Regulation of Angiopoietin-2 mRNA Levels in Bovine Microvascular Endothelial Cells by Cytokines and Hypoxia

Stefano J. Mandriota, Michael S. Pepper

Abstract—Angiopoietin-2 (Ang2) is a ligand for the endothelial cell tyrosine kinase receptor Tie2 and counteracts blood vessel maturation/stability mediated by angiopoietin-1 (Ang1), the other known ligand of Tie2. Using degenerate oligonucleotides and reverse transcriptase–polymerase chain reaction, we have screened bovine microvascular endothelial (BME), aortic, lymphatic, pulmonary artery, and transformed fetal aortic endothelial cells, as well as rat smooth muscle cells for Ang1 and Ang2 expression. Except for high Ang2 mRNA levels found in BME cells, none of the endothelial cell types studied expressed appreciable levels of Ang1 or Ang2 mRNAs, whereas smooth muscle cells expressed both Ang1 and Ang2. BME cell Ang2 mRNA levels were increased by vascular endothelial growth factor (1.9- to 2.9-fold), basic fibroblast growth factor (1.6- to 2-fold), both cytokines in combination (2.9- to 4-fold), and hypoxia (3.1- to 5.6-fold) and were decreased by Ang1 (31% to 70%) or transforming growth factor-β (64% to 81%). Ang2 also decreased (60% to 82%) BME cell Ang2 mRNA. mRNA levels for the Tie1 or Tie2 receptors were only slightly modulated under the conditions described above. These findings suggest that the angiogenic effect of a number of these functions may be achieved in part through the regulation of an autocrine loop of Ang2 activity in microvascular endothelial cells. (Circ Res. 1998;83:852-859.)

Key Words: angiopoietin-2 ■ angiogenesis ■ hypoxia ■ cytokine ■ vascular remodeling

During angiogenesis, previously quiescent endothelial cells are induced to degrade their basement membrane and to invade the surrounding stroma as cell cords. Later, these cords form a lumen, deposit a new basement membrane, and recruit perivascular cells such as smooth muscle cells (SMCs) and pericytes, thus resulting in functional new vessels. This sequence of events predicts that angiogenesis is dependent on the concerted regulation of distinct cell functions. From a descriptive point of view, it is useful to attribute these functions to a phase of “activation” or to a phase of “maturation.” Activation includes increased vascular permeability and extravascular fibrin deposition, induction of endothelial cell migration and proliferation, and controlled proteolysis of the extracellular matrix. Maturation includes cessation of endothelial cell migration and proliferation, reduction of extracellular proteolytic activity, new basement membrane deposition, lumen formation, junctional complex maturation, and recruitment of perivascular cells. After maturation, endothelial cell quiescence and blood vessel stability are thought to be actively maintained by a complex interaction between endothelial and SMCs or pericytes, which in turn is mediated by a balance between positive and negative regulators of angiogenesis.

Although the molecular mechanisms underlying the phase of activation have been extensively characterized, those governing the phase of maturation are only beginning to be understood. Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF, referred to as VEGF in this article) and basic fibroblast growth factor (bFGF) stimulate migration, proliferation, and extracellular proteolytic activity in cultured endothelial cells and have thus been classified as key regulators of the activation phase. However, although a role for VEGF in the endogenous regulation of angiogenesis has been clearly demonstrated, the role of bFGF as an endogenous angiogenesis regulator requires further clarification.

The recent discovery of angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) has provided novel and important insights into the molecular mechanisms of blood vessel formation. Ang1 and Ang2 share about 60% amino acid identity and bind with similar affinity to the endothelial cell tyrosine kinase receptor Tie2/Tek (referred to as Tie2 in this article). However, although Ang1 induces Tie2 autophosphorylation, Ang2 is a naturally occurring antagonist of Ang1, in that it competes with Ang1 for binding to Tie2 and blocks Ang1-induced Tie2 autophosphorylation. Intriguingly, Ang1 does not induce endothelial cell proliferation, despite intense autophosphorylation of Tie2. In vivo analysis of Ang1 function by targeted gene inactivation in the mouse has revealed lethal embryonic defects highly reminiscent of those observed in Tie2 knockout mice. These defects consist of a poorly organized subendothelial matrix, loosening of endothelial cell contacts with the basement membrane, and generalized lack of perivascular cells. Of note, endothelial

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From the Department of Morphology, University Medical Center, Geneva, Switzerland.
Michael S. Pepper, Department of Morphology, University Medical Center, 1 rue Michel Servet, 1211 Geneva 4, Switzerland. E-mail michael.pepper@medecine.unige.ch
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cells are present in normal number in Ang1 null mice. These findings imply that Ang1 is not necessary for endothelial cell differentiation and proliferation and demonstrate instead that Ang1 is required for correct vascular assembly and recruitment of perivascular cells. Tie1, another endothelial cell receptor with no ligand described to date, which is closely related to Tie2, is also essential for correct vascular assembly. Tie1 null mice die of edema and hemorrhage, resulting from poor structural integrity of the vasculature.

Several additional interesting features of Ang2 have been reported. Consistent with its role as an antagonist of Ang1, overexpression of Ang2 in endothelial cells of transgenic mice results in lethal embryonic defects reminiscent of those observed in Ang1 and Tie2 knockout mice. During embryogenesis and adult life, Ang2 expression occurs almost exclusively at sites of vascular remodeling and is most marked at the invading front of vascular sprouts, where its expression coincides with that of VEGF. However, Ang2 expression is also pronounced in atretic follicles and in aged corpora lutea, in which blood vessels regress, and VEGF mRNA is almost undetectable. Taken together, these findings have led to the proposal that, by virtue of its capacity to counteract Ang1-mediated blood vessel maturation/stability, the function of Ang2 may be context-dependent. When acting in the absence of angiogenic inducers such as VEGF, Ang2-mediated loosening of cell-matrix contacts may induce endothelial cell apoptosis with consequent vascular regression. When acting in concert with VEGF, Ang2 may facilitate endothelial cell migration and proliferation, thus serving as a permissive angiogenic signal. Although attractive, this hypothesis requires additional clarification. For example, virtually nothing is known about the molecular mechanisms that regulate the expression of Ang1 or Ang2 or of the endothelial cell receptors Tie1 or Tie2. In this study, we cloned partial cDNAs coding for bovine and rat Ang1 and Ang2 and used them as probes (1) to assess their expression in a variety of bovine endothelial cell lines as well as rat SMCs and C6 glioma cells and (2) to study the regulation of this expression by a variety of well-characterized angiogenic stimuli, including hypoxia. Expression of Tie1 and Tie2 was also studied in microvascular endothelial cells.

Molecular Cloning of Bovine and Rat Ang1 and Ang2 Partial cDNAs

Partially degenerate oligonucleotides were designed from the amino acid sequences QHLEHVM and MEGKHKE (for Ang1) and WEGFYVS (Ang2) and used to amplify cloned partial cDNAs coding for bovine and rat Ang1 and Ang2 cDNAs, respectively. The primer sequences used were as follows: Ang1, forward, 5′-CACGATCTGGA(A/G)CAT(C/T)CA(G/A)TA-3′; reverse, 5′-TTC(T/C)TGTGTTT(A/G)AC(T/C)TCC(T/C)TCA-3′; Ang2, forward, 5′-GT(G/A)C(T/C)TCA(G/A)TA-3′; reverse, 5′-CGA(A/G)TA GG(T/G)GA(A/G)AT(C/T)CC(T/C)TTCCA-3′. Total cellular RNAs (2 μg) from BME, BAE, BLE, CPAE, GM7373, rat SMC, and C6 glioma cells or from adult bovine liver or 17-day rat placenta were reverse-transcribed using random exonucleotides (Boehringer Mannheim) and Moloney murine leukemia virus reverse transcriptase (Promega). One twentieth of each reverse transcriptase (RT) product was amplified with degenerate Ang1 or Ang2 oligonucleotides using PymgLyse DNA polymerase (Biometra). Polymerase chain reaction cycles were (for all cDNAs) as follows: 95°C, 3 minutes (1×); 95°C, 30 seconds; 55°C, 1 minute; 72°C, 45 seconds (35×); and 72°C, 5 minutes (1×). Approximately 350-bp reverse transcriptase–polymerase chain reaction (RT-PCR) products amplified with degenerate Ang1 oligonucleotides from adult bovine liver or rat placenta or ~450-bp RT-PCR products amplified with degenerate Ang2 oligonucleotides from BME cells or rat placenta were cloned into pGEM-T Easy (Promega) and sequenced on both strands.

Northern Blot, RNase Protection, and Semiquantitative RT-PCR

Twenty-four to 36 hours after the last medium change, bFGF (10 ng/mL), VEGF (30 ng/mL), or both cytokines together, TGF-β1 (1 ng/mL), rAst (500 ng/mL), eAst (10 μg/mL), Ang1 (10 or 100 ng/mL), or Ang2 (10 or 100 ng/mL) were directly added to the culture medium of confluent monolayers of BME cells. Conditions of hypoxia (for both BME cells and SMCs) were achieved using an airtight Plexiglas container, in which O2 was replaced with a 95% N2/5% CO2 gas mixture. Under these conditions, the difference in pH values between the medium of controls and hypoxia-incubated cultures at the end of the incubation period did not exceed 0.08 units. Total cellular RNAs, purified using Trizol reagent (Gibco), were analyzed by Northern blot or RNase protection analysis as described using the 32P-labeled bovine Ang1 or Ang2 cRNAs as probes. For analysis of Tie1 or Tie2 mRNAs, a cDNA fragment corresponding to nucleotides 2076 to 2520 of the bovine Tie1 coding sequence or a cDNA fragment corresponding to nucleotides 2037 to 2484 of the bovine Tie2 coding sequence was amplified by RT-PCR, cloned, sequenced to confirm identity, and used to synthesize cRNA probes for use in Northern blot or RNase protection assays. For semiquantitative RT-PCR, 2 μg of total RNA from rat SMCs was reverse-transcribed using oligo-dT18 (Boehringer) and Superscript II (Gibco). For each RT product, one twentieth of the final reaction volume was amplified in three parallel PCR reactions using the Ang2 oligonucleotides described above, a pair of partially degenerate primers for the acidic ribosomal phosphoprotein P0 (forward: CCGGAATTCTACGGAAAGACGCGG-GACCTGG; reverse: CGCGGATCC(C/T)G/GAT(A/G)GCCT- GAG(C/A)(G/C)TAGCC(T/G)GA(A/G)TCC(T/C)TTCCA. Total cellular RNAs, purified using Trizol reagent (Gibco), were analyzed by Northern blot or RNase protection analysis as described using the 32P-labeled bovine Ang1 or Ang2 cRNAs as probes. For analysis of Tie1 or Tie2 mRNAs, a cDNA fragment corresponding to nucleotides 2076 to 2520 of the bovine Tie1 coding sequence or a cDNA fragment corresponding to nucleotides 2037 to 2484 of the bovine Tie2 coding sequence was amplified by RT-PCR, cloned, sequenced to confirm identity, and used to synthesize cRNA probes for use in Northern blot or RNase protection assays. For semiquantitative RT-PCR, 2 μg of total RNA from rat SMCs was reverse-transcribed using oligo-dT18 (Boehringer) and Superscript II (Gibco). For each RT product, one twentieth of the final reaction volume was amplified in three parallel PCR reactions using the Ang2 oligonucleotides described above, a pair of mouse oligo-dT18 (Boehringer) and Superscript II (Gibco). For each RT product, one twentieth of the final reaction volume was amplified in three parallel PCR reactions using the Ang2 oligonucleotides described above, a pair of mouse oligo-dT18 (Boehringer) and Superscript II (Gibco). For each RT product, one twentieth of the final reaction volume was amplified in three parallel PCR reactions using the Ang2 oligonucleotides described above, a pair of mouse oligo-dT18 (Boehringer) and Superscript II (Gibco). For each RT product, one twentieth of the final reaction volume was amplified in three parallel PCR reactions using the Ang2 oligonucleotides described above, a pair of mouse oligo-dT18 (Boehringer) and Superscript II (Gibco). For each RT product, one twentieth of the final reaction volume was amplified in three parallel PCR reactions using the Ang2 oligonucleotides described above, a pair of mouse oligo-dT18 (Boehringer) and Superscript II (Gibco). For each RT product, one twentieth of the final reaction volume was amplified in three parallel PCR reactions using the Ang2 oligonucleotides described above, a pair of mouse oligo-dT18 (Boehringer) and Superscript II (Gibco). For each RT product, one twentieth of the final reaction volume was amplified in three parallel PCR reactions using the Ang2 oligonucleotides described above, a pair of mouse oligo-dT18 (Boehringer) and Superscript II (Gibco).
Regulation of Angiopoietin-2 Expression

Results

In situ hybridization has revealed punctate Ang2 expression at the invading front of vascular sprouts, where it appears to be expressed by endothelial cells and/or pericytes. This raised the possibility that cultured endothelial cells might produce Ang2 and might therefore provide a model to study the regulation of Ang2 expression in vitro. To determine whether cultured bovine endothelial cells express Ang2, partially degenerate oligonucleotides were designed from the amino acid sequences VDFQRTW and WKGSGYS, which are conserved in the human and mouse Ang2 cDNAs, and used to screen bovine endothelial cell total cellular RNAs by RT-PCR. After 35 PCR cycles, a band of ~450 bp, thus corresponding in size to the RT-PCR product expected from total RNA from adult bovine liver (Figure 1A, upper panel), which is consistent with previous results but not from rat C6 glioma cells (Figure 1B, bottom panel). In all samples, the band was undetectable when RT was omitted (Figure 1A and 1B, bottom panels, and data not shown), demonstrating that when present, Ang2 was not amplified from genomic DNA.

To explore the possibility that some of the endothelial cell types studied also express Ang1, partially degenerate oligonucleotides were designed from the amino acid sequences QHLHEHVM and MEGKHKE, which are conserved in the human and mouse Ang1 cDNAs. By RT-PCR, these primers amplified a band of ~350 bp, thus corresponding in size to the RT-PCR product expected from human and mouse Ang1 mRNAs (372 bp), from total RNA from adult bovine liver (Figure 1A, upper panel), which is consistent with previous results but not from BME, BAE, BLE, CPAE, or GM7373 cells (Figure 1A, upper panel). The same pair of oligonucleotides amplified a band of identical size from total RNA from 17-day rat placenta, SMCs, and C6 glioma cells (Figure 1B, upper panel), which is consistent with previous results. Taken together, these results suggested that, with the exception of Ang2 expression by BME cells, none of the bovine endothelial cell types tested expressed significant levels of either Ang1 or Ang2 mRNAs.

To determine whether the RT-PCR products shown in Figure 1 actually correspond to the bovine or rat counterparts of Ang1 or Ang2 cDNAs, these products were cloned in pGEM-T Easy (Promega) and entirely sequenced on both strands (Figure 2A and 2B). A high degree of identity was found with human and mouse Ang1 or Ang2 cDNAs (Table), confirming that these RT-PCR products were the bovine or rat counterparts of Ang1 or Ang2 and thus could be used as probes to study Ang1 or Ang2 mRNA levels in bovine and rat cells and tissues.

To assess the mechanisms that might regulate angiopoietin expression, confluent monolayers of BME cells were incubated for 15 hours in the presence of a variety of well-characterized angiogenic regulators. These included bFGF (10 ng/mL), VEGF (30 ng/mL), or both cytokines in combination, TGF-β (1 ng/mL), rmAst (500 ng/mL), ehAst (10 μg/mL), Ang1 or Ang2 (both at 100 ng/mL), or hypoxia (95% N2/5% CO2). At the end of the incubation, total cellular RNA was purified and analyzed by RNase protection using 32P-labeled cRNA probes synthesized from the bovine Ang1 or Ang2 partial cDNAs shown in Figure 2. As an internal control, an equal amount of 32P-labeled cRNA from bovine P0 partial cDNA was included in all samples. Consistent with

Figure 1. RT-PCR screening of bovine endothelial, rat SMCs, and rat C6 glioma cells with partially degenerate oligonucleotides for Ang1 or Ang2. Two micrograms of total RNA from adult bovine liver, from BME, BAE, BLE, CPAE, and GM7373 cells (A) or from 17-day rat placenta, rat SMCs, or C6 glioma cells (B) was reverse-transcribed using random exanucleotides. One twentieth of the volume of the RT products, or an equivalent volume of H2O, was subjected to 35 PCR cycles in the presence of partially degenerate oligonucleotides for Ang1 (upper panels in A and B) or Ang2 (bottom panels in A and B). Where indicated, RT was omitted.
RT-PCR results (Figure 1A), Ang2 mRNA was readily detectable in BME cells (Figure 3) but not in BAE cells (data not shown). BME cell expression of Ang2 was increased 2-fold by bFGF, 2.9-fold by VEGF, 4-fold by bFGF and VEGF in combination, and 5.6-fold by hypoxia (Figures 3 and 4). In contrast, it was decreased by 81% by TGF-β, 70% by Ang1, and 82% by Ang2 (Figures 3 and 4). Angiostatin had little or no effect on Ang2 expression (Figure 3 and data not shown). The bovine Ang1 cRNA probe revealed Ang1 expression in total RNA from bovine adult skeletal muscle (Figure 2).

### Percent Identity Between Bovine, Rat, Human, and Mouse Ang1 or Ang2 cDNA and Amino Acid Sequences

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* b indicates bovine; r, rat; h, human; and m, mouse. Values in bold type are for cDNA; values not in bold type are for amino acid.

### Figure 3. RNase protection analysis for Ang1 and Ang2 mRNA in BME cells.

Purified 32P-labeled bovine Ang1 and Ang2 cRNAs (probe) were hybridized to hybridization mix (probe 1h.m.), yeast tRNA (tRNA), to 10 μg of total RNA from bovine adult skeletal muscle (Sk. muscle) (only for Ang1, upper panel), or to 10 μg of total RNA from BME cells incubated for 15 hours in the presence of normal growth medium (control) or in medium containing bFGF (10 ng/mL), VEGF (30 ng/mL), both cytokines in combination (F/V), TGF-β (1 ng/mL), rmAst (Ast, 500 ng/mL), Ang1 or Ang2 (both at 100 ng/mL), or a gas mixture containing 95% N2/5% CO2 (hypoxia). As an internal control, an equal amount of the P0 cRNA probe was included in all samples. SP6 control template marker. One of two experiments with similar results is shown.

### Figure 2. Nucleotide and deduced amino acid sequences of bovine and rat Ang1 and Ang2 partial cDNAs.

Approximately 350-bp RT-PCR products amplified with degenerate Ang1 primers from adult bovine liver or rat placenta (upper panels, Figure 1A and 1B) or ~450-bp RT-PCR products amplified with degenerate Ang2 oligonucleotides from BME cells or rat placenta (bottom panels, Figure 1A and 1B) were cloned and sequenced on both strands. Bovine and rat Ang1 (A) and Ang2 (B) were compared with human and mouse Ang1 (A) and Ang2 (B) amino acid sequences. Sequences of degenerate oligonucleotides have been omitted. (GenBank accession Nos., bAng1: AF032923, bAng2: AF032924, rAng1: AF030376, and rAng2: AF030378).

### Table

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* b indicates bovine; r, rat; h, human; and m, mouse. Values in bold type are for cDNA; values not in bold type are for amino acid.
Figure 3, which is consistent with previous results, but not in BME cells, either when cultured under normal growth conditions (Figure 3), which is consistent with RT-PCR results (Figure 1A), or when stimulated with the angiogenic regulators listed above (Figure 3). Ang2 mRNA was undetectable in rat SMCs by RNAse protection when the cells were grown under either normal or hypoxic conditions (data not shown), which may reflect the low level of Ang2 mRNA in these cells (Figure 1B). Semiquantitative RT-PCR analysis revealed that hypoxia had little or no effect on SMC Ang2 mRNA levels (Figure 5; compare the Ang2 band with the P0 band in control versus hypoxia-treated cells); hypoxia did not induce Ang2 mRNA expression in BAE or C6 glioma cells in which the Ang2 gene is however probably silent (Figure 1 and data not shown). In contrast, hypoxia strongly increased VEGF expression or slightly decreased Ang1 expression in both SMCs and C6 glioma cells (Figure 5 and data not shown), which is consistent with previous results.

Because RNase protection does not distinguish between transcripts of different sizes, total BME cell RNAs from experiments similar to those shown in Figures 3 and 4 were analyzed by Northern blot, using the 32P-labeled bovine Ang2 cRNA as a probe. BME cells were found to express three major Ang2 transcripts of 2.3, 2.8, and 5.8 kb (Figure 6). An additional band of 1.6 kb was detectable in cultures incubated under hypoxic conditions (Figure 6). All of the Ang2 mRNA transcripts were modulated in a similar manner by the different treatments used, although the effect of hypoxia was most marked on the 2.3-kb transcript, and that of Ang1 was more marked on the 5.8-kb transcript (Figures 6 and 7).
these exceptions noted, scanning densitometry gave values largely superimposable on those obtained by RNase protection (Figure 7). When replicate RNA blots were hybridized with bovine Tie1 or Tie2 cRNA probes, small modulations of Tie1 or Tie2 mRNA levels by the angiogenic regulators listed above were found (Figure 6). In particular, Tie1 mRNA was slightly increased by bFGF (1.6±0.08-fold) or by the combination bFGF/VEGF (1.8±0.15-fold), and Tie2 mRNA was slightly increased by bFGF (1.6±0.24-fold), Ang1 (100 ng/mL, 1.5±0.09-fold), or Ang2 (100 ng/mL, 1.9±0.29-fold) or decreased by hypoxia (62±4%), alone or in combination with VEGF (Figure 6 and data not shown) (for all of these modulations, errors represent SEM with n=4 experiments). Similar results were obtained by RNase protection using the same probes (data not shown).

**Discussion**

The recent discovery and characterization of two ligands (Ang1 and Ang2) for the endothelial cell tyrosine kinase receptor Tie2 has increased our understanding of the molecular basis of the angiogenic process. Ang1 was the first to be described and appeared as an unusual angiogenic factor in that it was unable to stimulate endothelial cell proliferation.5

Targeted inactivation of the Ang1 gene in the mouse revealed the requirement for this factor for recruitment of perivascular cells and correct vascular assembly, rather than for endothelial cell differentiation and proliferation,6,10 thus providing a novel and important insight into the mechanisms that govern the late phases of angiogenesis. Because Ang1-mediated blood vessel maturation/stability is likely to interfere with the extensive vascular remodeling that takes place during angiogenesis, it has been proposed that Ang2, a natural antagonist of Ang1,6 may be an important proangiogenic factor, in that it may counteract Ang1-mediated blood vessel stability, thus maintaining the endothelium in a more plastic state and promoting the response of endothelial cells to angiogenic inducers.6,11 To assess this hypothesis, we cloned partial cDNAs coding for bovine or rat Ang1 and Ang2, with the aim of studying the mechanisms that regulate their expression in a number of cultured bovine and rat cell types. We found that Ang1 is expressed in SMCs but not in five different types of bovine endothelial cells nor in BME cells exposed to a variety of angiogenic stimuli. This is largely consistent with the pattern of Ang1 expression in vivo.5,6 Rat C6 glioma cells also expressed Ang1, as previously reported by others.22 Relative high levels of Ang2 mRNA were found in endothelial cells of microvascular origin but not of large vessel or lymphatic origin, and lower levels were seen in SMCs. This

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**Figure 7.** Quantitation of Ang2 mRNA levels in BME cells as assessed by Northern blot analysis. For each experimental condition (bFGF: 10 ng/mL, VEGF: 30 ng/mL, F/V: bFGF 10 ng/mL + VEGF 30 ng/mL, TGF-β: 1 ng/mL, Ang1: 100 ng/mL, and Ang2: 100 ng/mL), the graph shows values obtained by scanning densitometry (±SEM, n=4 experiments) for the 5.8-kb, 2.8-kb, or 2.3-kb Ang2 transcripts, respectively. An arbitrary value of 1 was assigned to controls in all cases.

**Figure 8.** Dynamic interplay between Ang1 and Ang2 in the regulation of blood vessel maturation/stability.6,11 At the onset of angiogenesis, the local induction of Ang2 expression in endothelial cells by signals such as VEGF, bFGF, and/or hypoxia may inactivate the stabilizing Ang1 signal, thus promoting the loosening of endothelial-perivascular cell contacts (vessel wall disassembly) and allowing endothelial cells to respond to angiogenic inducers by the formation of sprouts. In the final phases of angiogenesis, a local diminution of Ang2 expression, possibly mediated by Ang1 itself and/or TGF-β1, may reestablish the Ang1 signal, whose expression is, in contrast to that of Ang2, constitutive in many organs,6 thus promoting the recruitment of perivascular cells and the maturation of the newly formed vessels (vessel wall assembly). The recruitment of perivascular cells to the vessel wall has been hypothesized to be mediated by endothelial cell–derived polypeptides such as TGF-β1 and platelet-derived growth factor (PDGF).26
is also consistent with the pattern of Ang2 expression in vivo, in which Ang2 mRNA was detected in SMCs, pericytes, and endothelial cells at the invading front of vascular sprouts. No Ang2 expression was observed in C6 glioma cells.

Of interest was the finding that microvascular endothelial cells express Ang2, for it suggested that (1) regulation of endothelial cell Ang2 levels during angiogenesis may give rise to and modulate an autocrine loop of Ang1 inactivation that would operate selectively at sites of vascular remodeling and (2) regulation of Ang2 expression in endothelial cells could be directly governed by angiogenic inducers/repressors, thus representing part of their mechanism of action. In this study, we report for the first time that three well-characterized angiogenic inducers, namely, VEGF, bFGF, and hypoxia, increase BME cell expression of Ang2. This suggests that an increase in Ang2 expression is a common pathway by which different angiogenic inducers act, and that Ang2-mediated inactivation of the stabilizing Ang1 signal is an important step in the vascular remodeling that occurs during angiogenesis, independently of the nature of the angiogenic stimulus (Figure 8).

The finding that Ang2 expression is increased by hypoxia was particularly striking. Tissue hypoxia is a fundamental angiogenic stimulus, characteristic of malignant tumors, healing wounds, and a number of other pathological or physiological situations associated with neovascularization. The VEGF gene has been shown to contain specific hypoxia-responsive elements and consistent with these features is upregulated in response to low oxygen tensions in a variety of experimental models.3 In the present study, we report that Ang2 mRNA is also increased by hypoxia in microvascular endothelial cells. This phenomenon was apparently specific for these cells, because Ang2 mRNA levels appeared not to be significantly altered by hypoxia in SMCs. In addition to VEGF, our findings identify Ang2 as another hypoxia-inducible angiogenic factor, point to Ang2 as a potential important component of the angiogenic switch that characterizes the passage of a tumor from the avascular to the vascular phase, and provide strong evidence for a collaboration between VEGF and Ang2 in the regulation of neovascularization in ischemic tissues.

The finding that Ang2 mRNA is rapidly and dramatically induced in tumor vasculature but not in the surrounding tumor tissue in which VEGF is upregulated (G.D. Yancopoulos, unpublished data, 1998) strengthens our conclusion that tumor-derived signals such as hypoxia and/or VEGF or bFGF may specifically induce Ang2 expression in tumor endothelium, and that this event may in turn be an important component of the angiogenic switch and/or of the subsequent phases of the formation of an endogenous tumor microcirculation.

If increased Ang2 activity is part of the mechanism of action of angiogenic inducers, one would expect those factors that are required for the maturation/stability of blood vessels to act in part by decreasing/repressing Ang2 activity (Figure 8). Consistent with this view, Ang1 decreased Ang2 mRNA levels in BME cells. Recent genetic studies have indicated that TGF-β1 is an important mediator of correct vascular assembly24; we found that TGF-β1 also decreased BME cell Ang2 mRNA levels. It is noteworthy that the vessels of the yolk sac of TGF-β1 null mice25 show defects that are somewhat reminiscent of those observed in Ang1 or Tie2 knockout mice26–28 or in transgenic mice overexpressing Ang2,29 which suggests that these defects could be due in part to an inappropriate overexpression of Ang2, which in turn is due to the lack of TGF-β1. A similar molecular basis may contribute to the phenotype of Ang1 null mice. Although these correlations are likely to be an oversimplification of what occurs in the whole organism, they are nonetheless intriguing.

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References


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