Nitric oxide modulates caveolin-1 and matrix metalloproteinase-9 expression and distribution at the endothelial cell/tumor cell interface

Patricia G. Phillips1,2,3 and Linda M. Birnby1

1 Research Service, Samuel S. Stratton Veterans Affairs Medical Center; and 2 Department of Medicine and 3 Center for Cardiovascular Sciences, Albany Medical College, Albany, New York 12208

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Phillips, Patricia G., and Linda M. Birnby. Nitric oxide modulates caveolin-1 and matrix metalloproteinase-9 expression and distribution at the endothelial cell/tumor cell interface. Am J Physiol Lung Cell Mol Physiol 286: L1055–L1065, 2004; 10.1152/ajplung.00262.2003.—We used a two-compartment coculture model comprising human endothelial cells (EC) and non-small cell lung carcinoma (CA) cells to study capillary formation. Elevated NO concentrations, contributed in part by CA cells, lead to inhibited capillary formation (Phillips PG, Birnby LM, Narendran A, and Milonovich WL. Am J Physiol Lung Cell Mol Physiol 281: L278–L290, 2001). Here we demonstrate using gelatin substrate zymography that high NO concentrations, whether produced endogenously or by NO donor spermine-NONOate or peroxynitrite-generating compound SIN-1, significantly inhibit MMP-9 expression and activation. Furthermore, high NO concentrations decrease Cav-1 abundance and alter its cellular distribution in EC. Cav-1 is essential for capillary formation in this model because Cav-1 antisense treatments targeted to EC significantly inhibit capillary formation. Laser confocal microscopy demonstrated extensive colocalization of MMP-9 with Cav-1 in sprouting EC, primarily at the basolateral surfaces of EC in focal structures associated with directed migration. This codistribution was NO concentration dependent, and elevated NO concentrations lead to marked dissociation of these two proteins. We propose that compartmentalization of MMP-9 within caveolar structures does occur, and that this could facilitate directed proteolysis essential for early migratory and invasive processes. Our data suggest elevated NO concentrations could impact on capillary formation via a combination of direct effects on MMP activation and by altering the distribution or abundance of Cav-1. Consequences of Cav-1 alterations may include impaired activation of proteolytic enzymes that utilize caveolar structure for stabilization and/or compartmentalization of MMP-9 as well as other putative members of an ECM proteolytic cascade.

angiogenesis; extracellular matrix; proteolysis; reactive nitrogen oxide species

SOLID TUMORS CANNOT GROW BEYOND a few millimeters in diameter unless they recruit a blood supply to furnish the rapidly growing cells with nutrients and oxygen (12). Microvessel density within a tumor, an end point reflecting the extent of angiogenesis, is an important prognostic indicator for solid tumors, including lung non-small cell carcinomas (CA) (13, 16). Recently, a number of studies have shown that levels of total nitric oxide synthase (NOS) activity are significantly elevated in many tumors, providing impetus for investigation of the role of this molecule in tumor growth, metastasis, and angiogenesis (reviewed in Refs. 6, 9, and 21). The process of angiogenesis is a focal phenomenon as demonstrated by the presence of “hot spots” of localized angiogenic activity in tumor tissues in animals and humans (39). Blood vessels within a tumor mass are tortuous, saccular, and dilated, and their responsiveness to pharmacological stimulation by vasoconstricitors and vasodilators is compromised (2, 18, 30). Therefore, it is feasible that localized concentrations of nitric oxide (NO) could impact on the initiation of angiogenesis in such a microenvironment.

We have demonstrated rapid induction of capillary networks (2–3 days) using cocultures of human endothelial cells (EC) and human CA cells (27). This EC morphogenic transformation is markedly diminished by elevated NO concentrations in the microenvironment, provided at least in part by the lung CA cells that constituently produce relatively high levels of NO. In our investigations of the mechanisms involved in NO modulation of capillary formation, we have observed that this molecule influences the expression and/or activation of two matrix metalloproteinases (MMPs), MMP-2 and -9, in EC (27). MMPs play a major permissive role in early capillary sprouting by degrading the underlying extracellular matrix (ECM) that holds the cells stationary. MMPs are expressed as inactivezymogens that require distinct activation processes to convert them to active enzymes. Activation requires disruption of a coordination bond formed between a highly conserved unpaired cysteine in the NH2-terminal propeptide of the pro-MMP molecule and the zinc ion at the active center. Disruption of this bond can be mediated by chemical and/or proteolytic mechanisms and results in the removal of the cysteine-containing propeptide domain and generation of a catalytically active MMP (31). NO modifies cysteine thiol and transition metal centers of a broad range of proteins with varying functions (37), and peroxynitrite (ONOO−) has been shown to release zinc from zinc-thiolate groups (8). The presence of high NO concentrations in the EC/tumor microenvironment in our model system, together with the potential sensitivity of the zinc-thiolate active site of MMPs to modification by reactive nitrogen oxide species (RNOS), suggested the possibility of direct localized effects on MMP activity in this setting.

Generalized proteolysis is counterproductive for directed cell migration. Therefore, these enzymes are usually localized to receptors, adhesion sites, or invasive protrusions of cells where ECM degradation takes place. This localization concentrates their activity in close proximity to their substrates in interactions with the cytoskeleton (3). By concentrating proteolytic events at or near the cell surface, these processes can be effective even in the presence of high concentrations of inhibitors (41). The caveolar membrane system comprises unique lipid and protein domains, and these structures serve to

Address for reprint requests and other correspondence: Patricia G. Phillips (151), Research Service, Samuel S. Stratton VA Medical Center, 113 Holland Ave., Albany, NY 12208 (E-mail: Patricia.Philips3@med.va.gov).

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compartmentalize and integrate a wide range of signal transduction processes (22, 32). Compartmentalization of urokinase-type plasminogen activator and its receptor uPAR within caveolae promotes efficient cell surface plasminogen generation (36), whereas caveolin and uPAR operate within adhesion sites to organize kinase-rich lipid domains in proximity to integrins for efficient signal transduction (40).

Subcellular compartmentation has not been definitely established for MMP function. However, specialized surface protrusions of invasive cells, termed invadopodia and lamelopodia, have differential localization of membrane type 1 (MT1)-MMP, MMP-2, and tissue inhibitors of metalloproteinases (TIMP)-2, and extension of these structures is regulated by expression of caveolin-1 (Cav-1) (44). Furthermore, EC shedding of sequestered MMPs and TIMPs as membrane vesicles suggestive of caveola-like structures have recently been reported (38). These vesicle-associated proteolysis units contain MMP-2, -9, and MT1-MMP, their production is induced by angiogenic growth factors, and they stimulate differentiation of EC into capillary networks. Recently, Puyraimond et al. (29) have demonstrated colocalization of MMP-2 and MT1-MMP with Cav-1 at the basolateral surface of EC using confocal microscopy, consistent with their roles in proteolysis of subcellular matrices.

The aim of the present studies was to investigate the effects of high NO concentrations on MMP-9 activation, on Cav-1 distribution, and the possible interrelationships between these two molecules in EC grown in coculture with lung cancer cells. We utilized a model established in our laboratory in which EC and lung cancer cell populations separated by a 10-μm membrane interact, resulting in the formation of capillary networks (27). Because individual cell populations remain separated over the first 30 h, we were able to study biochemical changes in isolated EC during the early sprouting phase of angiogenesis. We hypothesized that high NO concentration-induced changes in Cav-1 would have an impact on the distribution of MMP-9 in sprouting EC, the first in a series of steps essential for new vessel formation. We demonstrated that EC Cav-1 is essential for lung CA cell-induced capillary formation and 2) colocalization of MMP-9 with Cav-1 is NO concentration dependent, suggesting that elevated NO concentrations could have an impact on the activation of proteolytic enzymes that utilize caveolar structure for stabilization and/or activation.

MATERIALS AND METHODS

The Transwell culture system used for angiogenesis studies consisted of 6.5-mm-diameter polycarbonate inserts with 8-μm pores (cat. no. 3422; Costar, Cambridge, MA). RPMI 1640, F12K medium, penicillin, streptomycin, gentamicin, trypsin-EDTA, Dulbecco’s PBS, HBSS, trypsin-EDTA (both 0.05% and 0.25% trypsin-1 mM EDTA), and amphotericin B (fungizone) were obtained from GIBCO (Grand Island, NY). Media for growth of human umbilical vein endothelial cells (HUVEC) were obtained from Vec Technologies (Rensselaer, NY). FCS was obtained from Hyclone (Logan, UT). Costar Transwell culture wells were obtained from Fisher Scientific (Springfield, NJ). Analysis of lung CA cell cultures for constitutively produced NO was performed using the Cayman nitrite/nitrate colorimetric assay kit (Alexis Biochemicals, San Diego, CA). Sterile solutions of rat tail collagen type I and Matrigel [reduced growth factor (RFG) formulation] for coating surfaces of the Transwells were obtained from Collaborative Research (Bedford, MA). Gelatin (from calf skin, type III) for substratezymography was obtained from Sigma (St. Louis, MO). Spermine-NONOate and 3-morpholinosydnonimine (SIN-1) were obtained from Calbiochem (San Diego, CA). Purified MMP-9 standard was obtained from Chemicon International (Temecula, CA). Primary antibodies for visualization of Cav-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and for MMP-9 from Chemicon International. Secondary antibodies Alexa 488 and Alexa 568 were obtained from Molecular Probes (Eugene, OR). Enhanced chemiluminescence (ECL) reagents for detection on Western blots were purchased from Amersham Biosciences (Arlington Heights, IL). Phosphorothioated oligonucleotides for antisense treatments were obtained from Oligos Etc. (Wilsonville, OR). Lipofectamine reagent for transfection of oligonucleotides was obtained from Invitrogen Life Technologies (Grand Island, NY).

Cell Culture

HUVEC were obtained at passage 2 from Vec Technologies. Three human CA cell lines were obtained from American Type Culture Collection (Rockville, MD). The tumor cell lines were NCI-H157, squamous carcinoma (SQ); NCI-H125, adenosquamous (AS); and NCI-H522, adenocarcinoma (AD). HUVEC were cultured inVEC Technologies MCDB-131 complete media with FBS and antibiotics. EC were cultured on 0.2% gelatin-coated flasks. Lung cancer cell lines were cultured in lung CA medium RPMI 1640 supplemented with final concentrations of 5% FCS, 100 U/ml penicillin-streptomycin, 50 μg/ml gentamicin, and 0.5 μg/ml fungizone. All cells were routinely cultured in T-25 or T-75 flasks and subcultured by trypsinization (0.05% for HUVEC and 0.25% for CA cells). For angiogenesis studies, cells were subcultured in Transwells as described in detail previously (27), with all morphology studies performed on the Transwell filter insert membranes. Briefly, the upper surface was coated with basement membrane-like RGF-Matrigel, and the lower surface was coated with collagen type I, respectively, to simulate the composition of the in vivo microenvironment for EC and CA growth. Each Transwell insert was suspended in an individual well of a 24-well cluster dish, with 1-mm clearance between the bottom of the well and the culture dish. To confirm the identity of capillary-forming cells in cocultures as EC, and to quantify the capillary networks by digital image analysis, cultures were stained for EC-specific marker CD31/platelet endothelial cell adhesion molecules. No staining of lung CA cells was observed with either Di-1 acylated LDL or CD31. Because the two cell types remained essentially segregated, at least for the first 30 h of coculture, separate populations could be isolated for further biochemical analyses of cells in the early phase of capillary formation, as described below for substratezymography and immunocytochemistry studies.

Manipulation of NO Concentrations

Cocultures. Experiments to study the role of NO in the early sprouting time period were performed using 100 μM aminoguanidine (AG) added to RPMI in the lower chamber at the time of coculture to decrease NO production. In this culture setting, AG was more effective than NO3-nitro-L-arginine methyl ester in lowering NO concentrations in the cocultures, so AG was used for all studies.

Purified MMP-9 studies. To determine direct effects of NO or ONOO− on MMP-9 activity, purified MMP-9 enzyme (Chemicon International) was incubated for 1 h at 37°C with 1 mM aminophenyl mercuric acid (APMA) buffered with 0.05 M Tris-Cl, pH 7.5. Samples were then incubated for 5 h at 37°C with either spermine-NONOate (0.5 and 1 μM) or SIN-1 (20 μM, 200 μM, and 2 mM). The rate of NO production from spermine-NO, as determined spectrophotometrically with NO-specific electrode, was 5 or 10 nmol/min for 0.5 and 1 μM, respectively (23). SDH generates equivalent amounts of O2 and NO, which interact to form ONOO−. Owens et al. (23) reported that 100 μM SIN-1 produced ~30 μM ONOO− after a 30-min incubation time at 37°C assayed by monitoring rhodamine formation. We have
previously reported that the lung cancer cell lines produce micromolar amounts of NO (27).

**Evaluation of MMP Activity by Substrate Zymography**

HUVEC/CA cell cocultures were used to provide total cell extracts for evaluation of MMP activity after 16 h of culture, in the early sprouting phase of capillary formation. Cultures were set up to evaluate activity in HUVEC alone or HUVEC that had been cocultured with CA cells. Cultures were either untreated or treated with 100 μM AG to inhibit NO production. For preparation of samples in the EC/CA cell cocultures, CA cells were scraped off the bottom of Transwells, and both the lower surface of the well and EC in the upper compartment were washed extensively before extraction. This extraction procedure included scraping of wells to include EC-associated focal adhesion structures. Because few cancer cells migrate into the upper compartment of the Transwell (<1%), there are only EC in the upper compartment (27). Cells were extracted with electrophoresis sample buffer without mercaptoethanol, samples were activated with 1 mM APMA, and the peptides were separated on a 7.5% SDS-PAGE gel copolymerized with 0.05% gelatin (final concentration) as substrate for MMP activity, as described previously (27). Areas of proteolytic activity were visualized as light bands in dark Coomassie-stained gels. Metallo-dependence of proteolytic activity was confirmed by lack of proteolysis in gels incubated in 10 mM sodium EDTA in 50 mM Tris–Cl, pH 8.5, before Coomassie staining. For studies to determine direct effects of NO or ONOO− on MMP-9 activity, purified MMP-9 enzyme was incubated with SIN-1 or spermine-NONOate, as described above, and activity was evaluated by substrate zymography without an additional APMA activation step.

**Western Blot for Cav-1**

To determine the effects of NO concentration on Cav-1 abundance in EC, total cell lysates were obtained from EC growing alone or in coculture with CA cells in the presence or absence of AG to inhibit NO production. Before performing lysis of EC monolayers, CA cells were first scraped away, and the lower surface of the Transwell was washed carefully so that the lysate contained only EC-associated Cav-1. Lysate buffer composition was 0.125 M Tris–Cl, pH 6.8, 5% SDS, 2.5% β-mercaptoethanol, and 5% glycerol (19). Replicate cultures were harvested at 2 and 20 h, and peptides were separated on 12% SDS-PAGE gels based on equal protein loads. Bands were detected using ECL reagents as described previously (27).

**Immunocytochemistry**

**Cav-1 distribution in EC.** For visualization, EC growing on Transwells were washed and then fixed in ice-cold methanol for 10 min. After being rehydrated and blocked in 2% BSA, cells were incubated with rabbit anti-caveolin (Santa Cruz) at a 1:100 dilution for 1 h at 37°C followed by incubation with a 1:1,000 dilution of secondary antibody Alexa 568 (red) for 30 min. For double staining to visualize both Cav-1 and MMP-9, cells were fixed with methanol and stained for Cav-1 first as described above using Alexa 568-conjugated secondary antibody. Cells were blocked with 2% BSA and then stained with Chemicon mouse anti-human MMP-9 dilute 1:100 followed by incubation with a 1:1,000 dilution of secondary antibody Alexa 488 (green). For scanning confocal microscopy, the upper portion of the Transwell was sliced off with a heated scalpel, and the remaining well with stained cells attached to the polycarbonate membrane was placed on a 75 × 100-mm coverslip on the microscope stage. Photomicrographs shown are representative examples from 8–10 field observations per Transwell and from studies performed at least three times.

**Microscopy**

Conventional. An Olympus IMT2 microscope (equipped for phase, light, and fluorescence microscopy) interfaced with digital SPOT CD camera was used for initial examination of Transwell cultures.

**Laser scanning confocal microscopy.** Studies were performed utilizing the Leica TCS NT SP inverted laser scanning confocal microscope (DM IRB) equipped with three lasers with range from ultraviolet to far-red: green HeNe (543 nm), argon (457, 476, 488, and 514 nm), and red HeNe (633 nm), high-resolution z-stage, and software for 3-D reconstruction of optical sections and calculations of intensities and ratios. Optical sectioning was performed to generate a series of images, x and y planes along the z-axis. Typically, each section was 0.5–1 μm thick. Monolayers were sectioned from apical through basolateral planes. In the graphic representations, the x- and y-axes delineate the area of endothelial cell monolayer, i.e., 187.13 μm × 187.13 μm. The z-axis represents fluorescence intensity of red, green, or merged images within an individual section in that monolayer. Images illustrate distribution and colocalization of red (Cav-1) and green (MMP-9) fluorescence in representative sections within the monolayers. With the use of this technology, it is possible to define areas within a cell where Cav-1 and MMPs colocalize and, therefore, likely areas for potential interactions.

**Antisense Experiments**

**Sequences.** Translation of Cav-1 mRNA was inhibited using two separate Cav-1 antisense oligonucleotides complementary to human Cav-1: 1) 5′-HN-1, ATGTCCTTCGAGTCTA, directed against nucleotides 20–36 of the open reading frame and 2) 5′-HN-2, ATACTCTATCTCTTGAA, directed against nucleotides 579–595 of the 3′-untranslated region (14). Two nonsense oligonucleotides: 1) 5′-Non-1, ATGATGAAGAACGACGAATCTGTCG, and 2) Non-2, CTCTGCCTCGTCCGC, were used as controls to assess nonspecific effects of oligonucleotide treatments. All sequences were phosphothioated oligonucleotides purchased from Oligos Etc.

**Transfection experiments.** EC were seeded in serum-containing medium at subconfluent density (3 × 105 cells) in the upper compartments of Transwells prepared as described previously for angiogenesis studies (27) and allowed to attach and spread overnight (16–20 h) before treatment with oligonucleotides. Serum-containing medium was removed, and EC were treated with serum-free solutions containing a final concentration of 20 μg/ml of Lipofectamine reagent and a final concentration of 1 μM oligonucleotide for 4 h at 37°C. “Untreated” controls were exposed to the Lipofectamine reagent without added oligonucleotides. After 4 h, the serum-free Lipofectamine/oligonucleotide media were removed and replaced with 10% serum-containing media. Replicate wells of EC were extracted with Cav-1 extraction buffer at 6 and 24 h posttreatment, as described above, to evaluate Cav-1 abundance by Western blotting. Replicate wells stained with whole cell stain acridine orange to evaluate effects of treatments on cell density 24 h posttreatment showed minimal differences in the pattern or distribution of cells with any of the treatments. Western blotting demonstrated significant reduction of Cav-1 at 24 h posttransfection treatment, so CA cells were then seeded on the bottom of these EC wells (time 0 for initiation of coculture). Capillary formation by CD31-stained EC was evaluated 72 h later as described previously (27). Data shown are representative of two experiments performed in duplicate, showing very similar results.

**RESULTS**

**Effect of NO Concentration on MMP Activities in EC**

Elevated NO concentrations in the EC/tumor cell microenvironment markedly inhibit the activation of MMP-9, as shown in Fig. 1 for EC cocultured with each of two representative CA cell lines, AD and SQ cells. The MMP-9 purified standard shows predominant latent or proenzyme form at 92 kDa, and little activated enzyme was apparent even in the presence of optimum concentrations of APMA used to activate samples before zymography. In contrast, EC growing in coculture with
CA cells for 16 h demonstrate almost entirely the activated form at 82 kDa of MMP-9, with an intermediate form at 86 kDa. Although some activity was visible in untreated groups, when cocultures were treated with AG to inhibit NO production, this activity was markedly increased.

MMP-2 is constitutively produced in EC, and EC in these cultures show the presence of both proenzyme as well as the activated forms. In these cultures, AG treatment increased the amount of proenzyme form in EC/tumor cell cocultures but had little effect on the activated forms. For this reason, we concentrated on MMP-9 in the remaining studies.

**Direct Effect of RNOS on MMP-9 Activity**

MMP activity is regulated at multiple levels that include gene transcription, protein abundance, activation state, and endogenous inhibitors (41). MMP proenzymes are activated when active site zinc is exposed via a cysteine switch mechanism. NO modifies cysteine thiols and transition metal centers of a broad range of proteins with varying functions (37). Because the EC/tumor microenvironment could be the site of focally elevated NO concentrations, we investigated whether RNOS could directly inhibit MMP-9 activity (Fig. 2). Purified standard MMP-9 was incubated with varying concentrations of either NO donor spermine- NONOate or SIN-1 that produces ONOO⁻. Both agents markedly inhibited both the proenzyme and the activated forms of MMP-9 in a dose-dependent manner.

**Effect of Elevated NO Concentrations on the Cellular Distribution of Cav-1**

Caveolar structures could serve to localize and coordinate focal proteolysis essential for vascular remodeling. Cav-1 distribution was evaluated in EC growing either alone or in coculture with lung CA cells. In some cultures, AG was used to inhibit NO production. Figure 3A illustrates Cav-1 localization in a control EC monolayer in the absence of CA cells. Cav-1 is abundant in these cells and particularly prominent at the borders of contiguous cells. Figure 3B shows a pattern of focal changes that occurs in EC growing in coculture with high NO-producing SQ cells. In Fig. 3B (taken at the same magnification as Fig. 3A), Cav-1 staining is more diffuse and shows increased concentration in perinuclear areas consistent with Golgi localization. Figure 3C shows Cav-1 distribution in EC/SQ cocultures that have been treated with AG to reduce NO production. Cav-1 staining is more intense, and short sprouts enriched for Cav-1 are evident. Figure 3, D and E, illustrate the direct effects of either 500 μM spermine-NONOate or 200 μM SIN-1, respectively, on EC monolayers (EC alone). EC exposed to spermine-NONOate or SIN-1 show focally diminished staining for Cav-1. Spermine treatment is associated with some perinuclear Cav-1 localization as well as punctate staining in peripheral regions. SIN-1-treated cells show a fade out of protein in the periphery of the cells. Western blotting was performed to evaluate total Cav-1 protein in EC cultured alone or with lung CA cells in the Transwell model (Fig. 4). For these studies, as for those described previously (27), EC are seeded at confluent densities, 1 × 10⁵ cells/well. Samples were taken either at 2 h, representing confluent EC, or at 20 h in the initial sprouting phase of capillary formation. EC Cav-1 expression at 2 h is high, consistent with abundant localization at borders of confluent cells. There is no difference in Cav-1 expression with or without AG at 2 h. At 20 h, there is a decrease in Cav-1 abundance in HUVEC that are cocultured with lung CA cells. Treatment with AG increases Cav-1 expression in EC in all treatment groups. We conclude that Cav-1 concentrations fluc-

**Effects of Time in Culture and NO Concentration on Cav-1 Expression in EC**

Western blotting was performed to evaluate total Cav-1 protein in EC cultured alone or with lung CA cells in the Transwell model (Fig. 4). For these studies, as for those described previously (27), EC are seeded at confluent densities, 1 × 10⁵ cells/well. Samples were taken either at 2 h, representing confluent EC, or at 20 h in the initial sprouting phase of capillary formation. EC Cav-1 expression at 2 h is high, consistent with abundant localization at borders of confluent cells. There is no difference in Cav-1 expression with or without AG at 2 h. At 20 h, there is a decrease in Cav-1 abundance in HUVEC that are cocultured with lung CA cells. Treatment with AG increases Cav-1 expression in EC in all treatment groups. We conclude that Cav-1 concentrations fluc-

![Fig. 1. Elevated nitric oxide (NO) concentration at the endothelial cell (EC)/tumor cell interface inhibits matrix metalloproteinase (MMP)-9 activation in EC. Gelatin substrate zymography was used to evaluate MMP activities in 16‐h cultures of EC alone or cocultured with EC/carcinoma (CA) cells. Cells were cultured in the presence or absence of 100 μM aminoguanidine (AG) to inhibit NO production. EC lysates were obtained after scraping away CA cells, as described in MATERIALS AND METHODS, and peptides were separated on 10% SDS-PAGE gels copolymerized with gelatin. Light bands in dark background represent proenzyme form (higher-molecular-weight band) or activated form (lower-molecular-weight bands) for each MMP. Purified MMP-2 or MMP-9 standards are shown in first and last lanes, respectively. Arrowheads indicate molecular weight for proenzyme form. Arrows point to activated forms of MMP-9 at 82 kDa and intermediate form at 84–86 kDa. Shown is a representative gel from 3 separate studies with identical results. SQ, squamous carcinoma; AD, adenocarcinoma; HU, human umbilical vein endothelial cells.](image)

![Fig. 2. Direct effects of reactive nitrogen oxide species on MMP-9 activity. Purified MMP-9 standards were activated with aminophenyl mercuric acid then exposed to either spermine-NONOate (S-NONOate) or 3-morpholinosydnonimine (SIN-1) for 5 h at 37°C.](image)
tuate over the time course involved in early capillary formation. Lowering NO levels with AG increases Cav-1 expression in EC at 20 h. These preparations represent Cav-1 protein from total cell lysates and not only those cells that are involved in capillary formation, and it is likely that focal changes in Cav-1 occur in sprouting EC, as shown in Fig. 3C. Increased Cav-1 abundance with AG treatment may correspond to Cav-1/caveola augmentation that occurs before capillary sprouting as reported by others (11, 20, 33).

Effects of EC-Targeted Cav-1 Antisense Treatments on Capillary Formation

Cav-1 has been shown to inhibit cancer cell growth (32). To evaluate directly the importance of Cav-1 in EC in the process of capillary formation, we specifically targeted antisense treatments to EC by taking advantage of the two-compartment culture system. EC were treated in situ in upper compartments of Transwells with either antisense or nonsense oligonucleotides before seeding of CA cells. Experiments demonstrated significant downregulation of Cav-1 24 h after treatment with 1 μM antisense, but not with nonsense, oligonucleotides (Fig. 5). Cultures evaluated for capillary formation by CD31 staining of EC showed highly significant inhibition of angiogenesis (~90%) with two separate antisense oligonucleotides, whereas nonsense oligos had minimal effects on this process (Fig. 5).

Fig. 3. Effect of elevated NO concentrations on the cellular distribution of caveolin-1 (Cav-1). Cav-1 distribution was evaluated in EC growing either alone or in coculture with lung CA cells. In some cultures, AG was used to inhibit NO production. A: Cav-1 localization (arrows) in parts of a confluent EC monolayer. Shown is control monolayer in the absence of CA cells. Cav-1 is abundant in these cells and particularly prominent at the borders of contiguous cells. B: a pattern of focal changes that occur in EC growing in coculture with high NO-producing SQ cells. In B, Cav-1 staining is more diffuse and shows increased concentration in perinuclear areas (arrowheads) consistent with Golgi localization. C: Cav-1 distribution in EC/SQ cocultures treated with AG to reduce NO production. Cav-1 staining is more intense, and short sprouts enriched for Cav-1 are evident (circled areas). D and E illustrate the direct effects of either 500 μM spermine-NONOate or 200 μM SIN-1, respectively, on EC monolayers (EC alone). EC exposed to spermine-NONOate or SIN-1 show diminished staining for Cav-1 structure. Spermine treatment is associated with some perinuclear Cav-1 localization as well as punctate staining in peripheral regions. SIN-1-treated cells show a fade out of protein in the periphery of the cells. Bar = 50 μm.

Fig. 4. Effects of time in culture and NO concentration on Cav-1 expression in EC. Western blotting was performed to evaluate total Cav-1 protein in EC cultured alone or with lung CA cells in Transwell model. Samples were taken either at 2 h, representing confluent EC, or at 20 h in the initial sprouting phase of capillary formation. Lane designations for either 2 or 20 h in the absence of AG: lane 1, human umbilical vein endothelial cells (HUVEC) alone; lane 2, HU from HU/SQ cocultures; lane 3, HU from HU/adenosquamous (AS) cocultures; lane 4, HU from HU/AD cocultures. In the presence of AG: lane 5, HUVEC alone; lane 6, HU from HU/SQ cocultures; lane 7, HU from HU/AS cocultures; and lane 8, HU from HU/AD cocultures. HU Cav-1 expression at 2 h is high, consistent with abundant localization at borders of confluent cells. There is no difference in Cav-1 expression with or without AG at 2 h. At 20 h, there is a decrease in Cav-1 abundance in HU that are cocultured with lung CA cells. Treatment with AG increases Cav-1 protein abundance.
Morphological observations were confirmed by quantification of sprout/capillary lengths using SAMBA software and demonstrated markedly shortened EC sprouts in the antisense-treated cultures. We conclude that EC Cav-1 plays an essential role in capillary formation in this lung CA-induced angiogenesis model.

Effects of NO Concentration on the Distribution and Colocalization of MMP-9 and Cav-1

Data from Fig. 1 demonstrated that high NO concentrations at the EC/tumor interface significantly diminish EC-associated MMP-9 activation. This NO environment also influences Cav-1 abundance (Fig. 4), with increased expression of Cav-1 following inhibition of NO production. These studies were performed with whole EC lysates, but because angiogenesis is a focal phenomenon, it is important to assess the potential relevance of fluctuations in these molecules locally, in EC involved in the process of sprouting. Confocal microscopy was employed to evaluate codistribution of Cav-1 and MMP-9 in EC after 16 h in culture. Transwell cultures of HUVEC that had been cocultured with human lung non-small cell AD cells were stained to demonstrate distribution of these molecules in doubly stained EC.

To compare cellular distributions of these proteins in the presence and absence of high NO concentrations, EC monolayers were examined for structural features consistent with a sprouting phenotype, i.e., clustering associated with extension of processes. Figure 6 illustrates the features of sprouting EC in the EC/tumor cell model. Few of these features were observed in a high NO atmosphere at the time point studied. However, numerous sprouting EC were observed in cultures in which NO production had been inhibited by treatment with the NOS inhibitor AG. To make valid comparisons between treatment groups, observations were made of EC monolayer areas that had similar cellular densities, e.g., both areas showed a similar state of confluence. Optical sectioning was performed using a Leica confocal scanning microscope and software to generate a series of images, x- and y-planes along the z-axis. Typically, each section is 0.5–1 μm thick. Monolayers were sectioned from apical through basolateral planes. Figure 7 shows 3-D reconstructions of image series (maximum projections) for red, green, and true-color overlays (merged).
Figure 7A shows EC from an untreated EC/AD coculture (NO production is not inhibited). EC are exposed to high NO concentrations due, at least in part, to high constitutive production and release of NO from lung cancer (AD) cells. Although few sprout-type structures were seen in this group, some clustering of cells was seen, but evidence of directed migration is not apparent. Cav-1 and MMP-9 proteins occupy distinct locations within the cell, and little colocalization is observed (merged). In contrast, Fig. 7B shows EC from EC/AD coculture treated with 100 μM AG. Early sprouting structures show extensive colocalization of the two proteins (arrows in merged image). C: direct effect of chemically generated NO was evaluated by exposing EC monolayers to 500 μM spermine-NONOate. This NO donor changed both the cellular distribution and abundance of Cav-1, and there was scant evidence of colocalization of MMP-9 with Cav-1. Magnification, ×800.

Fig. 7. Effect of NO concentration on colocalization of MMP-9 and Cav-1. Confocal imaging was used to examine distributions of MMP-9 and Cav-1. Transwell cocultures of EC and human lung non-small cell AD cells were stained to demonstrate distribution of Cav-1 and MMP-9 in EC at 16 h in culture. To compare cellular distributions of these proteins in the presence and absence of high NO concentrations, EC monolayers were examined for the presence of structural features consistent with a sprouting phenotype, i.e., clustering associated with extension of processes. Few of these features were observed in a high-NO atmosphere at the time point studied (A). However, numerous sprouting EC were observed in cultures where NO production had been inhibited by treatment with the nitric oxide synthase inhibitor AG (B). Observations were made of EC monolayer areas that had similar cellular densities, e.g., both areas showed a similar state of confluence. Shown are 3-D reconstructions of image series for red, green, and true-color overlays (merged). A: untreated EC/AD coculture (NO production is not inhibited). Although some clustering of cells is seen, directed migration is not apparent. MMP-9 and Cav-1 are not colocalized in this setting. B: EC/AD coculture treated with 100 μM AG. Early sprouting structures show extensive colocalization of the two proteins (arrows in merged image). C: direct effect of chemically generated NO was evaluated by exposing EC monolayers to 500 μM spermine-NONOate. This NO donor changed both the cellular distribution and abundance of Cav-1, and there was scant evidence of colocalization of MMP-9 with Cav-1. Magnification, ×800.
show extensive colocalization of the two proteins. We have previously demonstrated that elevated NO concentrations at the EC/tumor interface impair capillary formation (25). The data in Fig. 7B suggest that colocalization of these two elements may be necessary for optimum activation of MMP-9, a crucial step in capillary morphogenesis. Finally, the direct effect of chemically generated NO was evaluated by exposing EC monolayers to 500 μM spermine-NONOate (Fig. 7C). As shown in Fig. 3E, exposure of EC to this NO donor changed both the cellular distribution and abundance of Cav-1, and there was scant evidence of colocalization of MMP-9 with Cav-1.

Three-Dimensional Representation of Fluorescence Intensity

Three-dimensional reconstructions of the image series shown in Fig. 7 provide information about colocalization of MMP-9 and Cav-1. These data can also be represented in a manner that provides insight into relative location within individual cells as well as the fluorescence intensity of individual molecules.

In Fig. 8, the x- and y-axes delineate the area of the EC monolayer, i.e., 187.13 μm × 187.13 μm. The z-axis represents fluorescence intensity of red, green, or merged images within an individual section in that monolayer. Images illustrate distribution and colocalization of red (Cav-1) and green (MMP-9) fluorescence in two representative sections within the monolayers. Section 3 represents an area closest to the apical surface of the monolayer. Section 10 represents fluorescence intensity proximal to the basolateral and matrix-associated surface of the cells. These graphic representations correspond to the 3-D image reconstructions shown in Fig. 7, A and B, with the orientations of graphics identical to that of the corresponding photomicrographs. These data show that codistribution of MMP-9 and Cav-1 proteins is greater in the AG-treated group and furthermore, that the highest concentration of these colocalized molecules is at the basolateral surface of the cell. This is consistent with optimization of proteolytic activity in close proximity to its substrate ECM.

DISCUSSION

The role of NO in the process of angiogenesis itself is controversial, with conflicting reports that it is either an angiogenesis inhibitor or stimulator (reviewed in Ref. 10). NO has been shown to function as both an upstream and a downstream mediator of angiogenesis (24, 45). This molecule performs numerous physiological regulatory roles when acting at low concentrations in short, controlled bursts. However, elevated and sustained levels of NO, such as would result from activation of inducible NOS (iNOS), produce indirect effects in the form of nitrosative or oxidative stresses and may lead to genotoxic, cytostatic, or cytotoxic sequelae (43). Given the complex chemistry of the NO molecule and the large number of potential cellular targets, it is not surprising that diametrically opposing roles have been reported in cancer and in angiogenesis (reviewed in Refs. 10 and 43). Investigators who demonstrate angiogenesis stimulation by NO often utilize the rabbit corneal pocket model in which capillary growth is stimulated in an avascular tissue (46). Angiogenesis inhibition is reported from laboratories using the chick chorioallantoic membrane assay (28), a well-vascularized embryonic tissue. It has been suggested that tissues with different levels of baseline vascularization utilize different mechanisms for angiogenesis stimulation that depend, at least in part, on the balance of positive and negative factors within a given tissue (15), with NO concentrations serving a modulatory function.

MMPs are classified into subgroups according to their substrate specificity and structure as collagenases, stromelysins,
gelatinases, and membrane-type MMPs. Collectively, the MMP family can degrade all known ECM components. Expression of these enzymes in tissues is usually low but is induced when remodeling of ECM is needed. MMPs are primarily regulated at the transcriptional level, but some modulation of mRNA stability occurs with cytokines and growth factors. Posttranslational control of MMP function is thought to occur by various activators of the latent proenzyme forms and by specific inhibitors (TIMPs and α2-macroglobulin). MMP activation can occur through intracellular, extracellular, and cell surface-mediated proteolytic mechanisms. MMP-2 appears to be constitutively expressed by many cell types in culture, whereas MMP-9 expression is induced by cytokines, growth factors, and cell stroma interactions (25, 31). Despite close structural homology between MMP-2 and -9, the mechanism(s) of activation of the proteases is different. MMP-2 activation occurs through formation of a ternary complex with TIMP-2 and MT1-MMP on the surface of cells (3). MMP-9 (gelatinase B) does not appear to be activated by the same mechanism, as it remains azymogen under identical cellular conditions where a majority of coexpressed MMP-2 is activated by the cell surface mechanism (31).

Our culture system provides the opportunity to study relevant protease activities at the endothelium/tumor interface. Migration of individual cell populations into the adjacent compartment during the time period of these studies does not occur during the first 30 h. However, the presence of 8-μm pores in the Transwell membrane allows diffusion of soluble components across the membrane barrier, leading to a complex proteolytic milieu in the EC compartment. Such soluble proteases in the extracellular space may play a limited role on EC cell-associated proteolysis. Our studies here with EC/tumor cell cocultures focused on EC-associated MMPs as a source of proteolytic activity in close proximity to underlying ECM and, therefore, most likely to be involved in directed proteolysis necessary for the remodeling of capillary formation. We utilized substrate zymography to evaluate gelatinase activities in EC cell total cell lysates, employing methodology that led to inclusion of EC focal contact proteins that we have previously shown are enriched for MMP-2 and -9 (25). Samples were activated with APMA before zymography so that both latent and activated forms of the enzymes could be visualized within the gels. In a cytokine- and oncprotein-enriched tumor tissue environment such as our EC/cancer cell cocultures, upregulation of MMP expression would not be unexpected. Indeed, MMP-2 expression (latent form) was increased in EC/CA cell cocultures that had been incubated with AG to suppress NO production (Fig. 1), but NO concentration-dependent activation of MMP-2 was not observed. In contrast, decreased NO concentrations in the cocultures were associated with a significant increase in expression of MMP-9, found almost entirely in the activated form. Elevated levels of NO have been shown to influence the expression of MMPs. However, the concentration of NO and the system involved determine whether MMP expression is increased (5, 26) or decreased (35). It is possible that even in the presence of upregulated proenzyme MMP protein, the rate-limiting step in matrix remodeling is localized activation of MMP in cells involved in capillary sprouting.

MMP-9 is a relatively stable zymogen, and whereas a number of proteolytic enzymes have been shown to activate pro-MMP-9 in vitro, the natural activator(s) of MMP-9 is unknown. Activation of this proenzyme in one model of tumor cell invasion has been shown to require converging exogenous serine protease and MMP cascades (31). MMP activity is inhibited when TIMPs bind to the highly conserved zinc-binding site of active MMPs at molar equivalence. The balance between MMPs and inhibitory TIMPs would affect not only MMP-9 but also any other MMP that would be required to activate it. This titering of activity may occur only in very focalized areas, such as regions of wound repair or at the tumor/stromal cell interface (31). The proteolytic cascades involved in MMP-9 activation in our system as well as the effects of elevated NO concentrations on TIMP-1 and -2 expression are currently under investigation. It is possible that EC-associated MMP-9 may be activated by one or more proteases produced by the lung CA cells growing at the tumor cell/EC interface.

The marked effect of NO concentration on EC-associated MMP-9 activation suggested the possibility of localized and/or direct effects of RNOS on this protease. MMP proenzymes are activated when active site zinc is exposed via a cysteine switch mechanism. NO modifies cysteine thiols and transition metal centers of a broad range of proteins with varying functions (37), and ONOO− has been shown to release zinc from zinc-thiolate groups, suggesting that it might alter MMP activity (8). To evaluate the sensitivity of MMP-9 to RNOS, we exposed purified MMP-9 to chemically generated NO or ONOO− in ranges utilized by others to study the effects of these species on MMP-2 (23). As Owens et al. (23) had shown for MMP-2, we observed that ONOO− significantly decreased MMP-9 activity, with both the latent and active forms affected. In contrast to their observations, NO generated by spermine NONOate inhibited MMP-9 activities in a dose-dependent manner. Differences in incubation times or their use of fibroblast-conditioned media rather than purified MMP as sources of proteases could have contributed to some of the differences observed. It is also possible that inherent differences in sensitivity to RNOS may exist for MMP-9, despite close structural homology with MMP-2.

In our cocultures, the CA cells are separated from the EC by a porous (8-μm) membrane that is 10 μm thick, and they therefore could serve as a continuous source of RNOS. In the development of this model system, we did not observe an NO effect in conditioned media (predictably, because of the short half-life of these species) or if the CA cells were cultured on the bottom of the culture dish (1 mm away) instead of on the under-surface of the Transwell. We have shown previously that these cells produce micromolar amounts of NO and express both endothelial NOS and iNOS isoforms (27). Reactive oxygen species, such as hypochlorous acid, hydrogen peroxide, and xanthine-xanthine oxidase-generated hydroxyl radicals have been shown to be potent and direct activators of neutrophil collagenase MMP-8 (34). But in our system, focal fluxes of NO and O2•− may help to regulate matrix degradation during the process of ECM remodeling. Our data demonstrate significant colocalization of MMP-9 with Cav-1 in sprouting capillaries that is dependent on the NO concentration in the microenvironment. Elevated NO concentrations could inhibit MMP-9-associated matrix remodeling both via direct effects on MMP activation and by decreasing compartmentalization of proteolytic machinery necessary for optimum activity.
A limited number of studies have addressed the role of Cav-1 in angiogenesis, and the molecular mechanisms involved have been only partially defined. Brouet et al. (4) demonstrated that Cav-1 and heat shock protein 90 are key targets for the effects of statins on NO-dependent angiogenesis, with decreasing Cav-1 abundance in this setting associated with augmented capillary formation. Studies from another laboratory showed that Cav-1 was essential for capillary formation but played different roles depending on the stage in the process of angiogenesis (19, 20). Downregulation of Cav-1 using an antisense approach decreased the number of capillary structures in this model of VEGF-induced angiogenesis (20). Studies from another laboratory using antisense oligonucleotides to decrease Cav-1 expression resulted in disrupted caveolae- and inhibition of angiogenesis in vitro and in vivo (14).

Because downregulation of Cav-1 using antisense technologies affects multiple cell types, and Cav-1 has been shown to have tumor suppressor activity, we investigated the importance of this molecule, specifically in EC, by targeting oligonucleotides to EC grown in situ. In this setting, we demonstrate significantly attenuated angiogenesis due to direct downregulation of this molecule, specifically Cav-1.

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Because EC sprouting is the first in a series of steps essential for new vessel formation, a number of MMP inhibitors are in clinical trials to evaluate their effectiveness against this component of pathological angiogenesis. Some therapeutic approaches targeting MMP activity utilized general class inhibitors that are selective, but not specific, for individual MMPs. This has resulted in moderately severe but reversible musculoskeletal complications, emphasizing the need for better understanding of the specific MMPs and their precise role in the angiogenic response (17). Localization of key members of the enzymatic machinery leading to activation of pro-MMPs within caveolae might afford the cell a tighter control of the activation and repression of signaling than would be possible if all players diffused freely throughout the cytoplasm. These domains might represent novel targets for manipulating the invasive potential of tumor cells or tumor-induced angiogenesis.

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REFERENCES


