Endostatin Causes G₁ Arrest of Endothelial Cells through Inhibition of Cyclin D₁*

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Endostatin, a type XVIII collagen fragment, is a potent antiangiogenic molecule that inhibits endothelial cell migration, promotes apoptosis, and induces cell cycle arrest in vitro. We have investigated the mechanism by which endostatin causes G₁ arrest in endothelial cells. Endostatin decreased the hyperphosphorylated retinoblastoma gene product and down-regulated cyclin D₁ mRNA and protein. Importantly, endostatin was unable to arrest cyclin D₁ overexpressing endothelial cells, suggesting that cyclin D₁ is a critical target for endostatin action. Next, we analyzed cyclin D₁ promoter activity in endothelial cells and found that endostatin down-regulated the cyclin D₁ promoter. Using a series of deletion and mutant promoter constructs, we identified the LEF1 site in the cyclin D₁ promoter as essential for the inhibitory effect of endostatin. Finally, we showed that endostatin can repress cyclin D₁ promoter activity in cells over-expressing β-catenin but not in cells over-expressing a transcriptional activator that functions through the LEF1 site and is insensitive to β-catenin. Collectively, our data pointed to a role for cyclin D₁, and in particular, transcription through the LEF1 site as critical for endostatin action in vitro and suggest that β-catenin is a target for endostatin.

Endostatin (ES),¹ the carboxyl-terminal 184 amino acids in the NC1 domain of collagen XVIII, is a recently discovered antiangiogenic molecule (1). Recombinantly produced ES or gene transfer of ES into tumor cells has been shown to markedly inhibit tumor growth and angiogenesis in mice. In ongoing Phase I clinical trials, ES administration appears to be without toxicity and has produced disease stabilization in an occasional patient. In vitro, ES possesses several activities, including inhibition of endothelial cell migration, induction of endothelial cell apoptosis, and G₁ cell cycle arrest (2–4). Molecular signaling mechanisms responsible for these events are under intense investigation. At the cell surface, integrins (5) and glypicans (6) have been implicated in the antimigratory effects of ES. Using suitable chimeric receptors, ES has been shown to trigger events that antagonize intracellular signals induced by the proangiogenic agents VEGF and bFGF, but the precise molecular targets remain to be discovered (6–8). To date, c-myc is the only gene whose expression is inhibited by ES (9). Of note, the introduction of this gene into endothelial cells abrogates the antimigratory effect of ES (9).

To date, signaling events that mediate the cell cycle effects of endostatin are unknown. Cell cycle progression occurs through distinct phases of the cell cycle, regulated by both intracellular and extracellular mechanisms. Growth factors are primarily involved in the exiting of a cell from a quiescent G₀ state, and these factors are necessary until cells reach a so-called restriction point “R.” Phosphorylation of retinoblastoma protein is a marker for the R point, and this step is regulated by G₁ cyclin-dependent kinases (CDKs). In turn, CDK activity is regulated by complexing with cyclins. Cyclin D₁ plays a key role in the transition of cells from G₁ to S, and abrogation of its expression leads to G₁ cell cycle arrest.

Based on our initial observation that ES causes endothelial cell cycle arrest in G₁, we investigated the molecular events. We show here that cyclin D₁ is a relevant target gene and that the LEF1 site in its promoter is critical for mediating the repressive effect of endostatin. Moreover, we use this target to analyze events affected by ES upstream of cyclin D₁, such as the important intracellular mediator β-catenin. A unifying hypothesis that emerges is that ES targets β-catenin and subsequently transcription of genes such as c-myc and cyclin D₁ containing LEF1/TCF binding sites in their promoters.

EXPERIMENTAL PROCEDURES

Reagents and Materials—The mouse and human endothostatin proteins (mES and hES, respectively) and mouse endostatin mutant (mES3.1) were produced and purified from yeast as described previously (4, 6). VEGF and bFGF were obtained from R&D Systems, Inc (Minneapolis, MN). The Dual-Luciferase Reporter Assay System was purchased from Promega (Madison, WI). The Calphos transfection kit was obtained from CLONTECH (Palo Alto, CA) and LipoFectAMINE 2000 was purchased from Invitrogen.

Plasmid Constructs—ES cDNA with K-cadherin signal peptide sequence in the amino terminus was amplified by PCR and cloned into pCS2 (10). This construct was referred to as pCS2-ES. Plasmids
containing the luciferase reporter under control of the wild type cyclin D1 promoter as well as numerous deletions and point mutants have been described previously (11, 12). The pCS2-TVP construct (a gift from A. Vonica) (13) has been described previously. The full-length human cyclin D1 sequence was amplified by PCR and cloned using MluI and NotI sites into the retroviral vector pCMMP-RGS7-IRES GFP (a gift from R. Mulligan and T. Benzing), confirmed by sequencing, and referred to as the cyclin D1 retrovirus construct. The TVP sequence was excised from a pCS2-TVP plasmid by partial digestion and cloned into the EcoRI- and NotI-digested retroviral vector pLXSHD2 (a gift from Miller and A. Kazlauskas) (6), confirmed by sequencing, and referred to as the TVP retroviral construct.

Cell Culture and DNA Transfection—Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (San Diego, CA). Bovine pulmonary arterial endothelial cells (C-PAEs) were obtained from American Type Culture Collection (Manassas, VA). HUVECs and C-PAEs were used between passages 2 and 3. HUVECs were maintained in EGM2-MV medium (Clonetics) containing endothelial basal medium (EBM-2) supplemented with 5% fetal bovine serum, gentamicin, amphothericin B, hydrocortisone, ascorbic acid, and the following growth factors: VEGF, bFGF, hEGF, and IGF-1. C-PAEs were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin. All cell lines were grown at 37 °C in a 100% humidified incubator with 5% CO2. Cells were grown to 80–90% confluency, harvested with trypsin, and resuspended to the cell density required for each assay. For transient transfections, 50–60% confluent cells in 6-well plates were transfected using LipofectAMINE 2000 (Invitrogen).

Retrovirus Production—Retrovirus production was performed as described before (14). Briefly, 20 μg of retroviral vector cDNA was transfected into the 293 GPG packaging cell line using the Calphos transfection kit. 48 h after transfection, the packing cell line supernatant was collected and used to infect target cells (HUVECs) in complete medium (EGM-2MV).

Cell Cycle Analysis—C-PAE and HUVEC cells were growth-arrested by contact inhibition for 48 h. The 0 h value refers to the percentage of cells in S phase at this time point. The cells (0.1 × 10^6 cells/well) were harvested and plated into a 6-well plate in 1% FCS/DMEM (for C-PAEs) or 1% FCS/EGM2-MV (for HUVECs) with recombinant VEGF or bFGF with or without ES. The cells were harvested at various time points and then fixed in ice-cold ethanol. Fixed cells were dehydrated at 4 °C for 30 min in phosphate-buffered saline containing 2% FCS and 0.1% Tween 20 and then centrifuged and resuspended in 0.5 ml of the same buffer. RNase digestion (5 μg/ml) was carried out at 37 °C for 1 h, followed by staining with propidium iodide (5 μg/ml). The cells were analyzed using a FACScan BD PharMingen flow cytometer.

Luciferase Assay—After transient transfection of the cDNAs, cells were incubated for 20 h, and luciferase activity in the cell lysates was determined using a luminometer and normalized using Renilla luciferase activity under the control of the thymidine kinase promoter.

Immunoblotting—Collected cell lysates were separated by polyacrylamide gel electrophoresis (precast gels, Bio-Rad) followed by electroblotting onto a polyvinylidene difluoride membrane. After blocking with 2% bovine serum albumin in Tris-buffered saline/Tween-20 (TBS-T) for 1 h, the polyvinylidene difluoride membrane was incubated overnight with each primary antibody. After washing with TBS-T, the membrane was incubated with the secondary anti-mouse Ig at a 1:5000 dilution for 30 min. Protein bands were detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

RESULTS

Cell Cycle Arrest of Endothelial Cells by ES—We have previously reported that ES causes G1 arrest of endothelial cells

![Fig. 1. Time course of the effect of ES on the endothelial cell cycle. C-PAEs (A) and HUVECs (B) were growth-arrested by contact inhibition for 48 h in complete medium. 0.1 × 10^6 cells were seeded in each well of 6-well plates in 1% FCS/DMEM or 1% FCS/EGM2-MV supplemented with VEGF (10 ng/ml) and/or ES (10 μg/ml) as indicated. Cells were harvested, and cell cycle analysis was performed at various points.](image-url)
We began our mechanistic studies by defining the time course of this effect and its dependence on ES dose. At various times after treatment with VEGF with or without ES, we monitored the percentage of cells in S phase in both C-PAEs and HUVECs. VEGF induced both cell types into S phase, whereas addition of ES markedly reduced S phase entry (Fig. 1, A and B). Similar data were obtained with bFGF (data not shown). Moreover, mutant ES3.1, which has been shown not to bind to the cell surface, did not cause cell cycle arrest (data not shown). At the 18–21 h time points, which demonstrated this effect most clearly, we assessed ES dose dependence (Fig. 2, A and B). The half-maximal effect was noted at about 5 μg/ml ES.

**ES Inhibits Cyclin D1 Protein Expression**—We next examined the phosphorylation state of pRb protein in endothelial cells since it reflects the ability of a cell to exit G1. ES inhibited the hyper-phosphorylation of pRb induced by VEGF (Fig. 3A) and bFGF (data not shown), thus providing molecular confirmation of a G1 arrest induced by ES. To clarify the mechanism of this effect, we examined the protein expression level of several cyclins and of cyclin-dependent kinases. Consistent
with our retinoblastoma data, the cyclin D1 protein level in HUVECs was increased by treatment with VEGF or bFGF, and this up-regulation was inhibited by ES. Cyclin D1 was decreased as early as 8 h in HUVECs (Fig. 4A) and in C-PAEs at later time points (Fig. 4B). After cyclin D1 down-regulation (>14 h), cyclin A levels began to decrease; no changes were noted in the expression of cdk-2 or cdk-4 (data not shown).

ES Causes G1 Arrest of Endothelial Cells through Cyclin D1—To critically address the significance of the cyclin D1 changes observed upon ES addition, we generated cyclin D1 over-expressing HUVECs (cyclin D1-HUVECs) by retroviral infection and tested the effects of ES on these cells using cycle analysis. ES inhibited progression into S phase induced by VEGF in control HUVECs (infected with a LacZ-carrying retrovirus) but did not cause G1 arrest in cyclin D1-HUVECs (Fig. 5). These data point to cyclin D1 as a critical ES target in mediating its effects on the cell cycle.

ES Affects Expression of Cyclin D1 mRNA and Inhibits Cyclin D1 Promoter Activity—Northern blot analysis revealed that ES decreased cyclin D1 mRNA levels in control cells, but this down-regulation was not observed in cyclin D1-HUVECs, consistent with observations in Fig. 4. The decrease of cyclin D1 mRNA by ES was clear after 8 h with significant inhibition seen by 14 h. These data suggest that the changes noted in cyclin D1 protein expression are reflected by antecedent changes in cyclin D1 mRNA and could be accounted for either by effects on cyclin D1 mRNA transcription and/or on mRNA stability. To address this issue, we transfected a cyclin D1 promoter reporter construct into endothelial cells and assessed the effect of ES on this promoter. VEGF and bFGF up-regulated promoter activity, whereas ES down-regulated this activity in a dose-dependent fashion (Fig. 7). The dose dependence noted earlier on G1 arrest (Fig. 2) was paralleled in the dose dependence noted earlier on G1 arrest (Fig. 2). This data suggest that ES affects cyclin D1 transcription.

ES Decreases Cyclin D1 Promoter Activity via the LEF1 Site—To determine site(s) in the cyclin D1 promoter responsive to ES, we used a series of deletion mutants of the cyclin D1 promoter. As shown in Fig. 8A, the responses to VEGF and ES were lost after the deletion of nucleotides between −141 and −66 (numbers are from the ATG start codon). The cyclin D1 promoter has both SP1 and LEF1 sites between −141 and −66. Since the SP1 site is thought to be the primary VEGF-responsive element (15), we utilized point mutants in the LEF1 site in the context of the −1745 and the −163 constructs. The results of these experiments (Fig. 8B) point to the LEF1 site as the target for down-regulation of the cyclin D1 promoter by ES.

ES Can Inhibit β-Catenin-induced Cyclin D1 Promoter Activity—Since the LEF1 site was important for the inhibitory effect of ES on the cyclin D1 promoter, we focused on intracellular signaling events upstream of transcription through the LEF1 site. β-catenin is known to interact with LEF1/TCF transcription factors to activate transcription through this site. We, therefore, assessed the effects of ES on the −163 cyclin D1 promoter construct when β-catenin was over-expressed. Fig. 9A shows that β-catenin enhanced luciferase activity from the −163 promoter construct and that ES inhibited this increase. Nearly identical results were obtained with the −1745 construct (Fig. 9B). These data suggest that ES either acts on β-catenin or targets an event “downstream” of β-catenin.

To further define the site of action of ES, we utilized an expression vector (TVP) coding for a constitutive activator (insensitive to β-catenin) acting through the LEF1 site. TVP expresses a fusion protein consisting of the transcriptional activator VP16 and (Xenopus) TCF-3 in which the β-catenin binding region has been deleted. As shown in Fig. 9, ES could not inhibit TVP-stimulated promoter activity from either the −163 or −1745 cyclin D1 promoter constructs, suggesting that ES does not block LEF1/TCF binding to DNA.

DISCUSSION

We have used the fact that ES causes G1 arrest of endothelial cells to dissect part of its molecular mechanism of action. Our novel observations are that ES inhibits cyclin D1 RNA and protein expression in endothelial cells, that this suppression is via transcriptional inhibition through the LEF1 site in the cyclin D1 promoter, and that endostatin acts at the level of β-catenin or downstream of it. Finally, we have shown that over-expression of cyclin D1 protein overrides the effects of ES on the cell cycle.
FIG. 8. The LEF1 site is critical for the effect of ES on cyclin D1 promoter activity. A, transcriptional activity of the cyclin D1 promoter in C-PAE cells measured by using deletion mutants as indicated. The bold line in the schema represents the approximate location of LEF1 and SP1 sites. In constructs with these sites deleted, there was no activation by VEGF (20 ng/ml), and inhibition by ES could not be assessed. B, transcriptional activity of a point mutant affecting the LEF1 site in the context of the −1745 or −163 cyclin D1 promoter constructs. VEGF (20 ng/ml) was able to induce promoter activity, but there was no inhibition by ES (20 μg/ml) on the LEF1 site mutated promoter constructs.

FIG. 9. ES inhibits β-catenin-induced cyclin D1 promoter activity but is ineffective in the presence of TVP. The indicated cDNAs were transfected into C-PAEs. −163 CyD LUC and −1745 CyD LUC refer to the −163 and −1745 cyclin D1 luciferase constructs, respectively. Cells were harvested 21 h after transfection, and luciferase activity was measured. TVP represents a fusion of VP16 and TCF3 that lacks a β-catenin binding site.
The importance of cyclin D1 in transitioning cells from G1 into S has been amply demonstrated (reviewed in Ref. 16). Though cyclin D1 null animals are viable, abrogation of cyclin D1 expression in vitro (e.g. by antisense methods) causes cell cycle arrest or marked inhibition of cell proliferation (17–23). Of note, a dominant negative form of TCF causes cell arrest in G1 and can be rescued by cyclin D1 over-expression (24). Thus, our observation that cyclin D1 is an endostatin target is consistent with the known importance of this protein in cell cycle progression. Moreover, in cells over-expressing cyclin D1, endostatin was no longer efficacious in causing cell cycle arrest, indicating that there is no target downstream of cyclin D1 critical for ES action.

Identification of cyclin D1 (and the LEF1 site in its promoter) as an ES target has provided an inroad into intracellular signaling events triggered by ES, namely the role of β-catenin. Another line of investigation has also converged with the data presented here. Very recently, we have shown that ES can block axis duplication in Xenopus embryos induced by Wnt agonists. In particular, we have demonstrated that transcriptional targets of Wnt signaling (endogenous ones such as Siamois as well as synthetic constructs, e.g. TOP-FLASH containing multimerized LEF1 sites upstream of a reporter) can be inhibited by ES. Moreover, by utilizing numerous constructs in the canonical Wnt pathway upstream of β-catenin, we have shown that ES targets β-catenin through a Wnt-independent pathway. What this pathway is and how it connects to cell surface receptors for ES (e.g. the glypicans) remains to be elucidated.

There are several relationships among c-myc, a previously established ES target gene, cyclin D1, and cell cycle control. c-Myc promotes cyclin E-Cdk2 activity and E2F-dependent transcription (reviewed in Ref. 25) so that its expression is important for G1/S control. Moreover, c-Myc has been reported to induce cyclin D1 (26) (as well as cyclin D2 and E (25)) at the transcriptional level but can increase cyclin D1-kinase activity also (27, 28). Moreover, c-Myc (29) and cyclin D1 (11) are targets for β-catenin through the LEF1 sites they share in their respective promoters. Thus, our observation that ES can target β-catenin and LEF1 sites suggests the unifying hypothesis that ES might target other promoters containing such sites, accounting for the observation that c-Myc is down-regulated by ES. It should be noted, however, that not all genes containing core LEF1 site. These hypotheses will be the subject of future studies.

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