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The angiogenins

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Abstract. The angiogenic and other biological functions of the angiogenins, members of the pancreatic RNase superfamily of proteins, are reviewed in the context of their primary and tertiary structures. The ribonucleolytic activity and interactions with the placental ribonuclease inhibitor have seen much study in the last few years. The mechanism of the angiogenic activity of angiogenin has recently been postulated as involving multiple interactions with other proteins through specific regions on the molecular surface of angiogenin. These molecular partners include heparin, plasminogen, elastase, angiostatin, actin and most importantly a 170-kilodalton receptor on subconfluent endothelial cells. The existence of the latter receptor was established in conjunction with a mitogenic activity of angiogenin on subconfluent cells. The levels of angiogenin in various physiological and disease states are summarized, including various studies on pregnancy and angiogenin. Correlations are seen between states of enhanced angiogenesis and angiogenin levels. An overview of the relationship of angiogenin and the other RNases of the superfamily showed that their genes all are in relative close proximity on human chromosome 14. Examination of the many expressed sequence tags published in the public databanks, for angiogenin and the other RNases, revealed that angiogenin and RNase-4 (the most evolutionarily conserved RNase), share various identical 5′-untranslated regions on their sets of messenger RNAs, suggesting that their genes are in very close proximity on chromosome 14 and that they are products of differential splicing. This in turn suggests that, in both humans and mice, expression of these two proteins is under identical control, with obvious implications for their biological activities. The evolutionary history of the angiogenins is examined briefly on the basis of the protein sequences of the human, rabbit, pig, two bovine and four mouse angiogenins, and two mouse angiogenin pseudogene sequences. The discrepancy between the conventional requirement for conservatism in structure to allow multimolecule interactions, and the actual fast-changing sequence of the angiogenins, in concert with the wide-ranging activity even in birds, of human angiogenin, is discussed.

Key words. Angiogenin; RNase-4; ribonucleases; angiogenesis mechanism; differential splicing; ESTs; evolution; review.

Introduction

Angiogenin is a protein with a potent function in eliciting new blood vessel growth, angiogenesis. It was discovered and purified more than a decade ago from human tumour cell-conditioned media using an assay based purely on its function in angiogenesis [1]. The sequence was homologous to the ribonuclease A superfamily [2, 3], which led to the delineation of its inherent ribonucleolytic activity, distinct from that of the RNases-1 [4]. This activity is a necessary, but not sufficient, requisite for its angiogenic activity [5].

Today the actions of this protein and its mechanisms are still not clear despite a huge volume of work. This is due in large part to the complexity of the biochemistry underlying a biological process such as angiogenesis, much of it still unknown. In addition there is a lack of knowledge of its inherent structure-function relationships, and of its relationship to the other members of this RNase family.

A number of reviews have considered angiogenin in particular [6–9]. This overview of the current status of our knowledge of the angiogenins therefore will only briefly consider the latest studies on its structure, primary and tertiary, and its ribonucleolytic function. It will focus on its angiogenic and other biological functions, and especially its relationship to the other ribonucleases.

Ribonucleolytic function of angiogenins

The various angiogenins exhibit a very low ribonucleolytic activity when compared with the superfamily-defining RNases-1, using conventional RNA substrates (up to a millionfold less active). Angiogenin is, however, more active than RNases-1 when they are compared by their activity on the ribosomal RNAs in ribosomal particles [4, 10, 11] (see below). Angiogenin is therefore also by its enzymatic activity fully a member of the RNase A superfamily.

Specificity of dinucleotide cleavage by angiogenin is different from that by RNases-1 and also differs among the angiogenins. These are summarized by Riordan [9]. The specific activity differences of the various angiogenins can be considered as only general indicators of ribonucleolytic activity, and may not predict the actual enzymatic mode of action of angiogenin, which is bound to involve the 'natural substrate', that elusive Grail mentioned throughout the angiogenin literature.

Inhibition of angiogenin by the placental ribonuclease inhibitor (RI) is more potent than for RNase-1, being mediated by binding with a dissociation constant <1 fM [12], one of the strongest binding interactions found for proteins. The recent X-ray crystallographic study of this complex [13] found that the human angiogenin-ribonuclease inhibitor pair crystallizes as a dimer of the complex, in contrast to the RNase/porcine ribonuclease inhibitor pair, which forms a monomeric complex [14]. This dimer formation is mediated by the small N terminal extension of the human protein as opposed to the porcine inhibitor, but probably does not occur in solution [15].

RI has a strikingly symmetrical structure, consisting of 16 identically folded α-helix–β-sheet pairs arranged in a horseshoe structure. The overall orientation of angiogenin and RNase toward RI in the respective complexes is similar, with the RNase/angiogenin molecule sitting with about one-third of its mass inside the central cavity of the RI horseshoe, blocking the opening of the horseshoe, and the enzymatic active site contacting the C-terminal region of RI. However, the interaction of these protein pairs makes use of different residue contacts, and even the major contact residues are different. The binding of angiogenin to RI is mediated by an extensive rearrangement of the binding interface on angiogenin, especially for residues 85–89, much more so than seen for RNase-1. Thus three of the major contact residues of RNase-1 on RI, Tyr-434, Tyr-437 and Ser-460 are much less involved in interaction with angiogenin, as also shown by mutagenesis studies [16], as previously discussed [9]. The X-ray structure demonstrated that in many instances pairs of interactions visible in the one complex are not seen in the other complex. The one common interaction point is that of Lys-41, the active site Lys, which is probably the kingpin for all of the RNase family in their interactions with RI.

Small synthetic inhibitors of RNase activity, studied by Russo and Shapiro, similarly point to differences in their binding to angiogenin and RNase-1 [17–19], highlighting what are profound differences in the active sites of RNase-1 and Ang.

At the biological-functional level the ribonucleolytic activity of angiogenin is well known to be an integral part of its angiogenic activity, as previously reviewed [9]. Thus, site-directed mutagenesis studies have shown the intact RNase active site to be crucial for angiogenesis [20, 21], and RI to inhibit not only its ribonucleolytic activity but also angiogenesis and angiogenin-mediated tumour growth [22].

Angiogenic function

The angiogenins are very potently angiogenic in comparison with most other angiogenic factors, being active on the chicken chorioallantoic membrane to fractions of a nanogram. Angiogenesis manifests as a complex process starting with an intact blood vessel and ending in the formation of new vascular structures. A blood vessel is constituted of endothelial cells, which may be polarized. These cells are in contact on their outside surface with basement membranes (BM), which are specialized extracellular matrices. Angiogenesis then requires that endothelial cells, driven by some stimulus, migrate through the BM into the surrounding tissue towards the source of the stimulus, such as angiogenin [23]. This migration or invasion leads to the formation of new blood vessels. Proteolytic enzymes, such as metalloproteases and plasminogen activators [24, 25] are secreted by such endothelial cells to facilitate penetration of the BM. Plasmin is generated from plasminogen by the action of the plasminogen activators (tPA and uPA), and in turn both these enzymes digest the components of the BM and activate collagenases which are also metalloproteases [26].

Many initially disparate functions have been observed for angiogenin, but these are rapidly being integrated into an actual mechanism for the angiogenic function of angiogenin. A surprising number of the different aspects of angiogenesis can now be viewed as being influenced by angiogenin.

A specific activity of angiogenin is inhibition of protein synthesis [10]. This is seen in cell-free translation systems where ribosomal RNA (rRNA) is specifically cleaved at rates and with effects on protein synthesis larger than that of RNase-1. This inhibition acts at the peptide chain elongation or termination level [11]. After the initial finding that both 28S and 18S rRNA were digested by angiogenin [4], closer scrutiny showed the most specific cleavage of these two to be on the 18S particle of the intact 40S-ribosomal subunit [11]. This effect may yet be a clue to the actual substrate of angiogenin, perhaps in the cellular nucleus?

Adhesion of endothelial and fibroblast cells to both bovine and human angiogenin, but not RNase-1, coated on plastic [27] revealed yet another potential aspect of the angiogenic function of angiogenin. This binding property was independent of, although similar to, that of the extracellular matrix components such as laminin, fibronectin, collagen, vitronectin and fibrinogen. In addition, pretreatment of cells with angiogenin enhanced their adhesion on other substrates, such as gelatin. This suggests that angiogenin may well act as an integral part of the extracellular matrix, binding to it [28], as well as behaving as a matrix component. The human adenocarcinoma cell line, HT-29, a secretor of angiogenin [1], adheres more avidly to angiogenin than to the common adhesion molecules, and does this specifically through adhesion receptors on these cells, which were identified as cell surface proteoglycans [29]. This interaction is inhibited by heparin. One possible implication of this avid binding is the potential for enhancing metastases [29]. Copper has been implicated in angiogenesis [30–32] and may modulate angiogenin's binding to endothelial cells [28, 33, 34]. It is not likely that this influence is exerted by copper binding to angiogenin, since no obvious binding sites are present in the sequence, and the homologous residues for copper binding seen in RNase-1 [35] are not present in angiogenin.

A series of studies has highlighted the interactions of angiogenin with plasminogen, plasminogen activators, elastase, angiostatin and even actin, all interrelated in a potential regulatory system for angiogenesis/antiangiogenesis [36–39]. Actin was the first of these molecules to be tied to the action of angiogenin. It binds tightly to angiogenin, and antiactin antibodies also specifically interfere with angiogenesis by angiogenin [38]. Recently, Hu [37] found that human neutrophil elastase cleaves the Ile(29)-Met bond of angiogenin, yielding a product that does not undergo nuclear translocation as the native molecule does, although it still has ribonucleolytic activity. This cleavage is accelerated by plasminogen, while elastase also cleaves plasminogen [40] to produce an inhibitor of angiogenesis, angiostatin [41]. Since angiogenin, in complex with actin, activates tissue plasminogen activator to generate plasmin from plasminogen [36, 42], which in turn can activate the collagenases needed for angiogenesis, a potentially sensitive regulatory system is in place [37].

A peptide, ANI-E, was developed through use of a phage-display peptide library [43]. This peptide inhibits the interaction of angiogenin and actin, specifically inhibits angiogenesis by angiogenin and also blocks angiogenesis by human prostate adenocarcinoma cells which secrete angiogenin. Similarly, two peptide-antagonists of angiogenin were developed from the antisense RNA sequence corresponding to the actin-binding site of angiogenin, and have the same activity characteristics as ANI-E [44]. These studies provide additional rationale for implicating actin in the angiogenic action of angiogenin.

Since angiogenin circulates freely in plasma, and angiogenesis does not take place continuously, the question arises as to how it is kept inactive in the plasma, while extravascular angiogenin, such as from an invading tumour, facilely promotes angiogenesis. The recent discovery of the mitogenic activity of angiogenin in subconfluent endothelial cells and the existence of a 170-kD receptor, specific for angiogenin, has provided a key [45]. Since this receptor is expressed in the cell membrane only when the cells are not confluent, angiogenin cannot act as a stimulus inside the blood vessel. Only when an event such as physical damage or an external stimulus brings cells into a nonconfluent stage, can angiogenin act.

The actions of angiogenin now involve nearly all steps and phases of angiogenesis. Thus, binding to endothelial cells [28], second-messenger stimulation [46–49], activation of BM-proteases (thereby facilitating cell invasion [36, 37, 42]), mediation of cell adhesion [27, 29], construction of tubular structures of endothelial cells [50] and cell proliferation through mitogenesis [45], are all effects mediated by angiogenin. In addition, at the molecular level the actions of angiogenin are seen in its binding to extracellular actin [38, 39, 51], heparin [52], plasminogen [37] and the 170-kD receptor [45]. The entry of angiogenin into cells after endocytosis has been explored, and the nuclear translocation of angiogenin was established [53, 54]. This translocation is independent of microtubules and lysosomes, and the mechanism probably does not require activation of tyrosine kinase [55]. How to reconcile all these actions?

A current hypothesis for angiogenin's action, which includes many of these, can be summarized as follows [45]: The different actions of angiogenin take place at different stages in angiogenin-induced angiogenesis. The presence of cell surface actin in confluent cells, but not in subconfluent cells, the converse of the expression pattern of the 170-kD receptor, provides for the following scenario: Angiogenin binds to cell surface actin in a confluent endothelial cell. It dissociates with the actin, and activates the cell associated protease system and subsequent cell invasion. As the cells migrate into the basement membrane, cell density is of course less, triggering the expression of the 170-kD angiogenin receptor. This binds to more angiogenin, which is endocytosed and translocated to the nucleus. In this process the cells are stimulated mitogenically and proliferate, filling the space opened up by the migrating cells. Whenever the cells become confluent, the receptor expression is turned off, a process that regulates the new blood vessel network.

Protein structure and correlation with function

All of the angiogenins isolated to date are very basic proteins, more basic than any other RNases [56]. This property allows for their easy isolation from serum or milk, despite their relatively low abundance [57–60].

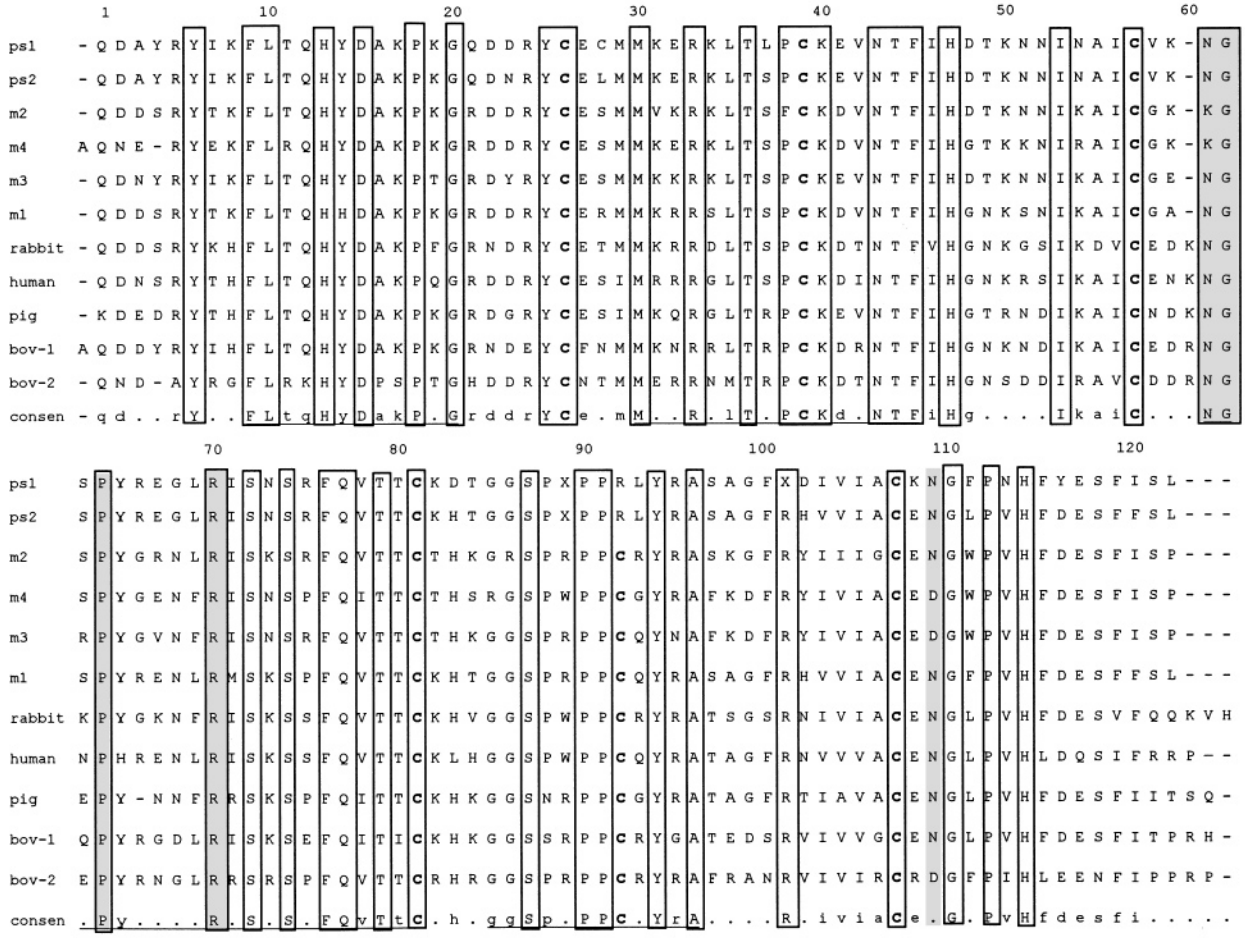

Figure 1. The protein sequences of angiogenins from various species. These are derived from protein and/or gene sequences where needed. They are: ps1,2, the pseudogenes mouse ps-1 (U22517), mouse ps-2 (U22518) [61], with X indicating positions of frame-shifts; m2, mouse Ang-related protein [61]; m4, mouse Ang-4 (consensus of ESTs AA220397, AA277314, AA277790, AA277758, AA469774, AA473916, AA500132, AA596231, AA611252); m3, mouse Ang-3 (U72672) [63]; m1, mouse Ang-1 [58]; rabbit Ang [59]; human Ang [3]; porcine Ang [59]; bov-1, bovine Ang-1 [60, 120]; bov-2, bovine Ang-2 [56]; consensus of the active sequences. Residues conserved between active angiogenins are boxed, Cys are in bold and the main residues thought to be involved with the receptor site of angiogenin are shaded. The four regions discussed in the text are indicated by underlining of the relevant consensus sequence parts. The sequences are numbered according to the sequence of human angiogenin.

Basicity may be necessary for its biological activity, suggestive of interactions with negatively charged macromolecules such as proteoglycans and the polynucleic acids.

The sequences of angiogenins from human, mouse, cattle, rabbit and pig sources are known (fig. 1). The mouse hosts the largest multiplicity of different sequences (proteins and genes), with only Ang-1 known to have angiogenic activity [58]. Ang-ps1 and -2 are pseudogenes [61], Ang-2 is an angiogenin-like protein with no angiogenic activity [62] and Ang-3 [63] has not been tested for angiogenic activity. Perusal of the EST databanks (GenBank) reveals another murine angiogenin, which for convenience is named Ang-4, a consensus sequence of a number of clones. A second angiogenin from cattle, Ang-2, was recently described [56]. Only one human, rabbit or porcine angiogenin has been seen.

Comparison of the sequences points to a number of regions of conservation (fig. 1). From the consensus sequence, a set of angiogenin signature sequences can be constructed: FLxxHxDxxPxG (region 1), MxxRxxTxPCKxxNTF (region 2), NGxPxxxxxRxSxSxFQxTTC (region 3) and GGSxxPPCxYxA (region 4). Some part of these patently are due to structural requirements for the folding of the protein, some parts are needed to maintain RNase activity and others are important for interactions with other molecules, such as the receptor(s) needed for aspects of the protein's angiogenic activity. A variety of studies, summarized in a previous review [9], show these regions to be of importance in what are postulated to be subaspects of angiogenic function. These are inhibition of cell-free protein synthesis [64] associated with region 1, nuclear translocation [54] with part of region 2, angiogenesis and the actin-binding site [5, 65, 66] partly with region 3, and inhibition of degranulation of polymorphonuclear leukocytes [67] with region 4. The underlying RNase activity is reflected by conservation of the catalytic residues His-13, Lys-40 and His-114, and of active site residues such as the RNase-1 signature region – the latter part of region 2.

The two angiogenins of cattle which have only 57% identity in sequence additionally differ markedly by a carbohydrate modification on Ang-2 and not Ang-1 [56]. This modification is on the homologous position of Asn-34 of the RNase-1 enzymes of a variety of species, which is the most frequently observed glycosylated site in this family [68, 69]. The nuclear-targeting sequence of angiogenin is immediately adjacent to this residue [54], and the carbohydrate is therefore in position to interfere with nuclear translocation of angiogenin. Although the significance of the carbohydrate for angiogenic activity is not known, this interference may be pertinent, perhaps steering this angiogenin to different activity pathways.

Bovine Ang-2 provides some substitutions that are unique in this group of angiogenins. Thus Lys is found to substitute for the otherwise conserved Gln-12 of this protein family, although in chicken and frog RNases, this same substitution is found [70–73]. Gly-7 replaces the basic residue of most other RNases, which is oriented towards the active site. This can be speculated [56] to cause a specificity or activity change towards the putative natural substrate of angiogenin, since the binding/steering interactions of that basic residue with the substrate are eliminated, as revealed by the X-ray structure. Another site that is of interest is the replacement of Asn-109 (bovine Ang-1) by an Asp. The same modification in human Ang-1 abolishes angiogenic activity, and Asn-109 is usually interpreted to be part of the actin-receptor-binding site [66]. This difference of Ang-2 probably reflects the complexity of the cellular interaction site. Finally, residue Ile-113 is unique to Ang-2, conservatively replacing a Val but reducing the number of totally conserved residues in the RNase superfamily by yet another residue.

X-ray crystallography has provided insight into various aspects of the angiogenin molecule, reviewed elsewhere [9]. First, the structure is indeed very similar to that of RNase-1, as evolutionary theory would require, and especially the catalytic residues of RNase-1 are well conserved in their spatial arrangement. Second, the differences between the structures point to possible reasons for the vast difference in conventional RNase activity, mostly effected by an active-site blockage by a specific residue in angiogenin, Glu- or Gln-115, which interacts with Thr-44. Much has been done in recent years to examine this interaction closely (Acharya, Shapiro and co-workers) [74, 75]. In addition, the much different character of the putative receptor (actin)-binding site of angiogenin (region 3; fig. 1), as compared with the homologous region of RNase-1 and -2, became clear. Nuclear magnetic resonance (NMR) studies on the structure showed that the general structure provided by X-ray crystallography also is valid in solution, including the occluded pyrimidine-binding-site cleft [76]. In addition, the orientation of the imidazole rings of both active-site histidines is unambiguously the same as in RNase-1, supporting the idea of conserved catalytic properties between these enzymes.

The receptor(actin)-binding site of angiogenin contains a unique sequence, present in all the active angiogenins. This is Asn-Gly (61–62), a potent off-switch in angiogenic function [66]. When Asn-61 deamidates, as this specific protein sequence very easily does (reviewed, e.g. in ref. 77), angiogenic activity is attenuated by the formation of an isoaspartyl residue. The resultant angiogenin variant will not bind to the receptor and will not inhibit receptor binding by intact angiogenin. Enzymatic rescue through conversion of the iso-aspartate by carboxymethyl transferases to an Asp residue [78] will in addition lead to inhibition of this receptor binding of

native angiogenin [66]. This region of angiogenin differs markedly from the homologous region of other RNases, which also contain an Asn-Gly sequence, albeit in a position usually not mutually aligned, but potentially alignable. Yet, as suggested by the deamidation and physiological control of the deamidation products of bovine seminal RNase (BSR) [78], that region in BSR is implicated in its interactions with other molecules, for carrying out its physiological function, a function that involves one or more of a variety of physiological effects [79]. Interestingly, all the RNases-1 (except for mouse) have conserved this sequence, and it does deamidate readily also in bovine RNase-1 [80]. Could this site then be a switch for a biological activity of these RNases?

Other biological activities of angiogenin

The major activities of angiogenin that have been investigated to date are the angiogenic and ribonucleolytic activities. In addition, a number of ostensibly different activities have been observed, some of which are perhaps pertinent to angiogenesis. The presence of angiogenin in a wide variety of cells has further led some to suggest that angiogenesis may not be the sole biological function of angiogenin [81, 82].

The view has been expressed that angiogenin may also act as part of a host-defence system, due to its high activity as a transfer RNase (tRNase) in serum [83]. This has been supported by the finding that angiogenin is one of the members of the ribonuclease A family for which the genes are expressed in adherent monocytes [84]. That RNase-4, possibly under identical promoter control as angiogenin (see below), is found in cytoplasmic granules of these monocytes, suggests that angiogenin may well itself be similarly segregated.

The cytotoxicity of angiogenin [83] and the enhancement of its entrance to the cytosol effected by retinoic acid and monensin [85] demonstrated the potential for using angiogenin as a human-specific (and therefore not immunogenic) cellular toxin. This has been followed up by different successful attempts at designing immunotoxins, specific for specific cells. Thus a recombinant fusion of angiogenin and a single-chain antibody to the human transferrin receptor was cytotoxic to different human tumour cell lines [86, 87]. Chemical coupling of angiogenin to a monoclonal antibody against carcinoembryonic antigen yielded a cytotoxin, effective against a human adenocarcinoma cell line [88]. In contrast, the investigation of complications in kidney diseases, indicative of impaired host defence, led to discovery of a degranulation inhibitory protein, which was identical to angiogenin [67, 89].

Intriguing has been the discovery that a member of the RNase family, *Xenopus*-secreted protein FRL2, activates the fibroblast growth factor (FGF) receptor of *Xenopus* and yeast, a tyrosine kinase [90] by binding to it. Since FGF is angiogenic, and the FGF receptor ligands such as FRL2 appear to play a role in development of the frog, similar roles of angiogenin and/or the other RNases in mammalian systems can be envisioned. The recent discovery of the 170-kD receptor for angiogenin [45] may be the first step in addressing this possibility, although this may not specifically be a tyrosine kinase [55].

Angiogenin is the second member of the family that shows immunosuppressive activity, as does bovine seminal RNase [91]. How this may be related to an angiogenic effect is not immediately apparent, although synergism with tumour angiogenesis may be of medical importance.

Clinical

Numerous studies are appearing on the potential use of angiogenin levels in the diagnosis and prediction of a variety of clinical states. The levels of angiogenin found in a variety of studies are summarized in table 1.

Carcinomas are naturally of interest, given the original postulate regarding vascularization of solid tumours [92]. Thus Li et al. [93] correlated the expression of angiogenin with angiogenesis in colonic adenocarcinomas and gastric cancers, but not for hepatocellular carcinomas. Increased angiogenin levels in serum predict the progression of pancreatic cancers [94], and also endometrial cancers [95]. Treatment of carcinomas is presaged by the exciting series of studies of Olson et al. [22, 96], which demonstrate the prevention of tumours using antiangiogenin monoclonal antibodies, actin or ribonuclease inhibitor. The latter molecule similarly was shown to be an antitumour substance in separate studies [97, 98].

Peripheral arterial occlusive disease leads to higher angiogenin concentrations in circulation, implying that angiogenin is an indicator of endothelial damage, caused by the progression of this disease [99]. Since angiogenesis is involved in rheumatoid arthritis, the presence of angiogenin was examined in synovial fluid and tissue [100] and no difference from normal synovia was found. The authors suggest that angiogenin may be a normal constituent in the physiology of the synovium.

Pregnancy and the large amount of angiogenesis associated with it have become the target of numerous studies on angiogenin levels (table 1). This includes maternal, foetal and perinatal serum levels. The levels measured by different laboratories vary widely, suggestive of methodological difficulties in obtaining absolute

Table 1. Levels of angiogenin determined for various physiological fluids.

Source	State	Level (μg/l)	Method A = assay I = isolation	Reference
Human:				
serum	maternal	226	A	[114]
	umbilical vein	119	A	[114]
	neonate day 1	166	A	[114]
	neonate day 4	241	A	[114]
serum	endometrial cancer	higher	A	[95]
serum	pregnancy 10 weeks	150	A	[115]
serum	pregnancy 40 weeks	250	A	[115]
serum	impaired placental function	no raise	A	[115]
serum	pancreatic cancer	567	A	[94]
serum	normal	359	A	[94]
serum	normal	0.358, 0.406	A	[99]
serum	endothelial damage in peripheral arterial occlusive disease	0.467	A	[99]
plasma	normal	>150	I	[116]
plasma	normal	250	A	[117]
amniotic fluid	normal	18	A	[118]
amniotic fluid	preterm delivery	30	A	[118]
sinovial fluid	normal, arthritis	249, 306	A	[100]
Bovine serum		100–180	I, A	[57, 119]
Bovine milk		4000–8000	A	[119]
Porcine serum		100	I	[59]
Rabbit serum		100	I	[59]
Mouse serum		40	I	[59]

The levels measured by different laboratories vary widely, suggestive of methodological difficulties in obtaining absolute measurements. It should, however, be possible to interpret these studies internally as showing relatively lower or higher levels in various sources or clinical states, as long as proper controls (normals) were assayed at the same time.

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Clinical use of the angiogenic activity per se has been foreshadowed by the study of King and Vallee [101]. They demonstrated the healing of wounded menisci (cartilage) in the rabbit knee.

Relationship of angiogenins to other ribonucleases

The evolutionary relationship of angiogenin and the other RNases has been explored via the protein structures, and some information on genetic relationships has been gleaned [56, 69, 79, 102].

With the advent of the Human Genome Project, we have seen an enormous increase in sequences, especially at present as expressed sequence tags (ESTs). Inspection of these databanks, so well established and kept up to date by the relevant organizations, has provided some insights into the organization of the angiogenin gene, and its relationship to the other ribonucleases.

We find that all the known RNase genes are within a relatively short distance of one another on human chromosome 14, and are probably the most centromeric genes on that chromosome. At the website of the National Center for Biotechnology Information, National Library of Medicine (NCBI), the Human Gene Map summarizes the arrangement of the ribonuclease superfamily as in table 2 [mostly from studies by the Stanford Human Genome Center (SHGC)]. The order of the genes for the RNase superfamily with reference to gene markers on chromosome 14 therefore is pTer-(RNase-1, ANG)-D14S261-(RNase-4, RNase-6)-D14S72-(EDN, ECP)-D14S283 (pTer, telomeric terminal of p-arm; D14Snn, Généthon marker sequences; commas indicate relative order unknown). Angiogenin (ANG) may be very close to marker D14S261. The mouse Ang-1 and RNase-1 genes have also been allocated to a common chromosome, chromosome 14 [103, 104], which is partially homologous to human chromosome 14.

The many ESTs for human angiogenin and RNase-4, maintained by Genbank [105], were inspected using the BLAST family of programs [106], as available at the NCBI. This has provided a new view of the relationship of these proteins.

Angiogenin messenger RNA (mRNA) is constructed from one exon that covers the whole of the translated protein and the 3′ untranslated region (UTR) [2], and another exon that codes for the 5′UTR. The 3′-end ESTs of human angiogenin are identical within the

Table 2. Arrangement of the human ribonuclease A superfamily on chromosome 14.

Protein	Location between specified Généthon markers	Reference number
RNase-1	14pTer–D14S261	SHGC-11531
RNase-5 (Ang)	14pTer–D14S261	SHGC-11728
	Near D14S261	stSG1614 (Sanger Center, UK)
RNase-4	14pTer–D14S72	A006H30 (Généthon, France)
	D14S261–D14S72	SHGC-11775
RNase-6	D14S261–D14S72	SHGC-15797
RNase-2 (EDN)	D14S72–D14S283	SHGC-10764
RNase-3 (ECP)	D14S72–D14S283	SHGC-10764
RNase-3b (ECP-related)	D14S72–D14S283	SHGC-10764

Summarized from the Human Gene Map, at the NCBI website: http://www.ncbi.nlm.nih.gov/, through the UniGene (Unique Human Gene Sequence Collection) page. SHGC, Stanford Human Genome Center, San Francisco.

experimental errors associated with the rapid sequencing methods employed (loss of single bases within a multiple stretch; stuttering of extra bases). ESTs covering the 5′UTR of the angiogenin mRNA, however, clearly form three different groups. Using consensus sequences for these groups, BLAST searches revealed no homology to the known 2000 bases of the human angiogenin gene 5′ to the main exon (coding region), probably representing the intron sequence, and no homology to any other DNA sequences, except for RNase-4 ESTs.

The three groups of mRNAs clearly are derived from a single angiogenin exon, preceded by a different exon for each of the three cases, that codes for a 5′UTR. These different UTRs are conveniently classified as types 1, 2 and 3. The type 2 and type 3 5′UTRs differ only in a deletion of 21 bases in type 3, while type 1 is totally different from them. The tissue source of all of the ESTs with type 2 5′UTRs is liver or fetal liver-spleen, and the related type 3 5′UTR likewise is from liver. In contrast, the type 1 5′UTRs were obtained from a variety of tissues. This tissue distribution could reflect a higher

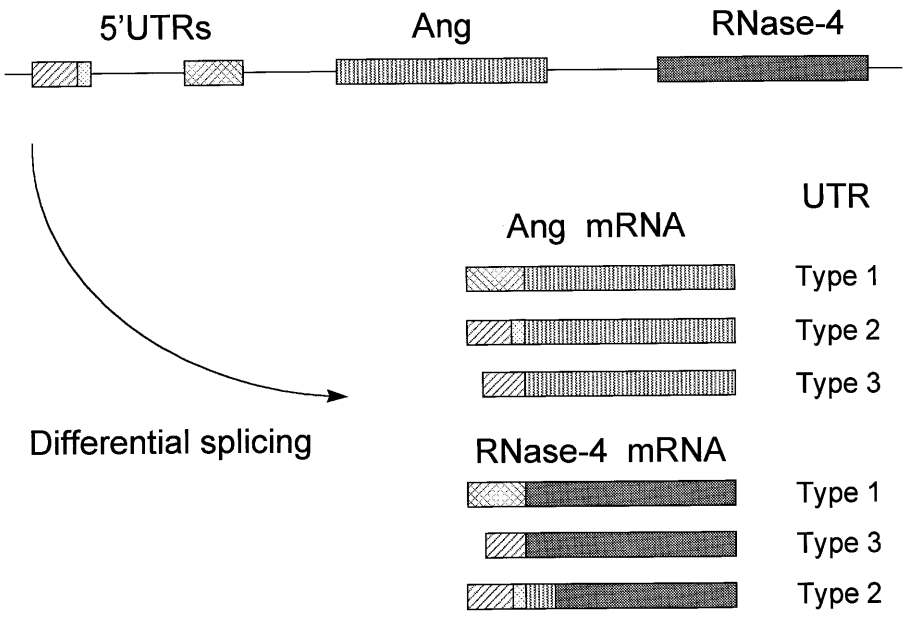

Figure 2. The arrangement of angiogenin and RNase-4 genes in the human genomic sequence. Differential splicing of the angiogenin or RNase-4 exons with either of the 5′UTR exons leads to multiple mRNAs, as illustrated. One of the RNase-4 mRNAs presents a hybrid with part of the angiogenin exon.

level of expression in liver cells, with controls exerted at the transcription level.

A number of the ESTs of human RNase-4 which were identified by a BLAST search with the RNase-4 sequence as probe extended beyond the 5′-exon boundary of the RNase gene. They contain identical sequences to the types 1, 2 and 3 5′UTRs of angiogenin. This strongly suggests that these mRNAs are spliced to contain the same first exon and two totally different proteins as second exon, and implies that these genes are closely arrayed on chromosome 14. Their mutual arrangement with reference to the types 1 and 2 5′UTR is of course not immediately apparent, since the requisite splicing can take place with both a UTR-ANG-RN4 and a UTR-RN4-ANG arrangement. However, in addition one of the clones (T92792) also included another stretch of bases between the type 2 5′UTR and the main RNase-4 exon, identical to the presequence of angiogenin, up to residue 6 of the mature angiogenin, and continued on as for RNase-4. This hybrid fixes an exon sequence for this part of the human genome of 5′UTR-ANG-RN4, since out-of-order splicing is not plausible. Figure 2 illustrates the proposed arrangement of these 5′-UTR exons and those for angiogenin and RNase-4 in the genomic sequence and in the mRNAs (D. J. Strydom, unpublished observations). Taken together with the order of markers and RNase genes inferred above, this arrangement suggests that marker D14S261 should be between the angiogenin and RNase-4 genes on chromosome 14, which may refine the previously presented gene order of the RNase superfamily in humans to pTer-RNase-1-Ang-D14S261-RNase-4-RNase-6-D14S72-(EDN,ECP)-D14S283.

This arrangement is one of the few known instances of two different proteins sharing the same 5′UTR exon. Another example is from the choline acetyltransferase (*ChAT*) gene, which protein is encoded by multiple mRNAs with different 5′ ends, at least two of which are shared by a vesicular acetylcholine transporter protein, encoded within an intron in the *ChAT* gene [107]. Since 5′UTRs are generally involved in control of protein expression, this means that identical controls operate at the mRNA level on the expression of angiogenin and of RNase-4. The different 5′UTR types of angiogenin point in addition to such controls being differently implemented for different mRNA populations. Patterns of tissue and cellular expression are also identical or highly similar for angiogenin and RNase-4 [84, 108].

The situation in the mouse genome is, of course, potentially more complicated, since more angiogenin genes are present. However, a similar gene arrangement for mouse RNase-4 and angiogenins -1 and -4 may be in place. RNase-4 mRNA has two different 5′UTRs (such as that from ESTs AA104176 and AA396298, respectively). Ang-4 has one 5′UTR (e.g. in AA277314), with some of these having a small ~10-base deletion (e.g. AA220397), while mouse Ang-1 has two 5′UTRs, one seen in the gene sequence U22516 and another in the known ESTs, exemplified by AA237829. The former region has no matches to other proteins. The other EST Ang-1 5′UTR shows very high similarity to that of Ang-4 (e.g. AA500132) and also to one of the RNase-4 5′UTRs (e.g. AA396298). This may suggest similar controls for all three of these genes, although the slight differences in EST sequences may be real, in which case at least the UTRs, if not the controls, may be discrete for each of these gene products.

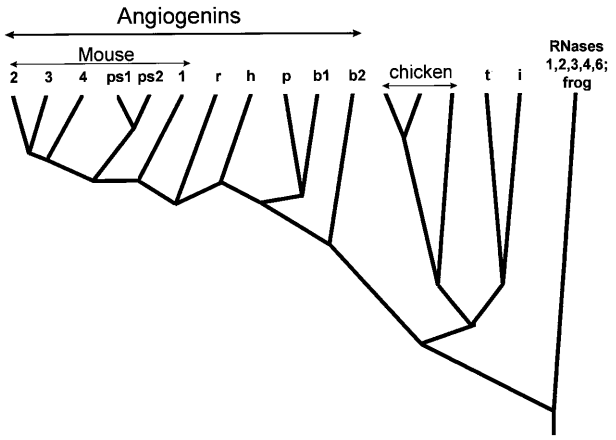

Figure 3. Phylogenetic relationship of the angiogenins. The sequences of known RNases were used for constructing a tree (Program PHYMOD [121]). The classification of the angiogenins (mouse 1, 2, 3, 4, pseudogenes ps1 and ps2, rabbit (r), human (h), porcine (p), and bovine (b1 and b2) and the RNases from frogs (f), chicken, iguana (I) and turtle (t) are graphed using all the rest of the known RNase sequences as a combined outlier.

How does this affect our understanding of the functions and mechanisms of action of these two very different though homologous proteins? Angiogenin has very low ribonucleolytic activity and exhibits potent angiogenic activity. RNase-4 is a very active RNase, with unusual specificity in this family, for uridyl residues. It is the most conserved between species of all the RNases in this family, suggesting a specific biological function, which is as yet unknown [84, 109]. If, as suggested by the association of their genes, these proteins are expressed in concert, perhaps they can also act in concert. Experiments to explore this possibility should include the interaction of RNase-4, alone and in combination with angiogenin, with RNAs, actin, cell-free translation, nuclear translocation, cell adhesion and protease activation.

A search for a cellular receptor for RNase-4 may also be useful. It may be especially pertinent that RNases-4,

RNases-1 (except mouse), the RNases-6 from ox and pig, but not humans, and the angiogenins all contain an Asn-Gly sequence in the general region of the angiogenin receptor(actin)-binding site. Since this sequence is thought to act as a control switch in angiogenin by deamidation [66], do not the other proteins also use this sequence in this area as a receptor-binding region with a built-in off-switch?

Evolution of angiogenin genes

The 11 sequences for angiogenins and the 5 sequences of bird and reptile RNases cluster clearly as a subgroup of the RNases (fig. 3). All four of the mouse angiogenins and their two pseudogenes form a subgroup of sequences, allied with the rabbit and human sequences separate from the pig and bovine-1 sequences. The bovine angiogenin-2 is clearly still in the angiogenin cluster, but well separated.

The overall clustering pattern is probably governed by the structural similarities between these proteins, in contrast to the other RNases. This is dominated by the three-disulphide pattern of this group, contrasted with the four disulphides of the mammalian RNases in general. It is of interest that this extra disulphide bond is the one that is most readily reduced, and selectively at that, in bovine RNase-1 [110]. This would suggest that that disulphide is a later addition to the structure during evolution of the RNases, in accord with finding that the only RNases with that disulphide bond are found in mammals.

The multiplicity of genes for the angiogenin pattern in mice is interesting, but does not yet allow for understanding of their roles. Activity measurements are still lacking – we only know that Ang-1 is angiogenically active, while Ang-2 is not active. Could Ang-3 and -4 be active? Their sequences are suggestive of activity for Ang-3, but Ang-4 would probably not be angiogenically active, due to changes in the receptor(actin)-binding site, most importantly with the cardinal Asn-61 change to Lys. On the other hand, Ang-4 is perhaps under the same translational control as Ang-1 (above), which implies coexpression, and perhaps then the need for another receptor or cellular interaction site. As RNases, both Ang-3 and -4 should be active, since they maintain all the active-site residues.

We should also keep in mind that molecules such as angiogenin and RNase-1 are among the fastest-mutating enzymes. RNase-1 has been hijacked in some species to act as a pancreatic-secreted catabolic enzyme, but in most species it is of little importance in that capacity [111]. Why and how such fast changes evolved, coupled with extremely tight control by a single inhibitor protein, is not yet clear. This whole family has evolved in vertebrates only, as far as known, and our current knowledge comes especially from mammalian species. It is as if this enzyme is facilely adaptable by evolutionary forces to take on new functions, based on its underlying RNase activity, but coupled with other interactions.

The presently proposed mechanism(s) for angiogenin's involvement in angiogenesis demands a variety of different actions mediated by the one molecule. This interpretation presupposes that various parts of the sequence are involved differently. Such multiple interactions would, in the light of our general understanding of protein evolution, be expected to require conservatism in sequence, with few changes between species, whereas in actuality the various mammalian angiogenins differ markedly. Compare this to the similar extent of variation in RNase-1 sequences, which are considered to be only the scaffolding for ribonucleolytic activity and thus acceptably divergent in sequences. Similarly, the extraordinary tight binding of angiogenin and RNases from different species to human RI, both types of protein being quite variable between species, has been counterintuitive; and indeed it is clear that RI and RNases-1, -2 and -5 (angiogenin) bind through different specific interactions at the molecular level, albeit between the same homologous surfaces on these proteins [13]. Additionally, the angiogenins, all from placental mammalian species, are all active on the chicken chorioallantoic membrane (CAM). This requires that most of the activities expected for angiogenin in and around mammalian cells also should be active in birds, a very different environment. The interesting implication is that it is rather the general action, than very tight matching of surfaces, that mediates the various activities, and therefore that the evolution and evolutionary maintenance of angiogenic activity of angiogenin has been influenced and allowed not by the conventional tight fit at the molecular level, but by general shapes and molecular environments.

Future studies

Angiogenin, by its function, has participated in demonstrating that RNases can be considered as more than mere catabolic enzymes [109, 112]. It acts both as a ribonuclease and as a messenger molecule, acting through at least one receptor. The complexities of its interactions are exemplified by interactions with actin, heparin and proteoglycans, and its influence in so many processes. Is it too far-fetched to expect that RNase-4, which shares a common control through common 5′ sequences in the various mRNAs of these molecules, could also interact in a variety of capacities – especially since RNase-4 is the most conserved molecule among these RNases? Comparative studies of these two en-

zymes would be expected to point to perhaps the true substrate of angiogenin, and one for RNase-4, and would there be a further commonality in those?

Angiogenin's receptor(s) will shed light on at least one aspect of angiogenesis, and perhaps lead to discovery of receptors (and functions) of other members of the RNase superfamily. After all, why should the region of that receptor-binding site on angiogenin have such a function only on angiogenin? Homology of function may well be expected.

The peculiarities of the mechanism of angiogenin's ribonucleolytic action, and the 'obstructed' active site, can be expected to lead to new insights and controls of not only angiogenin, but also of RNase-1 and the other RNases. Differential inhibition or activation of the various members of the superfamily could result from such studies, with opportunities to understand their biological functions in isolation from one another.

The variety of interactions of angiogenin can surely not be seen as uniquely evolved in only this one member of the superfamily. Its existence must also point the way to a search for the effects of all the other members on the physiology and life of the organs and cells within and with which they coexist, as exemplified by bovine seminal RNase [79], the frog lectins and onconase [113] (Irie et al., this issue) and the human eosinophil RNases-2 and 3 (Rosenberg, this issue). Interactions with the proteolytic and other members of basement membranes, especially, are potential areas of research. The Asn-Gly switch for angiogenic activity of angiogenin, and its conserved presence with no known associated activity change in all of the RNases-1 (except mouse), RNases-4 and RNases -6 (pig and ox, but not humans), strongly argues for searches for cellular partners for these other RNases. Such searches could be patterned on those for cellular receptors for angiogenin, using both confluent and subconfluent cells. Such receptors, when found, will provide the clues to actual physiological functions.

Finally, the Grail of angiogenin research, its natural substrate, remains an aim in the elucidation of angiogenin's mechanism of action. The absolute requirement for a ribonucleolytic activity has been established conclusively for nearly a decade, and perhaps the next few years may see the final search, with its wider implications for all the other members of the RNase A superfamily.

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