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Organogenesis and angiogenin

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Abstract. Our search for an angiogenesis-inducing factor in culture medium conditioned by human colon adenocarcinoma cells (HT-29) was inspired by the 'organizer' hypothesis originally postulated by Spemann. It led us to the isolation of angiogenin, a 14 kD protein homologous to pancreatic ribonuclease and one of the most potent stimulators of blood vessel formation known. This review summarizes the properties of angiogenin, its enzymatic and three-dimensional relationship to ribonuclease A (RNase A), those aspects of its structure that are critical for its biological function, and the therapeutic potential of angiogenin inhibition. Despite having the same arrangement of catalytic residues as RNase A, angiogenin has very low enzymatic activity. It lacks one of the four disulphide loops of RNase A; instead, the corresponding residues form part of a cell binding region. Both the catalytic activity and cell binding site are essential for angiogenesis. Angiogenin binds to cell-surface actin in confluent endothelial cells and to an as yet uncharacterized receptor on proliferating cells. Internalization and translocation to the nucleolus are also required for activity. Inhibitors of angiogenin can block angiogenesis in vitro and prevent tumour growth in vivo. Thus, a noncytotoxic neutralizing monoclonal antibody prevents the establishment of HT-29 human tumour xenografts in up to 65% of treated athymic mice. In those tumours that develop, the number of vascular elements is reduced. Actin also prevents the establishment of tumours while exhibiting no toxic effects at daily doses >50 times the molar amount of circulating mouse angiogenin. These antagonists also inhibit the appearance of tumours derived from two other human tumour cell lines. Inhibition of the action of angiogenin may prove to be an effective therapeutic approach for the treatment of malignant disease.

Key words. Angiogenin; angiogenesis; ribonuclease; nuclear localization; anti-angiogenesis; tumour therapy.

Abbreviations. BACE = bovine adrenal capillary endothelial; BHK = baby hamster kidney; CAM = chorioallantoic membrane; CPAE = calf pulmonary artery endothelial; DG = diacylglycerol; FGF = fibroblast growth factor; FITC = fluorescein isothiocyanate; HUVE = human umbilical vein endothelial; IP = inositol phosphate; mAb = monoclonal antibody; NLS = nuclear localization sequence; PBS = phosphate-buffered saline; PIP = phosphatidylinositol phosphate; PLA_2 = phospholipase A_2; PMA = phorbol myristate acetate; PRI = placental ribonuclease inhibitor; RNase A = bovine pancreatic ribonuclease A.

Introduction

The development of organisms and their organs has long been an attractive field for biologists to study, particularly embryologists. In the early part of the twentieth century, Hans Spemann and his students pioneered studies of development. This work was to become the benchmark of investigating the embryology of the salamander and frog. Spemann formulated the 'organizer' hypothesis which postulates that 'inducers' and 'organizers' regulate embryonic development and are responsible for the localization of organs. He supposed that they would bring about and control the formation of the germ layers which, in turn, would form organs

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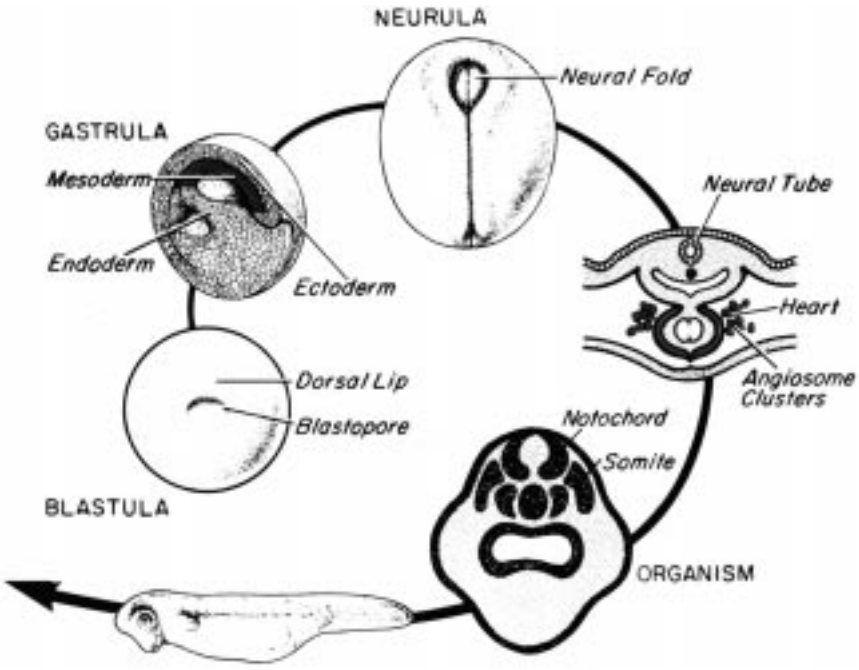

Figure 1. Stages of amphibian development and organogenesis. After fertilization of the oocyte and subsequent cell division, the resultant blastula undergoes gastrulation in which the cells rearrange into three germ layers: ectoderm, endoderm and mesoderm. Once these layers are established the cells interact to form organs by the process of organogenesis, which in vertebrates is initiated by formation of the neural tube.

whose location and anatomical characteristics are shown schematically in figure 1.

As is now well established, the fertilized egg first forms a blastopore by invagination of the dorsal lip into the interior of the fertilized egg, initiating differentiation into ectoderm, endoderm and mesoderm. From the very beginning, the features of the material contained in that dorsal lip determine its developmental/morphological future. The ectoderm encompasses formation of the skin, neural fold, brain, and neural tube which ultimately becomes the spinal cord and nerves. The endoderm gives rise to the internal organs: heart and blood vessels, lungs, liver, intestines, spleen and kidneys. The mesoderm generates muscles, bones, connective tissue and tendons, that ultimately become the support structure of the organism.

Spemann, together with his collaborators and contemporaries, proved that plans and instructions for organ formation are already contained in the fertilized ovum. This was recognized through systematic experiments demonstrating that a given organ system in one embryo could be transferred into another 'host' embryo. He took different parts of the dorsal lip at different stages of development and transplanted them into hosts whose progeny allowed observation of their subsequent fate. By this means, he clearly demonstrated that the capacity for organ formation pre-exists and can be dissected, identified, and transplanted into a host, thereby localizing and defining the origin, development and fate of the precursor. Spemann's procedures and findings have been summarized in great detail in a remarkable monograph [1].

In Spemann's critical experiment (fig. 2) the host embryo invaginates the transplanted dorsal lip into its interior where it undergoes development and differentiation similar or identical to that of the host itself, finally resulting in 'Siamese twins'. This generates a neurula with two ecto-, endo-, and mesoderms. Incidentally, Spemann also succeeded in tracking the identity of the transplants from the source to the host by choosing a pigmented axolotl variant for insertion into an unpigmented one, thereby differentiating the source of the host and guest tissues. This 'tracer' experiment in which pigmentation served as the marker is analogous to the present-day use of black and white mice to monitor targeted gene disruption experiments.

These experiments generated a great deal of excitement in biochemical and biological circles and forecast the future of biological experimentation. There was general awareness and belief that the experiments had delineated totally novel avenues of approach to the understanding of growth, development, differentiation, maturation and ultimately organ formation. There was hope that these complex processes would be understood

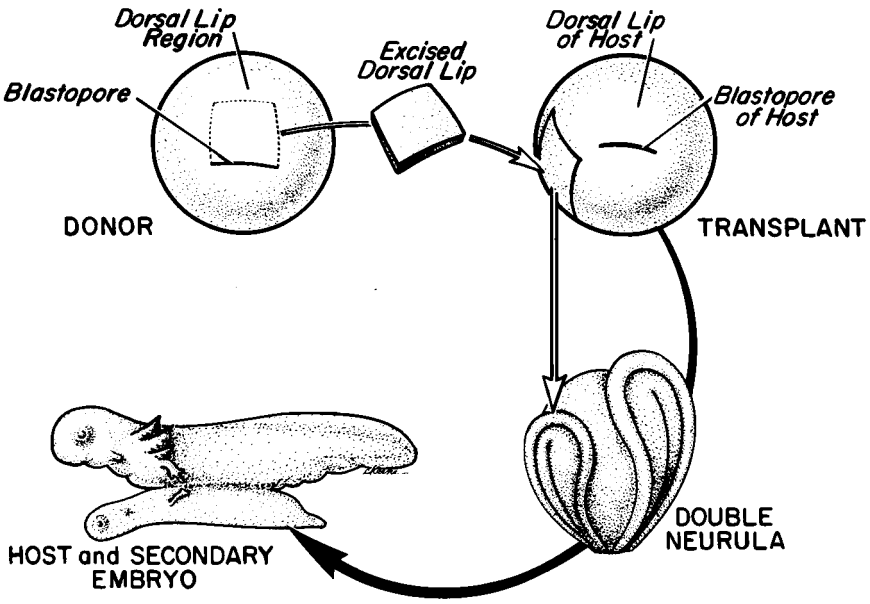

Figure 2. Biology of the 'organizer.' Cells from the blastula are removed from the dorsal lip region just above the blastopore and implanted into a second blastula which develops into a double neurula and eventually gives rise to 'Siamese twins'.

before long and would serve as a road map for biology throughout the remainder of the millennium. Needham, in particular, raised hopes for great events to come [2]. However, his optimism seemed exaggerated since progress actually was quite slow. There were many reasons for this delay; but in particular support for basic research was minimal at that time. National and international communication on science was quite poor and did not facilitate rapid exchange and propagation of information. Nationalism itself became an unexpected hindrance to scientific progress. Chauvinistic sentiments were abundant and proved divisive and counterproductive. The major political and economic upheavals that culminated in World War II accentuated all of these hindrances by evaluating scientific effort on the basis of national origin and political alignments rather than intrinsic merit.

All the same, the biological and philosophical questions that Spemann's work raised were lasting, despite the slowed evolution of experimental biology. Pressing scientific questions could not be answered easily by the means available. The required methods were not sufficiently sophisticated and sensitive. Molecular and cell biology, needed to recognize the structure and function of organelles and the cell skeleton, were just being discovered. However, the important questions survived: Is the hypothesis of the existence of inducer and organizer molecules reality or fiction? Are there discrete classes of molecules responsible for organogenesis? How will such molecules be recognized and found to orchestrate the events that ultimately initiate and control embryonic development and govern hierarchical features of cell function? What are the characteristics of organizer molecules and in what manner might they resemble or differ from molecules currently known.

Spemann's discovery opened the door to many new biological vistas and potentials, but the door was not sufficiently ajar to illuminate and uncover the sources, origins and consequences of relevant considerations. As we approach the next millennium we can still ask when and how it will become possible to undertake a successful inquiry, and what particular organ systems might lend themselves best to the experimental exploration of such ideas and the documentation of valid answers?

The search for an angiogenesis agent

The characteristics of the vascular system seemed particularly attractive to us for the study of organogenesis. Not only can this system be studied embryologically but its replacement, repair and renewal continues throughout life [3]. Blood vessels are composed of endothelial cells and are formed early in embryonic development to support growth and maintenance of tissues. They are capable of repair, as in wound healing, and of cyclical replacement, as in menstruation. Angiogenesis, the stimulation of endothelial cells to form new blood vessels, in any of these situations, would be an example of organogenesis offering opportunities for the examination of multiple mechanisms and approaches, including tumour formation, mechanisms of defense and distribu-

tion of important aspects of metabolic control and activity.

The recognition that organizer molecules could be involved in tumour angiogenesis was based on the fact that rapidly growing tissues call for large amounts of nutrients to maintain growth beyond the needs of the host. Angiogenesis would require an organizer or a group of organizers, while formation of tumours, in turn, would depend on angiogenesis. Indeed, it was proposed that tumour cells might secrete angiogenic molecules [4]. Such molecules might be detectable in and recoverable from media in which tumour cells are grown. Such considerations led us to attempt to isolate angiogenic molecules from the conditioned media of HT-29 colon carcinoma cells grown in culture. To do this a suitable bioassay was necessary.

Assays for angiogenesis

The chorioallantoic membrane (CAM) of the chicken egg has been a preferred means for studying blood vessel growth [5]. A window is cut into the shell of an egg, and the material to be tested for angiogenic activity is deposited on the surface of the exposed membrane. The appearance of blood vessels in response to the test substance is then measured several days later. An alternative but more tedious method is the implantation of an angiogenic substance into the cornea of a rabbit eye [6]. This has the advantage that the second eye serves as the control.

There have been numerous efforts to devise more accurate and simpler assays, because the difficulty of determining angiogenic activity quantitatively has been a great hindrance to rapid progress in this field, and remains a major technical problem even today. Although the corneal and CAM assays have been mainstays, they are far from satisfactory. The CAM assay generally requires substantial numbers of eggs. In our laboratory, a given sample is implanted on six to ten eggs for each sample and repeated at a series of increasing concentrations [7]. The treated eggs are observed after 68 hours and scored for a positive response. An equivalent number of eggs treated with sterile H_2O serve as the control. The number of eggs implanted and observed becomes very large but this is necessary to obtain statistically significant results. The measurements are nevertheless highly subjective and require careful judgement on the part of the scientist. On the basis of these assays, we announced the isolation of angiogenin from large scale cultures of HT-29 tumour cells in 1985 [8]. It was the first angiogenic factor to be obtained by a direct, systematic search. All other angiogenic factors/molecules were isolated initially with another objective in mind and only subsequently found to be angiogenic.

Properties of angiogenin

Human angiogenin is a 14 kD single polypeptide chain with three disulphide bridges (table 1). Its N- and C-termini are pyroglutamic acid and proline, respectively. It is angiogenic on the CAM at a dose of one nanogram/egg and exhibits enzymatic activity: it is a ribonuclease. Its amino acid sequence has identified it as a member of the ribonuclease superfamily [9, 10]. Ribonuclease A (RNase A) from bovine pancreas has been a benchmark in the course of evolution of enzymology from the 1930s to the present. Its characteristics are exceptionally well known. Four Nobel Prizes for groundbreaking investigations on RNase A have been awarded: to Moore, Stein, Anfinsen and Merrifield [11]. Its three-dimensional structure has been established both by X-ray crystallography [12] and NMR [13]. Much is known about its chemical and biological features, and there is seemingly endless information on its mode of action. It was clearly revealing and informative to compare the structure and conformation of angiogenin with that of RNase A. Beintema [14] has sequenced more than 40 RNases from various phyla and species. Their sequences are from $\sim$30 to 70% identical to angiogenin, but none of them have been shown to be angiogenic. Virtually all have four disulphide bridges except for angiogenin and turtle RNase which have only three. The ribonucleolytic activity of angiogenin is only about 10^{-5} to 10^{-6} that of RNase A [15], despite the fact that it contains all the residues known to be essential for the enzymatic action of RNase A (i.e. the equivalents of His 12, His 119 and Lys 41). The differences in substrate specificities of angiogenin and the ribonucleases are minor.

The most significant sequence difference between the RNases and angiogenin is the absence of the fourth disulphide bond (between Cys 65 and Cys 72) in the

Table 1. Properties of angiogenin[a].

Amino acids	123
Molecular weight	14,124
Subunit structure	single chain
Disulphides	3
Isoelectric point	>9.5
Molar absorptivity	12,500 M^{-1} cm^{-1}
Enzymatic function	cleaves RNA
Biological functions	angiogenesis, cell adhesion, cell migration, inhibition of PMNL, immune suppression
Inhibitors	RNase inhibitor, $K_i = 7 \times 10^{-16}$ M
Genetic location	chromosome 14q11

[a]For human angiogenin. Angiogenins have also been characterized from mouse, rabbit, pig and cow.

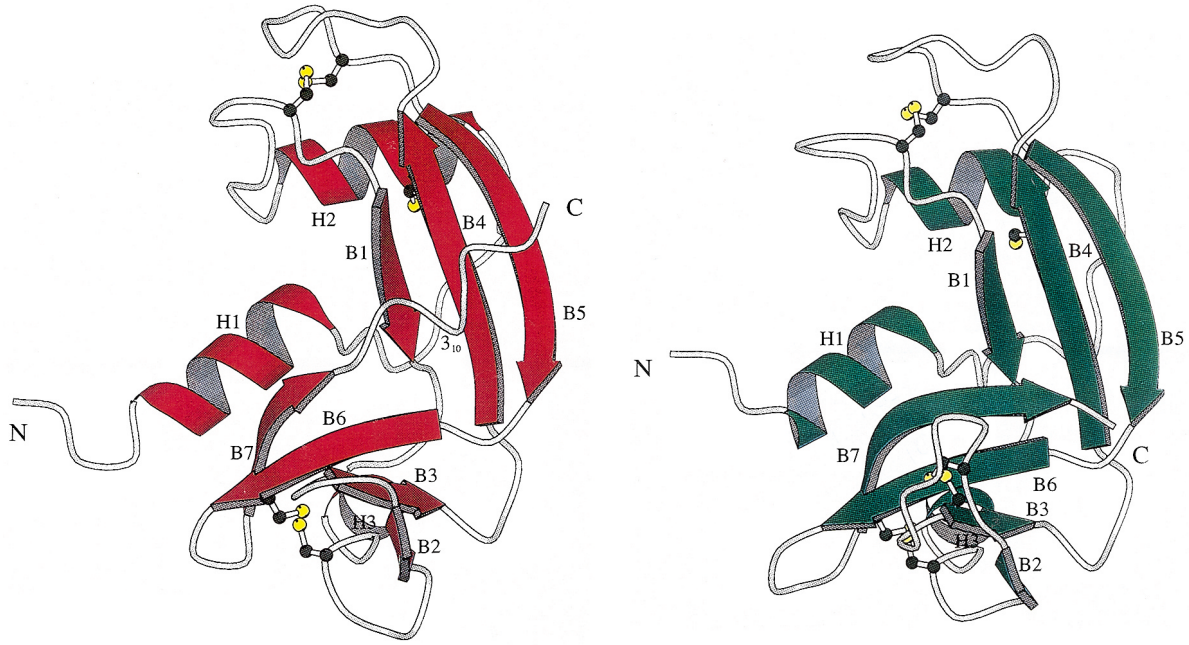

Fig. 3

Figure 3. The polypeptide fold for angiogenin (left) and RNase A (right). (Reprinted with permission from: Acharya K. R., Shapiro R., Allen S. C., Riordan J. F. and Vallee B. L. (1994) Crystal structure of human angiogenin reveals the structural basis for its functional divergence from ribonuclease. Proc. Natl Acad. Sci. USA **91:** 2915–2919, © 1997 National Academy of Sciences).

latter [14]. Both of these Cys residues are replaced in all angiogenins and moreover, two of the intervening residues are deleted. This region of the protein might therefore be predicted to serve a special role in angiogenin, as has proved to be the case. Other regions that differ from the RNases include residues 5–11 and 16–21, suggesting that the N-terminal region of angiogenin may also have a unique functional role. However, mutagenesis of angiogenin by replacing residues 8–22 with those of 7–21 of RNase A did not affect its enzymatic activity, but did increase its angiogenic potency as well as its capacity to inhibit cell free protein synthesis [16].

Tertiary structure

Subsequent to its recognition as a member of the RNase superfamily, a putative three-dimensional structure of angiogenin was calculated by energy minimization and compared with the known structure of RNase A [17]. Most of the structural predictions from this calculation turned out to be in remarkably good agreement with the subsequent results of crystallography of angiogenin [18]. There are a few major differences between angiogenin and RNase A however, which are in the surface loop regions, particularly of the C-terminal segment beginning at residue 116 (fig. 3). The region of the missing fourth disulphide bond (see below) also exhibits additional changes and realignments. However, the active site residues occupy essentially the same positions in both proteins, and hence they have the same P_1 site where cleavage of the scissile phosphodiester bond occurs. The most startling difference is at the B_1 (pyrimidine-binding) site. In RNase A there is an open pocket with threonine 45 at one side and serine 123 at the other. This pocket does not exist in angiogenin. It is occupied by the side chain of glutamine 117, a consequence of the markedly different orientation of the C-terminal residues of angiogenin vs that in RNase A. When the RNase inhibitor, uridine vanadate, is modeled into the putative B_1 site of angiogenin the side chain of glutamine 117 passes right through the uridine ring, indicating that binding of uridine vanadate in this position is incompatible with the structure of angiogenin. However, the substrate must bind to the B_1 site since it is hydrolysed, albeit weakly, and with essentially the same specificity as RNase A. In order for the substrate to be accommodated the protein would have to undergo a conformational change. To date the structure of an angiogenin/B_1 ligand complex that could confirm such a conformational change has not been obtained, but the functional consequences of mutagenesis of glutamine 117 are consistent with this postulate. Replacement of Gln by Ala or Gly increases RNase activity by 11 to 18 and 21 to 30-fold respectively, depending on the substrate [19]. Since the activity of these mutant angiogenins toward RNase substrates is still orders of magnitude lower than that of RNase A,

Table 2. Site directed mutagenesis of active site residues of angiogenin[a].

Residue	Mutant	Activity; % of control	
		tRNA	CAM
His-13	Ala	<0.01	<1
His-114	Ala	<0.01	<1
Lys-40[b]	Gln	<0.05	<1

[a]Enzymatic activity: formation of perchloric acid soluble fragments from yeast tRNA. Angiogenic activity: chick embryo chorioallantoic membrane using 7–17 eggs. From [21].
[b]From [47].

any extrapolation of kinetic data to confirm the validity of this postulate must remain tentative.

Substrate specificity

Evidence to date strongly suggests that RNA is the physiological substrate of angiogenin and that it is either a specific RNA or a particular class of RNAs, but in any case RNA. Angiogenin exhibits characteristic activity toward isolated 28S and 18S rRNA, with major products 100 to 500 nucleotides in length, and contrasting with the much smaller products generated by RNase A [20]. Despite the very low ribonucleolytic activity of angiogenin it is nevertheless *essential* for angiogenesis. Mutation of any one of the active site residues abolishes *both* ribonucleolytic and CAM activities (table 2). Although the active site mutants are not themselves active angiogenically, they do block the activity of native angiogenin [21]. This observation has important consequences in terms of the mode of action of angiogenin.

In the course of isolation of angiogenin from a recombinant expression system in BHK cells we discovered a modified form of the protein in which the Glu 67-Asn 68 peptide bond was cleaved [22]. This derivative, angiogenin E, was no longer angiogenic in the CAM assay, but still had ribonucleolytic activity. This was the first instance in which we were able to separate these two activities. The protease, endoproteinase Lys-C, also cleaves a single, specific bond (in this case between Lys 60 and Asn 61) and generates another new derivative termed angiogenin K [22]. As with angiogenin E, it is no longer angiogenic on the CAM but retains its ribonucleolytic activity. In both derivatives the site of cleavage is either close to or within the region of the 'missing' fourth disulphide loop (fig. 4). Importantly, unlike the active site mutants, the proteolytically modified angiogenins are unable to block the CAM activity of native angiogenin. These results suggested that this region of the protein might be the binding site for a putative receptor on the endothelial cell surface. It is an external loop region not only distinct from the catalytic site, but also relatively well conserved in the angiogenins from different species.

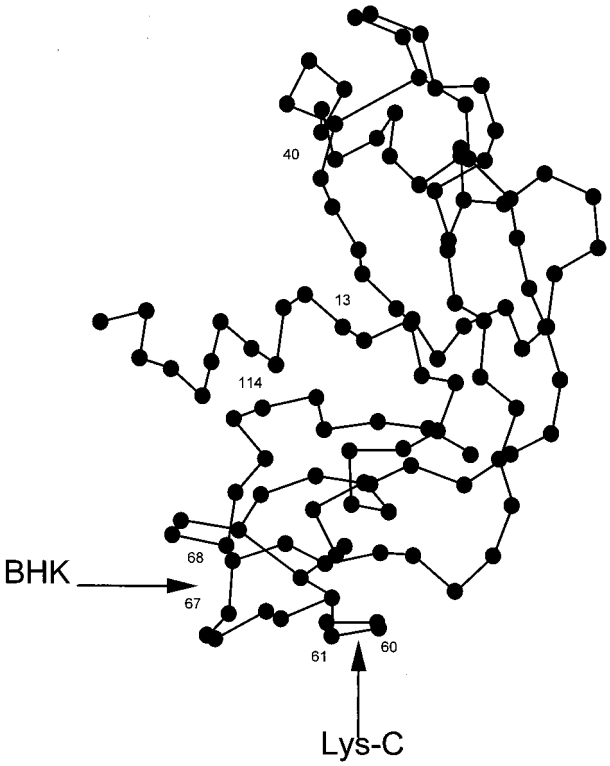

Figure 4. Three-dimensional structure of angiogenin (α-carbons) based on the energy-minimization calculation of Palmer et al. [17]. The active site region is identified by the catalytic residues Lys 40, His 13 and His 114 The putative cell-binding region includes residues 60 to 68. Arrows indicate the sites of cleavage for the BHK protease and endoproteinase Lys-C. See text for details. (Reprinted with permission from: Hallahan T. W., Shapiro R. and Vallee B. L. (1991) Dual site model for the antiogenic activity of angiogein. Proc. Natl Acad. Sci. USA **88:** 2222–2226, © 1997 National Academy of Sciences).

To summarize up to this point: 1. the ribonucleolytic activity of angiogenin is indispensable for the preservation of angiogenic activity, but of itself is clearly insufficient for angiogenesis; 2. the site where angiogenin presumably binds to endothelial cells is in the region of the proteolytic cleavage sites encompassing residues 60 to 68, and it is distinct from the enzymatic site; 3. both the ribonucleolytic activity and the binding site are required for angiogenesis. In angiogenin the area which would encompass the fourth disulphide bridge of RNase is replaced by a peptide which stretches from residues 65 to 72 where the BHK protease generates angiogenin E, and endoprotease Lys-C produces angiogenin K. This area is presumed to be the domain where angiogenin attaches itself to endothelial cells to bring

about angiogenesis. These facts provided the basis for our proposal that angiogenin has two functional sites, one for RNase activity and the other for endothelial cell binding [22] (fig. 4). Both sites and functions are necessary for angiogenesis.

Induction of second messengers

The interaction of angiogenin with CPAE cells stimulates a rapid but transient burst of diacylglycerol (DG) that reaches a maximum in 2.5 min and returns to baseline in 10 min [23]. The peak of the dose response occurs at 1 ng/ml angiogenin but varies with cell type. The appearance of DG is accompanied by a small and similarly transient increase in inositol phosphates, IP_3 and IP_2, followed by a more substantial and sustained increase in IP. There are also small temporary decreases in the phosphatidylinositol phosphates PIP_2 and PIP, and a more significant decrease of PI. A small increase in lyso-PI was also noted. These changes were interpreted to indicate that angiogenin activates an inositol-specific phospholipase C that acts briefly on PIP_2 and then on PIP. The amount of IP_3 produced is apparently not sufficient to induce a burst of cytosolic calcium. The appearance of lyso-PI suggests that phospholipase A_2 (PLA_2) is also activated, probably giving rise to arachidonic acid.

In a subsequent study Bicknell and Vallee confirmed that angiogenin stimulates endothelial cells to secrete prostacyclin, a product of arachidonate metabolism [24]. The time course and concentration dependence were similar to those observed for DG but the effect was not seen with CPAE, only with HUVE and BACE cells. In addition, the effect is inhibited by pertussis toxin but the DG response is not. A DG lipase inhibitor, indomethacin, quinacrine (a PLA_2 inhibitor), H7 (a protein kinase inhibitor), and an inhibitor of prostacyclin synthesis all block the stimulated secretion as does downregulation of protein kinase C by phorbol ester. The sensitivity to pertussis toxin suggests the involvement of a G protein in signal transduction to PLA_2. It was speculated that DG, released in response to angiogenin, activates protein kinase C which phosphorylates lipocortins and thus abolishes their ability to inhibit PLA_2. The inhibition of prostacyclin secretion by H7 and by phorbol myristic acid (PMA) argues for a role for protein kinase C in the angiogenin stimulatory effect.

Angiogenin receptor

The fact that angiogenin induces these responses, and the results of mutagenesis and limited proteolysis, all point to a possible cell surface receptor for angiogenin. Initial attempts to identify such a receptor led to the discovery of a 42 kD, endothelial cell surface protein that was subsequently characterized as a smooth muscle type of α-actin [25]. The physiological significance of this finding is still not clear since actin is known to bind to many proteins, but it can inhibit the angiogenic activity of angiogenin and prevent the growth of HT-29 cells subcutaneously implanted into nude mice [26].

For many years a classical transmembrane receptor remained elusive. Quite recently we were able to identify a 170 kD cell surface protein that may be the functional angiogenin receptor. It is only expressed on angiogenin-responsive endothelial cells (i.e. those that are growing in sparse cultures) and binds to and crosslinks with angiogenin [27]. Neither angiogenin nor heparin dissociate this protein from the cell surface. It is expressed in very small amounts in these sparse cell cultures and therefore although production is increasing we have not yet accumulated enough for characterization.

Nuclear translocation of angiogenin

What happens to angiogenin once it binds to the endothelial cell surface? Earlier studies with ^{125}I-labelled angiogenin at 4 °C and 37 °C had indicated that it was not internalized [28]. However, these studies were with confluent cells. In growing CPAE cells, immunofluorescence with an anti-human angiogenin monoclonal antibody identified an intracellular pathway for human angiogenin [29]. It rapidly undergoes endocytosis, is translocated from the cell surface to the nucleus, and accumulates in the nucleolus. It is not known whether the putative receptor to which angiogenin might bind is also internalized. Since internalization is not observed with confluent, nonproliferating cells, a specific nuclear translocation mechanism may only operate in proliferating cells. This conjecture may be especially significant in view of the fact that angiogenin is a circulating plasma protein, and the vast majority of the vascular endothelial cells are in a nonproliferating, confluent state. Only when perturbed by trauma, disease or some other change in local environment would these cells become responsive to angiogenin.

The nuclear localization of angiogenin could imply that substrates for its ribonucleolytic activity might be located within the nucleolus. The nucleolus in eukaryotic cells is a highly specialized subcompartment, where biogenesis of ribosomes takes place. Pre-rRNA is first transcribed here from rDNA genes, subsequently undergoing a series of processing modifications. The presence of angiogenin could perhaps enhance pre-rRNA transcription or processing.

CPAE cells do not internalize four enzymatically active angiogenin derivatives whose cell binding site is modified, but they do internalize two enzymatically

inactive mutants whose cell binding site is intact [29]. None of the mutants are angiogenically active so it seems that nuclear localization is essential for the biological function of angiogenin.

Proteins translocated to the nucleus of eukaryotic cells are targeted there by specific nuclear localization sequences (NLS) that typically contain a high proportion of basic amino acids. The prototype NLS is the sequence -Pro-$(Lys)_4$-Arg-Lys-Tyr of the SV 40 T-antigen. Angiogenin contains a sequence -Arg_{31}-Arg-Arg-Gly-Leu- that has this function [30]. Thus the Arg33Ala mutant of angiogenin, in which Arg_{33} is changed to Ala, is not translocated to the nucleus and lacks angiogenic activity. Moreover, a synthetic peptide -Cys-$(Gly)_2$-$(Arg)_3$-Gly-Leu-, when coupled to fluorescein isothiocyanate (FITC) albumin, to an antihistone monoclonal antibody (mAb), to an anti-human nucleolus mAb, or to FITC-Arg33Ala- angiogenin by crosslinking with maleimidobenzoyl-N-hydroxysuccinimide, enabled all of them to be translocated to the nucleus of permeabilized cells [30].

It is important to point out that angiogenin is not unique: other angiogenic factors also undergo nuclear translocation. Exogenous basic and acidic FGF, epidermal growth factor and platelet derived growth factor are endocytosed by target cells and translocated from the cell surface to the nucleus where they accumulate in the nucleolus. This appears to be a general nuclear transport pathway that is a critical step in the process of angiogenesis [31]. This view is supported by the observation that endocytosis and nuclear translocation of angiogenin, bFGF and aFGF are inhibited by two potent inhibitors of angiogenesis, protamine and platelet factor 4.

At this point we may conclude that angiogenin seems to have the characteristics we might expect from an organizer or inducer molecule as initially postulated by Spemann 70 years ago. In the framework of the RNase molecule, angiogenin carries with it a catalytic site which is responsible for its ribonucleolytic activity, it has a cell binding site which is responsible for its being attached to the endothelial cell surface, and it has a third site, the nuclear localization sequence, which is responsible for the transfer of the angiogenin molecule into the nucleus and nucleolus after it has been endocytosed. It remains to be seen whether or not angiogenin is actually a prototype for other organogenic molecules. This will become apparent as additional systems are studied in a manner similar to that employed here.

Mechanism of action

Angiogenin is normally extracellular: it is synthesized primarily in the liver and then released into the circulation. Since angiogenin is a potent inducer of neovascularization, its mode of action has been examined primarily with endothelial cells. For the sake of simplicity the details can be inspected from three separate yet interrelated points of view, namely its three distinct functional features: 1. it is an enzyme; 2. it is an organogenic molecule; 3. it has a nuclear localization sequence which aids in its transport to the nucleus and nucleolus.

The ribonuclease activity is essential for angiogenesis and probably operates within the cell, particularly within the nucleolus, most probably in conjunction with a ribonucleic acid substrate related to ribosome biogenesis. It could act outside of the cell if an extracellular RNA molecule were available for the purpose, but current evidence does not support this proposition. If it were to be active outside the cell it would be in competition with other blood plasma RNases. Since these are much more potent enzymes, an extracellular venue for angiogenin would not seem plausible.

The cell binding site of angiogenin is also critical for angiogenesis. Together with the observed second messenger responses, this suggests that it interacts directly with a target cell receptor to elicit a biological response. The competition observed in the alteration of the activity modified is compatible with this point of view. Since we have not found any RNA at the surface of cells, intracellular RNA seems to be the most probable substrate for angiogenin. Moroianu and Riordan [29] have shown by immunofluorescence that angiogenin is indeed transported into the cell, probably in an endocytotic vesicle, and perhaps as a receptor-ligand complex. Presumably it can reach RNA in the nucleus but how it is released from the endocytotic vesicle is unknown. It should be noted that when angiogenin is microinjected directly into the cytosol it is actually cytotoxic [32].

So far, the mechanism of endocytosis, the mode of transport to the nuclear membrane, and the mode of entry of angiogenin through the nuclear pores into the nucleus, have not been established. The fact that angiogenin contains an essential NLS strongly supports nuclear entry via the classical pore route. Nevertheless, the information currently available does not encourage speculation as to exactly what angiogenin might do once within the nucleus that would ultimately result in angiogenesis. Although the enzymatic activity of angiogenin is considerably less than that of RNase A, this activity is essential for the protein to exercise its angiogenic function. Even so angiogenin must, as noted above, undergo a conformational change to displace glutamine 117 from obstructing the B1 site. Perhaps nature has designed this ribonuclease to become enzymatically active only at the place where this activity will be most effective and not in a place e.g. (the cytosol) where it might cause ribonucleolytic havoc.

An extracellular role for angiogenin is also possible, of course, and might involve binding to cell surface actin

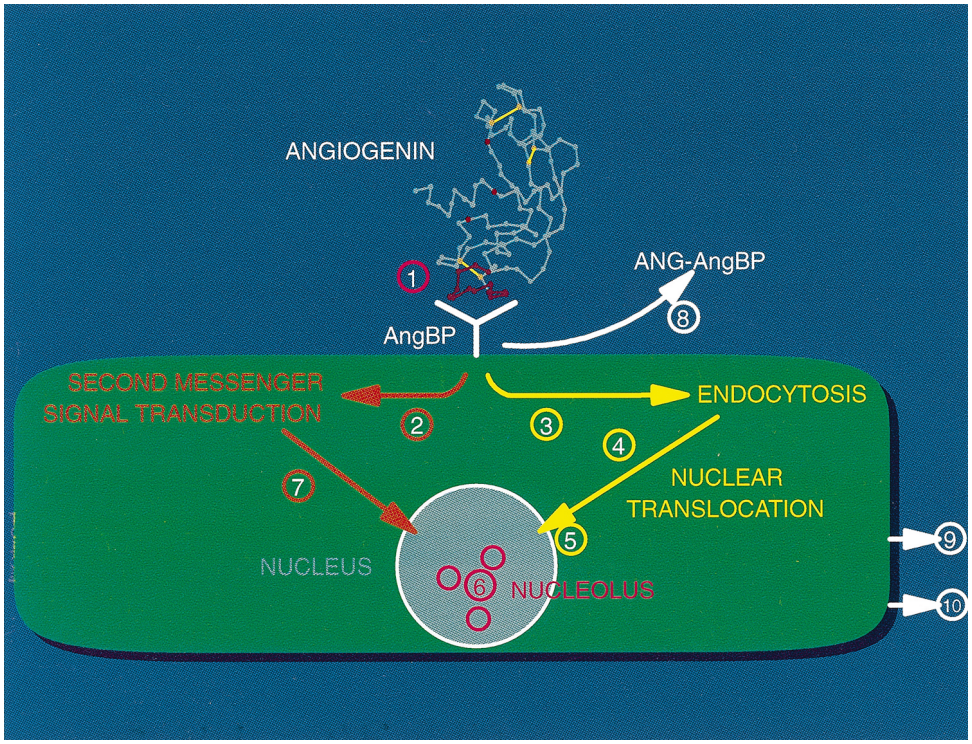

Figure 5. Hypothetical scheme for the mechanism of action of angiogenin. (1) Angiogenin binds to its cellular receptor, indicated here as the angiogenin-binding protein (AngBP; actin), but which may involve heparan sulphate proteoglycans as well as a more conventional receptor. Binding occurs through the cell-binding region as indicated. This results in (2) activation of phospholipases C and A_2, among others, with consequent second-messenger signal transduction, the details of which have not been elucidated. In addition, angiogenin is endocytosed (3) and undergoes translocation to the nucleus (4). It is then taken up by the nucleus (5) and localized to the nucleolus (6), where in conjunction with nuclear 'activation' signals initiated by some second messengers (7) it potentiates the endothelial cell for proliferation. Some of the angiogenin forms an extracellular complex with the angiogenin-binding protein (8), which assists in activating proteolytic cascades. Extracellular angiogenin also serves as a substratum that promotes cell adhesion and migration (9). The overall outcome of these processes is angiogenesis (10). (Reprinted with permission from: Riordan J. F. (1997) Structure and function of angiogenin. In: Ribonucleases: Structures and Functions, D'Alessio G. and Riordan J. F. (eds), pp. 445–489, Academic Press, New York, © 1997 Academic Press, Florida).

and/or heparansulphate proteoglycans, but this may be unrelated to its ribonucleolytic activity. Figure 5 summarizes our current, speculative scheme for the overall mechanism of action of angiogenin as it relates to angiogenesis outlined here: (1) angiogenin first binds to a cellular receptor through its cell binding region. (2) This activates phospholipases C and A2, among others, with consequent second messenger signal transduction, the details of which have not been elucidated. In addition, angiogenin is (3) endocytosed, and (4) undergoes translocation to the nucleus. It is then (5) taken up by the nucleus, and (6) localized to the nucleolus, where in conjunction with nuclear 'activation' signals initiated by the second messengers, it (7) potentiates the endothelial cells for proliferation. Some of the angiogenin may (8) form an extracellular complex with the angiogenin-binding protein, actin, which assists in activating proteolytic cascades [33]. Extracellular angiogenin has been shown to be an adhesion molecule [34] so (9) it can promote cell migration. The overall outcome of these processes, probably together with others not yet known, is angiogenesis (10).

Inhibition of angiogenin and ribonuclease A

Since angiogenin is responsible, at least in certain circumstances, for the formation of blood vessels, its control and modulation are of the utmost importance for development, repair and growth of normal and abnormal tissues. As a consequence, from the very beginning we have been concerned with any features of this molecule that relate to its inhibition, and have searched for agents that might affect its activity with physiological, pathological or therapeutic consequences.

Anti-angiogenic agents could potentially play important roles in the clinical treatment of a number of diseases including cancer, arthritis, psoriasis, hemangiomata and

Table 3. Inhibition of ribonuclease A[a].

Inhibitor	K_i, μM	Ref.
Thymidine 3′,5′-diphosphate	1.0	[48]
4-Thiouridine 3′-phosphate	2.7	[48]
Adenosine 3′,5-diphosphate	5.0	[48]
2′-CMP	7.0	[48]
2′-UMP	7.1	[49]
Uridine vanadate	10	[50]

[a]K_i values shown were measured at various pH values from 5.0 to 6.0 except for uridine vanadate which was at 7.0.

Table 4. Inhibition of the ribonucleolytic activity of ribonuclease A and angiogenin by nucleotides.

Nucleotide	K_i, μM	
	ribonuclease A[a]	angiogenin[b]
2′-CMP	7.0	8700
2′-UMP	7.1	13000
5′-AMP	80	7200
5′-ADP	1.2	1200
3′,5′-ADP	4.7	1300
2′,5′-ADP	9.0	400
3′-phosphoadenosine 5′-diphosphate	0.2	300
2′-phosphoadenosine 5′-diphosphate	0.5	150

[a]Assessed by determining effects of inhibitors on k_{cat}/K_m values for cleavage of CpA at pH 5.9. From [42].
[b]From [41].

diabetic retinopathy, among others. We first gave particular attention to an exceptionally potent protein RNase inhibitor which occurs in the tissues of numerous species and had been investigated by Blackburn and Moore [35]. Our interest in this inhibitor ultimately led us to isolate it from human placenta, characterize it and demonstrate that it potently inhibits both the ribonucleolytic and CAM activities of angiogenin [36]. It binds to both RNase A and angiogenin in 1:1 stoichiometry with K_i values of 4×10^{-14} M and 7×10^{-16} M, respectively. These are among the strongest protein-protein interactions known. Placental RNase inhibitor (PRI) is a 50 kD single chain polypeptide of 460 amino acids arranged in 15 tandem alternating 28 and 29-residue, leucine-rich repeats. The X-ray structure of the inhibitor from pig liver has been determined [37] as has that of its complex with RNase A [38]. The inhibitor is shaped like a horseshoe and when it binds RNase 28 of its residues are in contact with the enzyme and 24 from the enzyme contact the inhibitor. Many of the PRI-RNase A contact residues are not conserved in angiogenin. Since PRI inhibits all mammalian members of the RNase superfamily, it must be somewhat flexible so that it can accommodate them all with more or less the same remarkable affinity.

The tandem repeat structure seen in the sequence of PRI suggested that not all of the molecule would be necessary for interaction with either angiogenin or RNase A. Lee and Vallee [36, 39, 40] therefore expressed truncated versions of PRI and found that six of the 14 central repeats could be deleted without major detriment to binding to either protein. These studies suggested that a modified form of PRI could be constructed that would have therapeutic potential. Until now the effectiveness of PRI in preventing tumour growth has not been commensurate with its extraordinary affinity for angiogenin. As indicated below, other less tightly binding inhibitors of angiogenin have been much more potent. One reason for this poor result may be the instability of PRI. It contains 32 cysteine residues and if only a few are oxidized the protein becomes inactive. Mutants of PRI devoid of sulphhydryl groups may provide a more useful agent.

Meanwhile, an alternative strategy was to use low molecular weight RNase inhibitors instead of PRI. The most effective nucleotide inhibitors of RNase A previously reported are 2′-CMP, 2′-UMP, 4-thiouridine 3′-phosphate, thymidine 3′,5′-diphosphate, 5′-phosphoadenosine 3′-phosphate (pAp), and uridine vanadate (table 3). Even the best of these is not really adequate for animal studies. In an effort to prepare tighter binding inhibitors we began with pAp, since it should bind to the B_2 substrate binding site, which from their X-ray structures appear to be equally accessible in RNase A and angiogenin. We also note that pyrimidine nucleotides, which bind to the B_1 site and are relatively good inhibitors of RNase A, are ineffective with angiogenin whose B_1 site is occluded. We had found that addition of a second phosphate at the 5′ position of 5′-AMP to give 5′-ADP, reduced K_i 67-fold (table 4). Accordingly, addition of another phosphate to pAp to give ppAp reduced K_i almost 20-fold to 0.24 μM. This inhibitor, which indeed binds to the P_1-B_2-P_2 region of the active site of RNase (D. Leonidas, L. Irons and K. R. Acharya, personal communication) is the most potent, low molecular weight inhibitor of this enzyme to date [41].

We next tested ppAp with angiogenin and found that although it did inhibit, its K_i was 1000-fold higher than with RNase A (table 4). Studies of inhibition by a series of nucleotides revealed that in contrast to RNase, angiogenin prefers a 2′-phosphate to a 3′-phosphate [42]. This suggested that 5′-diphosphoadenosine-2′-phosphate might be more potent than the 3′-phosphate or any of the previously tested nucleotides. We therefore synthesized it and found that it has a K_i of 150 μM, which is actually 50 times lower than that of the best nucleotide previously reported, and 400 times better than the K_m of the best dinucleotide substrate. This may be adequate for crystallographic studies but not for therapeutic experiments with animals.

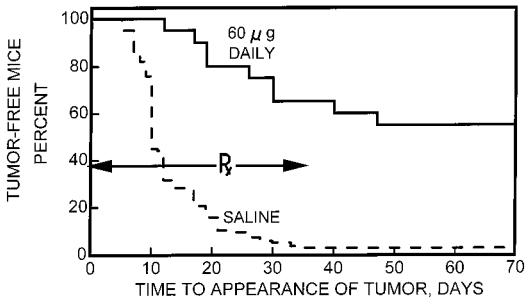

Figure 6. Kaplan-Meier survivor functions showing prevention of HT-29 tumour growth in athymic mice by treatment with mAb 26-2F. On day 0, tumour cells (125,000/mouse) are mixed with PBS or the mAb and injected sc. Daily injections are given for the next 35 days. ---, PBS (n = 145); ——, mAb 26-2F (60 μg; n = 20). (Reprinted with permission from: Olson K. A., Fett J. W., French T. C., Key M. E. and Vallee B. L. (1995) Angiogenin antagonists prevent tumour growth in vivo. Proc. Natl Acad. Sci. USA **92:** 442–446, © 1997 National Academy of Sciences).

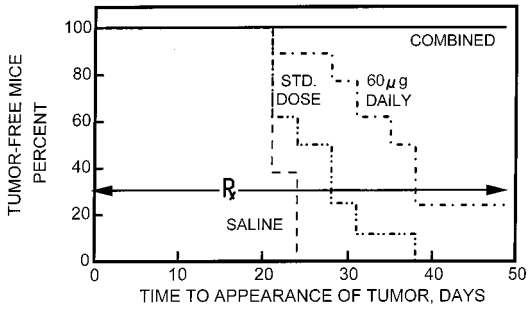

Figure 7. Prevention of PC-3 cell growth in athymic mice by treatment with mAb 26-2F (60 μg/dose, sc) (– · –); PBS plus cisplatin (4 mg/kg/dose, ip) plus suramin (60 mg/kg/dose, ip) (– ·· –); or mAb 26-2F plus cisplatin plus suramin as above (—). Control (---).

Angiogenin inhibition and suppression of tumour growth

Since angiogenin is a blood vessel inducing protein that is secreted by tumour cells, and since tumour cells require a blood supply for both growth and metastasis, we therefore examined the question of whether inhibition of angiogenin might inhibit tumour growth. We developed a subcutaneous (sc) tumour model in athymic mice in which a small number of HT-29 tumour cells are injected sc to mimic the early stage of metastatic disease. The low molecular weight inhibitors of angiogenin just described not only lack sufficient potency for in vivo studies, but were moreover unavailable when these studies began. We had prepared an anti-angiogenin monoclonal antibody, mAb 26-2F [43], which is not cytotoxic to tumour cells in vitro, and delays (or in up to 25% of cases prevents) the appearance of tumours when administered daily at the site of tumour cell implantation [44]. Subsequently, a more sensitive model was used and up to 65% of the treated animals remained tumour-free for at least 30 days [26] (fig. 6). A second neutralizing monoclonal antibody with an epitope specificity different from mAb 26-2F was also effective in inhibiting tumour growth. Two additional cell lines, a lung adenocarcinoma and a fibrosarcoma (which like HT-29 cells secrete angiogenin when grown in culture) were also sensitive to antibody treatment. We also tested actin, since it binds to angiogenin and inhibits its activity on the CAM, and it too was capable of preventing tumour growth in more than 60% of treated animals. It should be noted that actin, unlike mAb 26-2F, interacts strongly with mouse angiogenin in vitro, and yet it causes no toxic side effects in the treated animals.

Prostate cancer is an especially problematic disease owing to the high incidence of associated metastases that resist therapy. Since there seems to be a correlation between the degree of angiogenesis present in histological sections of an invasive prostate carcinoma and its metastatic spread [45], the use of anti-angiogenic agents for this condition could have profound therapeutic consequences. Olson and Fett, in our laboratory, investigated the effect of mAb 26-2F, alone and in combination with other cytotoxic drugs, on the growth of prostate cancer (PC-3) cells in the athymic mouse model. Under each of the six different conditions employed, the monoclonal antibody substantially delays and in some cases completely prevents the appearance of PC-3 tumours. Two drugs often administered separately to treat prostate cancer clinically, cisplatin and suramin, were examined at dosages comparable to those commonly employed in humans. The two drugs together were somewhat less effective than mAb 26-2F, but the combination of all three agents *completely prevented* the appearance of PC-3 tumours in all mice in the experimental group (fig. 7). Obviously this startling result requires repetition and confirmation.

Anti-angiogenin therapy seems to work by specific extracellular inactivation of tumour-secreted angiogenin and consequent inhibition of neovascularization. Histological examination of tumours removed from treated and nontreated animals revealed marked differences in staining for factor VIII, a marker for endothelial cells, and hence an indicator of vascular density [26]. These results clearly demonstrate that angiogenin has a critical role, at least in the early stages of tumour development, and that inhibition of its function could be an effective means of therapy for malignant disease. Although we have not pursued alternative therapeutic targets, it is conceivable that such inhibitors might also prove effective in other angiogenesis-related diseases such as

arthritis, psoriasis and hemangiomata. Conversely, angiogenin itself might be helpful in the treatment of fractures, cartilaginous trauma and wound healing in general, as well as in enhancing vascularization to alleviate occlusions and infarctions.

Conclusion

We have progressed from the identification of angiogenin as a blood vessel inducing protein secreted by tumour cells, and the surprising discovery of its homology to RNase, to an emerging understanding of its mechanism of action. The early days, or rather years, of this venture were quite correctly called an odyssey [46] as we had to overcome major obstacles, largely technological, caused by the dearth of adequate methods for dealing with minuscule samples. After the successful cloning of angiogenin we made great strides in characterizing it as a protein and an enzyme but were stymied in understanding its role as a growth factor. The remarkable results of anti-angiogenin therapy provided strong motivation to pursue its cell biology and finally recognize that it is a pleiotropic molecule with both intra- and extracellular activities. The imminent characterization of its receptor; the results of targeted gene disruption, also in progress; and the availability of specific, high potency inhibitors, all promise to add important and, no doubt, novel dimensions to the continuing story of angiogenin.

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