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Hypothesis

Extracellular 'communicator RNA'

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Evidence is presented that RNA molecules exist that act as extracellular messages important in the development of higher organisms.

Important metabolic and regulatory roles are occasionally discovered for classes of enzymes once thought to be interesting only to mechanistic enzymologists. For example, no sooner had enzymologists 'solved' all of the interesting catalytic problems presented by proteases than proteases were found to play important roles in genetic regulation. Now, proteases are known to control the expression of genes, to defend against viruses, and perhaps to control the induction and growth of tumors [1].

Ribonuclease (RNase) may provide a similar story. Today, this diminutive protein (13.5 kDa) from the digestive system of cows is thought to be interesting primarily as a model to study folding, catalysis and evolution in proteins [2]. However, recent discoveries promise to revolutionize this view.

Early evidence that RNases had interesting biological properties was uncovered 20 years ago. In studying the effect of actinomycin D on the growth of tumor cells, Sartorelli and his coworkers [3] noticed that the cytostatic effect of the drug was increased if pancreatic RNase was administered at the same time. The significance of this synergism was not immediately apparent, and the result was forgotten.

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10 years later, another RNase with anti-tumor activity was discovered. Vescia et al. [4] showed that bovine seminal RNase had strong anti-tumor activity both in vitro and in vivo. Seminal RNase is a dimer [5], and hydrolyzes double-stranded DNA-RNA hybrids [6]. Both properties contrast with those of pancreatic RNase, which is a monomer, hydrolyzes single-stranded RNA, and displays little anti-tumor activity. Nevertheless, the two proteins are highly homologous; their sequences are 81% identical, and over half of the amino acid substitutions are conservative.

Shortly afterwards, Bartholeyns and Moore [7] at the Rockefeller University chemically cross-linked pancreatic RNase to prepare dimers. The chemically dimerized enzyme displayed increased catalytic activity against double-stranded nucleic acids. Further, the dimeric enzyme had anti-tumor activity, both in vitro and in vivo [8].

These independent discoveries did not escape the attention of Dr Carl Levy of the Baltimore Laboratory of the National Cancer Institute, who wrote in 1980 [9]:

> "The finding of antitumor properties associated with [RNase] is exciting, for the protein offers what is in many respects the ideal type of therapeutic agent, that is, plentiful supply, low price, and minimal (at least in the animals tested) side effects. The dimeric RNase, moreover, is highly

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effective against a number of animal tumors in various chemotherapy testing programs. Many drugs have entered clinical trials with far fewer credentials than this enzyme has. Under the circumstances, serious consideration should be given to testing the dimer enzyme in extensive, formal preclinical trials."

Levy's suggestion was not pursued; indeed, the scientific community was less than enthusiastic when pursuit was suggested. Turning down a funding request to study the anti-tumor activity of RNase, an NIH consultant wrote in 1984: "If RNase had one tenth the potential as implied [by Levy's comment], dozens of scientists would already be busily investigating. This hasn't happened!" [10].

However, in recent years, evidence has accumulated to suggest that the suggestion had considerably more potential than Dr Levy's comment implied. This evidence comes from the fact that several proteins, first identified from their unusual biological activities, have been shown to be catalytically active as RNases and structurally homologous to bovine pancreatic RNase.

For example, solid tumors secrete substances to stimulate the growth of blood vessels, a process called 'angiogenesis'. The vascularization of solid tumors appears necessary for their viability; solid tumors unable to become vascularized simply do not grow. On these grounds, Folkman and his coworkers [11] argued for some time that inhibitors of angiogenesis would be potent pharmaceutical agents for managing solid tumors clinically.

One such tumor angiogenesis factor was recently isolated and sequenced by Vallee and his coworkers. The results were surprising; the protein's sequence is 40% identical to that of human pancreatic RNase [12] and the protein is catalytically active as an RNase [13]. Human placental RNase inhibitor, a protein with a molecular mass of 51 kDa, inhibits both the ribonucleolytic activity and angiogenetic activity of angiogenesis factor [14].

Even more recently, RNases with other unusual biological activities have been found elsewhere. For some 60 years, it has been known that human eosinophils are neurotoxic. When injected intracerebrally or intrathecally into laboratory animals, they produce a variety of neurological symptoms classified as a group as the 'Gordon phenomenon' [15], a syndrome associated with degeneration of brain tissues. At least two proteins from eosinophils are associated with their neurotoxicity, the eosinophil-derived neurotoxin (EDN) and the eosinophil cationic protein (ECP). These proteins were recently isolated [16] and partially sequenced [17]. Both were found to be homologous to digestive RNase; ECP was very recently shown to have catalytic activity as an RNase [18].

A role for these proteins in human pathology is not established. Proteins from eosinophils have antiparasitic action [19], and eosinophils are generally believed to play a role in killing foreign cells. However, the protein may be involved in human neurological disorders.

To this list must be added scattered reports of biological effects of pancreatic RNase, including the inhibition of protein synthesis by exogenous RNase [20], various effects of pancreatic RNase on the growth of tumors [21], and the use of pancreatic RNase to treat tick-borne encephalitis [22]. Further, there are a number of reports throughout the literature suggesting developmentally important roles for RNA molecules in many biological phenomena. For example, isolated reports in the early 1970's showed that another tumor angiogenesis factor loses its angiogenic activity upon treatment with RNase. This fact suggests that this angiogenic factor itself contains RNA [23].

There has been little speculation concerning the general significance of these scattered reports. Thus, it is intriguing to ask the obvious question: Is there any plausible reason why RNase homologs should appear in biology with angiogenetic properties, as a tumor growth inhibitor, and as a neurotoxin?

At the very least, the homology of tumor angiogenic factors, inhibitors of tumor growth, and neurotoxins is structurally interesting. Site-directed mutagenesis methods now permit us to correlate structural differences between these six proteins (pancreatic, artificially dimerized pancreatic, and seminal RNases, angiogenin, ECP and EDN) with their biological activities [24]. However, the question also suggests new hypotheses regarding RNA in biological processes.

The ribonucleolytic activities of these RNase homologs appear to be important for their biological action. For example, the anti-tumor activity of seminal RNase seems to correlate with

catalytic activity [4]. Further, as noted above, inhibition of the RNase catalytic activity of angiogenin inhibits its angiogenic activity as well. These facts are most consistent with the notion that the RNase catalytic activity of these proteins is relevant to their biological activities.

Only one example may contradict this notion. Chemically dimerized pancreatic RNase that had been 92% inactivated with iodoacetic acid retained anti-tumor activity; only after 98% of the catalytic activity had been destroyed by further chemical modification was anti-tumor activity abolished. The significance of this result is unclear, as extensive chemical modification with iodoacetate creates a species with a poorly defined chemical structure. Such experiments indicate the difficulty of drawing conclusions from chemically modified proteins (and the blessings of recombinant DNA techniques that permit clean structural modification of proteins).

The RNase homologs could exert their biological activity either extracellularly, where they are delivered, or intracellularly, following transport across the cell membrane. Each possibility must be considered. Labeling studies have shown that many RNase homologs are transported into cells. Once inside, RNase homologs could conceivably catalyze the hydrolysis of RNA molecules critical to cell processes, bind to DNA, or influence the expression of genes by influencing the rate of turnover of message.

A possible model for an intracellular target is the inhibition of translation in vitro by angiogenin [25]. Another possible model is the protein α-sarcin, a RNase that inhibits translation by catalyzing the hydrolysis of a single phosphodiester bond in ribosomal RNA [26]. As possible support for RNases as DNA-binding proteins, it is interesting to note that RNase A and gene 5 DNA-binding protein from bacteriophage fd, both helix-destabilizing proteins, have similar tertiary structures, and the two proteins may be homologous [27].

However, there are reasons for doubting an intracellular target for RNase homologs. For example, seminal RNase is transported with equal facility into transformed and non-transformed cells [4]. However, it inhibits the growth of only *transformed* cells. Further, intracellular RNases are already present in high concentrations; thus the rate of degradation of biologically active mRNA molecules inside cells may be independent of the concentration of RNases. If this is true, internalized RNase, even if it is delivered near the translation apparatus, is unlikely to affect the lifetime of mRNA. Finally, when RNases such as sarcin (vide supra) act intracellularly at the level of the ribosome, the biological activity appears to be non-specific with regard to cell type; thus, sarcins are generally rather toxic. This contrasts sharply with the rather specific biological effects produced by the RNase analogs discussed above.

The selectivity of the biological activity of the RNases discussed above, and the dependence of biological activity on catalytic activity when administered extracellularly, suggest an alternative hypothesis, that these RNase homologs exert their biological effects by extracellular action *as ribonucleases*. This suggestion is intriguing. It implies that the substrate for extracellular RNases, extracellular RNA, must play a biological role in angiogenesis, neurological development, and other biological processes. This suggests the general hypothesis that there exists extracellular RNA molecules having the role of carrying information over short distances between cells which act as intercellular communicators during the development of higher organisms [28]. Further, it is consistent with the suggestion that certain angiogenic factors contain RNA [23].

The hypothesis of a biological role for extracellular RNA offers an explanation for another fact. Extracellular fluid contains many proteins with RNase activity, and many proteins that are RNase inhibitors [29]. The biological significance of this balance between RNases and RNase inhibitors is not known. However, it makes little sense in any case in the absence of a substrate for the RNases, extracellular RNA. We hypothesize that a combination of RNA, RNases and RNase inhibitors assists in the control of the development of tissues in higher organisms. By perturbing the distribution of extracellular RNA, the addition of extracellular RNases influences development, including the stimulation and inhibition of cell growth.

Further, the chemical nature of RNA, especially the ease with which it forms tertiary structures and the ease with which it can undergo hydrolysis, makes it well-suited to play the role of a short

distance-short time messenger. Indeed, from a purely chemical point of view, the role of steroids as long distance-long time extracellular messengers, polypeptides as intermediate distance-intermediate time extracellular messengers, and RNA as a short distance-short time messenger seems to be a good match of chemistry and biological function.

One final interesting feature about the evolution of these homologous proteins is worth noting. Human angiogenin is structurally more similar to turtle RNase than to human pancreatic RNase [30]. This suggests that angiogenin and conventional RNase diverged before reptiles and mammals diverged. This places the evolutionary origin of angiogenins before the origin of land-based life and, significantly, perhaps at the time of the origin of specific physiology found in higher organisms. In contrast, bovine digestive RNase appears to be a relative late-comer evolutionarily. It appears as if digestive RNases had evolved from developmentally important RNases as an adaptive response to the evolution of ruminant digestion.

The hypothesis of extracellular 'communicator RNAs' involved in eukaryotic development is well worth testing, if only because a direct test is now possible. Should the hypothesis prove correct, it will add yet another chapter to the already growing resumé of RNA molecules, and resurrect RNase as a protein interesting to biologists as well as mechanistic enzymologists.

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