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Angiopoietin-3, a novel member of the angiopoietin family

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Abstract A cDNA clone encoding angiopoietin-3 protein (Ang3), a novel member of the angiopoietin family, was identified. Ang3 cDNA was cloned from a human aorta cDNA library. Ang3 is a 503 amino acid protein having 45.1% and 44.7% identity with human angiopoietin-1 and human angiopoietin-2, respectively. Ang3 mRNA is expressed in lung and cultured human umbilical vein endothelial cells (HUVECs). Ang3 mRNA expression in HUVECs was slightly decreased by vascular endothelial cell growth factor treatment, suggesting that the regulation of Ang3 mRNA expression is different from that of Ang2.

Key words: Angiopoietin; Tie; Vascular endothelial growth factor; Angiogenesis

1. Introduction

Angiogenesis is required for normal embryogenesis, and also is associated with several pathophysiological conditions such as the growth of solid tumor [1], neovascularization in the retina [2], and some inflammatory diseases [1]. Regulation of angiogenesis is thought to depend on a balance between positive and negative regulators [3]. The family of vascular endothelial growth factors (VEGFs), which are endothelial cell (EC) specific factors, are known to play a principal role in angiogenesis, including migration, proliferation [4], and expression of various genes related to angiogenesis in ECs. Moreover, the critical role of VEGFs and their receptors in vascular development of the embryo has been demonstrated using mutant mice with targeted disruption of VEGFs or the receptor gene [5–8].

Recently, angiopoietins (angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2)) were identified which constitute a novel family of EC specific factors [9,10]. Ang1 was discovered originally as a ligand for Tie2, a member of the tyrosine kinase with immunoglobulin and epidermal growth factor homology domains receptor (Tie) family [9]. The subsequently cloned Ang2 also binds Tie2 [10]. Tie2 and the originally cloned isoform, Tie1, belong to the Tie receptor family [11–13]. Although both Ang1 and Ang2 have been shown to bind Tie2 [9,10], no ligand for Tie1 has been identified. This suggests that the larger number of members constitutes the angiopoietin family. Here we report a novel member of the angiopoietin family, termed angiopoietin-3 (Ang3), which is expressed in lung as well as in cultured human umbilical vein endothelial cells (HUVECs).

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The nucleotide sequence reported in this paper has been submitted to GenBank with accession number AF074332.

2. Materials and methods

2.1. The high throughput genomic sequences (htgs) database screening

The htgs database was searched with human angiopoietin amino acid sequences as probes, using the tblastn program at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.znih.gov). One human genomic sequence (GenBank accession number AC004064) was found to contain eight DNA fragments homologous to the protein coding region of human Ang1 or Ang2 cDNAs. A part of these sequences was amplified by nested polymerase chain reaction (PCR) using two sets of oligonucleotide primers, designed based on the putative exons. The sequences of primers were: FW1, 5′-AGC TAC ACC TTC TTG CTG CC-3′; FW2, 5′-CCA ACA CCC TCC AGA GAG AA-3′; RV1, 5′-G TAT CAT CAT GCG AGA GGC A-3′; RV2, 5′-T GCC GTC CAT CTT GTA CTT G-3′. The first PCR was performed with a set of FW1 and RV1 and the human aorta cDNA library (λgt10, Clontech) as template, followed by the second PCR with a set of FW2 and RV2. A 1242 bp DNA fragment amplified by the PCR was subcloned into a pGEM-T vector and sequenced. This DNA fragment was further used for screening a human aorta cDNA library (Clontech).

2.2. cDNA cloning of human Ang3

To obtain the full-length cDNA, we then screened 1 440 000 plaques of the human aorta cDNA library under the standard hybridization conditions, using a ^{32}P-labeled cDNA fragment obtained by nested PCR as a probe. One clone encoded a partial angiopoietin- like protein (designated Ang3). This clone contained a poly A tail but lacked the 5′-terminus of the cDNA. The 5′-terminus of the cDNA was obtained by the PCR of the human aorta cDNA library. Both strands of cDNA were sequenced.

2.3. Northern blot analysis

Northern blot analysis was performed under the standard hybridization conditions with ^{32}P-labeled 1687 bp Ang3 cDNA (nt 235–1921) as a probe. A membrane (Human Multiple Tissue Northern Blot; Clontech) containing 2 μg of poly A RNA in each lane was hybridized for 16 h, was washed with 0.1×SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0), 0.1% SDS at 50°C for 1 h, and exposed to X-ray film with an intensifying screen at −80°C for 7 days.

2.4. Analysis of Ang3 expression in HUVECs under VEGF treatment

Confluent cultures of HUVECs in DMEM containing 1% fetal calf serum were stimulated with indicated concentrations of human VEGF. After a 4 h stimulation, total RNA was extracted, and Northern blot analysis was performed with ^{32}P-labeled human Ang3 cDNA, human Ets-1 cDNA, and human glyceraldehyde 3-phosphate dehydroxylase (GAPDH) cDNA as probes.

2.5. Radiation hybrid (RH) mapping of the human Ang3 gene

To determine chromosome localization of the Ang3 gene, we per-

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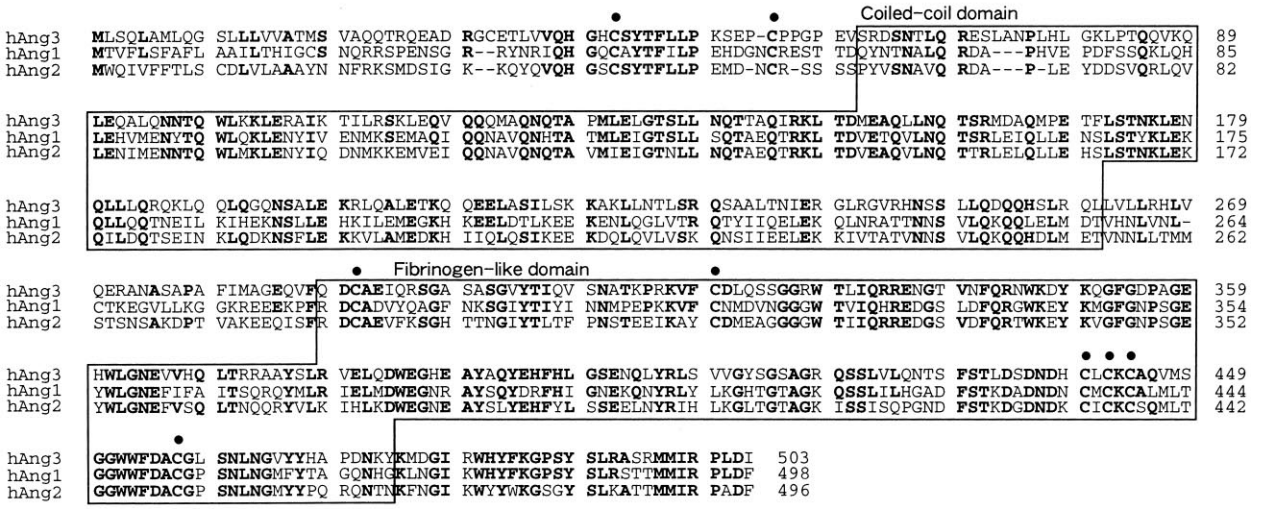

Fig. 1. Comparison of amino acid sequences of human Ang3, Ang1, and Ang2. Amino acids are indicated in single-letter code. Bold letters represent amino acids identical with Ang3. Dashes indicate gaps. Solid circles denote cysteine residues conserved among Ang3, Ang1, and Ang2. Coiled-coil domain and fibrinogen-like domain are boxed.

formed RH mapping using the GeneBridge 4 RH panel (Research Genetics, Huntsville, AL, USA) with two independent sets of primers. One set is: primer RH upst-1 (5′-GCT CTG AGC AGA CAT CCC TC-3′), and RH downst-1 (5′-TTC TCT CTG GAG GGT GTT GG-3′); the other is: RH upst-2 (5′-G GAC CCA GAT GCA AGA CAC T-3′), and RH downst-2 (5′ G-GTC TGT TCT CAG CCC ATT C-3′). The results obtained with these two sets of primers were consistent, and were then sent to the Whitehead Institute/MIT Center for Genome Research via internet (http://carbon.wi.mit.edu:8000/cgi-bin/contig/rhmapper.pl) for mapping of the gene relative to the RH map of the human genome.

3. Results and discussion

By searching the htgs database we found one genomic sequence (AC004064) encoding a novel member of the angiopoietin family, designated Ang3. To find if the transcript of the gene encoding Ang3 was present, we attempted to amplify the cDNA sequence from the human aorta cDNA library using nested PCR. As we expected, the sequence of the PCR product (1242 bp) was identical to that of the putative exons of the Ang3 gene in the database. Accordingly, we reasoned that there might be a novel member of the angiopoietin family.

By a combination of the PCR-based method and screening a human aorta cDNA library, we cloned a full-length cDNA (1957 bp) encoding Ang3. Ang3 is a 503 amino acid protein with a signal peptide, predicted by PSORT WWW Server (http://psort.nibb.ac.jp:8800/). The deduced amino acid sequence has 45.1% and 44.7% identity to human Ang1 and Ang2, respectively (Fig. 1). Ang1 and Ang2 have a characteristic feature of a coiled-coil domain and fibrinogen-like domain which are conserved in Ang3, further suggesting that Ang3 constitutes a novel member of angiopoietin family.

Northern blot analysis revealed two Ang3 transcripts of 5.6 kb and 4.0 kb at low levels in lung among the tissues examined (Fig. 2). Ang3 mRNA is also expressed in HUVECs (Fig. 3). Ang1 is expressed widely in adult tissues, including brain, skeletal muscles, prostate, ovary, uterus, placenta, and small intestine [10], while the expression of Ang2 is restricted to ovary, uterus, placenta, and endothelial cells [10]. Thus, the tissue distribution of Ang3 differs from those of Ang1 and Ang2, suggesting that Ang3 might have a role in angiogenesis distinct from those of Ang1 and Ang2.

Targeted disruption of the gene encoding Ang1 causes severe defects in vascular development; disturbance of angiogenesis, and lack of mural pericytes in the developing mouse embryo, leading to death by embryonic day 12.5 [14]. Transgenic overexpression of Ang2 disrupts blood vessel formation

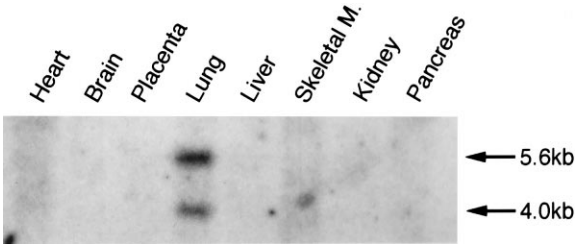

Fig. 2. Northern blot analysis of Ang3 in human tissues. The sizes of the transcripts are shown. Each lane contains 2 μg of poly A RNA. Skeletal M.: skeletal muscle.

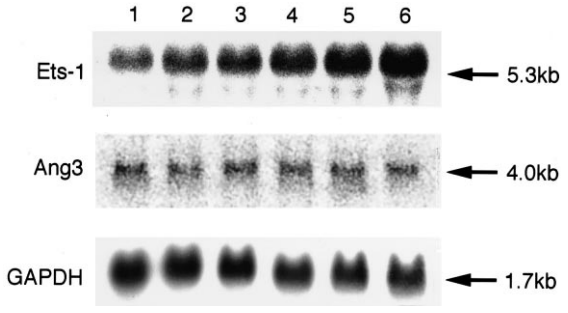

Fig. 3. Changes in Ang3 mRNA expression in HUVEC by VEGF treatment. After the stimulation with (lanes 2–6) or without (lane 1) VEGF at various doses, total RNA was isolated and the expression of Ets-1 (top) and Ang3 (middle) was analyzed by Northern blotting. As loading controls, the signals obtained with a probe for GAPDH (bottom) are shown. Lane 1: untreated control lanes. Lanes 2–6: treated with VEGF. The doses used were: 5 ng/ml for lane 2, 10 ng/ml for lane 3, 20 ng/ml for lane 4, 40 ng/ml for lane 5, 80 ng/ml for lane 6.

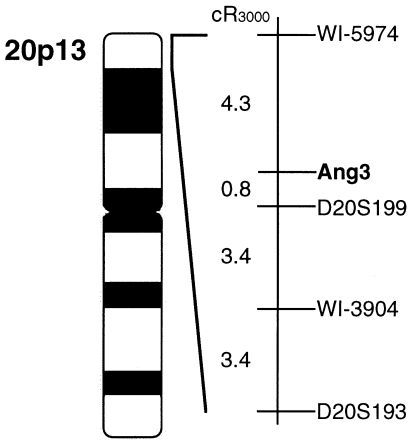

Fig. 4. RH mapping of human Ang3. Distances between STSs are given in cR_{3000}. Ang3 was mapped to the region 0.8 cR telomeric of D20S199, which locates at chromosome 20p13.

in the mouse embryo similarly to Ang1 knock-out mice [10]. In addition, targeted disruption of the Tie2 gene in mice revealed that angiopoietins and the Tie2 system play critical roles in embryonic vascular development [15–17]. Angiopoietins also play an considerable role in angiogenesis in adults, Ang1 inducing the maturation of neovessels while Ang2 elongates them in the presence of VEGF [18]. The effect of Ang3 on angiogenesis remains to be characterized.

Since a recent study has shown that Ang2 mRNA expression is up-regulated in bovine microvascular endothelial cells by stimulation of VEGF [19], we then examined the effect of VEGF on the expression of Ang3 mRNA in HUVECs. Although mRNA expression levels of Ets-1, a member of the ets gene family, were increased by VEGF treatment, as previously reported [20], we found that Ang3 mRNA expression in HUVECs is instead slightly decreased after the VEGF treatment (Fig. 3), suggesting that the expression of Ang3 is regulated by a mechanism different from that of Ang2.

Targeted disruption of the Tie1 gene, a homolog of Tie2, indicates that Tie1 is required for the maintenance of vascular integrity [16]. The ligand of Tie1 is not known at the moment. In this context, it is important to learn whether Ang3 binds to either Tie1 or Tie2.

Finally, RH mapping for Ang3 was performed with two independent primer sets. As shown in Fig. 4, Ang3 was mapped to the region 0.8 cR telomeric of D20S199 (LOD $>$ 3), which locates at chromosome 20p13.

Cloning, tissue expression and chromosomal mapping of a human angiopoietin-3 protein are required to understand its role in normal angiogenesis and its pathological states.

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