ESE-1 Is a Novel Transcriptional Mediator of Angiopoietin-1 Expression in the Setting of Inflammation*

Angiogenesis is a critical component of the inflammatory response associated with a number of conditions. Angiopoietin-1 (Ang-1) is an angiogenic growth factor that promotes the chemotaxis of endothelial cells and facilitates the maturation of new blood vessels. Ang-1 expression is up-regulated in response to tumor necrosis factor-α (TNF-α). To begin to elucidate the underlying molecular mechanisms by which Ang-1 gene expression is regulated during inflammation, we isolated 3.2 kb of the Ang-1 promoter that contain regulatory elements sufficient to mediate induction of the promoter in response to TNF-α, interleukin-1β, and endotoxin. Surprisingly, sequence analysis of this promoter failed to reveal binding sites for transcription factors that are frequently associated with mediating inflammatory responses, such as NF-κB, STAT, NFAT, or C/EBP. However, putative binding sites for ETS and AP-1 transcription factor family members were identified. Interestingly, among a panel of ETS factors tested in a transient transfection assay, only the ETS factor ESE-1 was capable of transactivating the Ang-1 promoter. ESE-1 binds to specific ETS sites within the Ang-1 promoter that are functionally important for transactivation by ESE-1. ESE-1 and Ang-1 are induced in synovial fibroblasts in response to inflammatory cytokines, with ESE-1 induction slightly preceding that of Ang-1. Mutation of a high-affinity ESE-1 binding site leads to a marked reduction in Ang-1 transactivation by ESE-1, inducibility by inflammatory cytokines, and DNA binding to the ESE-1 protein. Transcriptional profiling of cells transiently transfected with an ESE-1 expression vector demonstrates that the endogenous Ang-1 gene is directly inducible by ESE-1. Finally, Ang-1 and ESE-1 exhibit a similar and strong expression pattern in the synovium of patients with rheumatoid arthritis. Our results support a novel paradigm for the ETS factor ESE-1 as a transcriptional mediator of angiogenesis in the setting of inflammation.

Inflammation is an important component of several pathological conditions, including wound healing, atherosclerosis, and arthritis. Inflammation is often associated with the development of granulation tissue consisting of infiltrating mononuclear cells and blood vessels (1, 2). As this tissue proliferates there are zones of relative hypoxia where growth of the tissue has outstripped the blood vessel supply, triggering signals for further blood vessel development (3). The angiogenesis that results from these signals provides the necessary blood supply, oxygen, and nutrients to support the proliferating tissue, and plays an active role in the transport of inflammatory cells to the site of inflammation (4). The endothelial cells themselves also provide a source of proinflammatory cytokines.

One of the first steps in this process is the induction of angiogenic growth factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (5). These growth factors promote the proliferation and migration of endothelial cells to sites of inflammation, which is facilitated by the expression of matrix degrading enzymes and integrins on activated endothelial cells (6). Endothelial cells that have migrated to sites of inflammation then organize into small tubes. The formation of stable blood vessels requires the recruitment of surrounding mesenchymal cells and their differentiation into vascular smooth muscle cells. Vessel maturation is facilitated by the angiopoietins (7).

Ang-1 has the unique property of enhancing the stability of new blood vessels by recruiting surrounding mesenchymal cells and promoting their differentiation to vascular smooth muscle cells (7). In contrast to other angiogenic substances like VEGF or bFGF, Ang-1 is not a potent mitogen for endothelial growth. However, Ang-1 is a potent chemoattractant of endothelial cells and enhances endothelial cell migration. Other properties of Ang-1 include its ability to promote the adhesion of endothelial cells to fibronectin, as well as its ability to prevent endothelial cell death by inhibiting apoptosis (8, 9).

We and others (10–12) have recently demonstrated that Ang-1 mRNA and protein are expressed in the synovium of patients with rheumatoid arthritis (RA). Ang-1 is expressed at low levels in primary human synovial fibroblasts obtained from patients with RA, and can be markedly up-regulated in response to inflammatory cytokines such as TNF-α. However, the transcriptional mechanisms by which Ang-1 gene expression is regulated are currently unknown.

The purpose of this study was to begin to identify the molecular mechanisms of Ang-1 gene regulation in the setting of inflammation.

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Angiogenesis is a critical component of the inflammatory response associated with a number of conditions. Angiopoietin-1 (Ang-1) is an angiogenic growth factor that promotes the chemotaxis of endothelial cells and facilitates the maturation of new blood vessels. Ang-1 expression is up-regulated in response to tumor necrosis factor-α (TNF-α). To begin to elucidate the underlying molecular mechanisms by which Ang-1 gene expression is regulated during inflammation, we isolated 3.2 kb of the Ang-1 promoter that contain regulatory elements sufficient to mediate induction of the promoter in response to TNF-α, interleukin-1β, and endotoxin. Surprisingly, sequence analysis of this promoter failed to reveal binding sites for transcription factors that are frequently associated with mediating inflammatory responses, such as NF-κB, STAT, NFAT, or C/EBP. However, putative binding sites for ETS and AP-1 transcription factor family members were identified. Interestingly, among a panel of ETS factors tested in a transient transfection assay, only the ETS factor ESE-1 was capable of transactivating the Ang-1 promoter. ESE-1 binds to specific ETS sites within the Ang-1 promoter that are functionally important for transactivation by ESE-1. ESE-1 and Ang-1 are induced in synovial fibroblasts in response to inflammatory cytokines, with ESE-1 induction slightly preceding that of Ang-1. Mutation of a high-affinity ESE-1 binding site leads to a marked reduction in Ang-1 transactivation by ESE-1, inducibility by inflammatory cytokines, and DNA binding to the ESE-1 protein. Transcriptional profiling of cells transiently transfected with an ESE-1 expression vector demonstrates that the endogenous Ang-1 gene is directly inducible by ESE-1. Finally, Ang-1 and ESE-1 exhibit a similar and strong expression pattern in the synovium of patients with rheumatoid arthritis. Our results support a novel paradigm for the ETS factor ESE-1 as a transcriptional mediator of angiogenesis in the setting of inflammation.
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Our findings support a central role for the ETS transcription factor ESE-1 during this process. ESE-1 was originally identified as a transcription factor that, under basal conditions, is highly restricted to cells of epithelial origin (13). However, in response to inflammatory stimuli, this factor can be induced in a variety of non-epithelial cells, including vascular smooth muscle cells, chondrocytes, synovial fibroblasts, and endothelial cells (14). We recently showed that ESE-1 is highly expressed in the synovium of patients with rheumatoid arthritis (15). In this study we present direct evidence indicating a role for ESE-1 in Ang-1 gene regulation. ESE-1 can bind to specific ETS sites within the Ang-1 promoter that are functionally important for the transactivation by ESE-1, expression of ESE-1 in cultured cells leads to induction of the endogenous Ang-1 gene, and Ang-1 exhibits a similar and strong expression pattern in the synovium of patients with RA. In summary, our results support a role for the ETS factor ESE-1 as a novel transcriptional regulator of Ang-1 gene expression in the setting of inflammation.

EXPERIMENTAL PROCEDURES

Tissue Culture and Reagents—The cell lines HEK293 (human embryonic kidney), NIH3T3 (mouse fibroblast), and MCF-7 (human breast cancer) were grown in DMEM supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT) and antibiotics (penicillin and streptomycin; Invitrogen). Synovial tissue and bone were obtained at the time of arthroplasty or joint replacement surgery from patients with the clinical diagnosis of RA. Tissue procurement was approved by the Institutional Review Board. Dispersed synovial tissues were prepared by a previously published method (16). Briefly, synovial tissues were minced on tissue culture plates and treated with Type I collagenase (4 mg/ml; Worthington Biochemical Corporation, Lakewood, NJ), in DMEM (Invitrogen), incubated for 1 h at 37 °C, treated with 0.25% trypsin for 30 min, harvested, and centrifuged at 1,000 rpm for 10 min. Pellets were suspended in 0.05% trypsin-0.02% EDTA, centrifuged, and resuspended in 50% phosphate-buffered saline (Ca2+/Mg2+-free), 150 m M NaCl, 10 m M Tris pH 7.4, 1 m M phenylmethylsulfonyl fluoride, and 1 m M dithiothreitol. Whole cell lysates were obtained instead of nuclear extracts because the number of cells were limited and the primary synovial fibroblasts do not maintain their phenotype after 4–5 passages. 10 μl of whole cell extract or 2 μl of in vitro translation product and 0.1–0.2 ng of [32P]cDNA-labeled double-stranded oligonucleotide probes (5,000–20,000 cpm) in the presence or absence of antibody were run on 4% polyacrylamide gels containing 0.5% TBE buffer. For supershift assays, 1 μl of rabbit polyclonal anti-ESE-1 antibody raised against a glutathione S-transferase fusion protein of the N terminus of human ESE-1 (East Acres Biologics, Southbridge, MA) was used.

Oligonucleotides used as probes are as follows (ETS consensus binding site is underlined): 1) human Ang-1 promoter site –2586 to –2567, 5′-TTATAGGCACTGTAATCTC-3′; 3) AAGATTTAGTACATTGCAA-AATAAAA-5′; 2) human Ang-1 promoter site –2207 to –2188, 5′-TTCCTCT-CAGGAAATGTTGCC-5′ and 3′-TGCAAAATTTCTGAGGAAAGGAA-3′; 3) human Ang-1 promoter site –1573 to –1554, 5′-TGAATACTACGTTTACAC-3′ and 3′-GACTAAGAGGATGAACTCA-ACTG-5′; 4) human Ang-1 promoter mutated site –1573 to –1554, 5′-GCTTTGTGTAATCCATCTTGTTAGGCTTTCC-3′ and 3′-GAAAAGGCTAAACATGTAAT- TCCACAGAAGG-5′.

RT-PCR—Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNAs were generated from 2 μg of mRNA isolated from human synovial fibroblasts using oligo(dT)12-18, priming (Invitrogen). AML-1 (Mooney murine leukemia virus-retrovirus; Invitrogen). ESE-1, AML-1, and GAPDH RT-PCRs used equivalent amounts of 0.1 ng of cDNA, 4 ng/μl of each primer, 0.25 units of Taq polymerase (Promega), 150 μl of each dNTP, 3 m M of MgCl2 reaction buffer, and water to a final volume of 25 μl. Ang-1 RT-PCRs used equivalent amounts of 0.1 ng of cDNA, 4 ng/μl of each primer, 0.25 units of Advantage cDNA polymerase mix (Clontech), 150 μl of each dNTP, reaction buffer, and water to a final volume of 25 μl.

The sequences of the primers used in this study are as follows: 1) Human ESE-1: sense, 5′-ACCTGGATCCCAGCTGGAAGCTC-3′; and antisense, 5′-GGAGGAAGAGTTCCTACGATCGG-3′, with an expected amplification product of 457 bp. 2) Human Ang-1: sense, 5′-TCAGCCCTGCTGATCCCTGACC-3′ and antisense, 5′-ATCAGTGAATTTAATGCTG-5′; 3) human Ang-1: sense, 5′-TATGATGTTGCGTGTTGACTC-3′ and antisense, 5′-CTATGATGTTGCGTGTTGACTC-3′, with an expected amplification product of 497 bp. 3) Human AML-1: sense, 5′-AAATCGGCCACCTACACAGC-3′ and antisense, 5′-CTACAGTCTAGCTGATGTCG-5′, with an expected amplification product of 248 bp (21). 4) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): sense, 5′-CAAGGTTCATGGATGACC-3′ and antisense, 5′-CTAGGGGAGGTTGCTTGG-3′, with an expected amplification product of 200 bp.

RT-PCR amplifications were carried out using a MJ Research thermal cycler PTC-100 as follows: ESE-1, 32 cycles of 30 s at 94 °C and 1 min 30 s at 68 °C followed by 7 min at 68 °C; Ang-1, 35 cycles of 30 s at 94 °C and 1 min 30 s at 64 °C followed by 7 min at 64 °C; AML-1, 32 cycles of 30 s at 94 °C and 1 min at 60 °C, and 1 min 30 s at 72 °C; 26 cycles of 30 s at 94 °C, 1 min at 56 °C, and 1 min 30 s at 72 °C followed by 10 min at 72 °C. 5 μl of each RT-PCR was analyzed as a 2% agarose gel.

Western Blot Analysis—Whole cell lysates (50 μg) of MCF7 cells were boiled in Laemmli buffer and resolved on 4% polyacrylamide gel. Proteins were transferred on Hybond-polyvinylidene difluoride membranes and immunoblotted using the ECL Western blotting Detection reagents (Amersham Biosciences). Rabbit polyclonal anti-ESE-1 antibody raised against a glutathione S-transferase fusion protein of the N terminus of human ESE-1 (East Acres Biologics, Southbridge, MA) was used at a dilution of 1:1,000, and revealed with an anti-rabbit horseradish peroxidase antibody (Cell Signaling Technology, Beverly, MA) at a dilution of 1:1,000.

cDNA Microarray—Microarray experiments were performed as previously described (22). In brief, 1 × 108 human breast cancer MCF-7 cells were transiently transfected in duplicates with 8 μg of PCI-ESE-1 or PCI plasmid using the LipectofAMINE Plus system (Invitrogen). Total RNA was isolated separately from each plate 24 h after transfection using the RNeasy Kit (Qiagen) according to the manufacturer’s instructions. Atlas Human Cancer 1.2 cDNA microarray nylon membranes (Clontech), each containing 1176 different genes, were hybridized with [32P]cDNA-labeled cDNAs derived from 4 μg of total RNA according to the manufacturer at 68 °C in ExpressHyb solution (Clontech). Hybridization was performed on a Model 2000 Microarray Hybridization Incubator (Robbins Scientific, Sunnyvale, CA). The filters were washed according to the user’s instruction and exposed to Bio-Max MS film (Fisher) for different exposer times. Hybridizations were performed with duplicate experiments. The spot intensities reflecting gene expression levels on the
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RESULTS

Isolation and Characterization of the Human Ang-1 Promoter—The molecular mechanisms required for the induction of Ang-1 in response to inflammatory cytokines are currently unknown. To begin to identify potential mechanisms of Ang-1 gene regulation we isolated a series of genomic fragments (Fig. 1, A–C) of the human Ang-1 promoter, up to 3.2 kb in length, by long range PCR using human genomic DNA. The entire sequence of the Ang-1 gene is published in the human genome data base (accession NT 008046). Verification of the sequence was performed by automated sequencing. Comparison of the mouse and human Ang-1 promoter sequences revealed a relatively high overall degree of sequence homology. We examined the human Ang-1 promoter sequence for potential binding sites for transcription factors known to be involved in inflammation, such as NF-κB, JAK/STAT, PPAR, NFAT, and C/EBP. Surprisingly, no obvious binding site for any of these transcription factor families was identified. However, putative binding sites for members of the AP-1 and ETS families were identified. In addition, putative AML-1 and CBF-β binding sites were identified in the Ang-1 promoter.

Induction of the Ang-1 Promoter by Inflammatory Cytokines—To evaluate whether the Ang-1 promoter contains regulatory elements capable of mediating induction in response to inflammatory cytokines the three genomic fragments encoding the Ang-1 promoter (A–C) were incorporated into the pGL2 luciferase reporter construct. NIH3T3 fibroblasts were transiently transfected with each Ang-1 reporter construct, followed by stimulation with TNF-α (Fig. 2A). We have previously demonstrated that TNF-α consistently induces Ang-1 mRNA expression in cultured synovial fibroblasts (10). Stimulation of the 3.2-kb Ang-1 promoter construct C (−3040/+199) with TNF-α resulted in a 4-fold induction of the promoter. Similarly, stimulation of the 2-kb construct B (−1799/+199) with TNF-α led to a 4-fold increase in transactivation. However, further deletion to a 1-kb construct (Ang-1 A, −821/+199) markedly reduced the inducibility of the promoter to TNF-α, suggesting that the main regulatory elements responsible for mediating
induction of the Ang-1 gene reside in the nucleotide region between −1799 and −821. We also examined the inducibility of constructs A and B in NIH3T3 fibroblasts after stimulation with IL-1β and LPS (Fig. 2, B and C). A 4–5-fold level of induction of the activity of construct B was seen in response to IL-1β and LPS, but no induction was seen with construct A, suggesting the importance of regulatory elements between nucleotides −1799 and −821 in mediating this induction of Ang-1 gene expression. This region contains putative AP-1 and ETS binding sites (Fig. 1).

Transactivation of the Ang-1 Promoter by ETS Factors—As shown in Fig. 1, there are several putative binding sites for members of the ETS transcription factor family in the Ang-1 promoter. Selected members of the ETS transcription factor family have been shown previously to mediate the induction of other genes associated with inflammatory responses (23, 24). We therefore postulated that ETS factors might also be involved in the regulation of Ang-1 in response to inflammatory stimuli and tested the ability of a panel of ETS factors to transactivate the full-length Ang-1 promoter. Cotransfection experiments using mammalian expression plasmids encoding a panel of ETS factors and the 3.2-kb Ang-1 promoter luciferase construct were performed in HEK293 cells. We chose HEK293 cells because they do not express Ang-1 and can be transfected with high efficiency. As is shown in Fig. 3, only one of the ETS factors tested, ESE-1, was able to transactivate the Ang-1 promoter by −3-fold. These results suggest that ESE-1 is a potential transcriptional regulator of the Ang-1 promoter.

Binding of ESE-1 to Putative ETS Binding Sites—To further define the potential role of ESE-1 in the regulation of Ang-1, we evaluated the ability of ESE-1 to bind to putative ETS binding sites identified in the Ang-1 promoter by performing gel mobility shift assays. Three putative ETS binding sites were identified in the Ang-1 promoter corresponding to regions beginning at −2578, −2199, and −1565 (Fig. 1). The binding of in vitro translated ESE-1 protein was compared with control extracts in a gel mobility shift assay using radiolabeled oligonucleotides encoding the three putative ETS binding sites (Fig. 4). Whereas weak binding of ESE-1 to the first two sites (lanes 2 and 4) compared with the control extract (lanes 1 and 3) was observed, ESE-1 bound with high affinity to the third site (lane 6). Interestingly, this high affinity ESE-1 binding site (−1565/−1562) is located within the region of the Ang-1 promoter that is required for induction by inflammatory cytokines, whereas the low affinity sites are not.

Mutational Analysis of the Ang-1 Promoter ETS Binding Site—To assess the functional importance of the high affinity ESE-1 binding site, we mutated the ETS site corresponding to the region beginning at −1565/−1562 within the Ang-1 luciferase reporter construct C and evaluated the ability of ESE-1 to transactivate the mutant construct compared with the wild type Ang-1 promoter construct C (Fig. 5). Whereas ESE-1 was able to increase transactivation of the wild type Ang-1 pro-
Expression of ESE-1 and Ang-1 in Response to Inflammatory Cytokines—Having demonstrated the potential role for the ETS factor ESE-1 as a transcriptional regulator of the Ang-1 promoter, we wished to examine the temporal relationship between Ang-1 and ESE-1 expression in primary cells after stimulation with inflammatory cytokines. Primary human synovial fibroblasts have been shown to express low levels of Ang-1 at base line (10). The levels of Ang-1 expression can be markedly increased in response to TNF-α. We therefore evaluated the temporal expression of ESE-1 and Ang-1 in primary synovial fibroblasts after stimulation with IL-1β and TNF-α. As is shown in Fig. 6A, Ang-1 was weakly expressed at base line. After stimulation with TNF-α, Ang-1 expression levels increased from base line to 2 h and peaked at about 6–24 h. ESE-1 expression was weakly expressed at base line, and was markedly induced at 2 h, peaking at 6 h with some expression persisting up to 24 and 48 h. Similar results were observed in synovial fibroblasts after stimulation with IL-1β (Fig. 6B). The temporal onset of ESE-1 expression in response to inflammatory cytokines followed by increases in Ang-1 levels further support a potential role for ESE-1 in the regulation of Ang-1 gene expression.

Inflammatory Cytokine Stimulation Induces Binding of ESE-1 to a High Affinity Binding Site within the Ang-1 Promoter—To identify the specific protein binding to the ETS Ang-1 site, gel mobility shift assays were performed using whole cell extracts derived from human primary synovial fibroblasts stimulated with TNF-α. As shown in Fig. 7 (lanes 3–8), TNF-α stimulation is associated with an inducible change in binding pattern to the ETS Ang-1-binding site starting at position −1565. To identify the protein specifically binding to this site, EMSAs were performed in the presence or absence of antiserum to ESE-1. As shown in Fig. 7 (lanes 3–8), the ESE-1 antibody is able to block binding of the protein to the high affinity ETS Ang-1 site. Similar results were seen with IL-1β (data not shown). This suggests that the induction of Ang-1 is mediated by inducible binding of the ESE-1 protein to the Ang-1 promoter.

Microarray Analysis of Ang-1 Expression in ESE-1-transfected Cells—To further evaluate the role of Ang-1 as a potential target of ESE-1, the MCF-7 breast cancer cell line was transiently transfected with a mammalian CMV expression plasmid encoding ESE-1 (PCI-ESE-1). Several cell lines were screened for high transfection efficiency and for expression of ESE-1. The MCF-7 cell line was chosen because the transfection efficiency is routinely over 50% and ESE-1 is only weakly expressed in these cells prior to transfection. Microarray anal-

Fig. 3. Transactivation of the Ang-1 promoter by a panel of ETS factors. Transactivation of the 3.2-kb Ang-1 luciferase reporter construct C (−3040/+199) (300 ng) by a panel of ETS transcription factors (150 ng each) compared with the empty mammalian expression plasmid (PCI, 150 ng) in HEK293 cells. The change in luciferase activity is expressed as fold induction compared with PCI.

Fig. 4. Gel mobility shift assays for ESE-1 binding to putative ETS sites in the Ang-1 promoter. In vitro translated ESE-1 protein (lanes 2, 4, 6) or control extract (lanes 1, 3, 5) was used with end-labeled oligonucleotide probes encoding putative ETS sites starting at positions −2578 (lanes 1 and 2), −2199 (lanes 3 and 4), and −1565 (lanes 5 and 6).

moter more than 8-fold and in a dose-dependent manner, only a minimal increase in transactivation of the mutated Ang-1 promoter was observed, even at the highest doses of ESE-1. The effect of the ETS mutation on the inducibility of the promoter in response to inflammatory cytokines was also tested (Fig. 5B). This mutation led to a marked reduction in the inducibility of the promoter in response to the inflammatory cytokine TNF-α. We also tested the ability of ESE-1 to bind to a mutated ETS binding site through gel mobility shift assay analysis. As shown in Fig. 5C, the binding of in vitro translated ESE-1 protein compared with control extract was blocked by the mutated high-affinity ESE-1 binding site (−1565/−1562). An antibody to ESE-1 also blocked the binding of in vitro ESE-1 protein to the wild type high affinity ESE-1 binding site (−1565/−1562) compared with control extract, specifying that the protein binding to the high affinity site is indeed ESE-1. These results, together with Fig. 4, provide further support that the ETS binding site corresponding to the region beginning at −1565 is required for the transactivation of the Ang-1 promoter by ESE-1.
ysis was performed with total RNA extracted from the transiently transfected cells after 24 h. Following ESE-1 transfection, a 2.4-fold induction of Ang-1 mRNA expression was observed (Fig. 8A). The change in luciferase activity is expressed as fold induction compared with PCI. Transactivation studies of wild type (filled bars) and mutant ETS binding site −1565/−1562 (open bars) Ang-1 luciferase reporter construct C (300 ng each) in HEK293 cells with increasing doses of ESE-1 (4A). The change in luciferase activity is expressed as fold induction compared with PCI. Transactivation studies of wild type (filled bars) and mutant ETS binding site −1565/−1562 (open bars) Ang-1 luciferase reporter construct C (300 ng each) in NIH3T3 fibroblasts after stimulation with TNF-α (50 ng/ml) (B). The change in luciferase activity is expressed as fold induction compared with the promoter construct in unstimulated cells (DMEM supplemented with 10% FCS). In vitro translated ESE-1 protein (lanes 2, 4, and 6) or control extract (lanes 1, 3, and 5) in the presence (lanes 5 and 6) or absence (lanes 1–4) of ESE-1 antibody was used with end-labeled oligonucleotide probes encoding either the wild-type ETS binding site starting at position −1565 on the Ang-1 promoter (lanes 1, 2, 5, and 6) or the mutated ETS binding site starting at position −1565 (lanes 3 and 4) (C).

**Fig. 5. Mutational analysis of the Ang-1 promoter.** Transactivation studies of wild type (filled bars) and mutant ETS binding site −1565/−1562 (open bars) Ang-1 luciferase reporter construct C (300 ng each) in HEK293 cells with increasing doses of ESE-1 (A). The change in luciferase activity is expressed as fold induction compared with PCI. Transactivation studies of wild type (filled bars) and mutant ETS binding site −1565/−1562 (open bars) Ang-1 luciferase reporter construct C (300 ng each) in NIH3T3 fibroblasts after stimulation with TNF-α (50 ng/ml) (B). The change in luciferase activity is expressed as fold induction compared with the promoter construct in unstimulated cells (DMEM supplemented with 10% FCS). In vitro translated ESE-1 protein (lanes 2, 4, and 6) or control extract (lanes 1, 3, and 5) in the presence (lanes 5 and 6) or absence (lanes 1–4) of ESE-1 antibody was used with end-labeled oligonucleotide probes encoding either the wild-type ETS binding site starting at position −1565 on the Ang-1 promoter (lanes 1, 2, 5, and 6) or the mutated ETS binding site starting at position −1565 (lanes 3 and 4) (C).

AML-1 Is Constitutively Expressed in Human Synovial Fibroblasts and Interacts Synergistically with ESE-1 and CBF-β to Regulate the Ang-1 Gene—AML-1 (CBF-α/CBFA2) is a member of the runt transcription factor family that was recently shown to be one of the main factors regulating constitutive expression of Ang-1 in hematopoietic cells (25). Targeted disruption of AML-1 leads to a marked reduction in Ang-1 expression in hematopoietic cells, resulting in defective angiogenesis that can be rescued by administration of Ang-1. Because synovial fibroblasts constitutively express low levels of Ang-1, we postulated that AML-1 could also regulate the expression of Ang-1 in these cells. Evaluation of AML-1 expression by RT-PCR demonstrated constitutively high levels of AML-1 in the synovial fibroblasts that did not change in response to TNF-α (Fig. 9A). Similar results were observed with IL-1β stimulation (data not shown). Another transcription factor, core binding factor β (CBF-β), enhances the DNA binding activity of AML-1 (26, 27). This factor was also constitutively expressed at baseline in the synovial fibroblasts (data not shown).

We were also interested in determining whether AML-1 is capable of transactivating the Ang-1 promoter. Cotransfection experiments with the Ang-1 promoter luciferase construct C and increasing amounts of AML-1 resulted in a maximal 3-fold increase in the activity of the Ang-1 promoter (Fig. 9B). AML-1 has also been shown to interact synergistically with other ETS family members, including ETS-1, ETS-2, and PU.1, to promote the transactivation of several
AML-1 alone was observed in Fig. 9D because lower amounts of the AML-1 plasmid versus DNA (75 ng/ml) resulted in a 9-fold increase in Ang-1 promoter activity, whereas cotransfection with AML-1 or ESE-1 alone led to a 2–3-fold increase in Ang-1 promoter activity, cotransfection of both factors resulted in a synergistic 11-fold increase in Ang-1 promoter activity.

AML-1 also associates with CBF-β to enhance the DNA binding activity of AML-1. Similar synergistic transactivation of the Ang-1 promoter was observed in cotransfection experiments using all three transcription factors, ESE-1, AML-1, and CBF-β (Fig. 9D). Little to no induction of the Ang-1 promoter was seen with low doses (75–100 ng) of CBF-β (data not shown). These results are consistent with the fact that CBF-β does not bind DNA directly, but rather enhances the binding of AML-1 (CBF-α). Weaker induction of the Ang-1 promoter by AML-1 alone was observed in Fig. 9D than in 9C (3.5-fold versus 11-fold) because lower amounts of the AML-1 plasmid DNA (75 versus 100 ng) were used in these transfection experiments while maintaining the same total amounts of DNA. The combination of ESE-1, AML-1, and CBF-β resulted in a 9-fold induction of the Ang-1 promoter (Fig. 9D). Site-directed mutagenesis of the ETS binding site −1565 to −1562 in the Ang-1 promoter resulted in a marked reduction in the synergistic transactivation by the combination of ESE-1, AML-1, and CBF-β compared with the wild-type construct (Fig. 9D), further supporting the requirement of this high affinity ETS binding site in activation of the Ang-1 promoter. The results of these transactivation studies demonstrate that the interactions between AML-1, CBF-β, and ESE-1 are more than additive and result in synergistic regulation of Ang-1 gene expression.

Evaluation of ESE-1 and Ang-1 in Patients with Rheumatoid Arthritis—We have previously shown that Ang-1 is expressed in the synovium of patients with rheumatoid arthritis (10), and that ESE-1 is expressed in rheumatoid synovium (15). Because our results support a role for ESE-1 in regulating Ang-1, we were interested in determining whether ESE-1 and Ang-1 are expressed in similar regions of the rheumatoid synovium. Their expression was evaluated by immunohistochemistry in serial sections of tissue samples obtained from patients with RA. Representative examples of the immunohistochemistry are shown in Fig. 10. Ang-1 and ESE-1 exhibit very similar staining patterns, with strong expression in the synovial lining layer as well as in individual cells within the subsynovium and with a complete absence of staining in the isotype-matched control sample. These results further support a role for ESE-1 in the regulation of Ang-1 gene expression and angiogenesis in the setting of inflammation.
**DISCUSSION**

Angiogenesis is required for the growth and proliferation of inflammatory tissue in a number of diseases. Angiogenic growth factors such as VEGF, bFGF, and Ang-1 are critical mediators of angiogenesis. These angiogenic growth factors act upon endothelial cells, enhancing their proliferation and migration to sites of inflammation. The release of inflammatory cytokines and hypoxia promotes the local expression of these growth factors. Inflammatory cytokines such as IL-1 and TNF-α have been shown to induce the expression of both VEGF and bFGF (31–33). The results of our studies as well as others have recently shown that Ang-1 expression is also stimulated by TNF-α (10, 11). The primary goal of our study was to identify the transcriptional mechanisms responsible for mediating the induction of the Ang-1 gene in the setting of inflammation.

The regulation of other angiogenic growth factors in the setting of inflammation is dependent on several transcription factors. Members of the Rel/NF-κB family represent prototype transcription factors that mediate inflammatory responses. Activation of these factors does not require protein synthesis, as they are sequestered in the cytoplasm, where they are bound noncovalently to the endogenous inhibitors, the Iκ-B proteins. Upon stimulation by inflammatory cytokines or endotoxin, these inhibitors are phosphorylated and proteolytically degraded, allowing NF-κB to be translocated to the nucleus, where it binds to the regulatory regions of target genes as a homo- or heterodimer (34, 35). The induction of both bFGF and VEGF by TNF-α has been shown to be mediated at least in part by NF-κB (31). The promoters of both of these angiogenic growth factors contain binding sites for NF-κB. In contrast, we were unable to identify obvious binding sites for NF-κB within the proximal 3 kb of the Ang-1 promoter.

The absence of putative NF-κB binding sites in the Ang-1 promoter led us to look for alternative regulatory mechanisms. Several other transcription factors have been identified to play a role in mediating inflammatory responses, including ETS, Egr-1, C/EBP, STAT, and AP-1 (36, 37). Among these, only binding sites for AP-1 and ETS family members were identified within the Ang-1 promoter. The AP-1 family member c-Jun has been shown to be a critical transcriptional regulator of VEGF...
gene expression during inflammation and cooperate with the transcription factor HIF-1α to regulate the VEGF gene in the setting of hypoxia (38, 39).

The ETS genes are a family of at least thirty members that function as transcription factors (23). All ETS factors share a highly conserved DNA binding domain of 80–90 amino acids, known as the ETS domain. ETS factors play a central role in regulating genes involved in development, cellular differentiation, and proliferation. Several studies have demonstrated a role for members of the ETS factor family in inflammation and angiogenesis. For example, the role for members of the ETS factor family in inflammation and proliferation. Several studies have demonstrated a role for members of the ETS factor family in inflammation and angiogenesis. For example, the TNF-α gene is regulated by ETS-1 (24). TNF-α can also induce the expression of ETS-1 and is up-regulated in animal models of vascular inflammation (40). ETS-1 has also been shown to be enriched in angiogenic blood vessels and can cooperate with another transcription factor, HIF-1α, to regulate the expression of the VEGF receptor Flk-1 (41, 42). We have recently demonstrated a role for the ETS factor ESE-1 as a transcriptional mediator of inflammation factors AML-1 and CBF-β (43, 44). CBF-β has no direct DNA binding capability, but enhances binding of AML-1 to DNA (45). AML-1 has been shown to be involved in the regulation of a wide variety of genes in cells of hematopoietic origin, including several B- and T cell-specific genes, cytokines, and their receptors, and several enzymes including neutrophil elastase, granzyme B, and myeloperoxidase (46–48). Other members of the runt transcription factor family are also expressed in several other cell types. For example, AML-3 (CBFA1) is involved in the regulation of the osteopontin gene in skeletal tissues. Overlapping expression of osteopontin and AML-3 was observed in osteoblasts, chondrocytes, and periosteal fibroblasts (49). We observed constitutive expression of AML-1 in synovial fibroblasts that remained unchanged after stimulation with inflammatory cytokines. This is the first example that we are aware of demonstrating AML-1 expression in nonhematopoietic cells. Because Ang-1 is a known target of AML-1 under normal conditions in hematopoietic cells, this would also support a role for AML-1 in the regulation of Ang-1 in synovial cells (25). AML-1 forms a ternary complex together with CBF-β and ETS-1 to regulate the expression of the osteopontin gene, and AML-1 and ETS-1 have been shown to synergistically transactivate the osteopontin promoter (49). We similarly observed a synergistic response in transactivation of the Ang-1 promoter with the combination of ESE-1 and AML-1, suggesting that AML-1 may also act to promote the induction of the Ang-1 gene under inflammatory conditions through cooperativity with ESE-1, even though AML-1 expression levels remain unchanged.

Several studies have demonstrated that there is marked neovascularization within the rheumatoid synovium (50, 51). In addition to its role in supplying blood to the inflamed synovium, the endothelial cells derived from these new blood vessels actively participate in the inflammatory response by recruiting lymphocytes and releasing cytokines and angiogenic factors (4). We recently showed that ESE-1 is also highly expressed in the synovium of patients with RA (15). We and others (10) have also shown that Ang-1 is expressed in synovial tissues from patients with RA. Ang-1 and ESE-1 were detected not only around developing blood vessels but also within the synovial lining layer and other less vascular regions within the subsynovium. The similar expression pattern of ESE-1 and Ang-1 in patients with RA further supports a role for ESE-1 as a transcriptional regulator of Ang-1 expression in the setting of inflammation. In addition to its role in promoting vessel maturation, Ang-1 may also act to promote the migration of endothelial cells to sites of active inflammation. In summary, the results of our studies demonstrate that Ang-1 is one of several critical regulators of angiogenesis that is up-regulated in the setting of inflammatory conditions such as rheumatoid arthritis. In contrast to other angiogenic factors, the induction of the Ang-1 gene does not appear to be directly dependent upon NF-κB, but instead is mediated in part by the ETS factor ESE-1 acting cooperatively with the runt transcription factors AML-1 and CBF-β.

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