Angiogenesis Activators and Inhibitors Differentially Regulate Caveolin-1 Expression and Caveolae Formation in Vascular Endothelial Cells

ANGIOGENESIS INHIBITORS BLOCK VASCULAR ENDOTHELIAL GROWTH FACTOR-INDUCED DOWN-REGULATION OF CAVEOLIN-1*

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Angiogenesis is the process by which new blood vessels are formed via proliferation of vascular endothelial cells. A variety of angiogenesis inhibitors that antagonize the effects of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) have recently been identified. However, the mechanism by which these diverse angiogenesis inhibitors exert their common effects remains largely unknown. Caveolin-1 and -2 are known to be highly expressed in vascular endothelial cells both in vitro and in vivo. Here, we examine the potential role of caveolins in the angiogenic response. For this purpose, we used the well established human umbilical vein endothelial cell line, ECV 304. Treatment of ECV 304 cells with known angiogenic growth factors (VEGF, bFGF, or hepatocyte growth factor/scatter factor), resulted in a dramatic reduction in the expression of caveolin-1. This down-regulation event was selective for caveolin-1, as caveolin-2 levels remained constant under these conditions of growth factor stimulation. VEGF-induced down-regulation of caveolin-1 expression also resulted in the morphological loss of cell surface caveolae organelles as seen by transmission electron microscopy. A variety of well characterized angiogenesis inhibitors (including angiostatin, fumagillin, 2-methoxy estradiol, transforming growth factor-β, and thalidomide) effectively blocked VEGF-induced down-regulation of caveolin-1 as seen by immunoblotting and immunofluorescence microscopy. However, treatment with angiogenesis inhibitors alone did not significantly affect the expression of caveolin-1.

On the other hand, the observed VEGF-induced down-regulation of caveolin-1 may be an important step along the pathway toward endothelial cell proliferation.

Angiogenesis is the development of new capillaries from pre-existing blood vessels (1). In the adult, angiogenesis is primarily associated with pathological conditions such as tumor formation and wound healing (2–4). With regard to tumor formation, angiogenesis is essential for the rapid and continued growth of a variety of tumors (5–7). This makes anti-angiogenic therapy an attractive and promising treatment for cancer (8).

Several different angiogenic activators have been described thus far (2–4). These include, but are not limited to, fibroblast growth factor (FGF),1 vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF; also known as scatter factor) (9). These growth factors bind and activate specific receptor tyrosine kinases within endothelial cells that are couple to a variety of signal transduction pathways, most notably the Ras-p42/44 MAP kinase pathway (10, 11).

A number of endogenous angiogenesis inhibitors have been identified that antagonize the effects of VEGF and FGF. Angiogenesis inhibitors may be stored as inactive precursors within larger proteins and released upon appropriate proteolytic processing (12–16). A notable one is angiostatin, an 38–40-kDa fragment of plasminogen (12, 13, 17). Others include endostatin (an ~18-kDa fragment of collagen XVIII), TGF-β and thalidomide (14–16, 18–21).

Similarly, angiogenesis inhibitors derived from microbes (fumagillin, from Aspergillus fumigatus; minocycline, a tetracycline derivative) have been identified by a variety of screening approaches (22–26). Others are chemically synthesized compounds, such as 2-methoxyestradiol (an endogenous estrogen metabolite) and thalidomide (27–30). However, the mechanism of action of these anti-angiogenic agents remains largely unknown.

Here, we examine the effects of both angiogenesis activators and inhibitors on the expression of caveolin-1, a caveola marker protein that is known to be abundantly expressed in normal endothelial cells in the adult (31). We find that angio-

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1 The abbreviations used are: FGF, fibroblast growth factor; bFGF, basic FGF; VEGF, vascular endothelial growth factor; VEGF-R, VEGF receptor; MAP kinase, mitogen-activated protein kinase; TGF-β, transforming growth factor-β; HGF, hepatocyte growth factor; ERK, extracellular signal-regulated kinase, MEK, mitogen-activated protein kinase.
Genes and proteins have different regulatory mechanisms, and they have the potential to regulate the expression of caveolin-1. Caveolin-1 is thought to play a role as a negative regulator of signal transduction (31), down-regulation of caveolin-1 by angiogenic growth factors may be important for endothelial cell proliferation and subsequent angiogenesis.

**EXPERIMENTAL PROCEDURES**

**Materials—**Monoclonal antibodies directed against caveolin-1 (clone 2297) and caveolin-2 (clone 65) were the gift of Drs. John R. Glenny, Jr. and Roberto Campos-Gonzalez (Transduction Laboratories, Lexington, KY) (32, 33). Reagents and other supplies were obtained from the following sources: polyclonal anti-caveolin-1 IgG from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA); rhodamine-goat anti-rabbit IgG from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA); Slow-Fade anti-fade reagent from Molecular Probes, Inc. (Eugene, OR); HGF from Sigma; fumagillin, 2-methoxyestradiol, thalidomide, and PD98059 from Calbiochem; angiotatin from Angiogenesis Research Industries, Inc. (Chicago, IL); bFGF and TGF-β from Upstate Biotechnology (Lake Placid, NY); VEGF from PeproTech EC Ltd. (Rocky Hill, NJ), and the bicinchoninic acid protein assay kit from Pierce. The cDNA for the human VEGF-R (KDR) was as described previously (34). The cDNA for mutationally activated MEK-1 is included as a positive control in the PathDetect Elk-1 trans-reporting system (Stratagene, Inc.).

**Cell Culture and Treatment with Angiogenesis Activators and Inhibitors—**Human endothelial cells (ECV 304; CRL-1998) were grown in medium-199 (Life Technologies, Inc.) with 10% heat-inactivated fetal bovine serum (normal growth medium). Endothelial cells were seeded at a density of ~1.0 × 10^4 cells/ml in 24-well plates. After incubation in normal growth medium overnight, the medium was replaced by medium-199 containing 5% fetal bovine serum.

**Protein Analysis—**Expression of caveolin-1 and -2 was examined by Western blot analysis. Cells were solubilized with sample buffer containing 0.125 M Tris-HCl (pH 6.8), 5% (w/v) SDS, 2.5% (v/v) β-mercaptoethanol, 5% glycerol in double distilled water. After boiling for 4 min, proteins were separated by SDS-polyacrylamide gel electrophoresis (5–15% gradient gels), transferred to nitrocellulose, and subjected to Western blot analysis using enhanced chemiluminescence. Prior to loading, the protein concentration of the samples was measured with the bicinchoninic acid method using bovine serum albumin as a standard.

**Immunofluorescence Microscopy—**Briefly, cells were fixed with methanol at −20 °C for 10 min, blocked with 2% bovine serum albumin and stained with specific anti-peptide IgG directed against the unique N terminus of caveolin-1 (pAb N-20, directed against human caveolin-1 residues 2–21; Santa Cruz Biotechnologies, Inc.) (35). Bound primary antibodies were detected using rhodamine-conjugated goat anti-rabbit IgG. The immunostained cells were mounted in the presence of Slow-Fade anti-fade reagent. Immunostaining was visualized using a Zeiss Axios-fluorescence microscope.

**Electron Microscopy—**Transmission electron microscopy was performed as described previously by our laboratory (36, 37).

**In Vivo Assay for VEGF-R Signaling—**Coupling of the VEGF receptor tyrosine kinase (KDR) to the MAP kinase cascade was assessed using the PathDetect Elk trans-reporting system (Stratagene, Inc.). Briefly, confluent ECV 304 or Chinese hamster ovary cells were trypsinized and seeded at a density of ~8 × 10^4 cells/well for ECV 304 or 3 × 10^5 cells/well for Chinese hamster ovary in 6-well plates. After incubation overnight in growth medium, cells were transiently co-transfected with the following plasmids in various combinations, as indicated in a given experiment: pFR-luc (1 μg/well), pFA-Elk (0.1 μg/well), KDR (1 μg/well), caveolin-1 (1 μg/well), MEK-1 (0.1 μg/well), or pCB7 (1 μg/well) using the calcium/phosphate precipitation method (38, 39). 24 h post-transfection, cells were treated with or without angiogenesis inhibitors for an additional 20 h, as indicated in a given experiment. Finally, cells were lysed, and luciferase activity was measured as described previously (38, 39).

**RESULTS AND DISCUSSION**

**Angiogenesis Activators (VEGF, bFGF, and HGF) Down-regulate Caveolin-1 Expression—**ECV 304 cells are a well characterized human umbilical endothelial cell line (40–42). These cells express both caveolin-1 and -2, but fail to express caveolin-3, a muscle-specific caveolin protein (data not shown).

To evaluate the potential role of caveolins in angiogenesis, we studied the effect of angiogenesis activators (VEGF and bFGF) on caveolin expression in ECV 304 cells. In addition, we evaluated the effect of another growth factor (HGF, hepatocyte growth factor; scatter factor) that is known to activate the MET receptor tyrosine kinase and promote angiogenesis (9).

Fig. 1 shows the effects of these growth factors on caveolin expression. Treatment with VEGF, bFGF, or HGF all dramatically down-regulated the expression of the caveolin-1 protein (top panels). In striking contrast, the levels of the caveolin-2 protein remained essentially unaffected (bottom panels).

As all three growth factors yielded similar results, we decide to focus our efforts on the effects of VEGF, a well characterized stimulator of angiogenesis. We next determined the time and concentration dependence of the effects of VEGF. Interestingly, VEGF-induced down-regulation of caveolin-1 appeared after only 8 h of treatment, exerting its maximal effects at 24 h (Fig. 2A). VEGF induced the down-regulation of caveolin-1 at a minimal concentration of 3 ng/ml, and the effect became maximal at 10 ng/ml (Fig. 2B). In contrast, caveolin-2 levels remained unaffected at every time and dose investigated.

**Angiogenesis Inhibitors Block VEGF-induced Down-regulation of Caveolin-1: Involvement of the p42/44 MAP Kinase Cascade—**Angiogenesis inhibitors exert their effects by antagonizing the effects of endothelial growth factors, such as VEGF. However, their mechanism of action remains largely unknown.

Fig. 3A shows the effects of a variety of well studied angiogenesis inhibitors (angiotatin, fumagillin, 2-methoxyestradiol, TGF-β, and thalidomide) in combination with VEGF. In all cases examined, these angiogenesis inhibitors selectively
blocked the ability of VEGF to induce the down-regulation of caveolin-1. Caveolin-2 levels are shown for comparison.

The effects of these inhibitors were also analyzed by immunofluorescence microscopy (Fig. 3B). In untreated cells, caveolin-1 staining was abundant and localized primarily to the periphery of the cell. Growth factor stimulation using VEGF clearly down-regulated caveolin-1 immunostaining; similarly, we find that VEGF also induced the down-regulation of morphologically detectable caveolae as seen by transmission electron microscopy (Fig. 3C). Again, this down-regulation of caveolin-1 and caveolae organelles was prevented by treatment with angiogenesis inhibitors, such as angiostatin and thalidomide (Fig. 3, B and C).

Because caveolin-1 levels are down-regulated in Ras-transformed NIH 3T3 cells and can be restored to normal levels by inhibiting the p42/44 MAP kinase cascade, we next evaluated the effects of a well characterized MEK inhibitor, PD 98059. PD 98059 also blocked the ability of VEGF to down-regulate caveolin-1 expression, as seen by Western blotting (Fig. 3A) and immunofluorescence microscopy (Fig. 3B). Thus, inhibition of the p42/44 MAP kinase cascade by PD 98059 is sufficient to block growth factor-induced down-regulation of caveolin-1. Interestingly, several recent reports indicate that PD 98059 can function as an angiogenesis inhibitor (10, 11, 43, 44).

Angiogenesis Inhibitors Alone Do Not Affect Caveolin-1 Expression—Next we tested the effects of the angiogenesis inhibitors alone on caveolin-1 expression (Fig. 4). Interestingly, in most cases, the angiogenesis inhibitors alone had little or no effect on caveolin-1 levels, with one exception. In the case of fumagillin, caveolin-1 levels were slightly down-regulated. We
do not yet know the significance of this observation. Importantly, these results indicate that the angiogenesis inhibitors exert their effects on caveolin-1 expression primarily in response to endothelial growth factors stimulation via VEGF.

**Caveolin and VEGF-R Signal Transduction**—Caveolae have also been implicated in signaling through the p42/44 MAP kinase pathway. Morphological studies have directly shown that ERK-1/2 is concentrated in plasma membrane caveolae in vivo using immunoelectron microscopy (45). Evidence has been presented suggesting that other components of the p42/44 MAP kinase cascade are localized within caveolae membranes. These include receptor tyrosine kinases (EGF-R; PDGF-R; Ins-R) (35, 46–48), H-Ras (47, 49), Raf kinase (47), 14-3-3 proteins (48), ERK (37, 48), Shc (48), Grb-2 (48), mSos-1 (48), and Nck (48).

Recently, we examined the functional role of caveolins in regulating signaling along the MAP kinase cascade (39). Co-expression with caveolin-1 dramatically inhibited signaling

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**Fig. 4. Angiogenesis inhibitors alone do not affect caveolin-1 expression.** ECV 304 cells were treated with or without angiostatin (5 μg/ml), fumagillin (0.2 μg/ml), 2-methoxyestradiol (0.5 μg/ml), TGF-β (3 ng/ml), thalidomide (1 μg/ml), or PD98059 (20 μM). After the treatment, cells lysates were subjected to immunoblot analysis with isoform-specific antibodies that detect either caveolin-1 or caveolin-2. Each lane contains an equal amount of protein. Note that in most cases, the levels of caveolin-1 remain relatively constant with one exception (fumagillin).

**Fig. 5. Effects of caveolin-1 and angiogenesis inhibitors on VEGF-R-induced signaling to the nuclear transcription factor, Elk-1.** VEGF-R signaling causes activation of p42/44 MAP kinases (ERK 1/2), which translocate to the nucleus and activate the transcription factor Elk. We measured VEGF-R (KDR) induced signaling using a luciferase-based reporter system that reflects the activation state of Elk-1 (see “Experimental Procedures”). Chinese hamster ovary cells (panel A) or ECV 304 cells (panels B, C, and D) were transiently transfected with a plasmid encoding either VEGF-R (KDR) (panels A and B) or mutationally activated MEK-1 (panel C). In addition, these cells were co-transfected with a plasmid encoding wild-type full-length caveolin-1 (pCB7-Cav-1) or with empty vector alone (pCB7) as a negative control. Note that co-transfection with caveolin-1 significantly inhibited VEGF-R-induced and MEK-1-induced signaling. In panel D, ECV 304 cells were transfected with VEGF-R (KDR) and incubated in the presence or absence of a variety of angiogenesis inhibitors for an additional 20 h (detailed in the legend of Fig. 4). Note that treatment of ECV 304 cells with either PD98059 or thalidomide substantially reduced VEGF-R-induced signaling, whereas treatment with angiostatin or fumagillin had little or no effect. Data are the mean ± S.D.
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from EGF-R, Raf, MEK-1, and ERK-2 to the nucleus in vivo (39). Using a variety of caveolin-1 deletion mutants, we mapped this in vivo inhibitory activity to caveolin-1 residues 32–95. In addition, peptides derived from this region of caveolin-1 (i.e. the caveolin-scaffolding domain) also inhibited the in vitro kinase activity of purified MEK-1 and ERK-2 (39). Thus, caveolin-1 can inhibit signal transduction from the p42/44 MAP kinase cascade both in vitro and in vivo by acting as a natural endogenous inhibitor of both MEK and ERK.

To assess whether caveolin-1 expression or angiogenesis inhibitors can negatively regulate signaling from the VEGF receptor tyrosine kinase (VEGF-R) along the MAP kinase cas-

...receptor tyrosine kinases is sufficient to cause receptor dimer-

...tor, Elk-1. It is well known that transient overexpression of in vitro caveolin-scaffolding domain) also inhibited the this (39). Using a variety of caveolin-1 deletion mutants, we mapped (33, 52–54). Thus, down-regulation of caveolin-1 expres-

...tion and caveolae organelles may be a prerequisite for...sion levels are down-regulated in rapidly dividing NIH 3T3 cells and are dramatically up-regulated at confluency. These...els may be important to mediate normal contact inhibition and...