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In vivo and *in vitro* studies of angiogenin—a potent angiogenic factor

J. Badet, F. Soncin, T. N'Guyen and D. Barritault

Angiogenin is a potent blood-vessel-inducing polypeptide with a molecular weight of 14 000 that has a unique ribonucleolytic activity. First isolated from the conditioned medium of tumour cells, angiogenin has since been purified from normal plasma, which suggested that its propensity to induce neovascularization should be strictly controlled. Modulation of that activity might involve interaction of angiogenin with cell-surface receptors and extracellular matrix of endothelial cells, tight-binding inhibition of both its ribonucleolytic activity and cell binding property by ribonuclease inhibitor, as well as the overall influence of divalent copper, a modulator of angiogenesis.

Introduction

Angiogenesis, the growth of new vessels from the pre-existing vascular network, plays a key role in various normal and pathological processes. Under normal conditions, blood vessel proliferation occurs mainly during development, whereas in the adult, angiogenesis is a rare event limited to wound healing, formation of the placenta, corpus luteum and to tissues that show a cyclic growth pattern, such as the endometrium. This neovascularization process, is perfectly controlled and located. However, angiogenesis is also associated with the development of pathologies in which it is thought to play a preponderant role. Those included rheumatoid arthritis, diabetic retinopathy and vascular glaucoma. Furthermore, the growth of solid tumours and metastases are fully dependent on the proliferation of capillary blood vessels.¹ In both normal and pathological conditions, the morphological events resulting in the formation of new blood vessels are similar.² Consequently, a biochemical approach ought to be successful in underlining the peculiarities of the angiogenesis involved in pathology. Tumours produce a variety of diffusible factors³ which attract and activate endothelial cells such as basic fibroblast growth factor (bFGF),⁴ transforming growth factor alpha,⁵ platelet-derived endothelial cell

growth factor⁶ and glioma-derived vascular endothelial cell growth factor,⁷ and an angiogenic polypeptide with a ribonucleolytic activity, angiogenin.⁸

Biochemical characteristics and properties of angiogenin

Angiogenin, a polypeptide with a molecular weight of 14 000, was first purified from conditioned medium of human colonic adenocarcinoma cell line HT29 by monitoring its angiogenic activity in the chick embryo chorioallantoic membrane assay.⁸ It is a potent blood-vessel-inducing factor, since 0.5 ng (35 fmol) induces angiogenesis in that system and 50 ng (3.5 pmol) in the rabbit corneal assay.⁸ This single-chain protein has a high content of basic amino acids which results in a high isoelectric point (>9.5) and could account for its affinity for DNA. The cDNA of the angiogenin gene contains a sequence coding for a peptide signal, indicating that angiogenin is a secreted protein.⁹

Although angiogenin has 35% sequence identity and an overall homology of 68% with pancreatic RNase, it has been shown to be inactive in standard pancreatic RNase assays. However, angiogenin has an unusual

ribonucleolytic specificity toward ribosomal and transfer RNAs.¹⁰ The limited cleavage of 18S rRNA seems to be responsible for the inhibition of cell-free protein synthesis by specific inactivation of the 40S ribosomal subunit.¹¹ The physiological relevance of this enzymatic activity has been demonstrated by Vallee's group.¹² Thus, a functional enzymatic active site is necessary for its angiogenic property since placental ribonuclease inhibitor abolishes both ribonucleolytic and angiogenic activities. However, other ribonucleases such as bovine RNase A, a human tumour-cell-derived RNase and eosinophil-derived neurotoxin are not angiogenic, which emphasizes the peculiarity of angiogenin.

Unlike the other angiogenic polypeptides from tumour cells, angiogenin has no known direct effect on cell proliferation. But like the other factors, angiogenin is also present in normal tissues. It has been purified from normal plasma¹³ and milk,¹⁴ and its mRNA transcripts have been detected in normal and tumour cells,¹⁵ as well as predominantly in adult rat liver.¹⁶ Its presence in normal tissues and plasma indicates that its action should be well controlled. Furthermore, the apparent absence of a relationship between these tissues and their potential angiogenesis status suggest that angiogenin might have an unknown biological function.

Angiogenin cell surface receptors

Using human recombinant angiogenin produced in *Escherichia coli* and shown to be angiogenic *in vivo* both in the chick chorioallantoic membrane¹⁷ and in the rabbit cornea (Figure 1), we have demonstrated the presence of specific binding sites on endothelial cells from calf pulmonary artery (CPAE), bovine aorta, cornea and adrenal cortex capillary as well as association to endothelial extracellular matrix.¹⁸ Cellular binding of iodinated angiogenin at 4°C, was time- and concentration-dependent, reversible and saturable. This interaction was shown to be specific since a large excess of unlabelled angiogenin reduced binding by more than 80% whereas a similar concentration of RNase had no effect. Furthermore, iodinated RNase did not bind to endothelial cells. Scatchard analyses of binding data revealed two apparent types of specific interactions. A high-affinity binding site with an apparent dissociation constant (K_d1) in the nanomolar range, involved about 200 000 angiogenin molecules per cell and represented cell-specific interactions. The second component, comprising low-affinity/high-capacity binding sites with a K_d2 of 0.2 μ M was associated with pericellular components and involved several million molecules.

The high-affinity binding sites are probably cell surface receptors,¹⁸ since angiogenin, added to cultured cells, triggers a series of intracellular events. Indeed,

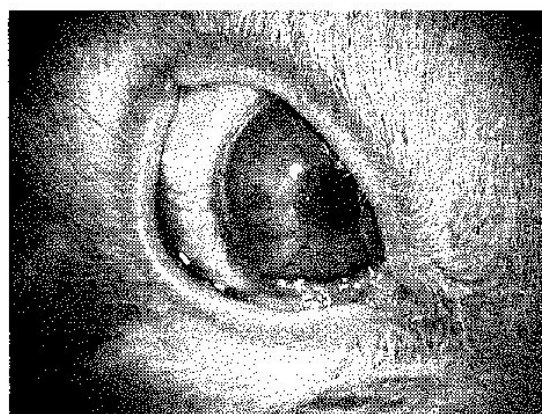


Figure 1. Induction of blood vessel growth by human recombinant angiogenin in the rabbit corneal assay. A 2 mm² piece of contact lens containing 200 ng of angiogenin was implanted in the cornea and angiogenesis observed 7 days later.

angiogenin has been shown to activate endothelial cell phospholipases¹⁹ and to induce the secretion of prostacyclin, an inhibitor of platelet aggregation and a potent vasodilator.²⁰ In vascular smooth muscle cells, besides activation of phospholipase C, angiogenin elicits a rapid cholesterol esterification.²¹ Furthermore, a primary mitogenic response has been reported for conditioned media from activated smooth muscle cells, 3T3 and capillary endothelial cells in correlation with *in vitro* activation of several plasma membrane enzymes resulting in *de novo* synthesis of phosphatidylinositol biphosphate known to play a key role in the intracellular signalling pathway of some mitogens.²²

Modulators of angiogenesis and angiogenin cell-surface receptors

Among the factors that modulate angiogenesis, divalent copper was shown to induce a several-fold increase in specific cell-bound angiogenin.¹⁸ Other metal ions such as Cu(I), Li(I) and Co(II), Ni(II) had no effect. Protamine, an inhibitor of angiogenesis,²² avidly affected angiogenin cell binding, whereas heparin interacted at a lower extent.

Placental ribonuclease inhibitor (PRI), a tight-binding inhibitor of both ribonucleolytic and angiogenic activities of angiogenin¹² abolished its binding to the CPAE cells at a molar angiogenin/PRI ratio of 1. However, its inhibitory effect on cell binding was no more observed in the presence of Cu(II) (Figure 2A). Metal ions did not irreversibly inactivate PRI since their action could be reversed by the addition of a metal chelator, the diethyldithiocarbamate (Figure 2B).

Although PRI is an intracellular regulatory protein,

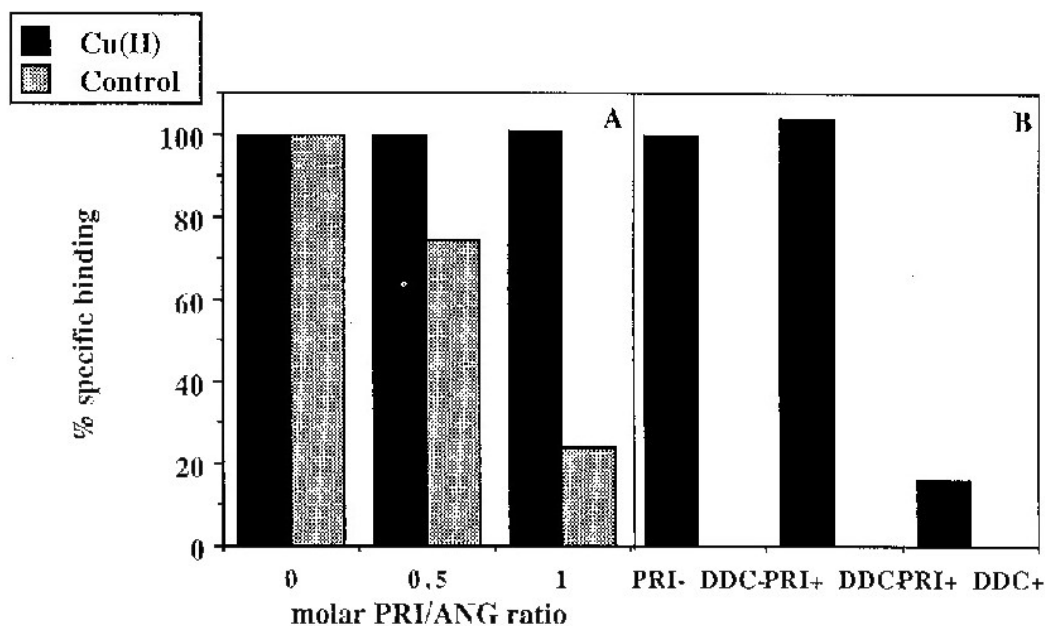


Figure 2. Modulation of ^{125}I -angiogenin cell-specific binding to calf pulmonary artery endothelial cells by placental ribonuclease inhibitor and copper sulphate. (A) Angiogenin was pre-incubated with an increasing amount of the inhibitor for 15 min at 4°C and then assayed for its binding property in the absence (control) or presence of $100\ \mu\text{M}$ $\text{Cu}(\text{II})$. Only cell-associated radioactivity is presented. (B) Effect of metal ions on the stability of angiogenin-inhibitor complex. Iodinated angiogenin was incubated for 30 min at 4°C in the presence of the ribonuclease inhibitor (PRI+) or in its absence (PRI-). Metal ion with 1 mM diethyldithiocarbamate (DDC+) or without (DDC-) were then added and incubations continued for 90 min before binding experiments were assayed.

immunoreactive forms have been detected in plasma, where it could be associated to angiogenin present in the circulation.²⁴ PRI contains seven homologous tandem repeats with a remarkable leucine-rich module. These repetitive motifs are a common structural feature of glycoproteins involved in important biological processes such as blood coagulation, signal transduction, embryonic development, cell morphogenesis and negative regulation of transforming growth factor.²⁵

As shown in Figure 2, copper reverses the tight PRI/angiogenin complex and increases angiogenin binding to its receptors. Although the mechanism is unclear, increased copper levels have been detected in cornea in response to angiogenic stimuli.³ These observations suggest a possible tight regulation of angiogenin action in the process of angiogenesis where cell surface receptors, pericellular components, copper metal ions and ribonuclease inhibitor or a still unknown member of the leucine-rich protein family would be partners.

Because of its specific properties, studies on the mechanism of action of angiogenin should lead the way to a better knowledge of the biochemical events involved in angiogenesis.

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