Acta Biochim Biophys Sin (2008): 619-624 | © 2008 Institute of Biochemistry and Cell Biology, SIBS, CAS | All Rights Reserved 1672-9145
http://www.abbs.info; www.blackwellpublishing.com/abbs | DOI: 10.1111/j.1745-7270.2008. 00442.x

Review

Mechanisms of action of angiogenin

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Angiogenin induces angiogenesis by activating vessel endothelial and smooth muscle cells and triggering a number of biological processes, including cell migration, invasion, proliferation, and formation of tubular structures. It has been reported that angiogenin plays its functions mainly through four pathways: (1) exerting its ribonucleolytic activity; (2) binding to membrane actin and then inducing basement membrane degradation; (3) binding to a putative 170-kDa protein and subsequently transducing signal into cytoplasm; and (4) translocating into the nucleus of target cells directly and then enhancing ribosomal RNA transcription. Angiogenin can also translocate into the nucleus of cancer cells and induces the corresponding cell proliferation. Furthermore, angiogenin has neuroprotective activities in the central nervous system and the loss of its function may be related to amyotrophic lateral sclerosis. This review intends to conclude the mechanisms underlying these actions of angiogenin and give a perspective on future research.

Keywords angiogenin; angiogenesis; cancer; amyotrophic lateral sclerosis

Angiogenin (ANG) was originally isolated from the conditioned medium of cultured HT-29 human colon adenocarcinoma cells based solely on its angiogenic activity [1]. The gene encoding ANG is present as a single copy per haploid genome, and localizes on chromosome 14q11 [2]. The mature ANG is a basic, single-chain protein containing 123 amino acids with a molecular weight of about 14, 400 Da [1], and is a homolog of bovine pancreatic ribonuclease A. Although its ribonucleolytic activity is rather weak, it is essential for angiogenesis and other functions. ANG has also been reported to induce the proliferation of cancer cells directly. Recently, *ANG* gene was identified to be a potential amyotrophic lateral sclerosis (ALS) related gene. In this review, we will overview the functions and mechanisms of ANG in these physiological and pathological processes.

Functions and Mechanisms of ANG in Angiogenesis

Angiogenesis, the process of new blood-vessel growth, plays an essential role in normal physiological processes, such as development and reproduction. However, pathological angiogenesis occurs in many angiogenesis-dependent diseases such as tumors and other non-neoplastic diseases [3]. As a key angiogenic factor, ANG is believed to be an ideal target for anti-angiogenesis therapy. Therefore, revealing the mechanism of action of ANG will facilitate not only the understanding of angiogenesis, but also the discovery of angiogenesis inhibitors.

It has been reported that ANG interacts with endothelial and smooth muscle cells to induce a wide range of cellular responses including cell migration, invasion, proliferation, and formation of tubular structures. Four aspects of ANG have been discovered to be necessary for the process of ANG-induced angiogenesis, including ribonuclease activity, basement membrane degradation, signaling transduction, and nuclear translocation.

ANG exerts its ribonucleolytic activity

ANG belongs to the ribonuclease superfamily with a 33% sequence homology to the pancreatic ribonuclease A [4]. Although the crystal structures of human ANG and pancreatic ribonuclease A have high similarity, there is notable difference in the ribonucleolytic active center. The pyrimidine binding site of ANG is "obstructed" by the glutamine (Gln)117 residue, which results in a very weak

Received: May 13, 2008 Accepted: June 10, 2008
This work was supported by grants from the National Natural Science Foundation of China (30171035, 30470670 and 30770470), the Program for New Century Excellent Talents in University (No. NCET-05-0521) and the Zhejiang Provincial Program for the Cultivation of High-level Innovative Health Talents (2007-191)
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ribonucleolytic activity, about 10^5–10^6 lower than that of RNase A. Movement of Gln117 and the adjacent residues may be required prior to or during catalysis for substrate binding to ANG [5]. Latter experiments showed that mutation of this residue greatly increased the RNase activity of ANG but without changing its specificity, which further supported the notion that Gln117 impeded the ribonucleolytic activity of ANG [6]. Although weak, the RNase activity is necessary for the functions of ANG. Mutations of His13, Lys40, or His114, key amino acids for the RNase activity of ANG, greatly decrease its angiogenic activity in the chick embryo chorioallantoic membrane (CAM) assay [7,8]. Moreover, human placental ribonuclease inhibitor (PRI) [9] and compound 65828 [10] targeting the ANG enzymatic active site abolish both the ribonucleolytic activity and the angiogenic activity of ANG.

ANG stimulates basement membrane degradation

Besides its ribonucleolytic activity, the binding of ANG with endothelial cell surface is also needed for its biological functions, and amino acid residues from 60 to 68 are critical in this process [11]. During an effort to identify the ANG receptor in endothelial cells, a 42-kDa cell surface protein was initially found as an ANG-binding molecule [12], and was later shown to be a smooth muscle type α-actin [13]. The cell surface actin seems to be involved in the basement membrane degradation. Upon binding of ANG to actin, some of the ANG-actin complexs dissociate from the cell surface. Thereafter, this complex accelerates tissue-type plasminogen activator (tPA)-catalyzed generation of plasmin from plasminogen [14]. Therefore, through the formation of its actin complex, ANG promotes the degradation of basement membrane and extracellular matrix and thus allows endothelial cells to penetrate and migrate into the perivascular tissue [15], an essential feature of angiogenesis (**Fig. 1**).

ANG activates signaling transduction

Because actin is not an ANG receptor for signal transduction, a 170-kDa molecule was later identified as a potential ANG receptor located on the endothelial cell surface, and expressed only on ANG-responsive but sparsely cultured endothelial cells (<2×10^4 cells/cm^2) [16]. Unfortunately, the nature of this molecule is still elusive.

Although there is a lack of knowledge on ANG receptors, several pathways have been proposed to be activated by ANG stimulation. In response to ANG treatment, extracellular signal-related kinase1/2 (ERK1/2) [17] as well as protein kinase B/Akt [18] were activated in human umbilical vein endothelial (HUVE) cells, and phosphorylation of stress-associated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) was observed in human umbilical artery

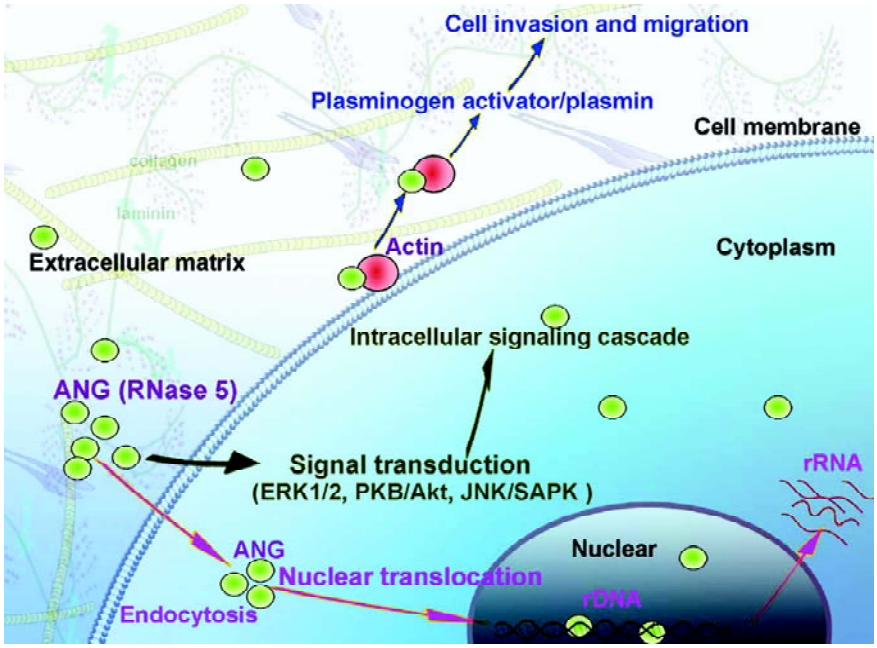

Fig. 1 Mechanism of action of angiogenin in the process of angiogenesis Angiogenin, also known as RNase 5, induces angiogenesis mainly through four pathways: its ribonuclease activity, basement membrane degradation, signaling transduction, and nuclear translocation.

smooth muscle (HuASM) cells [19] (**Fig. 1**). Activations of these signaling pathways by ANG are considered to be an important mechanism leading to cell proliferation and further angiogenesis.

It appears that the 170-kDa putative receptor and actin are not expressed concurrently on the endothelial cell surface. They seem to be expressed under different cell conditions and play roles at different stages of ANG-induced angiogenesis. In subconfluent cells, actin is expressed and binds to ANG specifically [13]. Binding of ANG to cell surface actin results in activation of a cell-associated protease system that promotes cell invasion [14]. After the cells start to migrate and invade into the basement membrane, the local density of the cells in the vicinity of the migrating cells decreases, thus triggering the expression of the 170-kDa putative ANG receptor on the remaining adjacent cells. These cells become responsive to stimulation of ANG and will therefore divide to fill the space created by the migrating cells. The expression of the receptor may then be turned off when the cell density increases. It is speculated that such density-dependent receptor expressions may regulate the ANG-induced growth of the new capillary network.

ANG undergoes nuclear translocation and enhances rRNA transcription

Angiogenin undergoes nuclear translocation in endothelial cells and smooth muscle cells [19], which has also been shown to be necessary for ANG-induced angiogenesis (**Fig. 1**). Inhibition of nuclear translocation of ANG [20] or mutagenesis of its nuclear localization sequence [21] both abolish its angiogenic activity. Nuclear translocation of ANG in endothelial cells is rapid [22], but is strictly dependent on cell density [22]. It decreases as cell density increases and ceases when cells are confluent.

The nuclear function of ANG has been found to enhance ribosomal RNA (rRNA) transcription [23] (**Fig. 1**). An ANG-binding element (ABE), known as CTCT repeats, has been identified from the intergenic spacer (IGS) region of rDNA. ABE binds ANG specifically and exhibits ANG-dependent promoter activity in the luciferase reporter system [24]. Now it is recognized that the nuclear ANG assumes an essential role in endothelial cell proliferation and is necessary for angiogenesis induced by other angiogenic factors, such as acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF) [25]. ANG-stimulated rRNA transcription in endothelial cells has been shown to serve as a crossroad in the process of angiogenesis induced by the other angiogenic factors.

However, the process of nuclear translocation of ANG is largely unknown. The first step required for the nuclear translocation of exogenous ANG is the internalization of the protein, and receptor-mediated endocytosis seems to be involved in the internalization [21]. A nuclear localization signal (NLS), which lies in 31-RRRGL-35 of the protein, is responsible for the nucleolus targeting of human ANG [26]. However, the ANG NLS does not confer nuclear import through the pathway used by conventional NLSs in that importins and Ran are not required [27]. The process is also independent of microtubules and lysosomes [28]. Since the molecular weight of ANG is less than the limit of nuclear pore size (50-kDa), the most probable mechanism for ANG nuclear/nucleolus import may involve passive diffusion of ANG through the nuclear pore and NLS-mediated nuclear/nucleolus retention [27].

Roles of ANG in Diseases

ANG induces tumor growth

It was reported that the expression of ANG was upregulated in various types of human cancers, including breast, cervical, colon, colorectal, endometrial, gastric, liver, kidney, ovarian, pancreatic, prostate, and urothelial cancers, as well as astrocytoma, leukemia (acute myeloid leukemia and myelodysplastic syndrome), lymphoma (non-Hodgkin's), melanoma, osteosarcoma, and Wilms' tumor [29]. This indicates a close relationship between ANG and tumor development.

Angiogenin was once thought to promote cancer progression by its angiogenic activity, and target HUVE and HuASM cells as described above. Recently ANG was reported to constantly translocate to the nucleus of HeLa cells in a cell density-independent manner. Downregulation of ANG expression in HeLa cells resulted in a decrease in rRNA transcription, ribosome biogenesis, proliferation, and tumorigenesis [30]. These results point to a direct effect of ANG on cancer cells for the first time with a similar action manner as in HUVE cells. Latter studies on prostate cancer cells showed that ANG could directly stimulate PC-3 proliferation, and underwent nuclear translocation in PC-3 cells grown both *in vitro* and in mice. Blockade of nuclear translocation of ANG by neomycin inhibited PC-3 cell tumor growth in athymic mice and was accompanied by a decrease in both cancer cell proliferation and angiogenesis [29]. In addition, ANG could be an effective substrate for HT-29 cells adhesion during metastasis [31]. Thus, it is clear that ANG takes part in cancer development by stimulating both angiogenesis and cancer cell proliferation. However, whether the mechanisms of ANG that act on

HUVE cells and cancer cells are similar is unknown yet.

ANG may be related with amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a progressive late-onset neurodegenerative disorder affecting upper and lower motoneurons (MNs). VEGF was the first angiogenic factor shown to contribute to the pathogenesis of ALS [32]. ANG was recently identified as the second angiogenic factor related to this disease. First, allelic association studies of Irish and Scottish ALS populations identified chromosome 14q11.2 where the ANG gene is located as a candidate region. Later a synonymous single nucleotide polymorphism (rs 11701) was found to be associated with Irish and Scottish ALS populations by sequencing 1629 ALS patients [33]. Thereafter, seven missense mutations in the ANG gene were identified in 15 patients with either familial or sporadic ALS by sequencing the same 1629 ALS patients [34]. To date, mutations of the ANG gene have been detected in the Irish, Scottish, Italian, and North American patients with ALS diseases [34-39].

Angiogenin may exert neuroprotective activities on motoneurons in the central nervous system. First, as an angiogenic factor, ANG protects MNs by increasing neurovascular perfusion. Studies on the functional consequence of those detected *ANG* mutations showed that the mutants diminished ANG's ribonucleolytic activity, nuclear translocation, or both. A correlative reduction in the HUVE cell proliferative and angiogenic activities was observed [35,36], which may contribute to the induction of ALS.

Second, ANG may protect MNs via its direct effects on the neurons themselves. Mouse ANG-1 (mAng-1) was found to be strongly expressed in motor neurons in the spinal cord and dorsal root ganglia as well as in post-mitotic MNs derived from P19 cells. Its expression was found in the growth cones and neurites. Inhibition of the ribonucleolytic activity of human ANG affected path finding by P19-derived neurons [40]. Cultured P19 EC cells could internalize both wild-type ANG and the variants implicated in ALS. However, wild-type ANG could induce P19 EC cell differentiation and the extending of the neuritis, whereas the variants lost these capacities. Wild-type ANG was able to protect neurons from hypoxia-induced cell death, but the variants lacked the neuroprotective activity [41]. These findings provide a causal link between mutations in ANG and ALS.

ANG acts in other diseases

Angiogenin may also play roles in a variety of non-malignant angiogenesis-dependent diseases such as endometriosis [42], peripheral vascular disease [43], inflammatory bowel disease (IBD) [44], rheumatoid arthritis [45], diabetes [46], and so on. In these disorders, ANG expression levels increase and ANG may contribute to the local pathological angiogenesis conditions.

Perspectives

Identifying the physiological RNase substrate of ANG

The ribonucleolytic activity of ANG is essential for its functions. Although ANG was reported to be able to catalyze degradation of 18S and 28S rRNA [47], tRNA from *Xenopus oocytes* [48], and 5S RNA from *Saccharomyces cerevisiae* and *Escherichia coli* [49], the physiological substrate of ANG is still unknown. It is unlikely that ANG has a specific recognition sequence for catalysis. Instead, the target may have a specific secondary structure, such as a hairpin or a pseudo-knot, or may be part of a protein-nucleic acid complex. The poor catalytic activity of ANG may have evolved to maximize specificity for the target substrate [50]. The natural substrate of ANG may reside in the nucleolus of its target cells where it accumulates. Since rRNA transcription is always coupled and coordinated with its processing, rRNA could be a candidate substrate of ANG. The identification of its physiological substrate should be a great help for the complete description of ANG's activities.

Developing ANG nuclear translocation inhibitors

The function of ANG in mediating both endothelial cell and cancer cell proliferation is related to rRNA transcription and depends on nuclear translocation [21,30]. Thus, the process of ANG nuclear translocation seems to be an ideal target for anti-ANG drug discovery. Neomycin seems to be such a promising drug because it blocks nuclear translocation of ANG in PC-3 cells and inhibits tumor establishment and growth in athymic mice by inhibiting tumor angiogenesis and prostate cancer cell proliferation, respectively. In other words, this drug has a combined benefit of chemotherapy and antiangiogenesis therapy. Now the Hu group is evaluating the therapeutic value of neomycin and its nontoxic derivative neamine against cancers [29]. We reason that elucidating the mechanisms of ANG nuclear translocation would provide new targets for developing such kinds of inhibitors.

Identifying ANG-interacting proteins

Since protein interactions are critical in every biological process, interactions between ANG and other proteins should mediate or modulate a series of biological activities in ANG-induced angiogenesis and tumor cell growth.

Unfortunately, few proteins have so far been identified as binding partners of ANG. To identify more mediators or modulators of ANG activity, yeast two-hybrid technology was used in our laboratory and 21 proteins were identified as potential ANG-interacting molecules from the human liver cDNA library and heart cDNA library, including cytoskeleton proteins such as alpha-actinin 2 (ACTN-2) [51], regulatory proteins such as follistatin (FS) [52], and extracellular matrix proteins such as fibulin-1 [53]. Through interacting with ACTN-2, ANG may regulate the movement or the cytokinesis of the cells. Follistatin may act as a regulator on angiogenin's actions. Interaction between ANG and fibulins may facilitate cell adhesion. The concrete significance of those interactions is under study now.

In summary, angiogenin plays important roles in many pathological states, and could be an ideal target for disease treatment. Although many molecules have been reported to exert antitumor effects, such as anti-ANG monoclonal antibodies [54], ANG-binding peptides [55], ANG antisense RNA [56], and its ribonuclease inhibitors [10], they all antagonize angiogenin itself, and may have significant side effects. An ideal angiogenin-oriented drug could only be made possible after fully elucidating the mechanism of action of angiogenin and identify a disease-specific process.

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