Angiostatin effects on endothelial cells mediated by ceramide and RhoA

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Received December 4, 2000; revised April 24, 2001; accepted April 26, 2001

Angiostatin is a cleavage product of plasminogen that has anti-angiogenic properties. We investigated whether the effects of angiostatin on endothelial cells are mediated by ceramide, a lipid implicated in endothelial cell signaling. Our results demonstrate that angiostatin produces a transient increase in ceramide that correlates with actin stress fiber reorganization, detachment and death. DNA array expression analysis performed on ceramide-treated human endothelial cells demonstrated induction of certain genes involved in cytoskeleton organization. Specifically, we report that treatment with angiostatin or ceramide results in the activation of RhoA, an important effector of cytoskeletal structure. We also show that treatment of endothelial cells with the antioxidant *N*-acetylcysteine abrogates morphological changes and cytotoxic effects of treatment with angiostatin or ceramide. These findings support a model in which angiostatin induces a transient rise in ceramide, RhoA activation and free radical production.

INTRODUCTION

Angiogenesis requires endothelial cell proliferation, migration, tube formation and vessel stabilization. These events are observed during normal development and repair of tissue injury, and are required for tumor growth beyond a few millimeters in diameter. Tumor vessels are dependent upon pro-angiogenic peptides such as vascular endothelial growth factor (VEGF), and members of the fibroblast growth factor (FGF) family (Ribatti *et al.*, 1999; Westphal *et al.*, 2000). In addition to promoting endothelial proliferation and migration, these proteins are reported to inhibit stress-mediated apoptosis in endothelial cells (Pena *et al.*, 2000). *In vivo* evidence suggests that inhibition of

VEGF-signaling pathways will inhibit tumor growth (Prewett *et al.*, 1999). A variety of naturally occurring anti-angiogenic proteins may also regulate angiogenesis in the context of tumor growth. For example, one of the first anti-angiogenic proteins identified, angiostatin, is a cleavage product of plasminogen that inhibits tumor growth (O'Reilly *et al.*, 1994). Although the signaling pathway of angiostatin is unknown, it is reported to produce endothelial cell death by an apoptotic mechanism or by divisional cell death (Claesson-Welsh *et al.*, 1998; Dhanabal *et al.*, 1999; Hari *et al.*, 2000).

A number of other stimuli are known to cause cell death in endothelial cells. Ceramide, a sphingolipid second messenger, is implicated in a pro-apoptotic pathway mediated by LPS, TNF α , ionizing radiation (IR) and hyperosmolarity in endothelial cells that leads to extensive cell death (Xu *et al.*, 1998). Ceramide-induced apoptosis can be abrogated by co-treatment with VEGF and bFGF, suggesting that these proteins function as survival factors in this setting (Gupta *et al.*, 1999). Therefore, inhibitors of angiogenesis, such as angiostatin, may promote cell death by increasing ceramide production. In this report, we provide evidence that the response to angiostatin is at least in part mediated by ceramide and RhoA. Early morphological changes and endothelial cell detachment following treatment with angiostatin or transient exposure to ceramide are blocked by a specific RhoA inhibitor, *Clostridium botulinum* C3 exoenzyme (Narumiya and Morii, 1993; van den Berghe *et al.*, 1996; Needham and Rozengurt, 1998; Ramakers and Moolenaar, 1998; Wang and Bitar, 1998; Beltman *et al.*, 1999; Fukata *et al.*, 1999; Masiero *et al.*, 1999; Stolz *et al.*, 1999). Additionally, an assay for RhoA activation that measures the phosphorylation of LIMK-2 demonstrates activation of RhoA following treatment with angiostatin or ceramide. The

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Fig. 1. Phase contrast micrographs show cell rounding following treatment with (A) 100 ng/ml angiostatin and (B) 1 h treatment with 15 μ M C_2 -ceramide.

Rho-activated kinase, ROCK, specifically phosphorylates LIMK-2 (Maekawa *et al.*, 1999; Sumi *et al.*, 2001). LIM-2 kinase is involved in regulating cytoskeletal reorganization through the phosphorylation and inhibition of the actin depolymerization protein cofilin (Sumi *et al.*, 1999). Most recently it was reported that the overexpression of LIM kinase restored actin stress fibers and inhibited the motility of ras transformed fibroblasts (Sahai *et al.*, 2001).

RESULTS AND DISCUSSION

The morphology of human umbilical vein endothelial cells (HUVECs) was studied during the first 24 h following angiostatin treatment (100 ng/ml at the start of treatment) (Claesson-Welsh *et al.*, 1998; Lucas *et al.*, 1998). Angiostatin treatment was associated with a gradual rounding of the cells that was detectable at 15 min and maximal at 4 h (Figure 1A). A subpopulation of cells that detached from the culture substrate were no longer clonogenic in colony forming assays (data not shown). These data are consistent with our previously published data on endothelial cell death following angiostatin exposure (Hari *et al.*, 2000). As ceramide is a mediator of endothelial cell death (Lin *et al.*, 2000), we treated HUVECs with 100 ng/ml angiostatin and measured intracellular ceramide. Ceramide rose within 5 min to a 2-fold increase at 30–60 min and declined by 2 h (Figure 2A). HUVECs treated with IR (10 Gy) were used as a positive control for ceramide induction. Although the amount of ceramide differed at the earlier time points, the maximum levels were similar at 30 and 60 min with a decline at 2 h. The levels of intracellular ceramide following exposure to angiostatin and IR as detected using TLC are shown in Figure 2B. HUVECs were exposed to 15 μ M C_2 -ceramide for 1 h and then placed in ceramide-free medium for 16 h. Under these conditions, HUVECs begin to round as early as 5 min, with maximal rounding at 30 min to 1 h (Figure 1B). The morphological data suggested that some of the effects of angiostatin may be mediated through ceramide signaling. To assess the role of ceramide in endothelial gene expression, we used cDNA macroarrays to screen for potential transcriptional targets of ceramide. Initial data showed

Fig. 2. Increase of endogenous ceramide following treatment with angiostatin (triangles) or IR (10 Gy) (squares). (A) Ceramide production was measured by counting the radioactivity of each TLC band, calculating pmols/ 10^6 cells from experimentally obtained calibration curves and normalizing to control levels. (B) TLC detection of intracellular ceramide following exposure to angiostatin or IR.

that administration of 20 μ M C_2 -ceramide to HUVECs leads to changes in expression among 10% of the genes presented on the array (58 out of 588 genes). Only 21 were upregulated. Among these upregulated genes was RhoA, a small GTPase involved in regulation of cell motility, focal adhesions, actin filament formation and related changes in cell morphology (Imamura *et al.*, 1998). Further DNA array experiments and application of cluster analysis revealed that upon application of stress stimuli to endothelial cells, RhoA is co-expressed together with several integrin molecules and p38 MAPK (data not shown).

RhoA is regulated primarily by post-translational modification, including translocation of the protein from the cytosol to the cell membrane (Allal *et al.*, 2000). We therefore investigated RhoA activation by angiostatin and ceramide. When treated with angiostatin, membrane-associated RhoA was downregulated by

Fig. 3. (A) Kinetics of RhoA translocation following treatment with 100 ng/ml angiostatin is delayed and attenuated compared with (B) translocation following 15 μ M C_2 -ceramide treatment, as would be expected for dependence on the production of endogenous ceramide. (C) Results of the LIM-2 kinase assay demonstrating the kinetics of RhoA activation following exposure to angiostatin compared with (D) RhoA activation following exposure to ceramide. (E) Kinetics of actin reorganization parallels the translocation of RhoA to the membrane for treatment with 100 ng/ml angiostatin and (F) with 15 μ M C_2 -ceramide. Ruffling denoted by thick arrows; stress fibers denoted by thin arrows.

30% at the 30 s and 5 min time points but increased to a 2.1-fold upregulation at 120 min (Figure 3A). Following treatment with exogenously added 15 μ M C_2 -ceramide, RhoA translocation to the membrane was detectable within 5 min and was maximal

Fig. 4. Cell rounding is abrogated by treatment with C3-exotoxin or NAC. Rounded cells were counted from phase-contrast micrographs. (A) Treatment with 100 ng/ml angiostatin alone (black bars), 72 h pretreatment with 20 μ g/ml C3-exotoxin before addition of angiostatin (hatched bars) and 14 h pretreatment with 2 mM NAC before addition of angiostatin (white bars). (B) Treatment with 15 μ M ceramide alone (black bars) and the same pretreatments as above.

(3.5-fold) at 30 min (Figure 3B). The LIM-2 kinase assay [which tests the ability of the Rho-activated kinase, ROCK, to specifically phosphorylate LIMK-2 (Sumi *et al.*, 2001)] was employed to confirm RhoA activation. The results of this assay are shown in Figure 3C and D, which provide further evidence of transient RhoA activation following exposure to angiostatin and C_2 -ceramide. The timing of RhoA translocation to the membrane was associated with reorganization of the actin cytoskeleton (Figure 3E and F). Phalloidin staining for F-actin and confocal microscopy showed that in HUVECs, the initial stages of response to angiostatin and ceramide are similar with formation or augmentation of actin stress fibers, membrane ruffling, disruption of cell-cell contacts and loss of focal adhesions. As a result, cell rounding is initiated and subsequent disorganization of the cytoskeleton occurs. These initial changes (stress fibers, membrane ruffling and loss of focal adhesions) are similar to initiation of cell motility (Rousseau *et al.*, 2000), while later stages (disruption of actin filaments and cell rounding) are more consistent with a cytotoxic response (Yamazaki *et al.*, 2000).

To determine whether the angiostatin and ceramide-induced cell shape changes are dependent upon RhoA, we used *Clostridium botulinum* C3 exoenzyme to inhibit the activation of RhoA. Incubation with 20 μ g/ml C3 exoenzyme for 72 h prior to angiostatin or ceramide treatment inhibited the effects on endothelial cell morphology (Figure 4). These effects were not due to a generalized suppression of cellular processes since little effect on cell viability was observed ($89.9 \pm 3.0\%$ as determined

by MTT assay; data not shown). Since Rac1, another GTPase associated with RhoA function, is associated with oxygen radical production (Kheradmand *et al.*, 1998), we also investigated the possibility that the RhoA-associated morphological changes following angiostatin and ceramide might also be tied to reactive oxygen species (ROS) production. The addition of the ROS scavenger *N*-acetyl-L-cysteine before angiostatin or C₂-ceramide abrogated cell rounding and detachment (Figure 4).

Our data support the involvement of ceramide signaling in the effects of angiostatin on endothelial cells. The results demonstrate that ceramide signaling includes activation of RhoA and is dependent on ROS. These data implicate RhoA and ROS in the responses of endothelial cells to angiostatin and to ceramide. The role of small GTPases of the Ras family (Rac1, RhoA and cdc42) in cell shape, adhesion and motility is well documented (Imamura *et al.*, 1998; Allal *et al.*, 2000; Rousseau *et al.*, 2000), but much less is known about their role in stress response and regulation of cell survival/death. Recent data implicate Rac1 in TNF-induced endothelial cell apoptosis as a survival factor, acting through ROS (Deshpande *et al.*, 2000). In contrast, our data suggest a function for RhoA in a cell death pathway. Our finding of ceramide as a mediator of angiostatin and inducer of RhoA provides a previously missing link between angiostatin and major up-stream regulators of cytoskeleton organization/focal adhesions in endothelial cells.

Recently, Moser *et al.* (1999) identified an angiostatin binding site on endothelial cells as the α/β subunits of ATP synthase. The authors propose that angiostatin might disrupt the production of ATP and thus render endothelial cells susceptible to hypoxic damage. Also, Troyanovsky *et al.* (2001) employed a yeast two-hybrid screen to identify angiostatin, a 75 kD protein that localizes to the lamellopodia and membrane ruffles of migrating endothelial cells and induces FAK activity. We speculate that changes in RhoA activity following angiostatin exposure in endothelial cells may mediate physiological effects of angiostatin as a downstream effector from one or both of the putative angiostatin receptors. These data are supported by the known role of the small GTP proteins in cellular functions such as migration and in the response to oxidative stress.

METHODS

Cell culture and imaging. HUVECs were obtained from Clonetics, and were maintained in culture in EGM-2 medium (Clonetics) at 37°C and 5% CO₂. Cells were photographed using a phase-contrast microscope (Nikon). For confocal microscopy, cells were stained for F-actin with phalloidin conjugated to rhodamine and visualized using an Olympus Fluoview Confocal Laser Scanning System and Fluoview software.

Ceramide assay. Ceramide was quantified using the diacylglycerol (DAG) kinase assay as previously described (Chmura *et al.*, 1996). As a positive control for ceramide production, cells were irradiated with 10 Gy delivered using a Gammacell 220 irradiator (Nordion).

Immunoblotting of membrane-associated RhoA. Cells were allowed to grow to 80% confluence on plates in complete medium. Medium was exchanged with fresh complete medium with 20 μ M C₂-ceramide or 100 ng/ml angiostatin at time zero. The cells were washed with ice-cold PBS at indicated time points and scraped in 0.3 ml of lysis buffer (50 mM Tris-Cl,

pH 7.3, 10 mM NaCl, 1 mM EGTA, 1 mM Na₃VO₄, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride). The extracts were sonicated for 5 s on ice and centrifuged at 250 g for 5 min to pellet unlysed cells, and the supernatants were further centrifuged at 100 000 g for 1 h. The pellet (membrane fraction) was resuspended in the same buffer with 0.1% Triton X-100 and left on ice for 15 min with frequent vortexing. Extracts were run on SDS-12% PAGE gels, transferred to 0.45 μ m PVDF membranes (Millipore), and then probed with a monoclonal anti-RhoA primary antibody (Santa Cruz), an anti-mouse IgG conjugated to horseradish peroxidase secondary antibody, and detected using a chemiluminescence detection kit (Pierce).

LIM-2 kinase assay. Cells were allowed to grow to 80% confluence on plates in complete medium. Medium was exchanged with fresh complete medium with 20 μ M C₂-ceramide or 100 ng/ml angiostatin at time zero. The cells were washed with PBS at room temperature. 0.6 ml ice-cold RIPA buffer with inhibitors was added to the plate. The cells were scraped and transferred to a microcentrifuge tube. The plate was rinsed with 0.3 ml RIPA buffer and combined with the first lysate. The lysate was passed through the 21-gauge needle to shear the DNA. A 10 μ l aliquot of PMSF (10 μ g/ml) was added and incubated for 30 min on ice. The cell lysate was centrifuged at 10 000 g for 10 min at 4°C. LIMK-2 is phosphorylated on Thr-505 when activated, so 10 μ g agarose-conjugated p-Thr (H-2) antibody (Santa Cruz) were added to the supernatant and incubated overnight at 4°C with mixing. The pellet was collected by centrifugation at 2500 r.p.m. for 5 min at 4°C. The pellet was washed 4x with PBS + 0.4% NP-40 and then resuspended in 40 μ l of 1x electrophoresis sample buffer, boiled for 3 min, and electrophoresed on a 12% Bio-Rad ReadyGel. The proteins were transferred to 0.45 μ m PVDF membranes (Millipore), and then probed with a polyclonal LIMK-2 (N-20) primary antibody (Santa Cruz), an anti-goat IgG conjugated to horseradish peroxidase secondary antibody, and detected using a chemiluminescence detection kit (Pierce).

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DOI: 10.1093/embo-reports/kve115