant-difference test.

RESULTS

Oversulfated exopolysaccharide enhances both FGF-2- and VEGF-induced HUVEC proliferation

HUVEC proliferation was increased by 52% by FGF-2 (after 3 days of treatment, $p<0.0001$) and 87% by VEGF (after 6 days of treatment, $p<0.0001$) compared to control (Fig. 1). Compared to FGF-2 alone, OS-EPS + FGF-2 increased HUVEC proliferation by about 30% ($p<0.05$). Compared to VEGF alone, OS-EPS + VEGF increased HUVEC proliferation by 70% ($p<0.01$). OS-EPS alone had no effect on HUVEC proliferation after 3 days, but increased it by 58% ($p<0.01$) after 6 days (Fig. 1).

EPS, used alone or combined with FGF-2 or VEGF, had no effect on HUVEC proliferation (Fig. 1).

Oversulfated exopolysaccharide modulates both FGF-2- and VEGF-induced HUVEC migration

In the presence of a FGF-2 or VEGF concentration gradient, HUVEC migration increased by about 40% ($p<0.0001$) compared to control (Fig. 2). OS-EPS + FGF-2 inhibited HUVEC migration by 20% ($p<0.05$) compared to FGF-2 alone. OS-EPS + VEGF did not modify HUVEC migration relative to VEGF alone. Compared to untreated controls, OS-EPS alone increased HUVEC migration by 28% ($p<0.05$) (Fig. 2).

EPS had no effect on HUVEC migration when used alone or in combination with FGF-2 or VEGF (Fig. 2).

Oversulfated exopolysaccharide enhances FGF-2-induced HUVEC differentiation

At the end of treatment on gelatin, HUVEC differentiation was estimated in terms of vascular tube formation on Matrigel and α_6 integrin subunit expression.

Untreated HUVEC did not form tubular-like structures after 18 h on Matrigel; HUVEC pretreated with OS-EPS alone fused and aligned but were unable to form measurable vascular tube network (Fig. 3A). When pretreated with FGF-2, HUVEC migrated, aligned and formed tubes that were partially organized into a vascular tube network. When added to FGF-2, OS-EPS enhanced the capillary-like network density and led to organization in closed areas (Fig. 3A). In the presence of OS-EPS, the total length of tubular structures was increased by 56% ($p<0.01$) relative to experiments with FGF-2 alone (Fig. 3B). The use of anti-α_6 blocking antibody showed an inhibition of FGF-2 or VEGF-induced tube formation in the absence or the presence of OS-EPS (Fig. 3A) whereas an irrelevant isotype-matched antibody induced no inhibition (data not shown).

Results of α_6 integrin subunit expression are shown in Fig. 4A and 4B. HUVEC treatment with OS-EPS alone or FGF-2 alone induced α_6 integrin subunit expression, with respectively a 0.5-fold or 3-fold increase, however only significant with FGF-2 ($p<0.0001$) compared to untreated control cells (Fig. 4B). When added to FGF-2, OS-EPS further increased α_6 integrin subunit expression by 72% ($p<0.001$) compared to FGF-2 alone (Fig. 4B).

EPS had no effect on HUVEC differentiation when used alone or in combination with FGF-2 (Fig. 3A, 4A and 4B).

Oversulfated exopolysaccharide enhances VEGF-induced HUVEC differentiation

After treatment on type I collagen, HUVEC differentiation was estimated by vascular tube formation on Matrigel and by α_6 integrin subunit expression.

On Matrigel, neither untreated HUVEC nor HUVEC pretreated with OS-EPS alone formed vascular tubes (Fig. 5A). HUVEC pretreated with VEGF formed a partially closed network on Matrigel. When added to VEGF, OS-EPS increased by 3.8 fold the density of this network, compared to VEGF alone (Fig. 5B).

Results of α_6 integrin subunit expression are shown in Fig. 6A and 6B. HUVEC treatment with VEGF upregulated α_6 integrin subunit expression 2-fold ($p<0.001$) compared to untreated control cells (Fig. 6B). When added to VEGF, OS-EPS increased α_6 integrin subunit expression by 54% ($p<0.001$) compared to VEGF alone. In the absence of VEGF, OS-EPS did not modify α_6 integrin subunit expression compared to untreated control cells (Fig. 6B). The use of anti-α_6 blocking antibody showed an inhibition of VEGF-induced tube formation in the absence or the presence of OS-EPS (Fig. 5A) whereas an irrelevant isotype-matched antibody induced no inhibition (data not shown).

EPS had no effect on HUVEC differentiation when used alone or in combination with VEGF (Fig. 5A, 6A and 6B).

DISCUSSION

In this study, we have shown the angiogenic properties of an oversulfated exopolysaccharide (OS-EPS) derived from a polysaccharide secreted by the mesophilic bacterium *Alteromonas infernus*. The original structure of this polysaccharide has been recently reported [18]. We have shown that OS-EPS increases angiogenic properties of FGF-2 or VEGF, i.e. endothelial cell proliferation and differentiation into vascular tubes; however it inhibits the effect of FGF-2-induced cell migration. The increased vascular tube formation is related to α_6 integrin subunit overexpression. These angiogenic properties of OS-EPS are

related to its sulfate content because no effect was observed with the non-oversulfated exopolysaccharide (EPS).

Angiogenesis involves endothelial cell survival, invasion, proliferation, migration and differentiation. Potent angiogenic growth factors and some extracellular matrix proteins via integrin subunits can trigger signaling pathways involved in these different steps of angiogenesis. In our study, HUVEC cultured with VEGF on type I collagen overexpressed α_6 integrin subunit and enhanced tube formation on Matrigel. To our knowledge, this is the first time that a link between α_6 integrin subunit overexpression and VEGF-induced tubular structure formation has been described. It has, however, been reported that α_6 integrin subunit associated with the β_4 subunit increases VEGF translation by inhibiting a translational repressor in breast carcinoma cells [25]. Moreover, α_6 integrin subunit plays an important role in type I collagen gel invasion [26]. Thus, positive feed-back control of the angiogenic effect of VEGF could be reinforced by α_6 integrin subunit expression. In our study, the key role of α_6 integrin subunit in vascular tube formation was underlined by the absence of vascular network after addition of the anti-α_6 antibody. This effect of α_6 was supported by its role in the interaction of the endothelial cells with two components of the extracellular matrix -- laminin (main component of Matrigel) and type I collagen -- and with two major angiogenic growth factors (FGF-2 or VEGF). In this study, we demonstrated that OS-EPS enhanced VEGF-induced vascular tube formation on Matrigel with an overexpression of α_6 integrin subunit. Like for VEGF, OS-EPS enhanced FGF-2 effects on vascular tube formation and overexpression of α_6 integrin subunit. These data confirm previous reports showing that FGF-2 alone induced α_6 integrin subunit expression on endothelial cells [27] and our previous data showing a key role of α_6 integrin subunit expression in the effect of other sulfated polysaccharides (LMW fucoidan or LMW heparin) on FGF-2-induced vascular tube formation on Matrigel [21].

Thus, OS-EPS via major angiogenic growth factors (FGF-2 or VEGF) promotes *in vitro* angiogenesis by its positive effects on vascular tube formation and cell proliferation despite its slight inhibitory effect on FGF-2-induced cell migration. Similar differential effects on the different steps of angiogenesis have also been reported with other polysaccharides. For example, in the presence of FGF-2, fucoidan (of algal origin) enhances vascular tube formation on Matrigel but has no effect on endothelial cell proliferation or migration [20, 24]. Likewise, tecogalan (of bacterial origin) inhibits endothelial cell migration and tube formation induced by VEGF, but has no effect on cell proliferation [28]. In the absence of angiogenic growth factor, OS-EPS significantly stimulated HUVEC proliferation (only after 6 days of treatment) and migration, whereas OS-EPS had no effect on HUVEC differentiation. It is not surprising that OS-EPS acts mainly via FGF-2 and VEGF. Indeed, as other sulfated polysaccharides [20, 29], OS-EPS, because of its negative charges, could be able to bind to heparin binding growth factors (such as FGF-2 and VEGF) and thus increase their proangiogenic properties.

Sulfate content, as molecular weight, is a key determinant of polysaccharide modulation of angiogenesis. Contrary to OS-EPS, EPS (the non-oversulfated species) has no effect on angiogenic activities, whether used with or without growth factors (FGF-2 or VEGF). The importance of sulfate groups in polysaccharide potentiation of growth factor-induced angiogenesis has been shown by a structure-activity relationship study with another polysaccharide, fucosylated chondroitin sulfate [30]. Moreover, unlike HMW fucoidan, oversulfated HMW fucoidan strongly inhibited FGF-2 or VEGF-induced HUVEC migration and the formation of vascular tubes [31, 32]. Modified heparin fractions made it possible to highlight sulfate groups positions of the polysaccharide involved in FGF-2 or VEGF-induced angiogenesis [33, 34]. Heparin interaction with FGF-2 requires the presence of 2-*O*-sulfate [33, 35], whereas its interaction with fibroblast growth factor receptor-1 and

thus signal transduction, requires the presence of 6-*O*-sulfate groups [33]. In contrast, 6-*O*-sulfate groups, contrary to 2-*O*-sulfate groups, are necessary for heparin to interact with VEGF [34]. Exopolysaccharide derivatives are sulfated in the 4-*O*- and 6-*O*-positions and disulfated in the 2,3-*O*-position [19]. Thus, they exhibit 6-*O*- and 2-*O*-sulfate groups which have been proved to be necessary for heparin binding to growth factors and their respective receptors. Moreover, the 4-*O*-sulfate groups could also play a role in VEGF - exopolysaccharide and fibroblast growth factor receptor-1 - exopolysaccharide interactions. Indeed, a recent study provided data on the binding of dermatan sulfate (a mammalian glycosaminoglycan) to hepatocyte growth factor/scatter factor, another potent angiogenic growth factor. The authors postulated that the 4-*O*-sulfate on the *N*-acetylgalactosamine in dermatan sulfate could adopt a similar spatial position to that of the 6-*O*-sulfate on the *N*-sulfoglucosamine of heparin, which allows this anticoagulant to bind to growth factors [36].

In conclusion, this study shows that OS-EPS acts synergistically with both FGF-2 and VEGF to induce α_6 integrin subunit overexpression and endothelial cell proliferation. OS-EPS has weak anticoagulant activity, and would therefore carry a low hemorrhagic risk. These findings imply that OS-EPS, in the presence of FGF-2 or VEGF, could be beneficial in clinical situations where active angiogenesis is required, such as wound repair and ischemia.

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FIGURE LEGENDS

Figure 1. **Effect of the exopolysaccharides on FGF-2- and VEGF-induced endothelial cell proliferation**. HUVEC were treated with FGF-2 (5 ng/ml) or VEGF (10 ng/ml), with or without OS-EPS or EPS (10 µg/ml), as described in Materials and Methods. HUVEC were counted after 3 days in FGF-2 experiments and 6 days in VEGF experiments. Each value represents the mean ± SEM of five determinations in FGF-2 experiments and four determinations in VEGF experiments. Results are expressed as percentages, 100% corresponding to untreated HUVEC (control) in each experiment. * $p<0.05$, ** $p<0.01$ and **** $p<0.0001$.

Figure 2. **Effect of the exopolysaccharides on FGF-2- and VEGF-induced endothelial cell migration**. HUVEC migration was measured using Transwell chambers. HUVEC, placed in the upper chamber, were treated with FGF-2 or VEGF, with or without OS-EPS or EPS (10 µg/ml), as described in Materials and Methods. After 6 h of incubation, cells that had migrated to the lower chamber were counted. Each value represents the mean ± SEM of duplicate determinations and is representative of three separate experiments. Results are expressed as percentages, 100% corresponding to untreated HUVEC (control). * $p<0.05$, **** $p<0.0001$.

Figure 3. **Effect of the exopolysaccharides on FGF-2-induced tube formation on Matrigel**. Experiments were performed as described in Materials and Methods. (A): photographs show vascular tube formation by HUVEC previously treated without FGF-2 (top row) or with FGF-2 at 5 ng/ml (middle and bottom rows), in the absence (left column) or in the presence of OS-EPS at 10 µg/ml (middle column) or EPS at 10 µg/ml (right column).

HUVEC previously treated with FGF-2 alone or FGF-2 plus OS-EPS or EPS were incubated with 10 µg/ml anti-α_6 antibody (bottom row). (B): in the presence of FGF-2 (alone or plus OS-EPS or EPS) corresponding to middle row of (A), tube formation was quantified with Mesurim software. Values are expressed in percentages, 100% corresponding to HUVEC treated with FGF-2 alone. Results are means ± SEM of three determinations. ** $p<0.01$.

Figure 4. **Effect of the exopolysaccharides on FGF-2-induced α_6 integrin subunit expression on HUVEC.** Experiments were performed as described in Materials and Methods. (A): an example representative of histograms of integrin α_6 subunit expression on HUVEC analyzed by flow cytometry after treatment without or with FGF-2 (5 ng/ml), in the absence or in the presence of OS-EPS (10 µg/ml) or EPS (10 µg/ml). (B): geometric mean fluorescence intensities are expressed in percentages, 100% corresponding to the value obtained with FGF-2 alone. Results are means ± SEM of four determinations. *** $p<0.001$.

Figure 5. **Effect of the exopolysaccharides on VEGF-induced tube formation on Matrigel**. Experiments were performed as described in Materials and Methods. (A): photographs show vascular tube formation by HUVEC previously treated without VEGF (top row) or with VEGF at 10 ng/ml (middle and bottom rows), in the absence (left column) or in the presence of OS-EPS at 10 µg/ml (middle column) or EPS at 10 µg/ml (right column). HUVEC previously treated with VEGF alone or VEGF plus OS-EPS or EPS were incubated with 10 µg/ml anti-α_6 antibody (bottom row). (B): in the presence of VEGF (alone or plus OS-EPS or EPS) corresponding to middle row of (A), tube formation was quantified with Mesurim software. Values are expressed in percentages, 100% corresponding to HUVEC treated with VEGF alone. Results are means ± SEM of three determinations. ** $p<0.01$.

Figure 6. **Effect of the exopolysaccharides on VEGF-induced α_6 integrin subunit expression on HUVEC.** Experiments were performed as described in Materials and Methods. (A): an example representative of histograms of integrin α_6 subunit expression on HUVEC analyzed by flow cytometry after treatment without or with VEGF (10 ng/ml), in the absence or the presence of OS-EPS (10 µg/ml) or EPS (10 µg/ml). (B): geometric mean fluorescence intensities are expressed in percentages, 100% corresponding to the value obtained with VEGF alone. Results are means ± SEM of four determinations. *** $p<0.001$.

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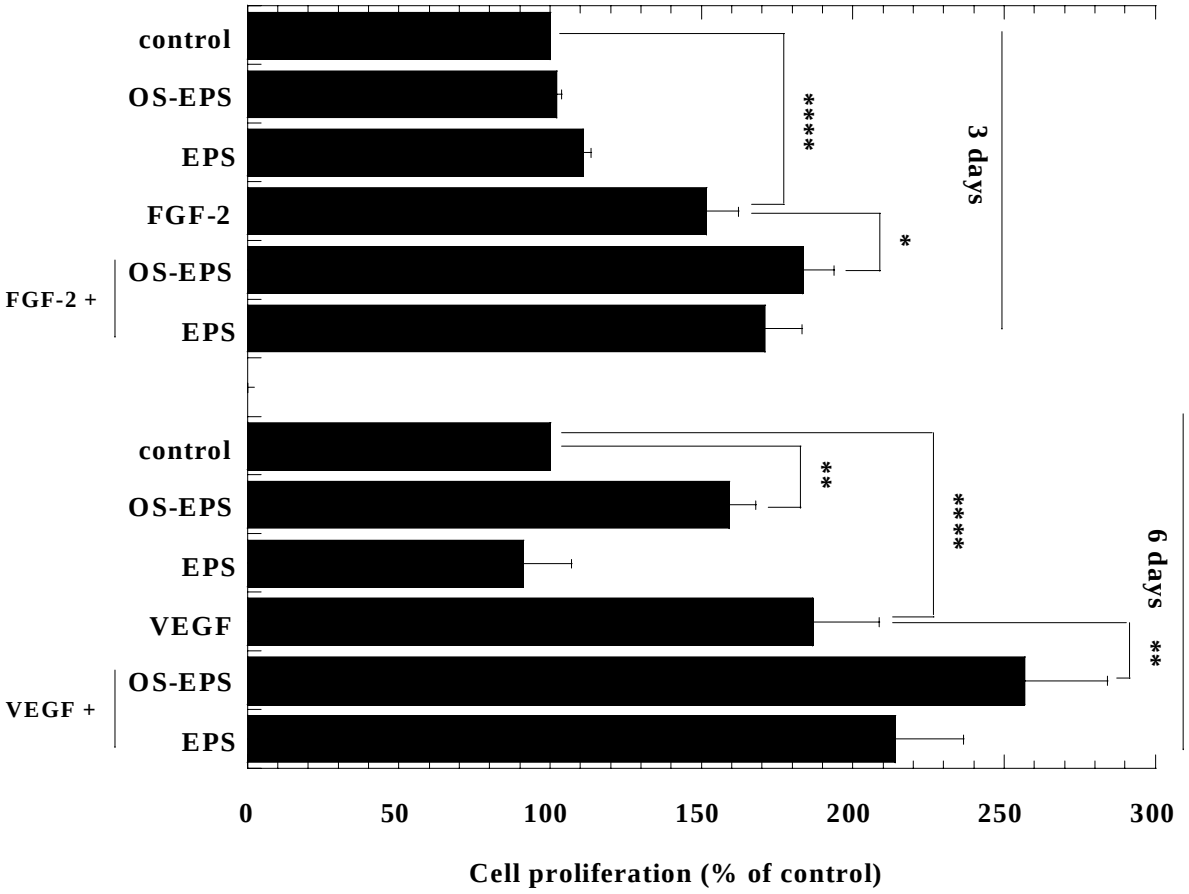

control
OS-EPS
EPS
FGF-2
FGF-2 +
OS-EPS
EPS
****
*
3 days
control
OS-EPS
EPS
VEGF
VEGF +
OS-EPS
EPS
**
****
**
6 days
0
50
100
150
200
250
300
Cell proliferation (% of control)

Figure 1

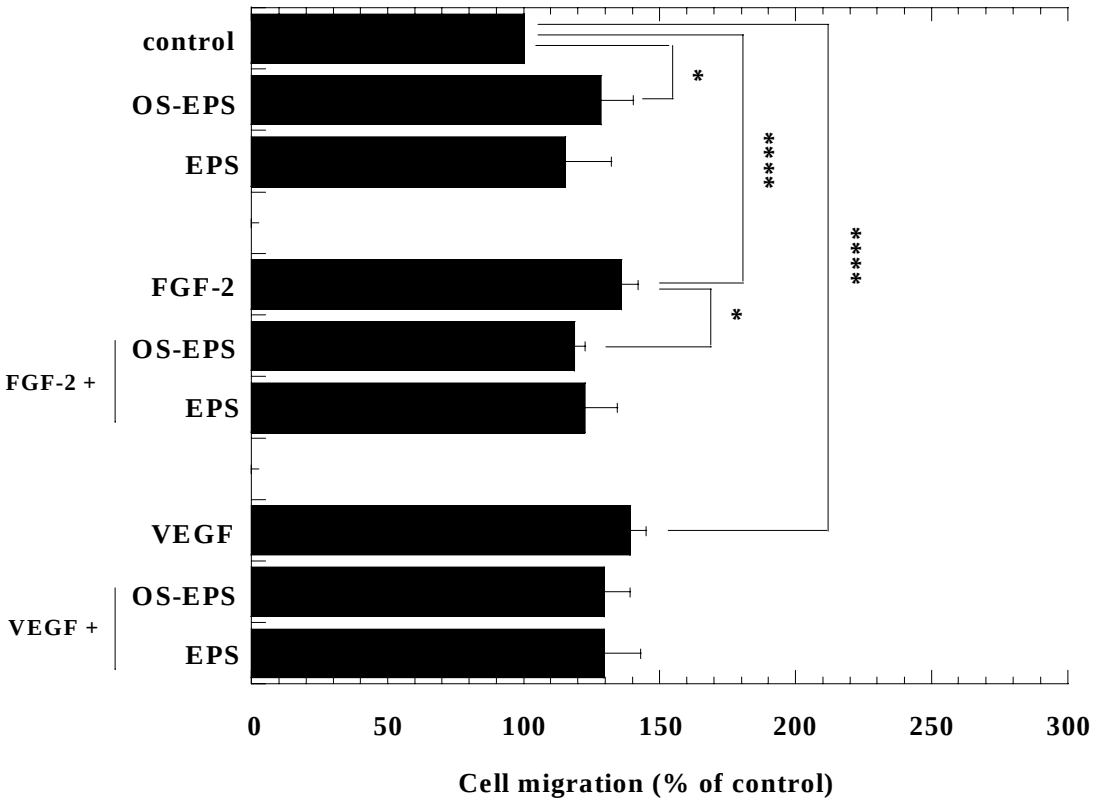
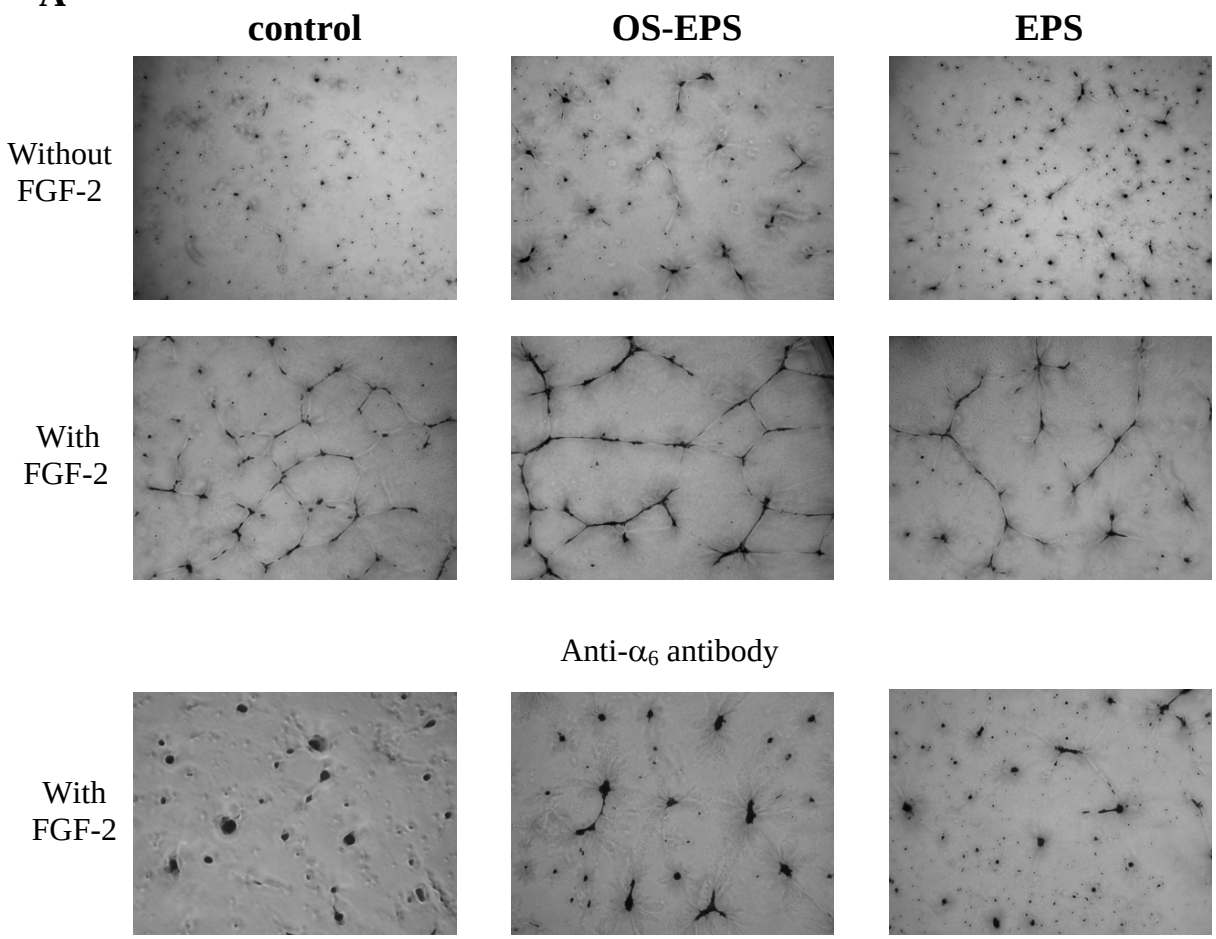

control
OS-EPS
EPS
FGF-2
FGF-2 +
OS-EPS
EPS
VEGF
VEGF +
OS-EPS
EPS
*
*
****
****
0
50
100
150
200
250
300
Cell migration (% of control)

Figure 2

A


control
OS-EPS
EPS
Without FGF-2
With FGF-2
Anti-α6 antibody
With FGF-2

B

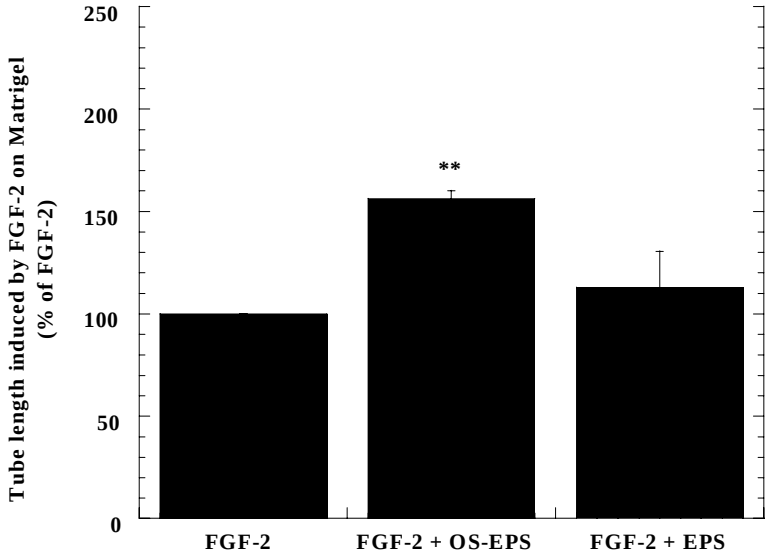
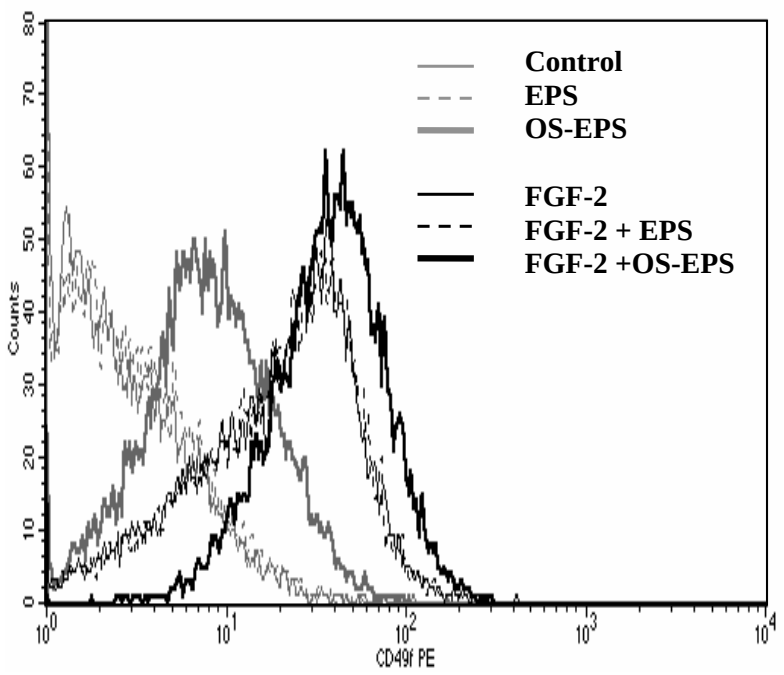

Tube length induced by FGF-2 on Matrigel (% of FGF-2)
250
200
150
100
50
0
**
FGF-2
FGF-2 + OS-EPS
FGF-2 + EPS

Figure 3

A


Control
EPS
OS-EPS
FGF-2
FGF-2 + EPS
FGF-2 +OS-EPS
Counts
CD49f PE

B

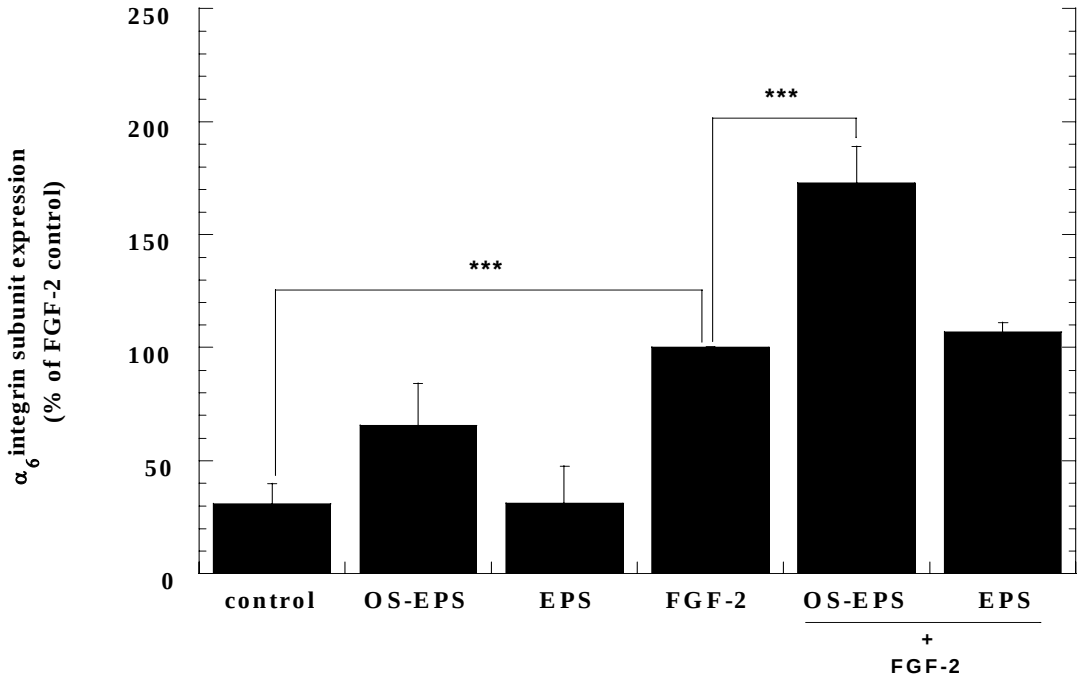
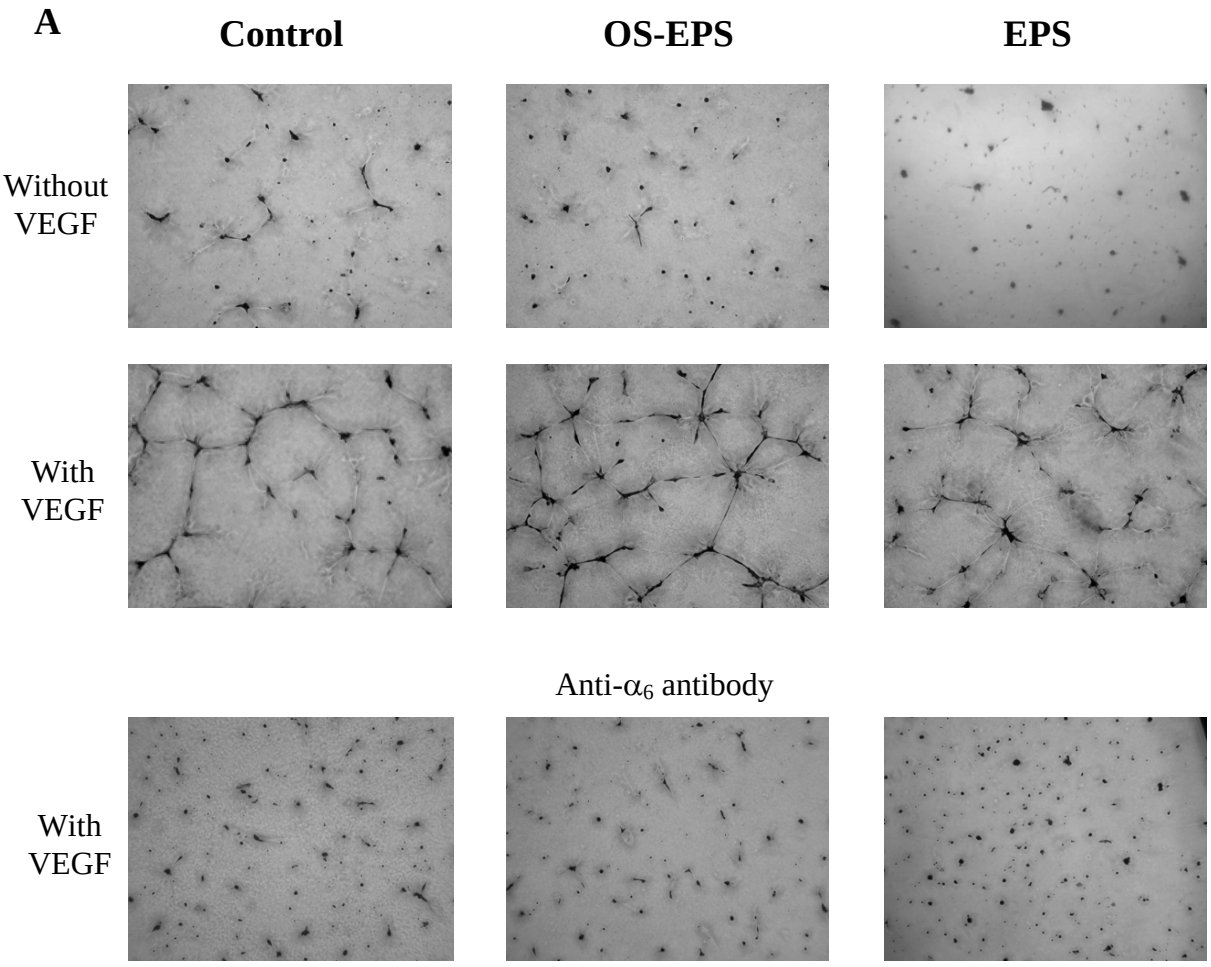

α6 integrin subunit expression
(% of FGF-2 control)
250
200
150
100
50
0
***
***
control
OS-EPS
EPS
FGF-2
OS-EPS
EPS
+
FGF-2

Figure 4


A
Control
OS-EPS
EPS
Without VEGF
With VEGF
Anti-α6 antibody
With VEGF

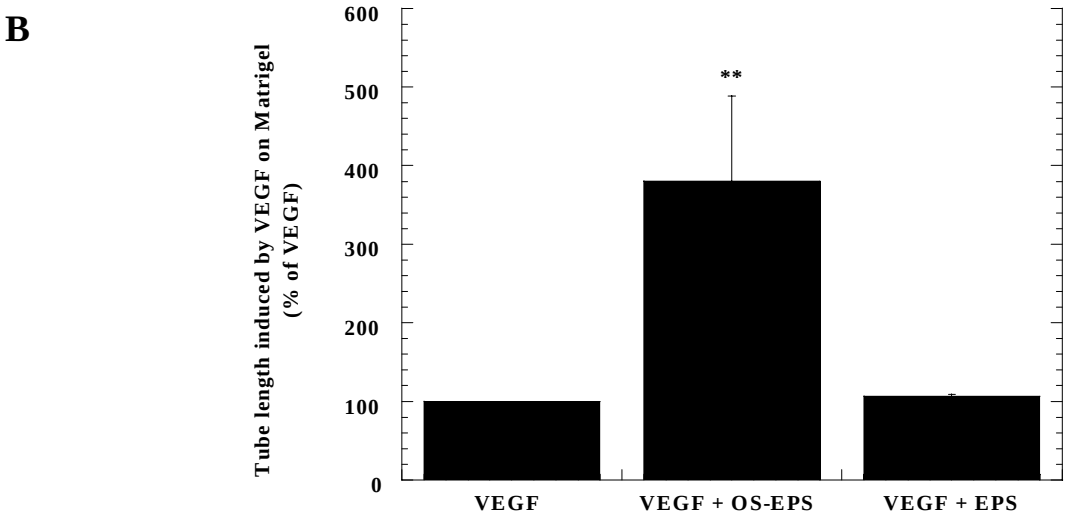
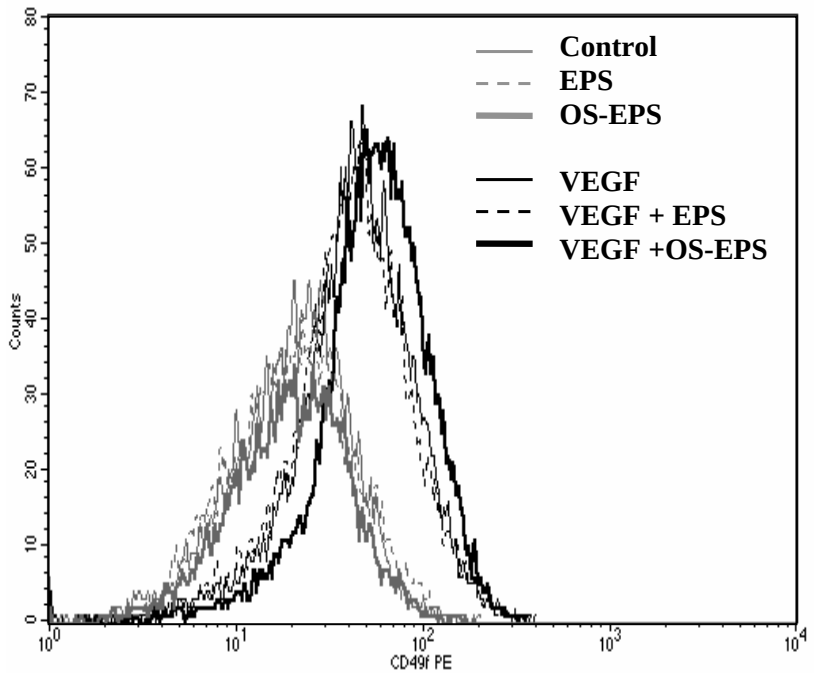

B
Tube length induced by VEGF on Matrigel (% of VEGF)
600
500
400
300
200
100
0
**
VEGF
VEGF + OS-EPS
VEGF + EPS

Figure 5

A


Control
EPS
OS-EPS
VEGF
VEGF + EPS
VEGF +OS-EPS
Counts
CD49f PE

B

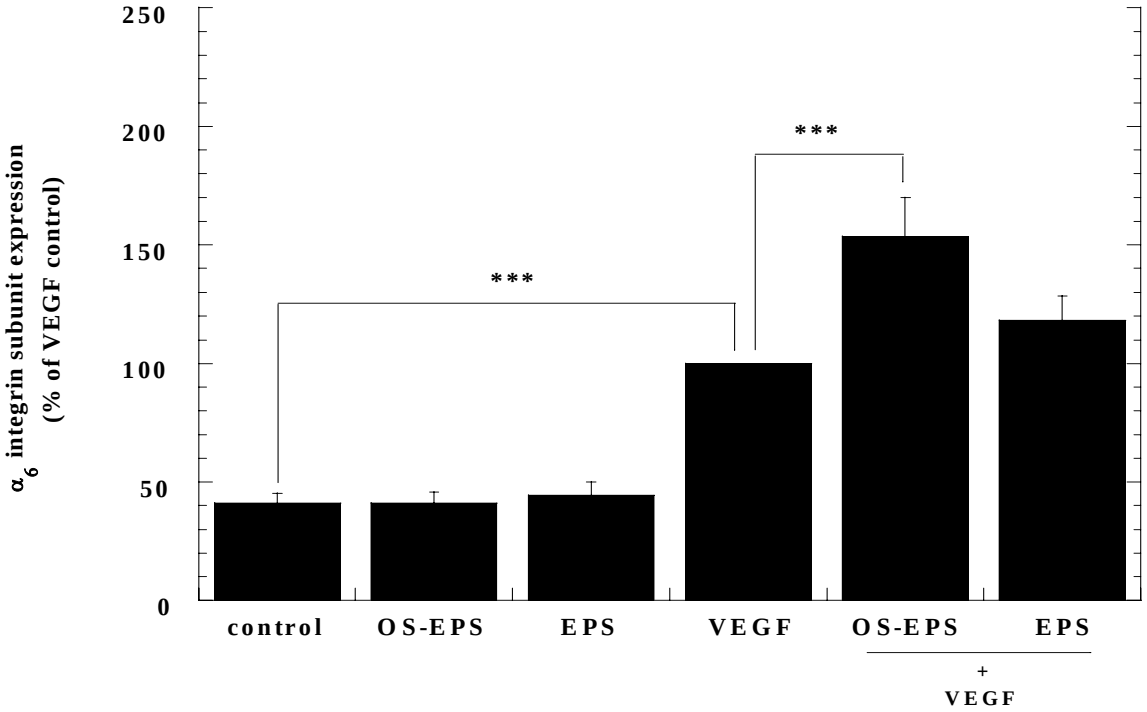

α6 integrin subunit expression
(% of VEGF control)
250
200
150
100
50
0
***
***
control
OS-EPS
EPS
VEGF
OS-EPS
EPS
+
VEGF

Figure 6